Clinical and histopathological correlations of melanoma in the molecular era

Louise A. Jackett

A thesis submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy.

Principal Supervisor: Professor Richard A. Scolyer AO BMedSc MBBS MD FRCPA FRCPath

Co-supervisor: Professor Georgina V. Long AO BSc PhD MBBS FRACP

Faculty of Medicine and Health

The University of Sydney

2025

ABSTRACT

In just over a decade the outlook for patients with melanoma, particularly those with metastatic disease, has radically altered due to a comprehensive understanding of melanoma's genomic topography and the development of effective targeted and immune-based treatments.[2,3] Foundations of this progress were laid by pathological observations of "fatal black tumours with metastases" that span centuries.[4-6] In the current 'molecular era', assessment of tissue specimens by pathologists continues to be central to understanding primary and metastatic melanoma and guiding management.

The humble light-microscopic evaluation of morphological features such as Breslow/tumour thickness, which is derived from observations that melanoma progression is a function of tissue depth of invasion, remains critical to prognostic estimates and surgical decision making, even in the face of advanced genomic data.[7-10] There is, however, a lack of rigorous study of variation of all prognostic parameters across tissue levels and features of residual disease in surgical beds.

At the other end of the spectrum, pathologists are faced with the promise, and challenges, of integrating novel complex genomic data with morphology to improve the diagnosis of melanocytic lesions. Such techniques offer a chance to improve over-diagnosis, under-diagnosis and misdiagnosis of melanoma and other similar tumours that exist in daily practice, including benign versus malignant melanocytic lesions, and melanoma versus tumours of other histogenesis. To grasp the potential of molecular data to improve the diagnosis of ambiguous lesions, there is a need to understand the sensitivity and specificity of these tests, interpreted in the context of clinical and pathological data.

Finally, as promising oncogenic therapeutic targets continue to be identified, pathologists will play an increasing role in the use of tissue to guide treatment selection and its effectiveness. An understanding of the biological and quality aspects of emergent predictive biomarkers will be key to ensure patient tissues are used appropriately for such purposes and will ultimately influence patient outcomes.

The ensuing body of work evaluates novel aspects of both established techniques and evolving technologies in the pathological evaluation of melanoma in our molecular era.

(Characters: 2000, limit 2000)

"As to the remote and exciting causes of melanosis, we are quite in the dark, nor can more be said of the methodus medendi*. We are hence forced to confess the incompetency of our knowledge of the disease under consideration, and to leave to future investigators the merit of revealing the laws which govern its origin and progress….and pointing out the means by which its ravages may be prevented or repressed."*

- Thomas Fawdington, The Manchester Royal Infirmary, 1826.[1]

TABLE OF CONTENTS

DECLARATION OF AUTHORSHIP AND FUNDING

I, Louise A. Jackett, declare that this thesis titled, **"Clinicopathological correlations of melanoma in the molecular era"** and the work presented in it are my own. This thesis is submitted to the University of Sydney in fulfillment of the requirement for the Doctor of Philosophy. The work described in this thesis was performed between March 2017 and February 2025 (part-time) in the Faculty of Medicine and Health, University of Sydney under the supervision of Professor Richard A. Scolyer AO.

The thesis contains original papers published in, or submitted to, international peer reviewed journals and a textbook. Declarations, with support of my co-authors, describing the contributions made by me to the research and manuscript preparation can be found in the appendix. A list of publications related to this thesis, to which I have contributed and which in turn have informed my collaborations, is also included before the main chapters.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text.

I hereby declare that I have not submitted any material presented in this thesis, either in part or in full, for a degree at this or any other institution.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in its preparation and sources have been acknowledged.

The research reported in this thesis was supported by the award of a Research Training Program scholarship to the PhD Candidate.

Signed:

Louise A. Jackett, 28 February 2025

ACKNOWLEDGEMENTS

To my **father**, who was of the gentlest nature and thoughtful wisdom. An incredible diagnostician who demonstrated exemplary care and kindness to generations of patients, he inspired in me a curiosity of the natural world and human condition. I am indebted to his quiet nudges and support along my journey in the beginnings of this PhD, as in all areas of my life, and I know he would be proud to see the culmination of these efforts.

To my **mother**, **brother**, **sister-in-law**, **partner** and countless **friends** who have always championed my studies and have unconditionally, and patiently, understood the division of time and mental load.

