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Predicting melanoma outcome using clinical and biological indicators

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MBBS

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Abstract

Globally, we are seeing an increasing rate of melanoma while melanoma mortality is reducing. This is significant as more patients with cancers including melanoma are now surviving longer, which increases their risk of being diagnosed with subsequent melanomas. In chapter 2, we examined patients with multiple invasive melanomas, looking at their pattern of progression towards sentinel node metastasis. 651 patients with primary invasive cutaneous melanoma who were referred to the Princess Alexandra Hospital melanoma clinic and underwent sentinel lymph node biopsy between 1994 and 2011 were included in this study. Information on their index melanoma as well as any melanomas before and after were collected and analysed. Logistic regression model was used to determine which factors predicted sentinel node metastasis. Additional stage II melanoma was an independent and significant predictor of sentinel lymph node metastasis. When we evaluated melanoma-specific survival in this cohort, patients with additional stage I melanoma had better melanoma-specific survival, whereas additional stage II and stage II melanoma prior to index melanoma had poorer melanoma-specific survival as expected. Patients with additional invasive melanomas fare poorer compared to those with single invasive melanoma, especially if the additional melanoma is of a later stage. This highlights the importance of patient education in the melanoma survivor population, as well as closer surveillance to diagnose any subsequent melanomas at an early stage.

Next, we explored the role of various biomarkers using immunohistochemistry, including P-STAT5 (phosphorylated signal transducer and activator of transcription 5) and a panel comprising SOX18 (SRY-related HMG-box 18), Ki67, CD31, D2-40, in predicting melanoma outcome in a cohort of clinical stage I and II melanoma patients. Identifying novel prognostic biomarkers in locally invasive melanomas to predict clinical outcomes is crucial with the new possibilities in adjuvant therapy. It enables further stratification of patients to reserve adjuvant therapy to high risk patients, provide closer follow up to improve survival, while preventing unnecessary exposure of low risk patients to toxicity from adjuvant therapy.

We studied cytokine receptor signalling via P-STAT5 to reflect tumour immune function as a potential prognostic marker of melanoma. We studied a cohort of stage Ib and II melanoma patients, with 189 melanoma tissue samples being analysed for active P-STAT5 level by immunohistochemistry and correlated this with patient and tumour characteristics and survival outcomes after an average of 10 years of follow-up. High P-STAT5 level was associated with poor prognostic factors of ulceration and increased tumour thickness. However, it was found to be independently and inversely associated with melanoma-specific death to 10 years (HR=0.25 [0.07-0.87] p=0.029). P-STAT5 allows to identify a subset of patients that have better melanoma outcomes despite having poor clinicopathological characteristics at diagnosis possibly through immune system activation. This finding could allow major improvements in stratification of the risk of progression.

Melanoma progression and metastasis via the vascular and lymphatic channels results in extremely poor prognosis. We evaluated the expression of biomarkers representing melanoma vascularisation and assessed their association with melanoma outcomes including SLN metastasis and melanoma death in a prospective cohort. The presence of Ki67+ nuclei in D2-40+ vessels was an independent predictor of SLN metastasis and melanoma death, which is a novel finding as a combination biomarker in a cohort of melanoma patients. Positive SOX18 was associated with lower SLN metastasis and better melanoma-specific survival.

Declaration by author

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No manuscripts submitted for publication.

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Contributions by others to the thesis

Professor Kiarash Khosrotehrani (principal advisor): Provided guidance and advice on overall study and editing of thesis.

Professor Adele Green (associate advisor): Provided guidance and advice on overall study.

Mr Clay Winterford: Provided assistance with development of staining protocols and staining of melanoma whole section slides.

Mr Jack Galbraith: Provided assistance with scanning of close to 2000 melanoma whole section slides to enable digital analysis of slides.

Ms Maryrose Malt: Provided assistance with data collection.

Statement of parts of the thesis submitted to qualify for the award of another degree

No works submitted towards another degree have been included in this thesis.

Research Involving Human or Animal Subjects

Ethics approval provided by the Metro South Health Service District Human Research Ethics Committee (HREC reference number: HREC/09/QPAH/217).

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melanoma, prognosis, survival, biomarker, immunohistochemistry, immunology, STAT5, lymphangiogenesis, Ki67, SOX18

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Abbreviations

ACD: adrenocortical dysplasia homologue

AJCC: American Joint Committee on Cancer

BAP1: BRCA1-associated protein-1

CDK4: cyclin-dependent kinase 4

CDKN2A: cyclin-dependent kinase inhibitor 2A

CI: confidence intervals

CISH: Chromogenic in-situ hybridization

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

EMT: epithelial-to-mesenchymal transition

FOXP3: forkhead box P3

HR: hazard ratio

IHC: immunohistochemistry

iNOS: inducible nitric oxide synthase

JAKs: janus kinases

LMM: lentigo maligna melanoma

MEF2C: myocyte enhancer factor 2C

MITF: microphthalmia-associated transcription factor

NK cells: natural killer cells

NM: nodular melanoma

OR: odds ratio

P-STAT5: phosphorylated signal transducer and activator of transcription 5

PD-1: programmed cell death protein 1

POT1: protection of telomeres 1

PUVA: psoralen-ultraviolet-A

REMARK: Reporting Recommendations for Tumour Marker Prognostic Studies

RR: relative risk

SCARB1/SR-BI: scavenger receptor class B type I

SD: standard deviation

SLN: sentinel lymph node

SLNB: sentinel lymph node biopsy

SOCS2: Suppressor of cytokine signalling 2

SOX: SRY-related HMG-box

SSM: superficial spreading melanoma

STAT5: signal transducer and activator of transcription 5

TERF2IP: telomeric repeat binding factor 2, interacting protein

TERT: telomerase reverse-transcriptase

TNM: tumour, node, metastasis

TRPC3: transient receptor protein channel 3

UVR: ultraviolet radiation

VCAM1: vascular cell adhesion molecule 1

VEGF-C: vascular endothelial growth factor-C

VEGF-D: vascular endothelial growth factor-D

VEGFR3: vascular endothelial growth factor receptor 3

Chapter 1:

Introduction and literature review

Melanoma epidemiology

Melanoma is the leading cause of skin cancer-related death primarily due to its ability and propensity to metastasize. The incidence of melanoma continues to rise globally[1], with an increase rate between 1.7-4.8% per year in the United States, United Kingdom, Canada, Sweden and Norway.[2] In Denmark however, melanoma incidence has stabilised since 2011, specifically in those younger than 40 years.[2] More locally, melanoma incidence in New Zealand has decreased 1.3% per year since 2008 whereas melanoma incidence in Australia has stabilised since 2002, especially in those who are younger than 60 years.[2] Despite this stabilisation and even decline in melanoma incidence in young Australians age between 15-24[3], it remains as the most common cancer in this age group, accounting for 15% of cancers diagnosed[4]. Melanoma survivors have an increased risk of developing a second cancer during their lifetime including melanomas.[5] It is projected that 62.5 per 100 000 males and 42.3 per 100 000 females will be diagnosed with melanoma in Australia, making up 15 229 new diagnosis of melanoma in 2019.[6]

The plateau and decline in melanoma incidence in Australia, Denmark and New Zealand could be a result from vigorous sun safety and early detection campaigns involving the entire community as well as implementing government policies such as tanning bed regulations.[7-9] Australia has been a global leader and pioneer in sun safety awareness. Public health campaigns since the 1980s such as ‘Slip Slop Slap’, ‘SunSmart’, and ‘National Skin Cancer Awareness Week’ have proven to be effective in educating the public regarding the link between ultraviolet radiation (UVR) exposure and skin cancer formation, instilling positive behavioural changes and encouraging early skin cancer detection and treatment.[10,11] This was reflected in the birth cohort pattern of invasive melanoma incidence between 1995-2014 where it was stabilising or decreasing in those under 60 years while increasing for those 60 years and above.[12]

Melanoma mortality was either stable or decreased in all age groups except men who are 60 years and above[12], and is projected to account for 1725 deaths in Australia in 2019.[6] Melanoma mortality and morbidity are typically less favourable in rural and regional Australia due to disparities in healthcare access and rural lifestyle, with rural patients having 20% higher mortality.[13] A way to distribute scarce

resources in regional and rural areas would be through better understanding of the risk of progression of melanoma patients. In this context, prognostic markers could be valuable to further stratify patients, identify those at high risk of progression and possibly prompt referral to tertiary centres for initiation of adjuvant therapy. Ideally this is a test that could be easily made available in all pathology laboratories including those in rural areas, and if successful this could potentially help close the mortality gap between rural and urban Australia.

Risk factors for melanoma

Melanoma is a multifactorial disease resulting from interplay between environmental factors and genetic predisposition.

Ultraviolet radiation

The general consensus is that exposure to UVR is the primary modifiable risk factor for melanoma, with different patterns of exposure contributing to different level of risk and distribution of melanoma.[14] In a meta-analysis looking at the association between sun exposure and melanoma, history of sunburn was found to have the highest risk (RR=2.03), followed by intermittent sun exposure (RR=1.61).[15] Chronic sun exposure is associated more with actinic keratosis and keratinocyte cancers, however is still associated with increased risk melanoma, as described in Whiteman et al's proposal on the divergent pathway model for melanoma development.[16] In their model, people with low melanocyte proliferation as characterised by low naevi counts require chronic sun exposure to induce melanoma and therefore typically have more solar damage and develop melanoma on sun-exposed sites. On the other hand, people with high naevi counts require sun exposure early on (eg. sunburn in childhood or youth) combined with host factors, and typically have less solar damage compared to the other group.[16] Artificial UV exposure such as tanning bed and psoralen-UVA (PUVA) therapy used for treating psoriasis also increase risk of melanoma.[17-18]

Phenotypic characteristics

In a meta-analysis of 60 observational studies by Gandini et al, phenotypic characteristics such as high naevi count (101-120 naevi vs <15, RR=6.89), high

atypical naevi count (RR=6.36), hair colour (red vs dark, RR=3.64), skin type (type I vs IV, RR=2.09), high freckle count (RR=2.10) and eye colour (blue vs dark, RR=1.47) were associated with increased risk of melanoma.[19-20] In a Queensland prospective cohort study, Olsen et al found self-reported naevus density at age 21 (many vs no moles, HR=4.91), inability to tan (no tan vs deep tan, HR=3.39), hair colour (red vs black, HR=3.11) was associated with higher melanoma risk.[21]

Family history and personal history

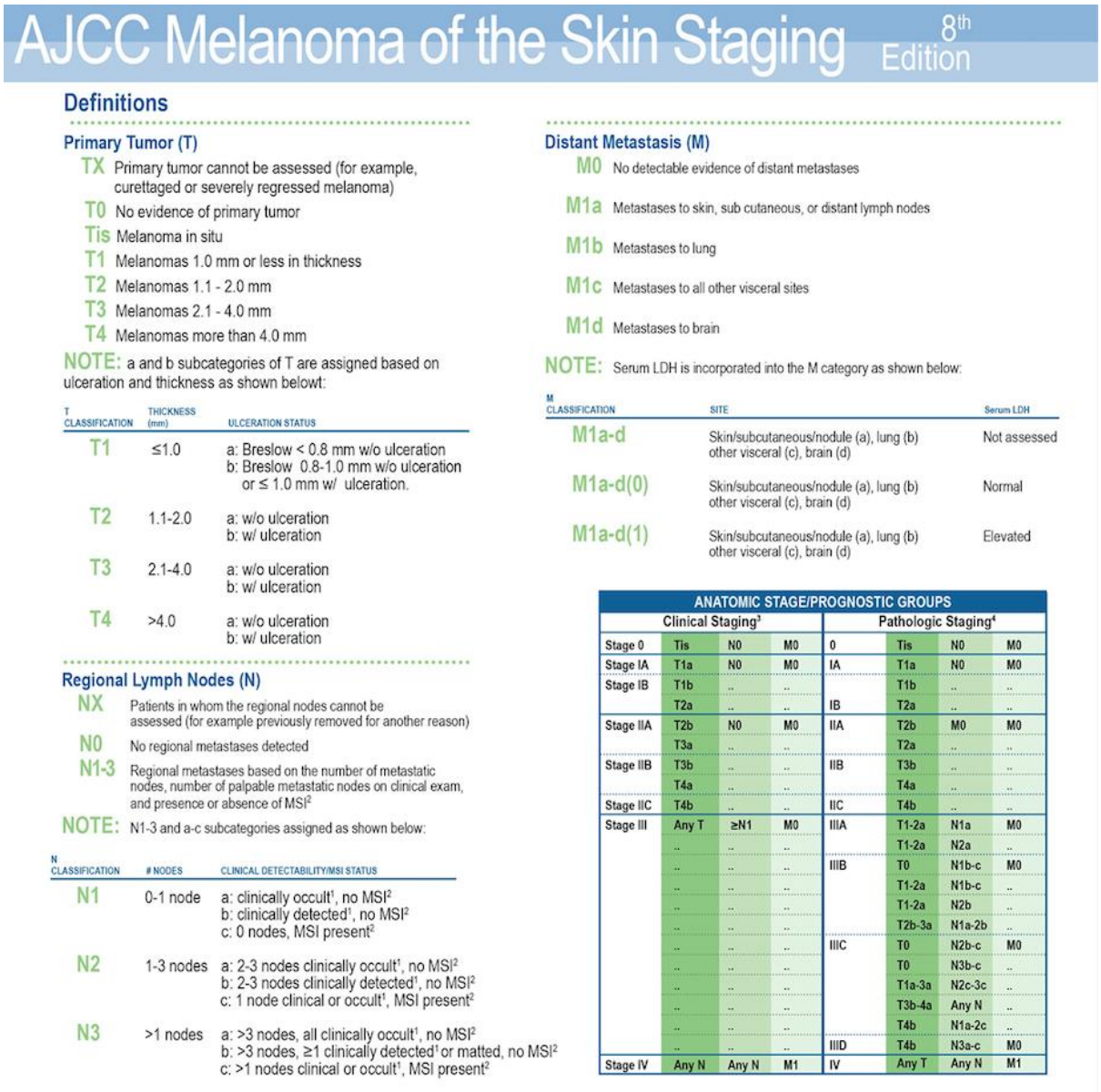
About 8-12% of melanoma patients have positive family history as defined by one or more first-degree relatives with melanoma, which contributes to higher relative risk of melanoma (RR=1.74).[19] About half of familial melanoma are caused by mutation in known high penetrance melanoma genes, the most common being mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A).[22] Other high penetrance genes include cyclin-dependent kinase 4 (CDK4), BRCA1-associated protein-1 (BAP1), microphthalmia-associated transcription factor (MITF), telomerase reverse-transcriptase (TERT), protection of telomeres 1 (POT1), adrenocortical dysplasia homologue (ACD) and telomeric repeat binding factor 2, interacting protein (TERF2IP).[23] Some of these mutations are also linked to other cancers, with associations found between CDKN2A (p16) and pancreatic cancer, CDKN2A (p14) and neural system tumours, MITF and renal cell carcinoma, POT1 and glioma.[23]

Similar to family history, having a personal history of melanoma also increases the risk of subsequent melanoma. Patients with in-situ or invasive melanoma are more likely to have subsequent invasive melanomas, with standardized incidence ratios of 4.59 and 5.42 respectively.[24] The subsequent melanoma was generally located at the same body site as initial melanoma.[24] Together, these have implications in terms of patient surveillance and follow up.