To **Prof Richard Scolyer AO**, who is the most remarkable person I know. I have been the recipient of unparalleled generosity in his time, mentoring and advocacy from someone who demonstrates humility in all avenues of collaboration, and possesses super-human resilience.

To **Prof Georgina Long AO** and colleagues at **Melanoma Institute Australia** and **Royal Prince Alfred Hospital, Sydney**, who together with Richard have created a culture where bold ideas are celebrated and the pursuit of understanding is limitless. I am privileged to have worked with these teams, in which brilliant minds seem to augment each other in ways that mean the results of collaborations are realised faster and faster every day.

To my **mentors** from my schooling and training years, and my consultant **peers** at **Austin Hospital** and **Peter MacCallum Cancer Centre**, from whom I continue to learn every day.

Finally, to our **patients**, whose selfless contributions to the research in the following pages allows others unknown to them to be better off by their generosity.

LIST OF PUBLICATIONS PRODUCED FOR THIS THESIS

Peer-reviewed publications accepted for publication

Jackett LA, Scolyer RA. A Review of Key Biological and Molecular Events Underpinning Transformation of Melanocytes to Primary and Metastatic Melanoma. Cancers (Basel). 2019 Dec 17;11(12):2041. doi: 10.3390/cancers11122041. PMID: 31861163; PMCID: PMC6966527.

Jackett LA, Gullifer JP, Scolyer RA. Evaluation of Multiple Tissue Levels Frequently Upstages Patients With Clinically Localized Thin Primary Cutaneous Melanoma. J Cutan Pathol. 2024 Oct 2. doi: 10.1111/cup.14726. Epub ahead of print. PMID: 39357975.

Jackett LA, Satgunaseelan L, Roper E, Lo SN, Thompson JF, Scolyer RA. Residual melanoma in wide local excision specimens after 'complete' excision of primary cutaneous in situ and invasive melanomas. Pathology. 2022 Feb;54(1):71-78. doi: 10.1016/j.pathol.2021.05.094. Epub 2021 Aug 13. PMID: 34392983.

Jackett LA, Mitchell C, Snell C, Hewitt C, Yellenki S, Snow H, Speakman D, Angel C, Khoo C, Pang J, Lo S, Scolyer RA, Fox S, Gyorki D. Molecular analysis of cutaneous sarcomatoid neoplasms frequently identifies melanoma driver variants. *Accepted to American Journal of Surgical Pathology, 22 February 2025 (manuscript reference AJSP-D-24-00590R1) .*

Jackett LA, Colebatch AJ, Rawson RV, Ferguson PM, Thompson JF, McCarthy SW, Wilmott JS, Scolyer RA. Molecular Profiling of Noncoding Mutations Distinguishes Nevoid Melanomas From Mitotically Active Nevi in Pregnancy. Am J Surg Pathol. 2020 Mar;44(3):357-367. doi: 10.1097/PAS.0000000000001406. PMID: 31743128.

Publications submitted to peer-reviewed journals

Jackett LA, Mitchell C, Chu J. Extraenteric gastrointestinal neuroectodermal tumour masquerading as mucosal melanoma of the bronchus. Submitted to: Pathology, February 2025

Jackett LA, Conway J, Thompson JF, Scolyer RA. Assessment of intrapatient homogeneity of PRAME immunohistochemistry expression in melanoma. Submitted to: Modern Pathology, February 2025.

Textbook publication

Jackett LA, Scolyer RA, Bishop JF, Thompson JF (2017) Primary melanoma of the lung, In: Raghaven D, Ahluwalia MS, Blanke CD, Brown J, Kim ES, Reaman GH, Sekered MA (eds) Textbook of Uncommon Cancer 5th Edition, Wiley-Blackwell, west Essex, pp 285–292

RELATED PUBLICATIONS PRODUCED DURING THIS THESIS

Machado P, Fuzzard S, Hamilton S, **Jackett L**, Fox C. An ear-piercing melanoma in situ. ANZ J Surg. 2024 Feb;94(1-2):272-273. doi: 10.1111/ans.18758. Epub 2023 Nov 3. PMID: 37921231.

Gupta R, Selinger CI, Ashford B, Chua MST, Clark JR, Damian DL, **Jackett LA**, James C, Johnson S, Ladwa R, Lambie D, McKenzie C, Tan ST, Scolyer RA. Implementing structured pathology reporting protocol for non-melanocytic skin cancers: practical considerations. Pathology. 2023 Oct;55(6):743-759.

Lam GT, Sorvina A, Martini C, Prabhakaran S, Ung BS, Lazniewska J, Moore CR, Beck AR, Hopkins AM, Johnson IRD, Caruso MC, Hickey SM, Brooks RD, **Jackett L**, Karageorgos L, Foster-Smith EJ, Malone V, Klebe S, O'Leary JJ, Brooks DA, Logan JM. Altered endosomal-lysosomal biogenesis in melanoma. Neoplasia. 2023 Sep;43:100924. doi: 10.1016/j.neo.2023.100924. Epub 2023 Aug 9. PMID: 37562257; PMCID: PMC10423698.

Wong AS, du Plessis JJ, **Jackett LA**. Merkel cell carcinoma: an unusual case with SOX10 nuclear staining. Pathology. 2023 Aug;55(5):718-721. doi: 10.1016/j.pathol.2022.12.352. Epub 2023 Feb 23. PMID: 36925343.

Lam GT, Prabhakaran S, Sorvina A, Martini C, Ung BS, Karageorgos L, Hickey SM, Lazniewska J, Johnson IRD, Williams DB, Klebe S, Malone V, O'Leary JJ, **Jackett L**, Brooks DA, Logan JM. Pitfalls in Cutaneous Melanoma Diagnosis and the Need for New Reliable Markers. Mol Diagn Ther. 2023 Jan;27(1):49-60. doi: 10.1007/s40291-022-00628-9. Epub 2022 Dec 7. PMID: 36477449.

Star P, **Jackett LA**, Cheung K, Wilmott JS, Ho G, Smith A, Long GV, Scolyer RA, Martin LK. Multiple eruptive squamoproliferative lesions during anti-PD1 immunotherapy for metastatic melanoma: Pathogenesis, immunohistochemical analysis and treatment. Dermatol Ther. 2022 Jun;35(6):e15472. doi: 10.1111/dth.15472. Epub 2022 Apr 5. PMID: 35347815.

Scolyer RA, Atkinson V, Gyorki DE, Lambie D, O'Toole S, Saw RPM, Amanuel B, Angel CM, Button-Sloan AE, Carlino MS, Ch'ng S, Colebatch AJ, Daneshvar D, Pires da Silva I, Dawson T, Ferguson PM, Foster-Smith E, Fox SB, Gill AJ, Gupta R, Henderson MA, Hong AM, Howle JR, **Jackett LA**, James C, Lee CS, Lochhead A, Loh D, McArthur GA, McLean CA, Menzies AM, Nieweg OE, O'Brien BH, Pennington TE, Potter AJ, Prakash S, Rawson RV, Read RL, Rtshiladze MA, Shannon KF, Smithers BM, Spillane AJ, Stretch JR, Thompson JF, Tucker P, Varey AHR, Vilain RE, Wood BA, Long GV. BRAF mutation testing for patients diagnosed with stage III or stage IV melanoma: practical guidance for the Australian setting. Pathology. 2022 Feb;54(1):6-19. doi: 10.1016/j.pathol.2021.11.002. Epub 2021 Dec 20. PMID: 34937664.

Klein O, Kee D, Gao B, Markman B, da Gama Duarte J, Quigley L, **Jackett L**, Linklater R, Strickland A, Scott C, Mileshkin L, Palmer J, Carlino M, Behren A, Cebon J. Combination immunotherapy with nivolumab and ipilimumab in patients with rare gynecological malignancies: results of the CA209-

538 clinical trial. J Immunother Cancer. 2021 Nov;9(11):e003156. doi: 10.1136/jitc-2021-003156. PMID: 34782426; PMCID: PMC8593709.

Robertson SJ, Orme L, Teixeira R, Shamassi M, Newell F, Patch AM, Yeh I, Gard G, Wilmott J, **Jackett L**, LeBoit P, Fellowes A, MacArthur G, Fox S, Hayward NK, Bastian B, Scolyer R, Waddell N, Penington A, Shackleton M. Evaluation of Crizotinib Treatment in a Patient With Unresectable GOPC-ROS1 Fusion Agminated Spitz Nevi. JAMA Dermatol. 2021 Jul 1;157(7):836-841. doi: 10.1001/jamadermatol.2021.0025.