Melanoma prognosis

Upon diagnosis of melanoma, the American Joint Committee on Cancer (AJCC) melanoma staging system is the gold standard guideline used internationally to stage patients, with the latest 8th edition published in 2017.[25]

Figure 1: AJCC melanoma of the skin staging 8th edition[25]



AJCC Melanoma of the Skin Staging 8th Edition

Definitions

Primary Tumor (T)

- **TX** Primary tumor cannot be assessed (for example, curettaged or severely regressed melanoma)
- **T0** No evidence of primary tumor
- **Tis** Melanoma in situ
- **T1** Melanomas 1.0 mm or less in thickness
- **T2** Melanomas 1.1 - 2.0 mm
- **T3** Melanomas 2.1 - 4.0 mm
- **T4** Melanomas more than 4.0 mm

NOTE: a and b subcategories of T are assigned based on ulceration and thickness as shown belowt:

T CLASSIFICATION	THICKNESS (mm)	ULCERATION STATUS
T1	≤1.0	a: Breslow < 0.8 mm w/o ulceration b: Breslow 0.8-1.0 mm w/o ulceration or ≤ 1.0 mm w/ ulceration.
T2	1.1-2.0	a: w/o ulceration b: w/ ulceration
T3	2.1-4.0	a: w/o ulceration b: w/ ulceration
T4	>4.0	a: w/o ulceration b: w/ ulceration

Regional Lymph Nodes (N)

- **NX** Patients in whom the regional nodes cannot be assessed (for example previously removed for another reason)
- **N0** No regional metastases detected
- **N1-3** Regional metastases based on the number of metastatic nodes, number of palpable metastatic nodes on clinical exam, and presence or absence of MSI[2]

NOTE: N1-3 and a-c subcategories assigned as shown below:

N CLASSIFICATION	# NODES	CLINICAL DETECTABILITY/MSI STATUS
N1	0-1 node	a: clinically occult[1], no MSI[2] b: clinically detected[1], no MSI[2] c: 0 nodes, MSI present[2]
N2	1-3 nodes	a: 2-3 nodes clinically occult[1], no MSI[2] b: 2-3 nodes clinically detected[1], no MSI[2] c: 1 node clinical or occult[1], MSI present[2]
N3	>1 nodes	a: >3 nodes, all clinically occult[1], no MSI[2] b: >3 nodes, ≥1 clinically detected[1] or matted, no MSI[2] c: >1 nodes clinical or occult[1], MSI present[2]

Distant Metastasis (M)

- **M0** No detectable evidence of distant metastases
- **M1a** Metastases to skin, sub cutaneous, or distant lymph nodes
- **M1b** Metastases to lung
- **M1c** Metastases to all other visceral sites
- **M1d** Metastases to brain

NOTE: Serum LDH is incorporated into the M category as shown below:

M CLASSIFICATION	SITE	Serum LDH
M1a-d	Skin/subcutaneous/nodule (a), lung (b) other visceral (c), brain (d)	Not assessed
M1a-d(0)	Skin/subcutaneous/nodule (a), lung (b) other visceral (c), brain (d)	Normal
M1a-d(1)	Skin/subcutaneous/nodule (a), lung (b) other visceral (c), brain (d)	Elevated

ANATOMIC STAGE/PROGNOSTIC GROUPS							
	Clinical Staging[3]			Pathologic Staging[4]			
Stage 0	Tis	N0	M0	0	Tis	N0	M0
Stage IA	T1a	N0	M0	IA	T1a	N0	M0
Stage IB	T1b	..	..		T1b	..	..
	T2a	..	..	IB	T2a	..	..
Stage IIA	T2b	N0	M0	IIA	T2b	M0	M0
	T3a	..	..		T2a	..	..
Stage IIB	T3b	..	..	IIB	T3b	..	..
	T4a	..	..		T4a	..	..
Stage IIC	T4b	..	..	IIC	T4b	..	..
Stage III	Any T	≥N1	M0	IIIA	T1-2a	N1a	M0
	..	..	..		T1-2a	N2a	..
	..	..	..	IIIB	T0	N1b-c	M0
	..	..	..		T1-2a	N1b-c	..
	..	..	..		T1-2a	N2b	..
	..	..	..		T2b-3a	N1a-2b	..
	..	..	..	IIIC	T0	N2b-c	M0
	..	..	..		T0	N3b-c	..
	..	..	..		T1a-3a	N2c-3c	..
	..	..	..		T3b-4a	Any N	..
	..	..	..		T4b	N1a-2c	..
	..	..	..	IIID	T4b	N3a-c	M0
Stage IV	Any N	Any N	M1	IV	Any T	Any N	M1

Patient prognosis is projected based on clinicohistological features of the primary tumour such as Breslow thickness, ulceration status, lymph node involvement and presence of distant metastasis. Elevated lactate dehydrogenase in the serum associated with active metastatic disease is also used as a monitoring tool. One of the changes in the 8th edition was removing mitotic rate from the staging criterion for T1 melanoma tumours, as review of survival data has shown it was not independent of ulceration and tumour thickness as a prognostic factor, and that thin tumours with mitotic rate ≥1/mm2 (T1b) had better prognosis than thicker tumours without mitotic figures (T1a).[26] Although no longer used to stage thin melanomas, there was still a significant correlation between mitotic rate and mortality therefore it was

recommended to be included in the pathology report for all tumour regardless of thickness. This updated framework includes evidence-based changes from analyses carried out on a database of over 46,000 patients from worldwide centres, and in line with the evolving scene of Stage IV melanoma treatment. This enables more accurate risk stratification, provide better information for clinical trial design and analysis, an assist in selecting patients to undergo targeted therapy and immunotherapy.

However, there are limitations to the AJCC staging system, for example it does not take into account whether or not patients have had multiple melanomas when determining their disease stage. The current literature showed multiple melanomas led to poorer prognosis, especially when compared to those with single melanoma of the same stage or lower.[27] Furthermore, there are still significant heterogeneity in melanoma survival within each AJCC stage despite the addition of sentinel lymph node biopsy (SLNB), which was introduced to address this issue.

Morton et al from the John Wayne Cancer Centre first described SLNB in 1992 as part of the staging process for melanoma.[28] The sentinel lymph node is the first node draining lymph from the primary tumour and is where tumour cells are most likely to spread first. SLNB involves targeted surgical removal and histological examination of this node and has been widely adopted as a standard of care for staging the regional lymph nodes of patients with stage Ib and II primary cutaneous melanoma. SLNB has been controversial since its conception and its role and value have been debated. While it provides accurate and important staging information that is independent of Breslow thickness and ulceration[29-30], only a small proportion of high risk patients eventually undergo the procedure due logistical reasons and patient comorbidities. A Queensland study of high risk melanoma patients showed only 30% underwent SLNB.[31] Carrying out the procedure requires a facility with appropriate and experienced surgical, nuclear medicine and pathology teams. As patients typically require general anaesthesia for the procedure, this may not be preferable in patients that have surgical and or medical comorbidities.

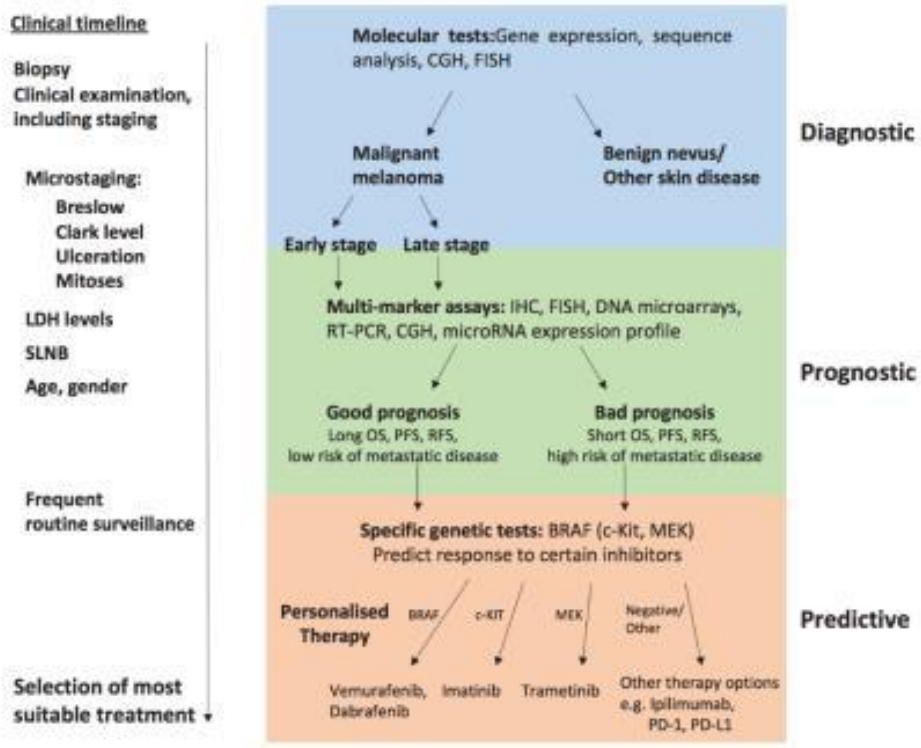
This highlights the need for a non- or less invasive test that could provide accurate information about melanoma prognosis, with less morbidity associated. The optimal

sub-staging approach for early stage melanoma remains unanswered. There is a need for prognostic tools beyond the current AJCC staging system to improve prognostication of early melanomas. The discovery of novel immune and checkpoint inhibitor therapies has led to a focus on the role of immune system and immune biomarkers in melanoma[32]. This coupled with a better understanding of tumour microenvironment and progression will assist in the discovery of further prognostic markers. Melanoma staging will undoubtedly continue to evolve as novel biomarkers are discovered and incorporated into clinical practice.

Prognostic biomarkers

Biomarkers are biological molecules found in body tissue or serum that reflect normal or abnormal processes, and may inform tumour behaviour, patient prognosis and response to treatment. These diagnostic, prognostic or predictive biomarkers (Figure 1) are obtained from the primary tumour sample or from a blood test at diagnosis, with no invasive tests needed.

Figure 2: Flow chart showing diagnostic, prognostic and predictive tests in melanoma management, Foth et al 2016[33]



The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) criteria is the gold standard for evaluating and reporting prognostic tumour biomarkers.[34] It provides a checklist of items (Figure 2) recommended to ensure the quality and consistency of these studies.

Figure 3: The REMARK checklist, Altman et al 2012[35]

INTRODUCTION	
1	State the marker examined, the study objectives, and any pre-specified hypotheses.
MATERIALS AND METHODS	
Patients	
2	Describe the characteristics (for example, disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.
3	Describe treatments received and how chosen (for example, randomized or rule-based).
Specimen characteristics	
4	Describe type of biological material used (including control samples) and methods of preservation and storage.
Assay methods	
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.
Study design	
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (for example, by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.
7	Precisely define all clinical endpoints examined.
8	List all candidate variables initially examined or considered for inclusion in models.
9	Give rationale for sample size: if the study was designed to detect a specified effect size, give the target power and effect size.
Statistical analysis methods	
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.
11	Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.
RESULTS	
Data	
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the number of patients and the number of events.
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.
Analysis and presentation	
14	Show the relation of the marker to standard prognostic variables.
15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (for example, hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.
16	For key multivariable analyses, report estimated effects (for example, hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.
17	Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.
18	If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.
DISCUSSION	
19	Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.
20	Discuss implications for future research and clinical value.

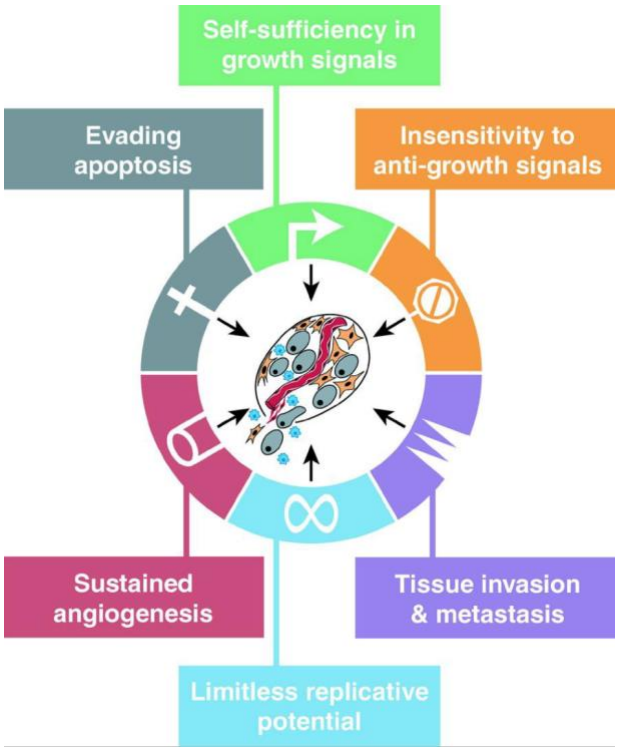
Rothberg et al conducted a meta-analysis of 102 published studies on immunohistochemistry (IHC) based prognostic protein biomarkers in melanoma and

found 37 studies with a total of 62 biomarkers collectively complied with the REMARK criteria.[36] Among the biomarkers that demonstrated significant association with melanoma-specific mortality were p16/INK4a representing insensitivity to antigrowth signals (HR=0.4), inducible nitric oxide synthase (iNOS) representing sustained angiogenesis (HR=4.63), metallothionein representing limitless replicative potential (HR=3.08), and matrix metalloproteinase-2 representing tissue invasion and metastasis (HR=2.60).

Melanomagenesis and tumour progression

Development of cancer including melanoma is a complex interplay involving multiple pathophysiological steps. Hanahan et al proposed six components that lead to malignancy – self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Figure 3).[37] Melanomagenesis and the its subsequent progression involves factors intrinsic to melanoma tumour cells as well as the interaction between tumour cells and the host microenvironment.

Figure 4: Acquired capabilities of cancer, Hanahan et al 2000[37]



In melanoma, malignant cells go through radial growth phase characterised by proliferation within the epidermis, then vertical growth phase whereby they invade through the basement membrane into the dermis and finally metastasise when they dissociate from the primary tumour and spread via vascular or lymphatic routes to distant sites.[38-39] Ki67 is a marker of cell proliferation (G1-S phase of cell cycle) and one of the most widely studied marker by immunohistochemistry to explore its involvement in the metastatic process. Previous studies have shown its prognostic value in thin and thick melanomas, where it was significantly associated with melanoma-specific mortality and all-cause mortality.[40,41,36] Beta3 integrin, an adhesion molecule, is associated with the shift from radial to vertical growth phase as well as metastatic melanoma, and was shown to have anti-metastatic effect in a murine model.[42] Of note this molecule is also expressed in Spitz naevi.

There have been cases of spontaneous melanoma regression and simultaneous onset of vitiligo indicating immunosurveillance activity in melanoma.[43] Furthermore, the development of immunotherapy that inhibits the PD-1 (programmed cell death protein 1) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) pathways indicate melanoma is an immunogenic tumour and the crucial role the immune system plays in the disease.[44,45] Realising this, more attention has been given to cells in the immune infiltrate in tumour microenvironment, specifically looking at their role as potential prognostic or predictive markers. By understanding the immune patterns of melanoma, we have the chance to discover immune markers with prognostic relevance, new therapeutic targets and predict response of patient to specific therapies.

The cutaneous immune system including T-cells, B-cells, natural killer (NK) tcells, neutrophils, macrophages, and dendritic cells perform homeostatic function and inflammatory responses, but also have shown to facilitate melanomagenesis and influence the evolution of tumours.[46,47] Pro-tumorigenic and immunosuppressive infiltrates from the tumour stroma include CD4+CD25+FOXP3+ regulatory T cells, myeloid-derived suppressor cells, M2 macrophages and N2 neutrophils, while NK cells, dendritic cells, and CD8+ cytotoxic T lymphocytes are some of the components recruited as anti-tumour defense.[47] The signal transducer and activator of

transcription 5 (STAT5) protein plays a role in T cell activation and proliferation, natural killer cell development and Treg induction.[48] It is a surrogate of cytokine receptor signalling and has been found to be associated with oncogenesis and tumour progression.[49]

Neovascularization and angiogenesis are crucial for tumour survival and metastasis. This is reflected in studies targeting antiangiogenic therapy in the field of cancer. CD31 (PECAM-1; platelet/endothelial cell adhesion molecule-1) plays a role in interaction between adjacent endothelial cells, leukocyte migration, and angiogenesis. Previous studies have shown prognostic significance of increased vascularity as determined by CD31 staining in intermediate-thickness and metastatic melanoma.[50-52] Another key regulator of endothelial cell differentiation, angiogenesis and lymphangiogenesis is the SOX group F transcription factor comprising SOX7, SOX17 and SOX18.[53] In adults SOX18 expression has been demonstrated in specific settings such as wound healing and tumour growth.[54]

Lymphovascular biomarkers are of interest as tumours can produce lymphangiogenic growth factors that promote formation of new lymphatic vessels, tumour cell adhesion and transmigration into vascular spaces which results in metastasis. Antibodies specific to lymphatic vessels such as D2-40, lymphangiogenetic markers such as vascular endothelial growth factor receptor 3 (VEGFR3), vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor-D (VEGF-D) together with proliferation marker (Ki67) have enabled detection of lymphovascular invasion, lymphatic vessel density and lymphangiogenesis. Pasquali et al conducted a systematic review of expression of lymphangiogenic markers in melanoma tumours.[55] They have similar findings to Rothberg et al[36] in terms of heterogeneous findings, wide methodologic variations and poor adherence to either the REMARK criteria or the First International Consensus on the Methodology of Lymphangiogenesis Quantification in Solid Human Tumours by Van Der Auwera et al[56]. Of note, the consensus by Van Der Auwera et al recommended double immunostaining with D2-40 and Ki67 monoclonal antibodies to better detect proliferating lymphatic vessel cells reflecting lymphangiogenesis, which has not been carried out in any studies in the existing literature. Lymphatic vessel density (LVD) especially in the peritumoral region and

lymphatic vessel invasion predicted sentinel lymph node metastasis and melanoma-specific mortality.[55] Intratumoral lymphovasular invasion (LVI) as detected by D2-40 was found to be an independent predictor of worse disease-free and overall survival.[55]

It is evident that we are still in need of novel prognostic markers or factors that can be translated into clinical application to improve melanoma patient stratification. Understanding tumour biology and its microenvironment has provided insight into melanoma progression and outcome. My study was designed to investigate potential clinical and prognostic markers in a cohort of melanoma patients.

Hypotheses

- Patients with multiple primary melanomas are at higher risk of sentinel lymph node invasion.
- P-STAT5 expression reflects tumour immune function and correlates with better melanoma survival.
- Melanoma vascularisation can predict patient outcome.

Aims

- To investigate effects of a previous diagnosis of invasive melanoma on sentinel node metastasis of a subsequent invasive melanoma.
- To assess activation of cytokine receptor signalling through activity of P-STAT5 and evaluate its association with melanoma survival and recurrence.
- To assess prognostic value of lymphovascular biomarkers in terms of SLN metastasis and melanoma-specific survival.

Chapter 2:

The pattern of progression of multiple invasive melanomas in a Queensland cohort

Introduction

Patients with a diagnosis of melanoma are at increased likelihood of having subsequent melanomas in their lifetime, both in-situ or invasive. This risk remains elevated for up to 20 years after diagnosis of the first melanoma.[5,57-60] In Australia, patients diagnosed with their first invasive melanoma have a 6- to 7-fold higher risk of a subsequent invasive melanoma compared to the general population.[5,24] This has become increasingly important as there are growing numbers of melanoma survivors in part due to earlier detection and improved treatment.[61]

The presence of an additional in-situ melanoma in patients with primary invasive melanoma, whether preceding, synchronous or subsequent, has been shown not to affect survival.[58] There have been conflicting findings with regards to survival in patients with multiple primary invasive melanomas, with previous studies showing it led to poorer, better or no change in prognosis versus single primary invasive melanomas.[27,62-66] Using delayed entry or time varying entry analysis, we and others have shown that once the survival bias is taken into account patients with multiple melanomas had poorer survival.[27] Of importance, this was only true for multiple melanomas of the same stage as compared to a single melanoma of the same stage.[27]

The mechanism by which an additional invasive melanoma affects survival remains unclear. In the present study, we looked at survival of patients with clinical stage Ib and II melanoma who have undergone sentinel lymph node (SLN) biopsy and asked if patients with multiple primary melanomas were at higher risk of SLN invasion and had a peculiar pattern of progression.

Methods

787 patients with primary invasive cutaneous melanoma who were referred to the Princess Alexandra Hospital melanoma clinic between 1994 and 2011 were considered for inclusion in this study. The eligibility criteria include being at least 18 years of age and able to provide consent, a diagnosis of clinical stage Ib and II melanoma and proceeded to have a SLN biopsy. We excluded patients where no

follow up data was available on the Queensland Cancer Registry as they were diagnosed in neighbouring states (n=82), patients under 18 years of age at time of diagnosis (n=10), patients with missing details or incompatible coding for tumour characteristics (n=44). 651 patients were included in the study cohort after written consent according to ethics protocol (HREC/09/QPAH/217) of Metro South Human Research Ethics Committee.

Study on a large population-based cohort in Queensland by Youlden et al[58] found that in patients with invasive melanoma, having a preceding, synchronous or subsequent in-situ melanoma did not contribute to significant differences in 10-year cause-specific mortality. Therefore, we chose to limit our study to only those with multiple invasive melanomas.

Patient demographics and clinicohistological characteristics of the index melanoma including sex, age at diagnosis, Breslow thickness, ulceration, histological subtype, tumour site, SLN status, recurrence and date of diagnosis were prospectively collected during visits at the Princess Alexandra Hospital melanoma unit clinic. Index melanoma was defined as the melanoma that led to the SLN biopsy, therefore excluding any melanomas diagnosed prior to or subsequent to this. Data on presence of additional melanomas before or after the index melanoma and their staging and update on survival status and date of death (when applicable) up to 31 December 2014 were obtained from the Queensland Cancer Registry. These were the latest validated data available for release from the Queensland Cancer Registry at the time of request.

Cohort characteristics and clinicohistological features of index tumour were analysed for association with additional invasive melanomas and SLN invasion using t-test and chi-squared test. Logistical regression model was used to determine predictors of SLN invasion. The Cox proportional hazards model was used to determine which demographic and tumour characteristics influenced the melanoma-specific mortality of patients. Survival interval was calculated from the time of diagnosis of the melanoma considered for the sentinel node biopsy to date of melanoma death, date of non-melanoma death, or date of last follow up (31 December 2014), whichever occurred first. We also included additional invasive melanoma prior to index

melanoma, additional stage I and stage II melanoma prior to index melanoma as covariates. All data were analysed using IBM SPSS Statistics (Version 25).

Results

Cohort characteristics

The characteristics of our cohort (n=651) at diagnosis of index melanoma are presented in Table 1. The cohort was stratified into those with single invasive melanoma (n=566, 86.9%), additional invasive melanoma (n=85, 13.1%), and within this, those with additional stage I melanoma (n=79, 12.1%) or additional stage II melanoma (n=11, 1.7%), with some overlap between the two groups. For those with multiple melanomas, only the information of index melanoma (melanoma that warranted the SLN biopsy) are listed in Table 1.

Out of 651 patients, 361 (55.5%) were males and 290 (44.5%) females. With an average age at diagnosis of 49 years, our study cohort was younger when compared to melanoma patients in the general Queensland population, as all of them had elected to undergo SLN biopsy. The average Breslow thickness of the study cohort was 2.3mm. 170 (26.1%) patients had tumour ulceration, and the main tumour subtype was superficial spreading melanoma (60.3%). The highest distribution of tumour site was the trunk (35.2%), followed by lower limb (29.6%), upper limb (28.6%) and head and neck (6.6%). 92 (14.1%) patients had positive SLN. 85 (13.1%) patients had an additional invasive melanoma, of which 36 (5.5%) were diagnosed prior to the index melanoma. 79 (12.1%) patients had an additional stage I melanoma, with 31 diagnosed prior to the index melanoma, and 11 (1.7%) patients had an additional stage II melanoma, of which 5 were diagnosed prior to the index melanoma (Table 1).

Table 1: Clinicohistological characteristics of index melanoma of the entire cohort and those with single versus multiple invasive melanomas

		Multiple invasive melanomas

	Total cohort (n=651) n (%)	Single invasive melanoma (n = 566) n (%)	Additional invasive melanoma (n = 85) n (%)	Additional stage I melanoma (n = 79) n (%)	Additional stage II melanoma (n = 11) n (%)	p-value
Sex						
Male	361 (55.5)	308 (54.4)	53 (62.4)	49 (62)	8 (72.7)	0.245
Female	290 (44.5)	258 (45.6)	32 (37.6)	30 (38)	3 (27.3)	
Age at diagnosis						
Mean ± SD	49 ± 14	48 ± 14	54 ± 14	53 ± 14	59 ± 14	0.022
Breslow thickness						
Mean (mm) ± SD	2.3 ± 1.6	2.3 ± 1.6	2.5 ± 1.6	2.3 ± 1.1	3.6 ± 3.3	0.227
Ulceration						
Yes	170 (26.1)	147 (26)	23 (27.1)	22 (27.8)	3 (27.3)	0.930
No	481 (73.9)	419 (74)	62 (72.9)	57 (72.2)	8 (72.7)	
Tumour site						
Head & neck	43 (6.6)	34 (6)	9 (10.6)	5 (6.3)	4 (36.4)	0.001
Trunk	229 (35.2)	202 (35.7)	27 (31.8)	27 (34.2)	2 (18.2)	
Upper limb	186 (28.6)	161 (28.4)	25 (29.4)	24 (30.4)	3 (27.3)	
Lower limb	193 (29.6)	169 (29.9)	24 (28.2)	23 (29.1)	2 (18.2)	
Histology						
SSM	348 (60.3)	301 (59.6)	47 (65.3)	45 (65.2)	6 (75)	0.003

NM	193 (33.4)	175 (34.7)	18 (25)	18 (26.1)	0	
LMM	6 (1)	4 (0.8)	2 (2.8)	1 (1.4)	1 (12.5)	
Desmoplastic	30 (5.2)	25 (5)	5 (6.9)	5 (7.2)	1 (12.5)	
SLN status						
Positive	92 (14.1)	83 (14.7)	9 (10.6)	6 (7.6)	4 (36.4)	0.033
Negative	559 (85.9)	483 (85.3)	76 (89.4)	73 (92.4)	7 (63.6)	
Melanoma death						
Yes	87 (13.4)	75 (13.3)	12 (14.1)	10 (12.7)	3 (27.3)	0.172
No	564 (86.6)	491 (86.7)	73 (85.9)	69 (87.3)	8 (72.7)	
Additional melanoma						
Before index melanoma	-	-	36 (42.4)	31 (39.2)	5 (45.5)	-
After index melanoma	-	-	49 (57.6)	48 (60.8)	6 (54.5)	

*For patients with multiple invasive melanomas, only the information of index melanoma (melanoma that warranted the SLN biopsy) are listed

Abbreviations: SD, standard deviation; SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; SLN, sentinel lymph node.

Association between clinicohistological features with additional melanoma diagnosis

Patients with multiple invasive melanomas, especially additional stage II melanoma were older at time of diagnosis and had a higher rate of tumour on head and neck sites (Table 1). Age at diagnosis (p=0.022), head and neck tumour site (p=0.001), and positive SLN status (p=0.033) were significantly associated with additional stage II melanoma (Table 1).

Association with SLN metastasis

A key benefit in assessing the sentinel node is the ability to gain understanding of the origin of metastatic deposits. We therefore looked at association of multiple invasive melanomas with SLN metastasis, with particular interest on those with a prior history of invasive melanoma. We found significant association between positive SLN and high Breslow thickness (3.0mm vs 2.2mm, p=0.001), ulceration (47.8% vs 22.5%, p<0.001), tumours on the trunk (45.7% vs 33.5%, p=0.016) and presence of additional stage II melanoma (4.3% vs 1.3%, p=0.033). There was a trend in the association with additional stage I melanoma (p=0.075), additional stage II melanoma before index melanoma (p=0.096) and nodular melanoma subtype (p=0.098) (Table 2).

When performing multivariate analysis using two sets of covariates listed in table 3, Breslow thickness, ulceration and additional stage II melanoma (OR=4.70 [1.12-19.68] p=0.034) remained significant as independent predictors of positive SLN. Additional stage II melanoma before index melanoma continued to show a strong trend for predicting positive SLN (OR=8.18 [0.47-143.19] p=0.150).

Table 2: The associations between clinicohistological features and sentinel lymph node metastasis

	SLN		
	Positive (n=92) n (%)	**Negative (n=559) n (%)**	**p-value**
Sex			
Male	50 (54.3)	311 (55.6)	0.818
Female	42 (45.7)	248 (44.4)	
Age at diagnosis			
Mean ± SD	48 ± 14	49 ± 14	0.481
Breslow thickness			
Mean (mm) ± SD	3.0 ±2.1	2.2 ±1.4	0.001
Ulceration			

Yes	44 (47.8)	126 (22.5)	<0.001
No	48 (52.2)	433 (77.5)	
Tumour site			
Head & neck	6 (6.5)	37 (6.6)	0.016
Trunk	42 (45.7)	187 (33.5)	
Upper limb	14 (15.2)	172 (30.8)	
Lower limb	30 (32.6)	163 (29.2)	
Histology			
SSM	50 (55.6)	298 (61.2)	0.098
NM	38 (42.2)	155 (31.8)	
LMM	1 (1.1)	5 (1)	
Desmoplastic	1 (1.1)	29 (6)	
Additional invasive melanoma			
Yes	9 (9.8)	76 (13.6)	0.314
No	83 (90.2)	483 (86.4)	
Additional invasive melanoma before index melanoma			
Yes	5 (5.4)	31 (5.5)	0.966
No	87 (94.6)	528 (94.5)	
Additional stage I melanoma			
Yes	6 (6.5)	73 (13.1)	0.075
No	86 (93.5)	486 (86.9)	
Additional stage II melanoma			
Yes	4 (4.3)	7 (1.3)	0.033
No	88 (95.7)	552 (98.7)	
Additional stage II melanoma before index melanoma			
Yes	2 (2.2)	3 (0.5)	0.096
No	90 (97.8)	556 (99.5)	

Table 3: Odds ratios and Confident Intervals for SLN metastasis

	Univariate analysis			Multivariate analysis			Multivariate analysis		
	OR	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Sex (Male)	0.95	0.61-1.48	0.818	0.89	0.56-1.41	0.616			
Age	0.99	0.98-1.01	0.480	0.99	0.98-1.01	0.276	0.99	0.97-1.01	0.214
Breslow thickness	1.27	1.13-1.43	<0.001	1.21	1.06-1.37	0.004	1.31	1.13-1.52	<0.001
Ulceration	3.15	2.00-4.96	<0.001	2.85	1.78-4.57	<0.001	2.33	1.43-3.79	0.001
Site (Trunk)	1.39	0.55-3.49	0.490						
Histology (NM)	1.46	0.92-2.33	0.109				1.01	0.61-1.67	0.983
Additional invasive melanoma	0.69	0.33-1.43	0.317						
Additional invasive melanoma before index	0.98	0.37-2.59	0.966						
Additional stage I melanoma	0.46	0.20-1.10	0.082	0.41	0.16-1.04	0.059			
Additional stage II melanoma	3.58	1.03-12.50	0.045	4.70	1.12-19.68	0.034			
Additional stage II melanoma before index melanoma	4.12	0.68-24.99	0.124				8.18	0.47-143.19	0.150

Discussion

Cutaneous melanoma is a deadly malignancy that occurs worldwide, with Queensland having the highest incidence rate globally.[67] Patients with a cancer diagnosis have a higher likelihood of developing subsequent cancers. Once a person has been diagnosed with a primary melanoma, regardless of in-situ or invasive, they are at a higher risk of developing further melanomas, with an estimated lifetime risk of up to 8.6%.[64,68-71] This could be attributed to factors including genetic predisposition, aging, impaired immunologic function, environmental and lifestyle factors, improved surveillance and follow up, and exposure to iatrogenic risks such as radiotherapy.[59-61,69]

There have been many studies looking at patients with multiple primary melanomas, with mixed reports with regards to their outcomes. Diagnosis of an additional invasive melanoma has been shown by Youlden et al to increase mortality risk, and the risk differs depending on the combination of stages of the melanomas in individual patients.[27] In their study, patients with two stage I melanomas or two stage II melanomas had significantly poorer outcome than those with single melanoma of the equivalent stage. They did not see any significant difference in survival for patients with a combination of one stage I and one stage II melanomas versus patients with a single stage II melanoma. Similarly, when we confined our cohort to those with only stage II index melanoma, additional stage I melanoma was not a significant predictor of melanoma specific survival.

In our study, those with additional invasive melanoma were more likely males and had poorer prognostic features such as older at diagnosis, higher propensity for head and neck sites which is consistent with previous studies.[59-60,72] The association between increased age at diagnosis and additional invasive melanoma is not surprising as it suggests increased cumulative UV damage, whereas the association with head and neck sites could be due to propensity for head and neck sites to undergo field UV damage leading to formation of more invasive melanomas.

Previously, the study on multiple primary melanomas have been largely looking at the natural history, clinicohistologic characteristics, risk factors and survival outcome, with no focus on SLN metastasis. We therefore explored further the relationship between multiple invasive melanomas and SLN metastasis. There were significant associations between increased Breslow thickness, ulceration and trunk tumour site with SLN metastasis. We found additional stage II melanoma to be a significant predictor of SLN metastasis, while additional stage II melanoma before index melanoma had an increased odds ratio of 8.18 but did not reach significance (p=0.150), probably due to a lack of power. One major limitation in our study is the small number of patients especially in the category of those with additional stage II melanoma (n=11) and additional stage II melanoma before index melanoma (n=5). Further studies with larger cohorts to validate our findings would be beneficial.

SLN metastasis is one of the most important prognostic factors in melanoma. Our findings are valuable in guiding future staging process, potentially in terms of more aggressive SLN surveillance in those with multiple invasive melanomas especially multiple stage II melanomas. This also highlights the need for more accurate prognostic biomarkers that can be used in conjunction or instead of SLN biopsy in this patient population and the general melanoma population.

The effect of an additional invasive melanoma on melanoma-specific mortality was also examined (supplementary results). Additional stage I melanoma had better outcome, whereas additional stage II and stage II melanoma prior to index melanoma had poorer outcome as expected, however these did not reach significance.

The risk of having multiple melanomas not only affect individual patients but is also of public health interest in Australia and around the world where there is combination of an ageing population and patients diagnosed with melanoma surviving longer. Our study validated that having additional stage II melanoma, with higher Breslow thickness and ulceration had worse outcomes for patients. Having multiple invasive melanomas should be considered as increased risk and factored into individual patient prognosis. Better understanding of the risks and pattern of progression of subsequent melanomas and a comprehensive surveillance is essential to detect any subsequent

melanoma at an early stage. Patient education, especially targeting the cancer survivor population is crucial to ensure positive and proactive attitudes towards follow up care.

Supplementary results

Survival analysis

Upon follow up for an average of 8 years, 87 (13.4%) patients died from melanoma. They were mostly males (66.7%, p=0.024) with higher Breslow thickness (3.1mm vs 2.2mm, p<0.001), ulceration (48.3% vs 22.7%, p<0.001), higher affinity for head and neck tumour sites (13.8% vs 5.5%, p=0.001) and higher rate of positive SLN (37.9% vs 10.5%, p<0.001) (supplementary table 1).

A summary of the Hazard Ratios (HR) and Confidence Intervals (CI) for all the covariates in melanoma-specific survival using cox regression analysis are shown in supplementary table 2. Sex, Breslow thickness, ulceration, SLN status, head and neck tumour sites were significant predictors of melanoma death on univariate analysis. The presence of additional stage II and additional stage II melanoma before index melanoma resulted in increased hazard ratios but did not reach significance (HR=2.60 [0.82-8.22] p=0.104, HR=2.69 [0.37-19.31] p=0.326). We did not observe increased hazard in patients with additional stage I melanoma (HR=0.87 [0.45-1.69] p=0.685).

On multivariate analysis using two sets of covariates listed in supplementary table 2, Breslow thickness, ulceration and sex remained as independent predictors of melanoma death, while additional stage II melanoma and additional stage II melanoma before index melanoma showed increased hazards but again did not reach significance (HR=2.80 [0.47-16.60] p=0.257, HR=1.68 [0.17-16.71] p=0.659).