Guo D, Lui GYL, Lai SL, Wilmott JS, Tikoo S, **Jackett LA**, Quek C, Brown DL, Sharp DM, Kwan RYQ, Chacon D, Wong JH, Beck D, van Geldermalsen M, Holst J, Thompson JF, Mann GJ, Scolyer RA, Stow JL, Weninger W, Haass NK, Beaumont KA. RAB27A promotes melanoma cell invasion and metastasis via regulation of pro-invasive exosomes. Int J Cancer. 2019 Jun 15;144(12):3070-3085.

Dodds TJ, Wilmott JS, **Jackett LA**, Lo SN, Long GV, Thompson JF, Scolyer RA. Primary anorectal melanoma: clinical, immunohistology and DNA analysis of 43 cases. Pathology. 2019 Jan;51(1):39-45.

Allanson BM, Weber MA, **Jackett LA**, Chan C, Lau L, Ziegler DS, Warby M, Mayoh C, Cowley MJ, Tucker KM, Long GV, Maher A, Anazodo A, Scolyer RA. Oral malignant gastrointestinal neuroectodermal tumour with junctional component mimicking mucosal melanoma. Pathology. 2018 Oct;50(6):648-653.

Liu RC, Sebaratnam DF, **Jackett L**, Kao S, Lowe PM. Subacute cutaneous lupus erythematosus induced by nivolumab. Australas J Dermatol. 2018 May;59(2):e152-e154

Dodds TJ, Lo S, **Jackett L**, Nieweg O, Thompson JF, Scolyer RA. Prognostic Significance of Periadnexal Extension in Cutaneous Melanoma and its Implications for Pathologic Reporting and Staging. Am J Surg Pathol. 2018 Mar;42(3):359-366.

Kakavand H, **Jackett LA**, Menzies AM, Gide TN, Carlino MS, Saw RPM, Thompson JF, Wilmott JS, Long GV, Scolyer RA. Negative immune checkpoint regulation by VISTA: a mechanism of acquired resistance to anti-PD-1 therapy in metastatic melanoma patients. Mod Pathol. 2017 Aug 4. doi: 10.1038/modpathol.2017.89.

Rocha L, Menzies SW, Lo S, Avramidis M, Khoury R, **Jackett L**, Guitera P. Analysis of an electrical impedance spectroscopy system in short-term digital dermoscopy imaging of melanocytic lesions. Br J Dermatol. 2017 Apr 19.

Griewank KG, Müller H, **Jackett LA**, Emberger M, Möller I, van de Nes JA, Zimmer L, Livingstone E, Wiesner T, Scholz SL, Cosgarea I, Sucker A, Schimming T, Hillen U, Schilling B, Paschen A, Reis H, Mentzel T, Kutzner H, Rütten A, Murali R, Scolyer RA, Schadendorf D. SF3B1 and BAP1 mutations in blue nevus-like melanoma. Mod Pathol. 2017 Jul;30(7)928-39.

ABBREVIATIONS

AFX	Atypical fibroxanthoma
AJCC	American Joint Committee on Cancer
ALM	Acral lentiginous melanoma
AI	Artificial intelligence
BT	Breslow thickness
CCS	Clear cell sarcoma
CCSLT	Clear cell sarcoma-like tumour
CEB	Complete excision biopsy
CNV	Copy number variations
CSD	Chronic sun damage
CT	Computed tomography
ctDNA	Circulating tumour DNA
DM	Dedifferentiated melanoma
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridisation
GNET	Gastrointestinal neuroectodermal tumour
H&E	Haematoxylin and eosin
IFN-y	Interferon-gamma
ICCR	International Collaboration on Cancer Reporting
IHC	Immunohistochemistry
LM	Lentigo maligna
LMM	Lentigo maligna melanoma
LNM	Lymph node metastasis
MANP	Mitotically active naevus of pregnancy
MAPK	Mitogen-activate protein kinase

MM	Metastatic melanoma
MRI	Magnetic resonance imaging
NGS	Next-generation sequencing
NM	Nodular melanoma
NM (chapter 6)	Naevoid melanoma
PDS	Pleomorphic dermal sarcoma
PEM	Pigmented epithelioid melanocytoma
PET	Positron emission topography
PM	Primary melanoma
PRAME	PReferentially expressed Antigen in MElanoma
pT	Pathological T stage (AJCC)
SABR	Stereotactic ablative body radiotherapy
SSM	Superficial spreading melanoma
TCR	T-cell receptor
TERT-p	TElomerase Reverse Transcriptase promoter mutation
UM	Undifferentiated melanoma
UPS	Undifferentiated pleomorphic sarcoma
UV	Ultraviolet
WLE	Wide local excision