Supplementary table 1: The associations between clinicohistological features and melanoma death

	Melanoma death		
	Yes (n=87) n (%)	**No (n=564) n (%)**	**p-value**
Sex			
Male	58 (66.7)	303 (53.7)	0.024
Female	29 (33.3)	261 (46.3)	

Age at diagnosis			
Mean ± SD	50 ±13	49 ± 14	0.538
Breslow thickness			
Mean (mm) ± SD	3.1 ± 2.0	2.2 ± 1.5	<0.001
Ulceration			
Yes	42 (48.3)	128 (22.7)	<0.001
No	45 (51.7)	436 (77.3)	
Tumour site			
Head & neck	12 (13.8)	31 (5.5)	0.001
Trunk	39 (44.8)	190 (33.7)	
Upper limb	13 (14.9)	173 (30.7)	
Lower limb	23 (26.4)	170 (30.1)	
Histology			
SSM	49 (58.3)	299 (60.6)	0.134
NM	34 (40.5)	159 (32.3)	
LMM	0	6 (1.2)	
Desmoplastic	1 (1.2)	29 (5.9)	
SLN status			
Positive	33 (37.9)	59 (10.5)	<0.001
Negative	54 (62.1)	505 (89.5)	
Additional invasive melanoma			
Yes	12 (13.8)	73 (12.9)	0.827
No	75 (86.2)	491 (87.1)	
Additional invasive melanoma before index melanoma			
Yes	4 (4.6)	32 (5.7)	0.683
No	83 (95.4)	532 (94.3)	
Additional stage I melanoma			
Yes	10 (11.5)	69 (12.2)	0.844
No	77 (88.5)	495 (87.8)	
Additional stage II melanoma			
Yes	3 (3.4)	8 (1.4)	0.172
No	84 (96.6)	556 (98.6)	

Additional stage II melanoma before index melanoma			
Yes	1 (1.1)	4 (0.7)	0.662
No	86 (98.9)	560 (99.3)	

Supplementary table 2: Hazard ratios and Confident Intervals for melanoma-specific survival

	Univariate analysis			**Multivariate analysis**			**Multivariate analysis**		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Sex (Male)	1.70	1.09-2.66	0.019	1.52	0.91-2.53	0.107	1.71	1.05-2.77	0.031
Age	1.01	0.99-1.03	0.242	1.00	0.98-1.02	0.891	1.00	0.99-1.02	0.754
Breslow thickness	1.23	1.14-1.33	<0.001	1.35	1.16-1.57	<0.001			
Ulceration	2.93	1.93-4.47	<0.001	2.53	1.53-4.19	<0.001	3.18	1.99-5.07	<0.001
SLN status	4.51	2.92-6.96	<0.001						
Site (Head and neck)	2.68	1.34-5.40	0.006						
Histology (NM)	1.31	0.81-2.10	0.275	0.82	0.48-1.40	0.463			
Additional invasive melanoma	1.03	0.56-1.90	0.915						
Additional invasive melanoma before index	0.91	0.34-2.49	0.859						
Additional stage I melanoma	0.87	0.45-1.69	0.685						
Additional stage II melanoma	2.60	0.82-8.22	0.104	2.80	0.47-16.60	0.257			

Additional stage II melanoma before index melanoma	2.69	0.37-19.31	0.326			1.68	0.17-16.71	0.659

Chapter 3:

Activation of cytokine signalling through STAT5 is associated with survival in patients with thick or ulcerated locally invasive melanomas

Introduction

Melanoma is the fifth most commonly diagnosed cancer in the United States, with an estimated 91,270 new cases in 2018.[73] Melanoma in its early stage can be cured by surgical resection, with survival rate of 95% at 10 years for stage I melanomas.[26] However, mortality increases dramatically in people with thicker locally invasive melanomas despite the initial complete post-surgical remission at diagnosis.[26]

Using the current American Joint Committee on Cancer (AJCC, 8th edition) melanoma staging system, the prognosis of patients with locally invasive melanoma depends on tumour thickness and presence of ulceration.[26] While this enables classification of locally invasive melanomas into stages with broad prognostic prospects, it lacks the ability to inform individual patient prognosis and personalize therapy. Indeed, within each TNM (tumour, node metastasis) tumour subcategory, a significant proportion of patients will progress to metastasis and death from melanoma without being identified in advance. It is important to differentiate patients with higher risk of progression immediately upon diagnosis to adjust management.

In recent years, the essential role of the immune system in controlling melanoma progression and survival has been revealed resulting in the use and efficacy of immune checkpoint inhibitors.[74-78] Furthermore, past studies have highlighted the importance of the immune infiltrate at diagnosis in predicting patient outcome.[79] For many years, cytokine production has been considered a hallmark of immune cell activity culminating in the use of interferon alpha in the treatment of melanoma.[80-81] In this study, we reasoned that in primary melanoma tumours, an active immune system would result in cytokine production such as interferon alpha and gamma. Following stimulation, cytokines receptors activate JAKs (janus kinases) that phosphorylate latent STAT proteins in the cytoplasm to translocate into the nucleus and regulate transcription of target genes.[82] In the family of STAT proteins, STAT1, STAT3 and STAT5a & 5b are found to be associated with oncogenesis and/or tumour progression.[49] We therefore analysed the activation of cytokine receptor signalling through the activity of STAT5[82-83], its downstream effector, and evaluated association of its phosphorylated form (P-STAT5) with melanoma recurrence and survival in a cohort of patients with locally invasive melanomas.

STAT5a and 5b (located on human chromosome 17q11.2) play a major role in regulating cell differentiation and growth including peripheral T cell proliferation and cell cycle progression, NK cell development, and the maintenance of haematopoietic stem cell population.[48] Its role in immune function such as T cell activation and Treg induction is demonstrated through its involvement in transduction of cytokine receptor signalling of γc-containing cytokines (IL-2, IL-4, IL-7, IL- 9, IL-15, IL-21). STAT signalling impairment has been observed in both peripheral T cells from melanoma patients and T cells at tumour sites, and this observation increases in frequency in those with more advanced or metastatic disease.[83]

Materials and Methods

Ethics statement

Ethical approval was obtained through the Metro South Health Human Research Ethics Committee, Brisbane, Australia to carry out this project.

Study population

Our study was carried out and evaluated according to the REMARK criteria.[35] All adult patients with locally invasive cutaneous melanoma who subsequently underwent SLN biopsy at the Princess Alexandra Hospital, Brisbane, between 1994 and 2007 were considered for inclusion in our study. Eligibility criteria include being at least 18 years of age and able to provide consent, a diagnosis of clinical stage Ib and II melanoma, proceeded to have a SLN biopsy, accessible melanoma tissue in the form of whole-section slides or formalin-fixed, paraffin embedded tissue blocks. 189 patients were included in the study. Each haematoxylin and eosin-stained melanoma tissue slide was reviewed by a pathologist to confirm the diagnosis.

Clinicopathological data including age, sex, date of diagnosis, site, tumour thickness, ulceration were collected up to 2007, and follow-up data on recurrence and survival were collected up to 31 December 2014. These were the latest validated data available for release from the Queensland Cancer Registry at the time of request. There was an exception of 17 patients who were followed up until February 2013 as their follow-up data

beyond this date were not available. Additional data on deaths to 2014 were collected through the National Death Index.[84]

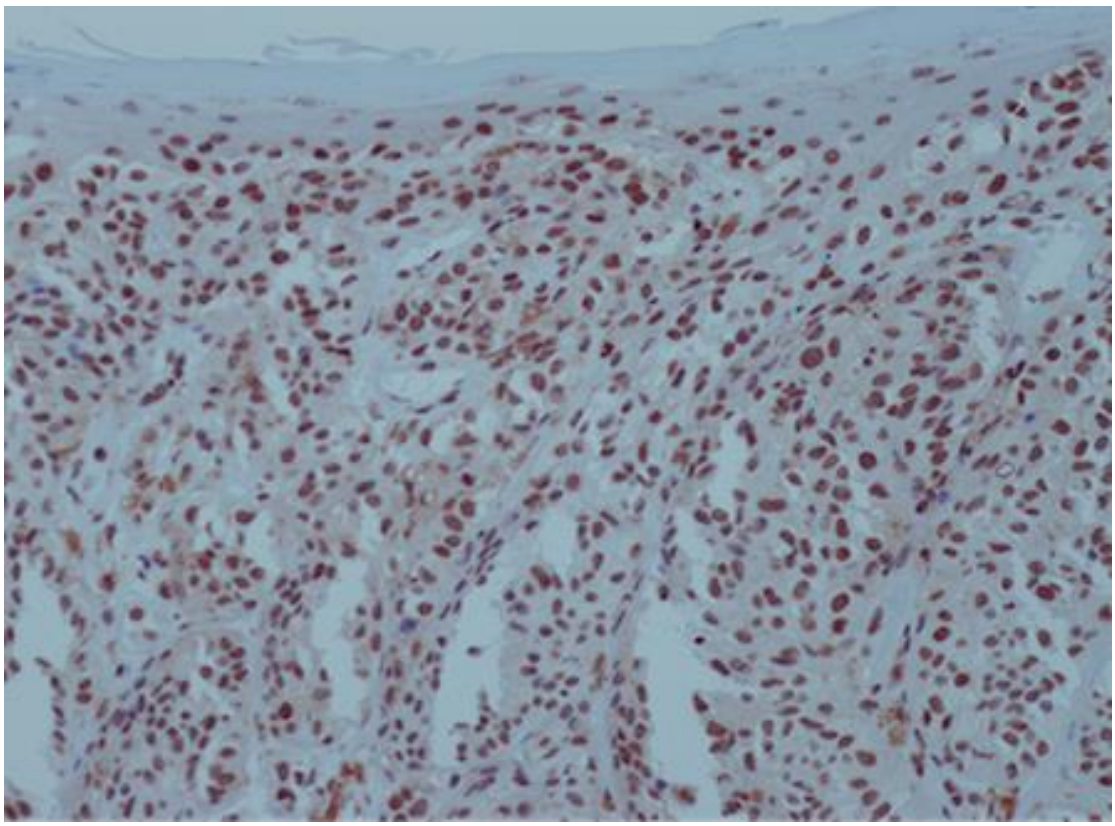
Immunohistochemical staining

Primary melanoma tissues were stained with the primary monoclonal antibody against STAT5 phosphorylated at Tyrosine residue 694 (Abcam ab32364, purified rabbit monoclonal anti-P-STAT5 (clone: E208) 220µg/ml, dilution 1:150). In addition to P-STAT5 we also used three previously validated biomarkers: Ki67, p16 and CD163[35,85]. Ki67, p16 and CD163 were chosen because they have been previously reported in REMARK compliant studies and validated in this same cohort[35]. Each antibody was matched with respective control samples to ensure staining quality and to determine positive and negative staining. Full details of the staining protocol are provided in the supplementary materials and methods section.

Evaluation of staining

P-STAT5 counts were performed across multiple high-power fields over each tumour section looking at nuclear uptake in melanoma cells (Fig. 1). Manual scoring was performed to ensure accuracy due to the state of some of our older melanoma tissue samples which did not stain particularly well. The percentage of positive cells on the section was recorded. Intensity of staining was also recorded for each tumour (0: none, 1: weak, 2: moderate, 3: strong). A staining index was calculated (staining index = intensity score x positive cell percentage) for each lesion and the mean (31) was used as a cut-off for analysis separating patients between two categories: High P-STAT5 level versus others. Evaluation was performed by two dermatopathologists. Differing scores were resolved by consensus during subsequent evaluation.

Figure 1: Photomicrograph depicts immunostaining of a melanoma section with anti-P-STAT5 antibody (red). As can be seen, P-STAT5 staining is abundant in this sample at high density and high intensity with characteristic nuclear staining reflecting the nuclear translocation of the STAT5 protein from the cytoplasm upon phosphorylation



Statistical analysis

The P-STAT5 level was tested for association with standard prognostic factors such as SLN status and ulceration using chi-square analysis, and Breslow thickness as a continuous variable using t-test.

Melanoma-specific survival was calculated from the date of histological diagnosis to date of melanoma death or last follow-up. Disease-free survival was determined by time between date of histological diagnosis and date of melanoma recurrence or last follow-up.

Using IBM SPSS statistics software version 25, Cox proportional hazards regression models were performed adjusting for age at diagnosis, sex, ulceration and Breslow thickness to determine the effect of clinicopathological factors and potential biomarkers on survival. Sentinel lymph node status was available for all patients but was not included in some models to account for scenarios where patients do not undergo SLN biopsy[31]. The Kaplan-Meier method was also used to analyse survival.

Results

Patient Characteristics

189 patients with primary invasive melanoma diagnosed between 1994 and 2007 who were eligible and underwent SLN biopsy were included in the analysis. The study cohort was younger compared to melanoma patients in the general Queensland population as all patients elected to undergo SLN biopsy as previously detailed[85]. However, there was no difference in terms of sex distribution and general tumour characteristics compared to the Queensland melanoma population. The cohort was composed of 55% male patients, with a mean age at diagnosis of 51 years and mean tumour thickness of 2.5mm (Table 1). 49 (25.9%) patients had ulceration. The most common site of melanoma was the trunk (38.1%) followed by lower limb (30.7%), upper limb (23.8%), head and neck (7.4%), while the most common melanoma subtype was superficial spreading melanoma (59.0%). SLN biopsy was performed in all cases and was positive in 33 (17.5%) patients. The average follow-up duration was 10 years. The median times to recurrence and death were 5.6 $\pm$ 2.9 years and 8.0 $\pm$ 3.8 years respectively. 52 (27.5%) patients had a recurrence and 41 (21.7%) died from melanoma during the follow-up period.

Table 1: Cohort Characteristics

	Total (n=189) n (%)
Sex	
Male	104 (55.0)
Female	85 (45.0)
Age at diagnosis	
0 – 40	37 (19.6)
41 – 60	104 (55.0)
61 – 80	48 (25.4)
mean ± SD	51.1 ± 13.6
Breslow thickness	
0 – 1.0 mm	14 (7.4)
1.0 – 2.0 mm	97 (51.3)
2.0 – 4.0 mm	53 (28.0)

>4.0 mm	25 (13.2)
Mean (mm) ± SD	2.5 ± 1.95
Ulceration	
Yes	49 (25.9)
No	140 (74.1)
Site of primary melanoma	
Head and Neck	14 (7.4)
Upper Limb	45 (23.8)
Lower Limb	58 (30.7)
Trunk	72 (38.1)
Histology	
SSM	111 (59.0)
NM	54 (28.7)
LMM	5 (2.7)
Desmoplastic	8 (4.3)
Other	10 (5.3)
SLN status	
Positive	33 (17.5)
Negative	156 (82.5)
Biomarker positivity#	
P-STAT5	28 (15.2)
Ki67	28 (15.2)
p16	90 (48.1)
CD163	73 (40.6)
Recurrence	
Yes	52 (27.5)
No	137 (72.5)
Melanoma death	
Yes	41 (21.7)
No	148 (78.3)

#16 unknowns (P-STAT5), 5 unknowns (Ki67), 2 unknowns (p16), 9 unknowns (CD163).

P-STAT5 activation in primary melanomas

In the cohort, 14.8% (n=28) had a high P-STAT5 level, while 16 patients could not be given

a reliable P-STAT5 score due to equivocal staining (Table 2). High P-STAT5 level was significantly and positively associated with Breslow thickness (high P-STAT5 mean Breslow: 3.79mm vs low P-STAT5: 2.36mm, t-test, p=0.035) and ulceration (high P-STAT5 ulceration rate 53.6% vs low P-STAT5 22.8%, chi square, p=0.001). Moreover, tumours with High P-STAT5 level tended to display a nodular histological subtype (46.4% vs 26.9%, p=0.056, non-significant). Overall, tumours harbouring signs of activated cytokine signalling had characteristics of more severe disease as they were thicker and more ulcerated. Of significant interest, despite reflecting more severe tumours, P-STAT5 level was not significantly associated with melanoma death (p=0.284) or recurrence (p=0.966), however it showed a trend for association with melanoma-specific survival on unadjusted Kaplan-Meier analysis (Figure 2). This prompted us to consider P-STAT5 in multivariate models.

Table 2: Association of P-STAT5 levels with patient and tumour characteristics

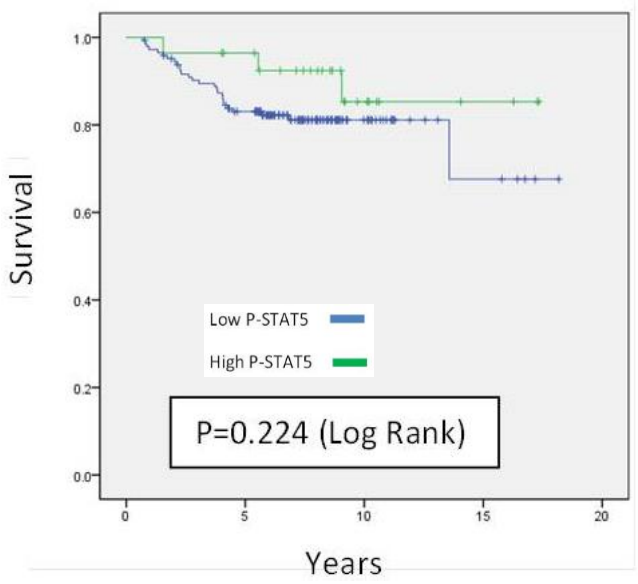
	High P-STAT5 (n=28) n (%)	**Low P-STAT5 (n=145) n (%)**	**p-value#**
Sex			
Male	19 (67.9)	77 (53.1)	0.150
Female	9 (32.1)	68 (46.9)	
Age			
0-40	5 (17.9)	32 (22.1)	0.909^
41-60	17 (60.7)	75 (51.7)	
61-80	6 (21.4)	38 (26.2)	
Mean ± SD	51.0 ± 12.1	51.3 ± 13.8	
Breslow thickness			
0 – 1.0 mm	1 (3.6)	10 (6.9)	0.035^
1.1 – 2.0 mm	13 (46.4)	74 (51)	
2.1 – 4.0 mm	8 (28.6)	43 (29.7)	
>4.0 mm	6 (21.4)	18 (12.4)	
Mean ± SD	3.79 ± 3.39	2.36 ± 1.46	
Ulceration			
Yes	15 (53.6)	33 (22.8)	0.001
No	13 (46.4)	112 (77.2)	

Histology			
SSM	11 (39.3)	89 (61.4)	0.056
NM	13 (46.4)	39 (26.9)	
LMM	1 (3.6)	3 (2.1)	
Desmoplastic	3 (10.7)	4 (2.8)	
Other	0	9 (6.2)	
Site			
Head and neck	2 (7.1)	10 (6.9)	0.790
Upper limb	8 (28.6)	35 (24.1)	
Lower limb	10 (35.7)	44 (30.3)	
Trunk	8 (28.6)	56 (38.6)	
SLN status			
Positive	7 (25)	24 (16.6)	0.286
Negative	21 (75)	121 (83.4)	
Recurrence			
Yes	8 (28.6)	42 (29)	0.966
No	20 (71.4)	103 (71)	
Melanoma death			
Yes	4 (14.3)	34 (23.4)	0.284
No	24 (85.7)	111 (76.6)	

#Pearson Chi-square test

^t-test

Figure 2: Unadjusted Kaplan-Meier actuarial curves for melanoma-specific death comparing survival of patients with high and low P-STAT5 staining



Association with melanoma death

Head and neck sites (p=0.025) and positive SLN status (p<0.001) were significantly associated with melanoma death, whereas Breslow thickness was approaching significance (p=0.067) (Table 3).

We tested whether P-STAT5 and three other biomarkers (Ki67, p16, CD163) in the primary melanoma would have independent predictive value and hence could improve predictive potential if combined. In multivariate model with all four biomarkers (P-STAT5, Ki67, p16, CD163) and adjusting for known prognostic indicators (age, sex, ulceration, Breslow and SLN status), P-STAT5 was inversely associated with melanoma-specific death (HR=0.30 [0.09-1.04] p=0.058), while SLN status was the main factor predicting mortality (Table 4A). To account for the fact that in a significant number of cases, patients do not undergo SLN biopsy we also conducted a multivariate analysis without adjustment for SLN status. In this model, the inverse association between P-STAT5 with melanoma death was even stronger and reached significance (HR=0.25 [0.07-0.88] p=0.031), whereas Breslow thickness was a significant predictor of melanoma death (HR=1.25 [1.07-1.45] p=0.004) (Table 4B).

Table 3: Association between cohort characteristics and melanoma death

	Melanoma death		
	Yes (n=41) n (%)	**No (n=148) n (%)**	**p-value#**
Sex			
Male	26 (63.4)	78 (52.7)	0.222
Female	15 (36.6)	70 (47.3)	
Age			
0-40	8 (19.5)	32 (21.6)	0.554^
41-60	26 (63.4)	75 (50.7)	
61-80	7 (17.1)	41 (27.7)	
Mean ± SD	50.0 ± 11.8	51.4 ± 14.0	
Breslow thickness			
0 – 1.0 mm	0	14 (9.5)	0.067^
1.1 – 2.0 mm	15 (36.6)	82 (55.4)	
2.1 – 4.0 mm	20 (48.8)	33 (22.3)	
>4.0 mm	6 (14.6)	19 (12.8)	
Mean (mm) ± SD	3.0 ± 2.0	2.4 ± 1.9	
Ulceration			
Yes	12 (29.3)	37 (25)	0.581
No	29 (70.7)	111 (75)	
Histology			
SSM	28 (68.3)	83 (56.5)	0.486
NM	11 (26.8)	43 (29.3)	
LMM	0	5 (3.4)	
Desmoplastic	1 (2.4)	7 (4.8)	
Other	1 (2.4)	9 (6.1)	
Site			
Head and neck	7 (17.1)	7 (4.7)	0.025
Upper limb	7 (17.1)	38 (25.7)	
Lower limb	9 (22)	49 (33.1)	
Trunk	18 (43.9)	54 (36.5)	
SLN status			

Positive	15 (36.6)	18 (12.2)	<0.001
Negative	26 (63.4)	130 (87.8)	

#Pearson Chi-square test

^t-test

Table 4A: Multivariate analysis for melanoma-specific death, including all 4 biomarkers and SLN status

	HR	**95% CI**	***p*-value**
Age	0.99	0.97-1.02	0.624
Sex	0.51	0.25-1.04	0.065
Breslow	1.16	0.98-1.37	0.091
Ulceration	0.62	0.26-1.48	0.284
SLN	4.84	2.14-10.93	<0.001
P-STAT5	0.30	0.09-1.04	0.058
Ki67	1.17	0.46-2.98	0.736
p16	1.27	0.64-2.53	0.488
CD163	1.79	0.88-3.64	0.107

Table 4B: Multivariate analysis for melanoma-specific death, including all 4 biomarkers, excluding SLN status

	HR	**95% CI**	***p*-value**
Age	0.98	0.96-1.01	0.231
Sex	0.55	0.27-1.10	0.092
Breslow	1.25	1.07-1.45	0.004
Ulceration	1.29	0.61-2.73	0.507
P-STAT5	0.25	0.07-0.88	0.031
Ki67	1.05	0.42-2.60	0.922
p16	1.24	0.62-2.47	0.551
CD163	1.79	0.90-3.54	0.096

Association with melanoma recurrence

Breslow thickness (p=0.001), ulceration (p=0.040) and SLN status (p<0.001) were significantly associated with melanoma recurrence (Table 5).

When all four biomarkers were fit in the cox proportional hazards regression model with clinicopathological covariates listed in Table 6A, P-STAT5 was inversely and significantly associated with melanoma recurrence (HR=0.39 [0.15-0.99] p=0.046). Breslow thickness (HR=1.16 [1.00-1.34] p=0.045), positive SLN (HR=5.44 [2.59-11.45] p<0.001) and Ki67 (HR=2.28 [1.14-4.56] p=0.020) were significant predictors of melanoma recurrence (Table 6A). In the model without SLN, P-STAT5 remained inversely associated with melanoma recurrence, but did not reach significance (HR=0.41 [0.17-1.02] p=0.056). Breslow thickness (HR=1.25 [1.11-1.42] p<0.001) and Ki67 (HR=2.02 [1.04-3.95] p=0.039) remained as significant predictors of melanoma recurrence (Table 6B).

Table 5: Association between cohort characteristics and melanoma recurrence

	Recurrence		
	Yes (n=52) n (%)	**No (n=137) n (%)**	**p-value#**
Sex			
Male	29 (55.8)	75 (54.7)	0.899
Female	23 (44.2)	62 (45.3)	
Age			
0-40	8 (15.4)	31 (22.6)	0.619^
41-60	31 (59.6)	71 (51.8)	
61-80	13 (25)	35 (25.5)	
Mean ± SD	51.8 ± 12.9	50.8 ± 13.8	
Breslow thickness			
0 – 1.0 mm	2 (3.8)	12 (8.8)	0.001^
1.1 – 2.0 mm	17 (32.7)	80 (58.4)	
2.1 – 4.0 mm	19 (36.5)	34 (24.8)	
>4.0 mm	14 (26.9)	11 (8)	
Mean (mm) ± SD	3.4 ± 2.3	2.2 ± 1.7	
Ulceration			

Yes	19 (36.5)	30 (21.9)	0.040
No	33 (63.5)	107 (78.1)	
Histology			
SSM	31 (59.6)	80 (58.8)	0.373
NM	17 (32.7)	37 (27.2)	
LMM	0	5 (3.7)	
Desmoplastic	3 (5.8)	5 (3.7)	
Other	1 (1.9)	9 (6.6)	
Site			
Head and neck	6 (11.5)	8 (5.8)	0.263
Upper limb	8 (15.4)	37 (27)	
Lower limb	17 (32.7)	41 (29.9)	
Trunk	21 (40.4)	51 (37.2)	
SLN status			
Positive	21 (40.4)	12 (8.8)	<0.001
Negative	31 (59.6)	125 (91.2)	

#Pearson Chi-square test

^t-test

Table 6A: Multivariate analysis for recurrence, including all 4 biomarkers and SLN status

	HR	**95% CI**	***p*-value**
Age	1.01	0.98-1.03	0.582
Sex	0.87	0.47-1.60	0.648
Breslow	1.16	1.00-1.34	0.045
Ulceration	0.63	0.30-1.31	0.217
SLN	5.44	2.59-11.45	<0.001
P-STAT5	0.39	0.15-0.99	0.046
Ki67	2.28	1.14-4.56	0.020
p16	1.33	0.73-2.40	0.352
CD163	1.76	0.93-3.33	0.084

Table 6B: Multivariate analysis for recurrence, including all 4 biomarkers, excluding SLN status

	HR	95% CI	*p*-value
Age	1.00	0.98-1.02	0.761
Sex	0.99	0.54-1.78	0.961
Breslow	1.25	1.11-1.42	<0.001
Ulceration	1.40	0.76-2.61	0.284
P-STAT5	0.41	0.17-1.02	0.056
Ki67	2.02	1.04-3.95	0.039
p16	1.28	0.70-2.35	0.418
CD163	1.60	0.87-2.93	0.132

Discussion

Upon diagnosis of invasive melanoma, often the first question asked by patients is about prognosis and management. The AJCC has established clear prognostic factors that allow a rapid categorization of patients into large outcome groups. However, for locally invasive melanoma that displays no sign of regional or systemic dissemination, within each AJCC stage, there remains significant variation in outcome. With continuous improvement of our knowledge into the intricate processes of melanomagenesis, its driver mutations, the metastatic process, and immune checkpoint proteins regulating the host response, one could speculate that advances in terms of prognostication could be within reach. We followed 189 patients with locally invasive cutaneous melanoma who underwent sentinel node biopsy, collecting clinicopathological data and mortality. Our study demonstrated that in spite of a high P-STAT5 level being present in more locally advanced melanoma, it was found to be independently and inversely associated with melanoma-specific death and recurrence. Furthermore, in combination with biomarkers Ki67, p16, CD163, a high P-STAT5 level remained strongly protective against melanoma death.

Currently, the main prognostic factors of early stage melanoma are Breslow thickness, presence of ulceration, and sentinel lymph node status.[86-87] Although SLN biopsy helps in further stratifying patients within an AJCC prognostic group, it is an invasive albeit low risk operative procedure that comes with logistical difficulties and may not be suitable for some patients. Furthermore, among patients with locally invasive melanoma, some develop systemic metastasis despite complete surgical excision and in the absence of SLN

invasion. This supports the efforts to use molecular information from the tumour microenvironment and host immune response to identify high-risk melanomas.[36]

Initial studies on STAT5a-/- and STAT5b-/- mice showed marked defects in their immune responses.[88-89] Pericle et al demonstrated that T and B cells isolated from immunocompromised tumour-bearing mice had a significantly decreased level of expression of STAT5, and there was a correlation between the decreased STAT5 level and tumour growth progression.[90] Similarly, Grange et al[91] found that activated STAT5 led to an increased effector T-cell accumulation in melanoma tumours, increased effector T-cell activation by tumour antigens and expression of cytolytic factor granzyme B, resulting in tumour regression. Indeed, accumulation of P-STAT5 in CD8 T cells and NK cells is an important element of a robust anti-tumour response.[87]

STAT5 also plays a role in other signalling cascades in melanoma such as the scavenger receptor class B type I (SCARB1/SR-BI) and transient receptor protein channel 3 (TRPC3).[92-93] SR-BI was found to predict melanoma progression and a knockdown of SR-BI disrupts the metastasis-associated epithelial-to-mesenchymal transition (EMT) phenotype in melanoma, in which STAT5 is an important mediator.[93]

Of interest, other experimental models have suggested an important role of STAT5 activation in tumour cell maintenance or progression[94] which are contradicted here by our human data. Indeed, in a clinical setting, high doses of interferon alpha used as adjuvant therapy in melanoma resulted in increased P-STAT5 and a reduction of STAT3[95]. Overall our findings are in strong concordance with previously published literature suggesting that activation of STAT5 reflects the activation of the immune system in the tumour resulting in better outcomes. Similarly, when looking at the genetic determinants of immune response in melanoma patients, Poźniak et al[96] found the subgroup of patients with stronger immune responses had better survival.

Although past studies have indicated the importance of tumour infiltrating lymphocytes[8] it has been difficult to establish if these cells are active, anergic or exhausted. Our study provides us with a new marker of effective immune activation in the tumour. P-STAT5 was mostly activated in thick and ulcerated tumours where one would expect a worse outcome.

Possibly ulceration despite being indicative of tumour progression allows more immune activation that in some cases is favourable. Overall, STAT5 activation strongly predicted survival, independently from classical prognostic indicators such as Breslow and ulceration. These findings indicate that this marker could be used to subdivide current staging groups. Indeed, those at risk of progressing may benefit from adjuvant therapy to stimulate their immune system. Our study incorporates immunohistochemical staining, a straightforward test routinely carried out at pathology laboratories, therefore this can be easily performed on melanoma tissues at time of diagnosis. The use of a phosphor-protein as a target may require more standardized tissue processing and it is therefore important to prospectively validate these findings or to examine the prognostic value of immunostaining of direct targets of STAT5 such as SOCS2 (suppressor of cytokine signalling 2) or CISH (chromogenic in-situ hybridization). Further, with the validation of any new prognostic marker, there is also prospect of translating that into novel therapeutic targets.

In conclusion, activation of STAT5 is a robust criterion highlighting activation of the tumour immune system and resulting in improved survival in a well-established and REMARK compliant cohort of melanoma patients. This finding opens new avenues in the subclassification of stage Ib and II patients for future adjuvant therapies.

Supplementary materials and methods

Staining protocol for P-STAT5 Immunohistochemistry

Antibody: Abcam ab32364, purified rabbit anti-p-STAT5 (clone: E208) 220ug/ml

Species reactivity: Human, mouse and rat

Positive control tissue: skin- normal or carinoma, placental trophoblasts, breast carcinoma

Positive control block: Multi tissue TMA, placenta or breast carcinoma x2128

Staining pattern: Nuclear

- Sections (3-µm) are affixed to Menzel Superfrost Plus adhesive slides and airdried overnight at 37^{o}C.
- Sections are dewaxed and rehydrated through descending graded alcohols to phosphate-buffered saline (PBS), pH 7.4, using standard protocol.
- Transfer to EDTA pH 9.0, and subjected to 20 minutes heat antigen retrieval at 95^{o}C using the Biocare Medical decloaking chamber. Completion of the cool down cycle remove container of slides and allow to cool for a further 20 minutes before transferring back to TBS.
- Wash in 3 changes of TBS.
- Endogenous peroxidase activity is blocked by incubating the sections in 3.0% H_2O_2 in TBS for 10 minutes.
- Sections are washed in three changes of TBS for 5 minutes each.
- Biocare Medical Background Sniper is applied for 30 minutes.
- Excess Sniper is removed and 10% normal goat serum is applied for 30 minutes.
- Excess normal serum is decanted from the sections and the primary antibody diluted 1:150 in Biocare Medical Da Vinci Green applied overnight at room temperature.
- Sections are washed in three changes of TBS for 5 minutes each, the first buffer change containing 0.5% (v/v) Triton X-100.
- Biocare Medical MACH2 anti-rabbit HRP is applied for 60 minutes at RT.
- Sections are washed in three changes of TBS for 5 minutes each.
- Control slide signals are developed in vector NovaRed for 5 minutes.
- Sections are washed in gently running tap water for 5-10 minutes to remove excess chromogen.
- Sections are lightly counterstained in Mayers' haematoxylin (program 6), then dehydrated through ascending graded alcohols, cleared in xylene, and mounted using DePeX or similar.

Chapter 4:

Analysis of lymphovascular biomarkers as prognostic marker of melanoma

Introduction

Cutaneous melanoma is a leading cause of cancer death, with a 5-year relative survival rate of 98% for patients with localised disease, which decreases dramatically to 23% for those with distant metastasis.[97] The current melanoma staging criteria based on the AJCC staging system takes into account the extent of invasion of the primary tumour, lymph node involvement and metastatic spread.[26] This system has several limitations as the survival outcome of patients within the same AJCC melanoma stage can vary widely, with some patients having thick melanoma that survive while others with thin melanoma that subsequently develop metastasis and die.