GENERAL INTRODUCTION

CHAPTER 1: OUTLINE AND AIMS OF THESIS

The outlook for patients with melanoma, particularly those with metastatic disease, has changed radically since two landmark drug therapies burst on to the scene in 2011, paving the way for an expanding repertoire of highly effective targeted and immune-based treatments.[2,3] The integration of clinical and pathological features determined by anatomical pathologists, trained in the art and science of observing the facets of melanoma morphology in tissue samples over time, has been fundamental to our understanding of the biological basis of melanoma, which has in turn paved the way for the profound advancements in treatment for melanoma patients.[11]

However, many aspects of melanoma pathological assessment continue to be difficult and subjective. Diagnostic certainty is prone to differences in opinion between pathologists, which is exacerbated for certain types of melanocytic lesion where there is a lack of clear diagnostic criteria.[12] There is an enduring need to improve objectivity in the pathological evaluation of melanoma and melanoma-like lesions in day-to-day practice; understanding correlations between clinical, pathological and genomic information is important to achieving to this outcome. *Critical inquiry into how novel genomic information should be integrated into the clinicopathological evaluation of melanoma and melanoma-like lesions, alongside the re-appraisal of some established processes in daily pathology practice of these lesions, are the principal subjects of this thesis.*

Pathologists have been firmly practicing in the era of molecular medicine for more than a decade, yet there is still much to be learned about the integration of molecular data into the diagnosis of melanoma.[13] The need for a better understanding of the utility and limitations of integrating genomic information with clinical and pathological features is even more urgent as the era of artificial intelligence (AI) begins to boom, and there already exists proof of principle for convolutional neural networks to be trained on digitised haematoxylin and eosin (H&E) slides.[14] But as data scientists

continue to discover, machine learning systems are contingent on the quality of the data on which they are trained.[15] AI systems will certainly not be immune to the problems of diagnostic error, diagnostic uncertainty and overdiagnosis if these issues continue to exist in regular histopathology practice and AI algorithms are trained on such data,[12] so it is crucial that pathological assessment continues to be verified for accuracy and validity in real-world settings.

The chapters presented in this thesis, therefore, focus on exploring quality issues of various instruments drawn from all compartments of the pathologist's toolkit. We methodically evaluate several time-honoured light microscope techniques; provide an evidence basis for the integration of molecular techniques into the assessment of a range of oft-encountered challenging melanocytic tumours and their mimics; and critically appraise biomarker expression relevant to the burgeoning field of targeted therapeutics (theragnostics) in melanoma.

The introductory literature review presented in **Chapter 2 "A review of key biological and molecular events underpinning transformation of melanocytes to primary and metastatic melanoma"** summarises contemporary knowledge of the molecular landscape for benign and malignant melanocytic tumours and discusses the role of genomic analysis as a potential tool for improved diagnosis, estimation of prognosis and management strategies. A sound understanding of the biological and molecular actions that transform normal melanocytes into their neoplastic counterparts (melanomagenesis) sets the stage for the practical works presented in subsequent chapters, the relevant contextual backgrounds for which are summarised below.

Re-appraising established practices in melanoma pathology

Tracking alongside improved community awareness and more prevalent skin checks across the population, Australia's age-standardised incidence of new cases of cutaneous melanoma has more than doubled in the last 4 decades, rising from 30 cases per 100,000 persons in 1982 to 65 per 100,000 in 2019.[16] This phenomenon is replicated, to varying extents, in nations with predominantly fair-skinned populations of European descent and high cumulative sun exposure, which has in turn

led to a substantial increase in melanoma specimens across the global pathology workforce.[17] The studies presented in **Chapters 3 and 4** aim to update pathologists on several practical aspects to improve melanoma evaluation, the latter being performed, at scale, every day across the world, for which there is a surprising paucity of contemporary, and historic, literature.