As part of the staging process, SLN biopsy is carried out in selected patient population as regional lymph node metastasis is one of the most important prognostic factors in melanoma. Although SLN biopsy has become a gold-standard procedure in melanoma for the purpose of staging, guiding treatment and recruitment to clinical trials[98-99], it comes with some post-operative complications. A recent systematic review demonstrated the overall post-operative complication rate for SLN biopsy was 11.3% with infection rates between 0.3%-19%.[100] In addition, about 15% patients with negative SLN will proceed to have disease progression.[98] Finally, SLN biopsy cannot be applied widely to all patients and in a recent study, only 30% of those with an indication of SLN biopsy underwent the procedure.[31] This has prompted the efforts to look for other prognostic markers that could add value to the current staging system and help further distinguish between aggressive and less aggressive forms of melanoma at the time of diagnosis.

Mortality in melanoma is primarily the result of tumour metastasis and this happens through invasion of the vascular and lymphatic system, spreading to regional or distant lymph nodes and organs. Tumour vascularization and lymphangiongenesis have shown to be prognostic indicators for the risk of lymph node metastasis in cutaneous melanoma and affect patient outcome and survival.[101-105] Previous studies revealed the important roles of vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet-derived growth factor, angiopoietin and SOX18 transcription factors in angiogenesis and lymphangiogenesis, and further studies would offer more prognostic and therapeutic potential in the form of anti-angiogenic and vascular-targeting agents.[54,106-111] Of particular interest is the role of SOX18, which together with SOX7 and SOX17 forms the SOX group F transcription factors, key regulators of endothelial cell differentiation, angiogenesis and lymphangiogenesis.[53]

SOX18 has been shown in preclinical studies to be expressed at early stages of tumour angiogenesis and metastasis.[112-115] Mutant forms of SOX18 prevent lymphatic metastasis suggesting its importance in this process. However, there have not been studies exploring the role of SOX18 in a prospective cohort of melanoma patients.

We aimed to evaluate the expression of lymphovascular markers and to assess the impact of these markers on the progression and prognosis of melanoma in a prospective cohort. We looked at SOX18 and a previously validated biomarker Ki67[85] in the endothelial cells of lymphatic and blood vessels as determined by vessels positively stained by CD31 and D2-40, and studied their association with melanoma outcomes including SLN status and melanoma death.

Materials and Methods

Ethics statement

Ethical approval was obtained through the Metro South Health Human Research Ethics Committee, Brisbane, Australia to carry out this project.

Patients

787 patients with invasive cutaneous melanoma who were referred to the Princess Alexandra Hospital melanoma clinic and underwent SLN biopsy between 1994 and 2011 were considered for inclusion in this study. Eligibility criteria include being at least 18 years of age and able to provide consent, a diagnosis of clinical stage Ib and II melanoma, proceeded to have a SLN biopsy, and accessible melanoma tissues in the form of whole-section slides or formalin-fixed, paraffin embedded tissue blocks. Melanoma tissues of 289 patients were retrieved. 18 patients were excluded due to poor staining while 1 patient was excluded as the follow-up information was unavailable, leaving 270 patients in the cohort. In a previous study by our group, this cohort has been shown to be equivalent to the general population undergoing SLN biopsy and younger than the general population with locally invasive melanoma thicker than 2mm.[85] All haematoxylin-eosin slides were reviewed by a pathologist to confirm the diagnosis. Clinicopathologic characteristics including sex, age at diagnosis, melanoma histologic subtype and site, Breslow thickness, ulceration, and SLN status were recorded and follow up data were collected until 31 December 2014 for recurrence and survival. These were the latest validated data available

for release from the Queensland Cancer Registry at the time of request. 11 patients were followed up until May 2013 and 8 patients until December 2010 as their follow-up data beyond this date were not available.

Immunohistochemistry

To identify lymphatic vessels, blood vessels and proliferating endothelial cells, the melanoma tissue samples were prepared according to tyramide-based Vectra protocol and stained with the primary monoclonal antibody against previously validated biomarkers D2-40 (Biocare Medical CM266A, mouse anti-D2-40, dilution 1:800), CD31 (Dako M0823, mouse anti-CD31, dilution 1:400), Ki67 (Dako M7240, mouse anti-Ki67, dilution 1:1400), and SOX18 (anti-SOX18 Mab, human IgG1, dilution 1:1000). The details of staining protocol are provided in the supplementary materials and methods section. Each antibody was matched with respective control samples to ensure staining quality and to determine positive and negative staining.

Evaluation of staining

Each slide was scanned first at low (x100) magnification to identify area with highest vascularity. The number of vessels stained by CD31 or D2-40 were counted in three high-power fields (x400) within the area of highest vascularity and the sum for each were recorded for each slide. The same was repeated for vessels stained by CD31 or D2-40 that contains Ki67+ or SOX18+ nuclei. Zero was used as a cutoff to divide the cohort into absent and present categories for the following five biomarker combinations: CD31+/SOX18+, CD31+/Ki67+, D2-40+/SOX18+, D2-40+/Ki67+, CD31+ Ki67+/D2-40+Ki67+. In addition, SOX18 positive and negative group was determined by the presence or absence of SOX18+ nuclei in both CD31+ and D2-40+ vessels. Scoring was performed by 2 people and results were corroborated.

Statistical analysis

All analysis was performed using IBM SPSS statistics software version 25. Melanoma survival was calculated from the date of histological diagnosis to date of melanoma death or last follow-up.

Presence of vessels stained by SOX18 was tested for association with standard prognostic factors including SLN status and ulceration using chi-square analysis, and t-test with Breslow thickness. Univariate and multivariate analyses were performed to determine

factors predictive of SLN metastasis and melanoma death. Statistical significance was defined by p-value of <0.05.

Results

Cohort characteristics

Our cohort included 151 (55.9%) men and 119 (44.1%) women, with an average age of 52 years and average Breslow thickness of 2.4mm. Majority of the melanomas were of the superficial spreading (60%) and nodular (31.9%) histological types and occurred most frequently on the lower limb (35.6%) followed by trunk (30.4%). 51 (19.2%) patients had positive sentinel lymph nodes and 53 (20%) patients had melanoma recurrence. The average duration of follow up was 7 years and death from melanoma occurred in 46 patients (17%). The data are summarised in table 1.

Table 1: Distribution of clinicohistologic characteristics and their relation to presence of SOX18+ nuclei in vasculatures

		SOX18		
	Total cohort (n=270) n (%)	**Negative (n=84) n (%)**	**Positive (n=186) n (%)**	**p-value**
Sex				
Male	151 (55.9)	48 (57.1)	103 (55.4)	0.787
Female	119 (44.1)	36 (42.9)	83 (44.6)	
Age				
Mean ± SD	52 ± 13.6	52 ± 14.4	51 ± 13.3	0.667#
Breslow thickness				
Mean ± SD	2.4 ± 1.8	2.8 ± 2.1	2.2 ± 1.6	0.022#
Ulceration*				
Yes	70 (26)	26 (31)	44 (23.8)	0.214
No	199 (74)	58 (69)	141 (76.2)	
Histology^				
SSM	156 (60)	47 (59.5)	109 (60.2)	0.458
NM	83 (31.9)	28 (35.4)	55 (30.4)	

LMM	6 (2.3)	1 (1.3)	5 (2.8)	
Desmoplastic	9 (3.5)	3 (3.8)	6 (3.3)	
Other	6 (2.3)	0	6 (3.3)	
Site				
Head or neck	19 (7)	6 (7.1)	13 (7)	0.480
Trunk	82 (30.4)	21 (25)	61 (32.8)	
Upper limb	73 (27)	22 (26.2)	51 (27.4)	
Lower limb	96 (35.6)	35 (41.7)	61 (32.8)	
SLN status**				
Positive	51 (19.2)	25 (29.8)	26 (14.4)	0.003
Negative	214 (80.8)	59 (70.2)	155 (85.6)	
Recurrence^^				
Yes	53 (20)	26 (31)	27 (14.9)	0.002
No	212 (80)	58 (69)	154 (85.1)	
Melanoma death				
Yes	46 (17)	19 (22.6)	27 (14.5)	0.101
No	224 (83)	65 (77.4)	159 (85.5)	
CD31+Ki67+				
Present	59 (21.9)	6 (7.1)	53 (28.5)	<0.001
Absent	211 (78.1)	78 (92.9)	133 (71.5)	
D2-40+Ki67+				
Present	13	3 (3.6)	10 (5.4)	0.521
Absent	257	81 (96.4)	176 (94.6)	
CD31+D2-40+Ki67+				
Present	66 (24.4)	8 (9.5)	58 (31.2)	<0.001
Absent	204 (75.6)	76 (90.5)	128 (68.8)	
STAT5				
High	27 (16.3)	13 (19.1)	14 (14.3)	0.407
Low	139 (83.7)	55 (80.9)	84 (85.7)	
CD163				
High	72 (41.4)	29 (41.4)	43 (41.3)	0.991
Low	102 (58.6)	41 (58.6)	61 (58.7)	

#T-test *1 unknown ^10 unknown **5 unknown ^^5 unknown

SOX18 distribution in cutaneous melanoma

SOX18 staining could be identified in the vasculature and more specifically in endothelial or lymphatic endothelial nuclei (Figures 1 & 2). SOX18 positive nuclei seemed to occur more frequently in tumour vessels of patients who were alive at the end of the follow-up period, compared to those who died from melanoma (mean 2.5 vessels ± 2.8 vs 1.7 vessels ± 2.0, p=0.017) (Table 2). When comparing patients who displayed any SOX18 positive nuclei to those with no detectable SOX18, the former had a lower rate of positive SLN (14.4% vs 29.8%, p=0.003) and recurrence (14.9% vs 31%, p=0.002) (Table 1). They also had better prognostic features such as lower Breslow thickness (2.2 ± 1.6mm vs 2.8 ± 2.1mm, p=0.022), lower rate of ulceration (23.8% vs 31%, p=0.214), and lower frequency of nodular subtype (30.4% vs 35.4%, p=0.458), although the latter two did not reach significance (Table 1). Interestingly even though the SOX18 positive group was associated with better prognostic features, they were also associated with higher rate of proliferative marker in their vasculature as demonstrated by the higher number of CD31+ vessels with Ki67+ nuclei (31.2% vs 9.5%, p<0.001) (Table 1). We correlated SOX18 level with immune biomarkers previously studied by our group on the same cohort[25] and noted those in the SOX18 positive group had a similar percentage of high STAT5 staining (14.3% vs 19.1%, p=0.407) and CD163 (Table 1).

Figure 1: Staining of anti-SOX18 antibody (red) in vascular (yellow) endothelial cells

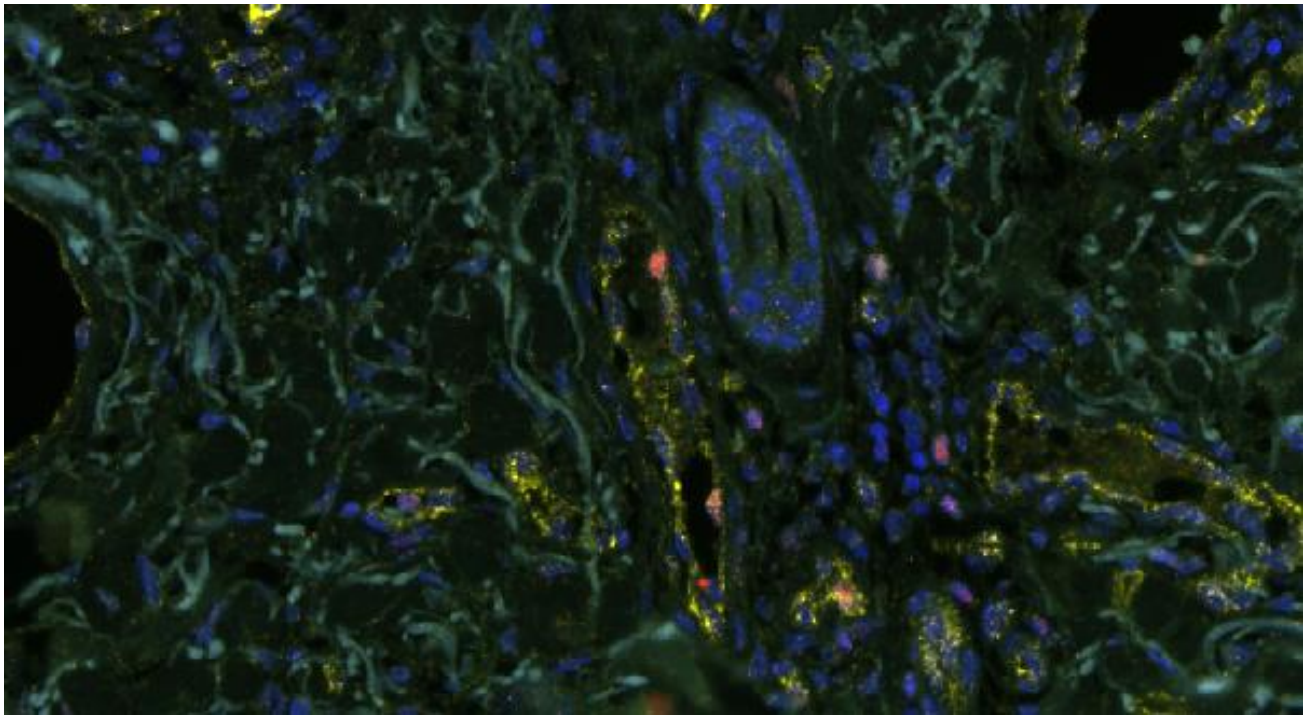


Figure 2: Staining of anti-SOX18 antibody (red) in lymphatic (cyan) endothelial cell and also seen in melanoma (purple) cell nuclei

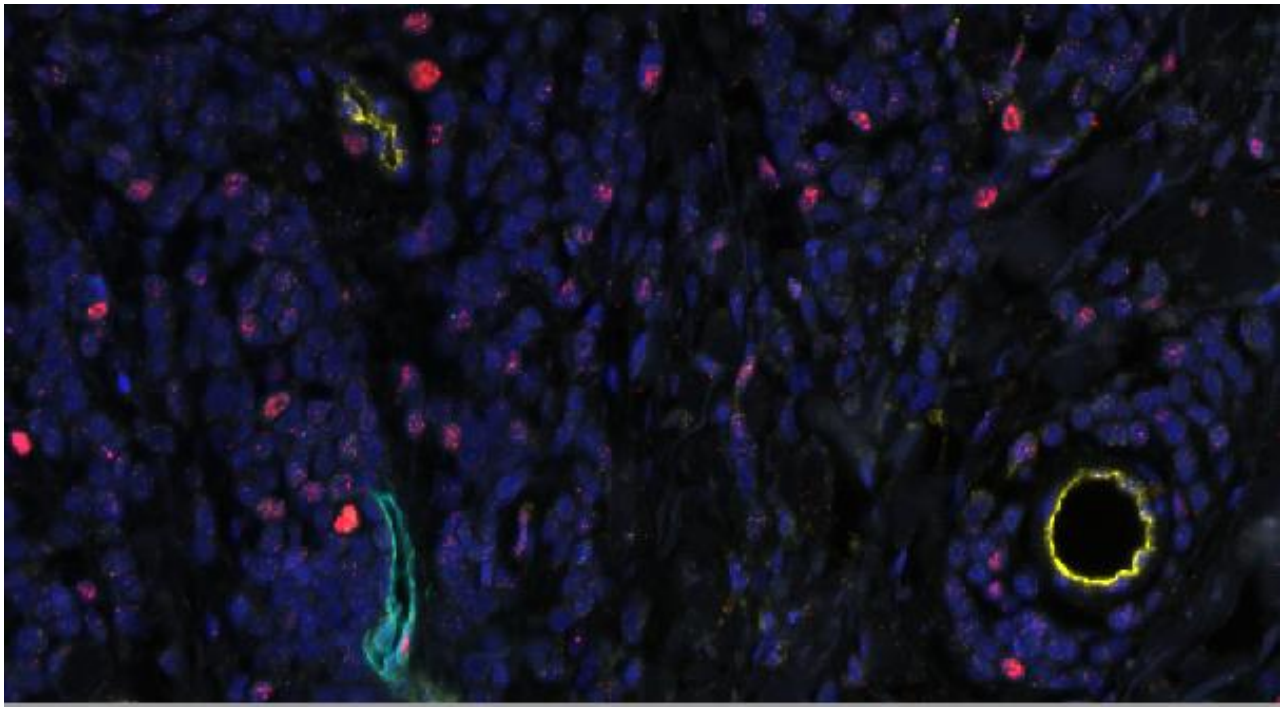


Table 2: Univariate analysis of the association with SLN status and melanoma death

	SLN status			Melanoma death		
Variables mean (SD)	**Pos n=51**	**Neg n=214**	**p-value**	**Yes n=46**	**No n=224**	**p-value**
CD31+ vessels	14.7 (11.4)	15.2 (9.8)	0.710	14.3 (10.1)	15.3 (10.0)	0.546
CD31+ vessels with SOX18+ nuclei	1.6 (2.1)	2.4 (2.6)	0.037	1.6 (2.0)	2.4 (2.6)	0.059
CD31+ vessels with Ki67+ nuclei	0.3 (0.5)	0.3 (0.7)	0.682	0.3 (0.6)	0.3 (0.7)	0.784

D240+ vessels	7.2 (4.2)	6.4 (4.4)	0.243	7.6 (4.7)	6.4 (4.2)	0.080
D240+ vessels with SOX18+ nuclei	0.1 (0.5)	0.2 (0.5)	0.335	0.1 (0.3)	0.2 (0.5)	0.134
D240+ vessels with Ki67+ nuclei	0.2 (0.7)	0 (0.2)	0.138	0.2 (0.6)	0.1 (0.3)	0.305
All vessels with SOX18+ nuclei	1.7 (2.4)	2.6 (2.8)	0.033	1.7 (2.0)	2.5 (2.8)	0.017
All vessels with Ki67+ nuclei	0.5 (1.2)	0.4 (0.8)	0.401	0.4 (1.0)	0.4 (0.8)	0.614

Association with SLN status

Tumour lymphangiogenesis has been linked to SLN metastasis and therefore we examined whether the presence of SOX18 and Ki67 in lymphatics and blood vessels can be used in predicting SLN status in melanoma patients. We divided the cohort into positive and negative SLN groups and expectedly found significant association between positive SLN and high Breslow thickness (p=0.002), presence of ulceration (p<0.001), recurrence (p<0.001), melanoma death (<0.001), absence of SOX18+ nuclei in CD31+ vessels (p=0.005), and absence of SOX18+ nuclei in CD31+ and D2-40+ vessels (p=0.003) (Table 3). There was a trend in the association between presence of Ki67+ nuclei in D2-40+ vessels (p=0.072) and positive SLN (Table 3).