In **Chapter 3 "Evaluation of multiple tissue levels frequently upstages patients with clinically localised thin primary cutaneous melanoma"**, we appraise the variability of the primary histopathological prognostic determinants of a cutaneous melanoma that occurs when increased tissue levels are taken at regular intervals. Nearly 60 years after Clark, McGovern and Breslow first predicted melanoma behaviour as a function of tumour size and phase of invasion,[7,8,18] and despite the discovery of vast genomic information since,[19] histopathological assessment of a primary melanoma remains the cornerstone of prognostic assessment and directs surgical management.[9,10] Breslow thickness, ulceration status and microsatellitosis are key prognostic determinants in the 8th edition of the AJCC Cancer Staging Manual (AJCC),[9] and despite their statistical importance derived from large datasets, very little work has actually been done to evaluate the variance of these parameters at an individual patient level.[20,21] In our study, we examined additional tissue levels in 100 µm increments with the aim to understand the frequency of upstaging of a patients' primary melanoma due to changes in these prognostic parameters. As the first study to consider all AJCC prognostic features in a single undertaking, this work is of importance because it provides information to help pathologists make decisions about the value of obtaining and assessing additional tissue levels at a very practical level, particularly when dealing with thin melanomas. This work is also relevant at a time where increasing resources are being directed to molecular and AI processes to stratify prognosis of thin melanomas (thickness <1 mm) beyond the AJCC 8th edition prognostic estimates.[22,23] The heterogeneity of prognostic parameters documented in our study suggest that under-staging of some patients occurs, and our findings may provide a relatively simple explanation to account for at least some of the 'bad behaving' thin melanomas.[24,25]

Our study in **Chapter 4 "Residual melanoma in wide local excision specimens after 'complete' excision of primary cutaneous in situ and invasive melanomas"** looks at the prevalence and clinicopathological features of melanoma persisting in the primary tumour bed. Wide local excision (WLE) is the standard definitive surgical treatment for patients with biopsy-proven melanoma, which aims to achieve adequate clearance margins around all in situ and invasive melanoma components, as well as any melanoma cells that might have spread beyond the primary tumour bed into surrounding skin and subcutaneous tissues.[10] By this theory and practice, surgery remains a stalwart of primary melanoma treatment, offering a potentially curative treatment.[10] The histopathological assessment of WLE specimens generated from surgical treatment is proportional to melanoma incidence, and therefore this test also represents a significant workload in pathology laboratories. In recent years, however, the need for current WLE margins has been questioned, with the concept of de-escalation of margin clearance gaining traction.[26] In our large cohort study of 640 patients, we aimed to determine the frequency of 'residual' melanoma occurring in WLE specimens after complete excision biopsy (CEB), and any clinical and pathological features associated with residual disease. The results of this study are important because they contribute to a better understanding of the relevance of residual melanoma in WLE specimens at a time when changes to the historical surgical treatment standard are being considered.

Evaluating applications of emergent technologies in melanoma pathology

In **Chapters 5-7**, we look at the role of molecular testing in the differential diagnosis of melanoma for a range of challenging cases and scenarios, including when benign mimics and tumours of a different histogenesis enter the picture. Accurately distinguishing a melanoma from benign and malignant mimics is a persisting diagnostic challenge for pathologists, including melanoma experts, with a host of tumours (both melanocytic and non-melanocytic) potentially requiring consideration. Studies designed to address issues of over- and under-diagnosis of melanoma are highly desirable because incorrect diagnosis can cause patient harm, often alters patient management and is costly to

healthcare systems.[12] The studies in this section are therefore unified by the aim of gaining a better understanding of how genomic ancillary information can practically assist pathologists, clinicians and patients in accurate diagnosis.

In **Chapter 5 "Molecular analysis of cutaneous sarcomatoid neoplasms frequently identifies melanoma driver variants"**, we look at the role of molecular testing for the express purpose of recognising undifferentiated and dedifferentiated melanoma (UM/DM) from other sarcomatoid primary cutaneous neoplasms, such as atypical fibroxanthoma (AFX) and pleomorphic dermal sarcoma (PDS). These tumours frequently present a diagnostic dilemma to pathologists due to clinical and morphological overlap, so assessment requires careful correlation of clinicopathological and molecular features.[27,28] Based on the concept of defining a melanoma by the presence of a classical MAPK pathway driver variant, the validity of which we discuss in our paper,[19,28,29]we hypothesised that a subset of patients with an immunohistologically unclassifiable cutaneous tumour might be re-classified as UM or DM after genomic testing, and aimed to understand the demographic and pathological features of such tumours. This work is of importance because it highlights how molecular testing of non-specific primary cutaneous sarcomatoid neoplasms has the potential to improve the detection of UM and DM, thereby opening-up alternative management pathways for some patients.