To identify risk factors for developing positive SLN, univariate and multivariate analysis were performed. All clinicopathologic characteristics and biomarkers were included in the univariate analysis. We found that age, Breslow thickness, ulceration, SOX18+ nuclei in CD31+ vessels, SOX18+ nuclei in CD31+ and D2-40+ vessels, Ki67+ nuclei in D2-40+ vessels were significant predictors of SLN status (Table 4).

When performing multivariate analysis using covariates listed in table 4, only Breslow thickness (OR=1.32 [1.10-1.60] p=0.004), ulceration (OR=5.02 [2.43-10.35] p=<0.001) and Ki67+ nuclei in D2-40+ vessels (OR=2.92 [1.26-6.79] p=0.013) were independent predictors of positive SLN. SOX18+ nuclei in CD31+ and D2-40+ vessels almost reached significance as a negative predictor (OR=0.86 [0.73-1.01] p=0.068).

Table 3: Association between clinicohistologic characteristics and SLN status

	Positive SLN (n=51) n (%)	**Negative SLN (n=214) n (%)**	**p-value**
Sex			
Male	34 (66.7)	115 (53.7)	0.094
Female	17 (33.3)	99 (46.3)	
Age			
Mean ± SD	48 ± 16.2	52 ± 12.9	0.050#
Breslow thickness			
Mean ± SD	3.3 ± 2.3	2.2 ± 1.6	0.002#
Ulceration*			
Yes	27 (54)	42 (19.6)	<0.001
No	23 (46)	172 (80.4)	
Histology^			
SSM	28 (54.9)	125 (61.3)	0.177
NM	22 (43.1)	59 (28.9)	
LMM	0	6 (2.9)	
Desmoplastic	1 (2)	8 (3.9)	
Other	0	6 (2.9)	
Site			
Head or neck	5 (9.8)	13 (6.1)	0.134
Trunk	15 (29.4)	66 (30.8)	
Upper limb	8 (15.7)	64 (29.9)	
Lower limb	23 (45.1)	71 (33.2)	
Recurrence			
Yes	24 (47.1)	29 (13.6)	<0.001
No	27 (52.9)	185 (86.4)	
Melanoma death			
Yes	19 (37.3)	27 (12.6)	<0.001
No	32 (62.7)	187 (87.4)	
CD31+SOX18+			
Present	26 (51)	153 (71.5)	0.005

Absent	25 (49)	61 (28.5)	
CD31+Ki67+			
Present	12 (23.5)	46 (21.5)	0.752
Absent	39 (76.5)	168 (78.5)	
D2-40+SOX18+			
Present	3 (5.9)	29 (13.6)	0.131
Absent	48 (94.1)	185 (86.4)	
D2-40+Ki67+			
Present	5 (9.8)	8 (3.7)	0.072
Absent	46 (90.2)	206 (96.3)	
CD31+D2-40+ SOX18+			
Present	26 (51)	155 (72.4)	0.003
Absent	25 (49)	59 (27.6)	
CD31+D2-40+ Ki67+			
Present	13 (25.5)	52 (24.3)	0.859
Absent	38 (74.5)	162 (75.7)	

#T-test *1 unknown ^10 unknown

Table 4: Univariate and multivariate regression analyses of the association with SLN metastasis

	Univariate analysis			**Multivariate analysis**		
	OR	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Sex						
Male	1.72	0.91-3.27	0.097	1.39	0.67-2.86	0.379
Age	0.98	0.95-1.00	0.024	0.95	0.93-0.98	0.001
Breslow thickness	1.33	1.13-1.57	0.001	1.32	1.10-1.60	0.004
Ulceration	4.81	2.51-9.21	<0.001	5.02	2.43-10.35	<0.001
Histology						
NM	1.67	0.88-3.15	0.118			
Site						
Head and neck	1.19	0.38-3.69	0.767			
CD31+SOX18+	0.86	0.74-0.99	0.040			

CD31+Ki67+	0.91	0.56-1.46	0.681			
D2-40+SOX18+	0.67	0.30-1.52	0.342			
D2-40+Ki67+	2.28	1.06-4.87	0.034	2.92	1.26-6.79	0.013
CD31+D2-40+SOX18+	0.86	0.75-0.99	0.037	0.86	0.73-1.01	0.068
CD31+D2-40+Ki67+	1.15	0.83-1.60	0.403			

Association with melanoma survival

The cohort was divided into two groups according to melanoma survival status (Table 5). There was significant association between melanoma death and the clinicopathologic characteristics Breslow thickness (p=0.020), head and neck site (p=0.009), SLN positivity (p<0.001) and recurrence (p<0.001), and they were significant predictors of melanoma death in univariate analysis (Table 6). There was an apparent trend in the association between melanoma death and absence of SOX18+ nuclei in CD31+ and D2-40+ vessels (p=0.101), absence of SOX18+ nuclei in CD31+ vessels (p=0.131) and presence of Ki67+ nuclei in D2-40+ vessels (p=0.177) (Table 5).

Multivariate analysis showed only Ki67+ nuclei in D2-40+ vessels was an independent predictor of melanoma death (HR=1.71 [1.03-2.83] p=0.037) (Table 6). Of note, in these models we did not include SLN status to explore the possibility of our biomarkers to replace SLNB. When SLN was included, none of the biomarkers remained independently associated with survival (not shown).

Table 5: Association between clinicohistologic characteristics and melanoma survival

	Dead (n=46) n (%)	**Alive (n=224) n (%)**	**p-value**
Sex			
Male	31 (67.4)	120 (53.6)	0.086
Female	15 (32.6)	104 (46.4)	
Age			
Mean ± SD	50 ± 12.2	52 ± 13.9	0.347#
Breslow thickness			

Mean ± SD	3.0 ± 1.9	2.3 ± 1.7	0.020#
Ulceration*			
Yes	13 (28.3)	57 (25.6)	0.704
No	33 (71.7)	166 (74.4)	
Histology^			
SSM	29 (64.4)	127 (59.1)	0.793
NM	14 (31.1)	69 (32.1)	
LMM	0	6 (2.8)	
Desmoplastic	1 (2.2)	8 (3.7)	
Other	1 (2.2)	5 (2.3)	
Site			
Head or neck	8 (17.4)	11 (4.9)	0.009
Trunk	9 (19.6)	73 (32.6)	
Upper limb	10 (21.7)	63 (28.1)	
Lower limb	19 (41.3)	77 (34.4)	
SLN status**			
Positive	19 (41.3)	32 (14.6)	<0.001
Negative	27 (58.7)	187 (85.4)	
Recurrence^^			
Yes	35 (76.1)	18 (8.2)	<0.001
No	11 (23.9)	201 (91.8)	
CD31+SOX18+			
Present	27 (58.7)	157 (70.1)	0.131
Absent	19 (41.3)	67 (29.9)	
CD31+Ki67+			
Present	10 (21.7)	49 (21.9)	0.984
Absent	36 (78.3)	175 (78.1)	
D2-40+SOX18+			
Present	4 (8.7)	28 (12.5)	0.467
Absent	42 (91.3)	196 (87.5)	
D2-40+Ki67+			
Present	4 (8.7)	9 (4)	0.177
Absent	42 (91.3)	215 (96)	
CD31+D2-40+ SOX18+			

Present		27 (58.7)	159 (71)	0.101
Absent		19 (41.3)	65 (29)	
CD31+D2-40+ Ki67+				
Present		11 (23.9)	55 (24.6)	0.927
Absent		35 (76.1)	169 (75.4)	
#T-test	*1 unknown	^10 unknown	**5 unknown	^^5 unknown

Table 6: Univariate and multivariate regression analyses of the association with melanoma-specific death

	Univariate analysis			Multivariate analysis		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Sex						
Male	1.76	0.95-3.25	0.074	1.66	0.89-3.12	0.115
Age	1.00	0.98-1.02	0.708	0.99	0.97-1.01	0.374
Breslow thickness	1.14	1.02-1.27	0.019	1.11	0.99-1.25	0.069
Ulceration	1.13	0.59-2.15	0.714	0.97	0.50-1.87	0.923
Histology						
NM	0.97	0.51-1.84	0.928			
Site						
Head and neck	2.37	1.03-5.42	0.042			
SLN	3.82	2.12-6.88	<0.001			
Recurrence	18.80	9.51-37.18	<0.001			
CD31+SOX18+	0.89	0.77-1.02	0.100			
CD31+Ki67+	0.96	0.61-1.50	0.855			
D2-40+SOX18+	0.67	0.28-1.58	0.362			
D2-40+Ki67+	1.64	1.00-2.71	0.051	1.71	1.03-2.83	0.037
CD31+D2-40+SOX18+	0.89	0.78-1.02	0.090	0.90	0.78-1.03	0.111
CD31+D2-40+Ki67+	1.11	0.81-1.54	0.514			

Association with melanoma outcome in SLN negative cohort

Despite its benefits, SLNB does not capture all melanoma related events and a significant number of patients die from the disease. We therefore examined SLN negative cohort only and performed statistical analysis for melanoma survival. Breslow thickness (p=0.032), head and neck site (p=0.038) and recurrence (p<0.001) were significantly associated with

melanoma death (Table 7). In univariate analysis, only D2-40+ vessel was a significant predictor of melanoma death and it remained significant in multivariate analysis (HR=1.10 [1.01-1.19] p=0.025) (Table 8).

Table 7: Association between clinicopathologic characteristics and biomarkers with melanoma survival in SLN negative cohort

	Dead (n=27) n (%)	**Alive (n=187) n (%)**	**p-value**
Sex			
Male	17 (63)	98 (52.4)	0.304
Female	10 (37)	89 (47.6)	
Age			
Mean ± SD	53 ± 11.7	52 ± 13.1	0.317#
Breslow thickness			
Mean ± SD	2.5 ± 1.2	2.2 ± 1.6	0.032#
Ulceration			
Yes	5 (18.5)	37 (19.8)	0.877
No	22 (81.5)	150 (80.2)	
Histology*			
SSM	18 (69.2)	107 (60.1)	0.805
NM	6 (23.1)	53 (29.8)	
LMM	0	6 (3.4)	
Desmoplastic	1 (3.8)	7 (3.9)	
Other	1 (3.8)	5 (2.8)	
Site			
Head or neck	4 (14.8)	9 (4.8)	0.038
Trunk	3 (11.1)	63 (33.7)	
Upper limb	9 (33.3)	55 (29.4)	
Lower limb	11 (40.7)	60 (32.1)	
Recurrence			
Yes	20 (74.1)	9 (4.8)	<0.001
No	7 (25.9)	178 (95.2)	

All CD31vessel_w_SOX18nuclei			
Present	19 (70.4)	134 (71.7)	0.890
Absent	8 (29.6)	53 (28.3)	
All CD31vessel_w_Ki67nuclei			
Present	5 (18.5)	41 (21.9)	0.687
Absent	22 (81.5)	146 (78.1)	
All D240vessel_w_SOX18nuclei			
Present	3 (11.1)	26 (13.9)	0.692
Absent	24 (88.9)	161 (86.1)	
All D240vessel_w_Ki67nuclei			
Present	1 (3.7)	7 (3.7)	0.992
Absent	26 (96.3)	180 (96.3)	
All_vessels_w_SOX18nuclei			
Present	19 (70.4)	136 (72.7)	0.798
Absent	8 (29.6)	51 (27.3)	
All_vessels_w_Ki67nuclei			
Present	5 (18.5)	47 (25.1)	0.454
Absent	22 (81.5)	140 (74.9)	

#T-test *10 unknown

Table 8: Univariate and multivariate regression analyses in SLN negative cohort for melanoma-specific death

	Univariate analysis			**Multivariate analysis**		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Sex	1.48	0.68-3.23	0.329	1.43	0.65-3.17	0.376
Age	1.01	0.98-1.04	0.686	1.00	0.97-1.04	0.815
Breslow	1.10	0.93-1.30	0.282	1.03	0.85-1.25	0.752
Ulceration	0.97	0.37-2.57	0.957	0.86	0.32-2.29	0.756
Histology						
NM	0.76	0.30-1.91	0.557			
Site						
Head and neck	1.94	0.62-6.09	0.259			
CD31+	1.01	0.97-1.05	0.693			

CD31+SOX18+	0.91	0.77-1.08	0.287			
CD31+Ki67+	0.90	0.50-1.63	0.724			
D240+	1.09	1.01-1.18	0.030	1.10	1.01-1.19	0.025
D240+SOX18+	0.78	0.29-2.11	0.627			
D240+Ki67+	0.84	0.13-5.39	0.857	0.78	0-12-4.95	0.795
CD31+D2-40+SOX18+	0.91	0.78-1.08	0.278	0.91	0.77-1.07	0.237
CD31+D2-40+Ki67+	0.90	0.51-1.57	0.700			

Discussion

Cutaneous melanoma is the most aggressive and deadly form of skin cancer, causing 80% of skin cancer deaths even though it represents less than 5% of all skin cancers.[116] Over the last two decades, there has been significant increase in education and awareness leading to risk reduction, prevention and early detection of melanoma. More recently, discovery of melanoma genetics, targeted therapy and immunotherapy have improved patient survival.[74-75] However, these adjuvant therapies come with their own toxicities and adverse effects. Therefore, it is important to be able to further stratify patients within the same AJCC stage beyond our current capabilities, and identify the ones with aggressive melanoma at the time of diagnosis to appropriately undergo adjuvant treatment. Currently adjuvant therapy decision is made mainly based on sentinel node status.

Lymphatic vasculature has emerged to play a key role in regional lymph node and distant metastasises. Key routes of spreading to regional and distant lymph nodes include direct invasion of existing lymphatic vessels by tumour cells or via lymphangiogenesis induced by the tumour itself through expression of various transcription or growth factors such as SOX18 and VEGF.[104-105,117] Lymphatic vessel density have also been associated with nodal metastasis.[117] We included D2-40 in our biomarker panel as it is an endothelial marker highly specific for lymphatics and has been shown to be more effective in detecting lymphatic invasion compared to conventional haematoxylin and eosin staining.[103,118-119] D2-40 has been utilised to highlight lymphatic vessel invasion in melanoma as well as other cancers such as breast, oesophageal and endometrial cancers.[120-122]

Our panel of protein biomarker also included SOX18, a transcription factor involved in the development of embryonic vascular and lymphatic vessels and hair follicles.[54] Consequently, a disruption in SOX18 expression has been demonstrated in hypotrichosis-

lymphoedema-telangiectasia syndrome, characterized by hair loss, swelling of the extremities due to lymphatic vessel leakage and widening of small blood vessels.[123] In adults, SOX18 expression has been demonstrated in specific settings such as wound healing and tumour growth, whereby it is seen in endothelial nuclei and tumour cell nuclei.[54] SOX18 is involved in increased endothelial cell migration and fusion. It activates endothelial cells through the PROX-1 (Prospero Homeobox 1) and VEGF-Flk1 pathway, activates VCAM1 (vascular cell adhesion molecule 1) expression required for endothelial cell function, and interacts with MEF2C (myocyte enhancer factor 2C), a muscle and endothelial transcription factor.[115,117,124,125] SOX18 expression has been observed in different types of cancers including melanoma, non-melanoma skin cancers, lung cancer and breast cancer.[113,126-129] Overman et al[130] found that supressing SOX18 activity interfered with vascular development and improved survival in an animal model of breast cancer by reducing vascular density and reducing metastatic spread. Duong et al[113] had similar findings where SOX18-mutant mice had a lower rate of lymphangiogenesis and metastasis.

In this study, we found that patients with SOX18 in their vasculature had better prognostic features such as lower Breslow thickness (p=0.022), lower rate of positive SLN (p=0.003) and recurrence (p=0.002). One theory for the different findings compared to existing literature is that SOX18 expression increased angiogenesis and lymphangiogenesis, resulting in increased migration of immune cells to tumour and lymph node sites, inhibiting tumour growth and subsequently led to better outcomes. To explore if SOX18 plays a role in tumour immune function, we examined the association between SOX18 and immune markers including STAT5 and CD163 and found no significant associations (Table 1), although we only had information on STAT5 staining for 166 patients and CD163 staining for 174 patients. Correlation with other immune markers would be beneficial to further explore this theory. Another theory is that the SOX18 expression seen is largely from mature endothelial cells, that is the definitive differentiated population rather than the endovascular progenitor population that has self-renewal and proliferative potential.[112,131] This is based on the findings from our group that endothelial cells have distinct hierarchal populations with different function - endovascular progenitor, transit amplifying and definitive differentiated populations.[112,131]

On multivariate analysis, the presence of Ki67+ nuclei in D2-40+ vessels was an independent predictor of SLN metastasis and melanoma death. This is consistent with the

existing literature on Ki67, a proliferation marker that has been previously studied as a potential biomarker of melanoma progression.[36,40,132] Ki67 is associate with poor melanoma survival.[36,40,132] This is a novel finding where combination of the two biomarkers Ki67 and D2-40 has been shown to independently and significantly predict SLN metastasis and melanoma death in a cohort of melanoma patients. Our study limitations include having a single person performing the IHC scoring and the lack of a validation cohort.

In conclusion, we studied a panel of biomarkers including SOX18, Ki67, D2-40, CD31 to explore their association with SLN metastasis and melanoma death. Positive SOX18 was associated with lower SLN metastasis and melanoma death, opening new avenues to explore the role of immune factors and the relationship with different subpopulation of endothelial cells. The presence of Ki67+ nuclei in D2-40+ vessels was an independent predictor of SLN metastasis and melanoma death, further reinforcing existing knowledge on the separate biomarkers Ki67 and D2-40, but is a novel finding as a combination biomarker in a cohort of melanoma patients. Most markers used in our study are routinely used in pathology laboratories, making their clinical implementation rather feasible.

Supplementary materials and methods

Staining protocol for multiplex panel

Antibodies:

1. SOX18
2. Melanoma
3. CD31
4. D2-40
5. Ki67
6. DAPI

Protocol:

1. Dewax slides. Standard dewax protocol (program 3 on the Leica XL stainer) Tissue treatment: Xylene: 1x3 min 2x1min, Ethanol: 100% 2x1min, 90% 1 x 1 min, 70% 1 x 1 min, running water 3 minutes and RO water briefly.
2. Condition slides with a 10 minutes' fix in 10% neutral buffered formalin followed by a 10-minute wash in running water.
3. Endogenous peroxidase is quenched by incubating sections in 0.5% hydrogen peroxide in TBS buffer for 5 minutes.
4. Wash slides with distilled water.
5. Place slides in 250ml 1X Biocare Medical DIVA Retrieval Buffer (Diva 10X DV2004MX) and microwave (LG microwave MS2540SR 1250W) for 2 minutes 20 seconds with 100% power and then continuing boil for 15 minutes with 20% power. On completion remove container of slides and allow to cool for a further 20 minutes on the bench.
6. Wash slides in TBS (Tris Base/NaCl pH7.6) plus 0.025% Tween 20 (Sigma - P1379-4L) (TBSTW) 3 x 1minute.
7. Cover tissue with blocking solution, Biocare Medical Background Sniper (BIC-BS96MM) + 2% BSA (Sigma A7906-100G) for 10 min.
8. Aspirate blocking solution and add primary antibody anti-Sox18 diluted 1:1000 in Biocare Medical Da Vinci Green PBS pH7.3 (PD900 M) to each slide for 60 minutes at room temperature.
9. Wash slides in TBSTW for 3 x 2minutes.
10. Incubate tissue with Biocare Medical Mach2 anti-Mouse HRP Polymer (BIC-MHRP520L) for 30 minutes.
11. Wash slides with TBSTW 3x 2 minutes.

12. Cover slides with TSA-OPAL 620(1:100) (Perkin Elmer - FP1495A) for 10 min.
13. Wash slides with TBSTW 3x 2 minutes.
14. Rinse slides with distilled water.
15. Place slides in 250ml 1X Biocare Medical DIVA Retrieval Buffer (Diva 10X DV2004MX) and microwave (LG microwave MS2540SR 1250W) for 2 minutes 20 seconds with 100% power and then continuing boil for 15 minutes with 20% power. On completion remove container of slides and allow to cool for a further 20 minutes on the bench.
16. Wash slides in TBS plus 0.025% Tween 20 (TBSTW) 3 x 1minute.
17. Cover tissue with blocking solution, Biocare Medical Background Sniper (BIC-BS96MM) + 2% BSA (Sigma A7906-100G) for 10 min.
18. Aspirate blocking solution and add primary antibody, mouse anti-Melanoma (Abcam - ab732) diluted 1:1400 in Biocare Medical Da Vinci Green PBS pH7.3 (PD900 M) to each slide for 30 minutes at room temperature.
19. Wash slides in TBSTW for 3 x 2minutes.
20. Incubate tissue with Perkin Elmer HRP conjugated Goat anti-Mouse (NEF22001EA) diluted 1:1000 in TBSTW for 15 minutes.
21. Wash slides with TBSTW 3x 2 minutes.
22. Cover slides with TSA-OPAL 690(1:100) (Perkin Elmer - FP1497001KT) for 10 min.
23. Wash slides with TBSTW 3x 2 minutes.
24. Rinse slides with distilled water.
25. Place slides in 250ml 1X Biocare Medical DIVA Retrieval Buffer (Diva 10X DV2004MX) and microwave (LG microwave MS2540SR 1250W) for 2 minutes 20 seconds with 100% power and then continuing boil for 15 minutes with 20% power. On completion remove container of slides and allow to cool for a further 20 minutes on the bench.
26. Wash slides in TBSTW 3 x 1 minute.
27. Cover tissue with blocking solution, Biocare Medical Background Sniper (BIC-BS96MM) + 2% BSA (Sigma A7906-100G) for 10 min.
28. Aspirate blocking solution and add primary antibody, anti-CD31 diluted 1:400 in Biocare Medical Da Vinci green PBS pH7.3 (PD900 M) to each slide for 60 minutes at room temperature.
29. Wash slides in TBSTW for 3 x 2minutes.
30. Incubate tissue with Perkin Elmer HRP conjugated Goat anti-Mouse (NEF22001EA) diluted 1:500 in TBSTW for 20 minutes.

31. Wash slides with TBSTW 3x 2 minutes.
32. Cover slides with TSA-OPAL 570(1:100) (Perkin Elmer - FP1488A) for 15 min.
33. Wash slides with TBSTW 3x 2 minutes.
34. Rinse slides with distilled water.
35. Place slides in 250ml 1X Biocare Medical DIVA Retrieval Buffer (Diva 10X DV2004MX) and microwave (LG microwave MS2540SR 1250W) for 2 minutes 20 seconds with 100% power and then continuing boil for 15 minutes with 20% power. On completion remove container of slides and allow to cool for a further 20 minutes on the bench.
36. Wash slides in TBSTW 3 x 1 minute.
37. Cover tissue with blocking solution, Biocare Medical Background Sniper (BIC-BS96MM) + 2% BSA (Sigma A7906-100G) for 10 min.
38. Aspirate blocking solution and add primary antibody, mouse anti-D2-40 (Biocare Medical - CM266A) diluted 1:800 in Biocare Medical Van Gough yellow PBS pH6.0 (PD902 M) to each slide for 60 minutes at room temperature.
39. Wash slides in TBSTW for 3x 2minutes
40. Incubate tissue with Perkin Elmer HRP conjugated Goat anti-Mouse (NEF22001EA) diluted 1:1000 in TBSTW for 30 minutes.
41. Wash slides with TBSTW 3x 2 minutes.
42. Cover slides with TSA-OPAL 650 (1:100) (Perkin Elmer - FP1496A) for 10 min.
43. Wash slides with TBSTW 3x 2 minutes.
44. Rinse slides with distilled water.
45. Place slides in 250ml 1X DAKO Target Retrieval Solution Citrate pH6.0 (10X S169984-2) and heat retrieve in a Biocare Medical Decloaking Chamber (DC2002) programmed to run for 5 minutes at 125^{o}C then cool to 90 ^{o}C. On completion remove container of slides and allow to cool for a further 20 minutes on the bench.
46. Wash slides in TBSTW 3 x 1 minute.
47. Cover tissue with blocking solution, Biocare Medical Background Sniper + 2% BSA for 10 min.
48. Aspirate blocking solution and add primary antibody, mouse anti-Ki67 (DAKO - M7240) diluted 1:1400 in Biocare Medical Van Gough yellow PBS pH6.0 (PD902 M) to each slide for 45 minutes at room temperature.
49. Wash slides in TBSTW for 3 x 2minutes.
50. Incubate tissue with Perkin Elmer Opal Polymer HRP (ARH1001EA) for 20 minutes.
51. Wash slides with TBSTW 3x 2 minutes.

52. Cover slides with TSA-OPAL 520 (1:50) (Perkin Elmer - FP1487A) for 10 min.
53. Wash slides with TBS_{TW} 3x 2 minutes.
54. Rinse slides with distilled water.
55. Transfer slides to 250ml citrate pH6.0 stripping buffer and microwave (LG microwave MS2540SR 1250W) for 2 minutes 20 seconds with 100% power and then continuing boil for 15 minutes with 20% power. On completion remove container of slides and allow to cool for a further 20 minutes on the bench.
56. Wash slides with 1x $TBST_w$ for 2x 3min.
57. Counterstain sections with DAPI (5mg/ml stock diluted 1:35000 in TBS pH7.6) for 2 min.
58. Rinse slides twice with 1xTBS.
59. Coverslip with Dako Fluorescent Mounting Medium (S3023).
60. Take to Vectra Imaging system for scanning.

Chapter 5:

Discussion and conclusion

Key findings

Overall, this study aimed to identify prognostic factors that could better inform melanoma prognosis beyond the current staging system. One of the challenges in melanoma is to accurately predict tumour behaviour and progression. In a study of 583 patients with melanoma less than 0.76mm thickness and without metastases by Slingluff et al[133], 4.8% progressed to metastatic disease after a mean follow-up of 3.6 years. Early stage melanoma can usually can be cured by complete surgical resection while patients with advanced melanoma typically require surgical resection and targeted or immunotherapy, with rather poor overall prognosis despite recent advancement in therapies. Therefore, it would be ideal if we are able to identify patients with thin melanoma that are at risk of progressing to metastatic disease at the time of diagnosis, to be able to intervene and prevent this progression. Although SLNB is a powerful prognostic tool, it has specific limitations, with significant morbidity and costs associated. Tumour-associated biomarkers are thought to have great potential in identifying early stage melanoma patients who are likely to develop advanced disease and therefore would potentially benefit from adjuvant therapy. By doing this study, we aimed to identify promising biomarkers that could warrant further large-scale studies and eventually be translated into clinical practice. We also investigated the association between having multiple invasive melanomas and survival outcome, with the hope that it will provide better prognostication for patients and guide melanoma surveillance in these selected patient population.

Chapter two examined the role of a previous primary invasive melanoma as an additional criterion beyond TNM-based melanoma staging by investigating its association with SLN invasion. One area that was lacking in the published literature was the effect of previous invasive melanoma on SLN metastasis of subsequent invasive melanoma. We explored this and found additional stage II melanoma was an independent and significant predictor of SLN metastasis. We also found association between additional stage II melanoma and poorer melanoma-specific survival, which was not found in those with additional stage I melanoma, consistent with findings from current literature.

Chapters three and four investigated protein biomarkers that reflect tumour immune response and vascularisation to gain insight into the disease behaviour that may predict melanoma outcome. In chapter three, we assessed STAT5 expression and found it was a protective factor, showing independent and inverse association with melanoma-specific death and recurrence. In chapter four, we assessed SOX18 and proliferation (Ki67) in the

endothelial cells of lymphatic and blood vessels as determined by vessels positively stained by CD31 and D2-40, and studied their association with SLN status and melanoma survival. Ki67+ nuclei in D2-40+ vessels reflecting proliferative lymphatic vessels was an independent predictor of SLN metastasis and melanoma death. SOX18+ nuclei in CD31+ and D2-40+ vessels tended to be associated with better outcomes. This confirms melanoma vascularisation can predict tumour and patient outcomes.

Our melanoma tissue samples comprised of whole-section slides and formalin-fixed, paraffin-embedded blocks collected between 1994-2011. Given the age of these samples, there were some variability in staining quality. We mitigated this issue by excluding patients whom tissue samples were of very poor staining quality, and also implemented manual scoring method as described below.

Commercially available systems providing automated analysis and scoring of digitalised slide images have been widely used to classify diseased tissue areas and quantify IHC staining.[134-135] Given the heterogeneous nature of our tumour samples and problems with some of the tissue sample quality which ultimately affected the staining quality, implementing an automated analysis and scoring system was slightly time consuming but more importantly, rather inaccurate. Manual scoring was performed to achieve better results. Areas of interest or hotspots within each whole slide section were selected for IHC scoring. This method of recognition of candidate hotspots could be somewhat subjective. To overcome this potential bias, multiple hotspots were selected for each whole slide section and the average and total scores from these hotspots were then used for statistical analysis.

Clinical implications

Our findings where patients with multiple invasive melanomas of at least the same stage have higher risk of SLN metastasis and melanoma death pose substantial implications in clinical practice. All patients should be encouraged to do regular self-examination, especially patients with a past history of invasive melanoma.[136] Patients with multiple invasive melanomas should be educated on their increased risk of having subsequent invasive melanomas, SLN metastasis, and the importance of early detection and treatment in improving survival. In addition to recognising new or changing lesions on their skin, they should also be taught to examine the draining lymph node sites for any lumps. It is

important that clinicians obtain a thorough history of patient's past melanoma diagnoses and take that into account when devising a follow-up plan. These high-risk patients would benefit from more frequent monitoring and any clinical suspicions should certainly trigger prompt investigations to detect disease progression sooner rather than later.

An ideal biomarker is one that is measurable and rapidly analysable in the tumour cell, sensitive, specific, cost effective, ideally non-invasive and should add value to current system. Our panel of biomarkers included CD31, D2-40, Ki67, SOX18, STAT5, p16 and CD163, all of which could be made accessible in pathology laboratories and tested on tumour tissue at the time of diagnosis. This prevents having to subject patients to further invasive procedures. Although we have some promising results, proper validation of these biomarkers in independent patient cohorts is needed to interrogate the behaviour of these biomarkers and allow further understanding of the molecular basis and function of these biomarkers. The discovery of new prognostic biomarkers could potentially guide patient selection for SLNB, replace SLNB in patients who are deemed not suitable to undergo the procedure, or used in conjunction with SLNB to select appropriate high-risk patients for adjuvant therapy. This study is a small step forward in the direction of improving risk stratification in melanoma patients.

Our study demonstrated investigations that can be easily implemented in existing histopathology routines and therefore is readily accessible to the broader community. Despite certain limitations such as a small study cohort, in general they are feasible tests that can be used in the general population.

Conclusion

In conclusion, we investigated clinical and protein factors in a cohort of melanoma patients to determine if any relationship with melanoma outcome exists. We made novel discovery in the pattern of melanoma progression in terms of sentinel lymph node metastasis in patients with multiple invasive melanomas. We also gained insight into the role of immune activation and tumour vascularisation in melanoma progression and their ability to predict tumour and patient outcome. This study forms the basis for further research with the hopes to ultimately improve patient outcome.

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Appendices

Appendix 1: Ethics approval

Princess Alexandra Hospital
Health Service District

Queensland Government

Queensland Health

Office of the Human Research Ethics Committee

Enquiries to: Ethics Manager
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Date 24 November 2009

A/Prof Kiarash Khosrotehrani
University of Queensland Centre for Clinical Research
Royal Brisbane Hospital, Bldg 71/918
Herston QLD 4029

APPROVAL LETTER – PRINCESS ALEXANDRA HOSPITAL

Dear A/Prof Khosrotehrani

HREC Reference number: HREC/09/QPAH/217

Project title: Molecular markers of sentinel node invasion in melanoma	
NEAF	**Version 2.0**
Participant Information and Consent Form:	**Version 1, Dated 14 September 2009**
Protocol	

At a meeting of the Metro South Health Service District Human Research Ethics Committee (MSHSD HREC) held on 01 September 2009, the Committee reviewed the above research Protocol. The Metro South Health Service District Human Research Ethics Committee is duly constituted, operates in accordance and complies with the current National Health and Medical Research Council's *National Statement on Ethical Conduct in Human Research 2007*.

On the recommendation of the Human Research Ethics Committee approval is granted for your project to proceed. This approval is subject to researcher(s) compliance throughout the duration of the research with certain requirements as outlined in the *National Statement on Ethical Conduct in Human Research 2007 and Australian Code for the Responsible Conduct of Research.*

The following links have been provided for your convenience:
http://www.nhmrc.gov.au/_files_nhmrc/file/publications/synopses/e72-jul09.pdf
http://www.nhmrc.gov.au/_files_nhmrc/file/publications/synopses/r39.pdf

Some requirements are briefly outlined below. Please ensure that you communicate with the HREC on the following:

- ***Protocol Changes:*** Substantial changes made to the protocol require HREC approval.
- ***Problems and SAEs:*** The HREC must be informed of any problems that arise during the course of the study which may have ethical implications. Serious adverse events must be notified to the HREC as soon as possible.
- ***Lapsed Approval***: If the study has not commenced within twelve months approval will lapse requiring resubmission of the study to the HREC.

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