Not only does molecular testing have the potential to assist the diagnosis of cutaneous lesions of different histogeneses, it is also increasingly being used to improve the diagnosis of ambiguous lesions within the melanocytic lineage. In **Chapter 6 "Molecular profiling of noncoding mutations distinguishes naevoid melanomas from mitotically active nevi in pregnancy",** we turn our attention to a common diagnostic dilemma between two types of challenging melanocytic lesions, namely naevoid melanoma (NM) and mitotically active naevi of pregnancy (MANP). NMs are subtle melanomas that morphologically simulate naevi and are therefore prone to under-diagnosis in both clinical and histopathological practice.[30] There is value in directly comparing the molecular features

of these tumours to mitotically naevi, which conversely are prone to over-diagnosis as melanoma.[31] In this study, we molecularly profile a cohort of NMs and MANPs to understand the variety of hotspot variants and noncoding mutations harboured by these tumours. Our work is one of the first studies to consider the role of molecular testing for an oft-recurring diagnostic dilemma,[32] and has relevance for the issue of over- and under-diagnosis in melanoma dermatopathology.

In **Chapter 7 "Extraenteric gastrointestinal neuroectodermal tumour masquerading as mucosal melanoma of the bronchus"**, we consider the concept of melanoma mimicry beyond the skin in unusual clinical settings. By presenting the molecular findings of a rare tumour with *EWSR1::CREB1* fusion that mimicked primary bronchial melanoma, we discuss the parallels between bona fide melanoma and melanoma-like tumours at rare and unusual locations, which are increasingly being discerned by molecular phenotypes.[33] This case is supported by a review of the literature in a published textbook chapter on the rare **"Primary melanoma of the lung"**, the very occurrence of which continues to be debated, particularly in our modern molecular era.

While the successes of currently available molecularly targeted and immune-based therapies have been remarkable, durable responses are not ubiquitous and there is still an impetus to develop new melanoma treatments. In the final section of this thesis, **Chapter 8 "Assessment of intrapatient intertumoural heterogeneity of PRAME immunohistochemistry expression in melanoma"**, we evaluate a transcriptional protein that has recently emerged as a potential 'theragnostic' target in melanoma.[34,35,36] The significance of this study lies in the fact that heterogeneity in PRAME expression could have implications for trial design and future clinical practice with regards to PRAME-directed therapies, and studies on inter-tumoural concordance of PRAME expression are currently lacking. We sought to examine the intra-patient inter-tumoural concordance of PRAME expression by immunohistochemistry (IHC) among a cohort of patients with multiple matched primary and metastatic melanoma tumour samples from individual patients. In looking at 94 melanoma specimens from 38 patients, we aimed to understand the degree of variability of expression of

PRAME IHC across combinations of matched primary, locoregional metastases and distant metastases, and determine what factors, if any, might contribute to heterogeneity. This study provides the first evidence of how PRAME expression varies across tumour samples and discusses how this phenomenon is potentially a function of inherent biological changes, but for which specimen quality may also play a part.

Finally, **Chapter 9** summarises all our results in the context of broader research across the field. We discuss the ways our findings impact current practice and how they might help to shape melanoma discovery into the future.

GENERAL INTRODUCTION

CHAPTER 2: LITERATURE REVIEW

The following literature review summarises our contemporary understanding of the molecular events that occur in the development of benign and malignant melanocytic tumours. It provides context for the role of genomic analysis in current diagnostic practice, estimation of prognosis and management strategies. This literature review outlines the foundational knowledge that underpins the practical works presented in subsequent chapters.

cancers

MDPI

Review

A Review of Key Biological and Molecular Events Underpinning Transformation of Melanocytes to Primary and Metastatic Melanoma

Louise A. Jackett [1,2,3,4] and Richard A. Scolyer [1,2,3,*]

1 Melanoma Institute Australia, 2065 Sydney, Australia; ljackett@gmail.com
2 Sydney Medical School, The University of Sydney, 2050 Sydney, Australia
3 Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, 2050 Sydney, Australia
4 Department of Anatomical Pathology, Austin Hospital, 3084 Melbourne, Australia
* Correspondence: Richard.Scolyer@health.nsw.gov.au; Tel.: +61-299117200; Fax: +61-299549290

Received: 3 October 2019; Accepted: 12 December 2019; Published: 17 December 2019

Abstract: Melanoma is a major public health concern that is responsible for significant morbidity and mortality, particularly in countries such as New Zealand and Australia where it is the commonest cause of cancer death in young adults. Until recently, there were no effective drug therapies for patients with advanced melanoma however significant advances in our understanding of the biological and molecular basis of melanoma in recent decades have led to the development of revolutionary treatments, including targeted molecular therapy and immunotherapy. This review summarizes our current understanding of the key events in the pathway of melanomagenesis and discusses the role of genomic analysis as a potential tool for improved diagnostic evaluation, prognostication and treatment strategies. Ultimately, it is hoped that a continued deeper understanding of the mechanisms of melanomagenesis will lead to the development of even more effective treatments that continue to provide better outcomes for patients with melanoma.

Keywords: diagnosis; melanoma; metastasis; pathology; progression; treatment; melanomagenesis

1. Introduction

Melanoma, a malignancy of the pigment-producing cells of the skin, is a major public health problem in most Western countries, where there is a predominance of Caucasians living in temperate climates. Despite awareness that most cutaneous melanomas are caused by ultraviolet (UV) irradiation from sun exposure and public health campaigns to promote "sun smart" behavior, the incidence of the disease has been steadily rising for many decades [1]. In Australia, melanoma is the third most common cancer in men and women and both the commonest cancer and commonest cause of cancer death in young adults [1]. Until recently, there were no effective drug therapies for treating patients with advanced melanoma and most patients with melanoma brain metastases would die within a few months [2]. However, over the past decade, a greater understanding of the molecular basis of melanoma disease pathogenesis and the immune system has led to the development of two new types of drug therapy, molecular targeted therapies and immunotherapies, the latter exerting its effect by harnessing the body's own immune system to fight the cancer [3]. These therapies have transformed the previously dismal outlook for advanced stage melanoma patients and are now also having a major impact in the management of many other cancer types. Indeed, immunotherapy has been described as cancer's "penicillin moment". Melanoma develops from the accumulation of heterogeneous molecular events that have been characterized over recent decades through epidemiological, clinical, pathological

and genetic studies [4,5]. The key mutational events, for which ultraviolet irradiation is frequently implicated as a triggering factor, are multifactorial and complex [6,7].

Melanomagenesis was historically conceived to occur in a serial linear model, starting with benign precursors, then moving through 'intermediate' lesions, eventually leading to malignant tumors with metastatic potential [4]. However, recent evidence suggests that not only are there multiple pathways by which a melanocyte may transform to a melanoma but also that some of the intermediate steps may be bypassed and that other non-linear biopathways exist [6]. Experimental studies have begun to unravel the aberrant mechanisms that promote melanoma progression and metastasis, including the factors involved in the dysregulation of melanocyte proliferation, impairment of the immune system, and extrinsic agents in the tumor microenvironment that promote primary and metastatic growth (Figure 1) [7]. Accurate biological models of these processes must reflect the great temporal and spatial diversity of the clinical patterns of the disease.

Recently, genetic studies have advanced our understanding of the molecular drivers behind the biological steps of melanomagenesis. Fundamentally, the mitogen-activated protein kinase (MAPK) signaling pathway plays a central role in the development of the vast majority of benign nevi and cutaneous melanomas [8,9]. A single driver mutation of the oncogenes *BRAF* or *NRAS* is a foundation genetic event that activates the MAPK signaling pathway to trigger melanocyte proliferation in approximately 60% of cases [9]. Most of the resulting tumors are benign and remain stable, kept in check by senescence due to functioning tumor suppressor genes [10]. A subset, however, acquire additional molecular alterations such as oncogenic driver mutations and copy number variations that alter tumor suppressor gene regulation [11–13]. These events may result in 'borderline' or 'intermediate' lesions which can mimic melanoma or be precursors of malignant transformation. Ultimately, the hallmarks of fully developed melanoma are the complete loss of tumor suppressor gene function and other mechanisms which confer traits for invasion and metastasis [14–16]. In turn, metastatic melanoma may acquire additional mutations that impart treatment resistance to molecularly targeted therapies and immunological agents [17–20].

This review summarizes our current understanding of the biological processes and molecular events in the pathway of melanomagenesis (Figure 1) and discusses the role of genomic analysis as a potential tool for improved diagnostic evaluation, prognostication and treatment strategies. Ultimately, such an understanding will lead to improved outcomes for melanoma patients.

The authors acknowledge that a comprehensive review of the histopathological diagnosis of melanocytic lesions is beyond the scope of this review and readers are referred to excellent textbooks on this subject [21–23].

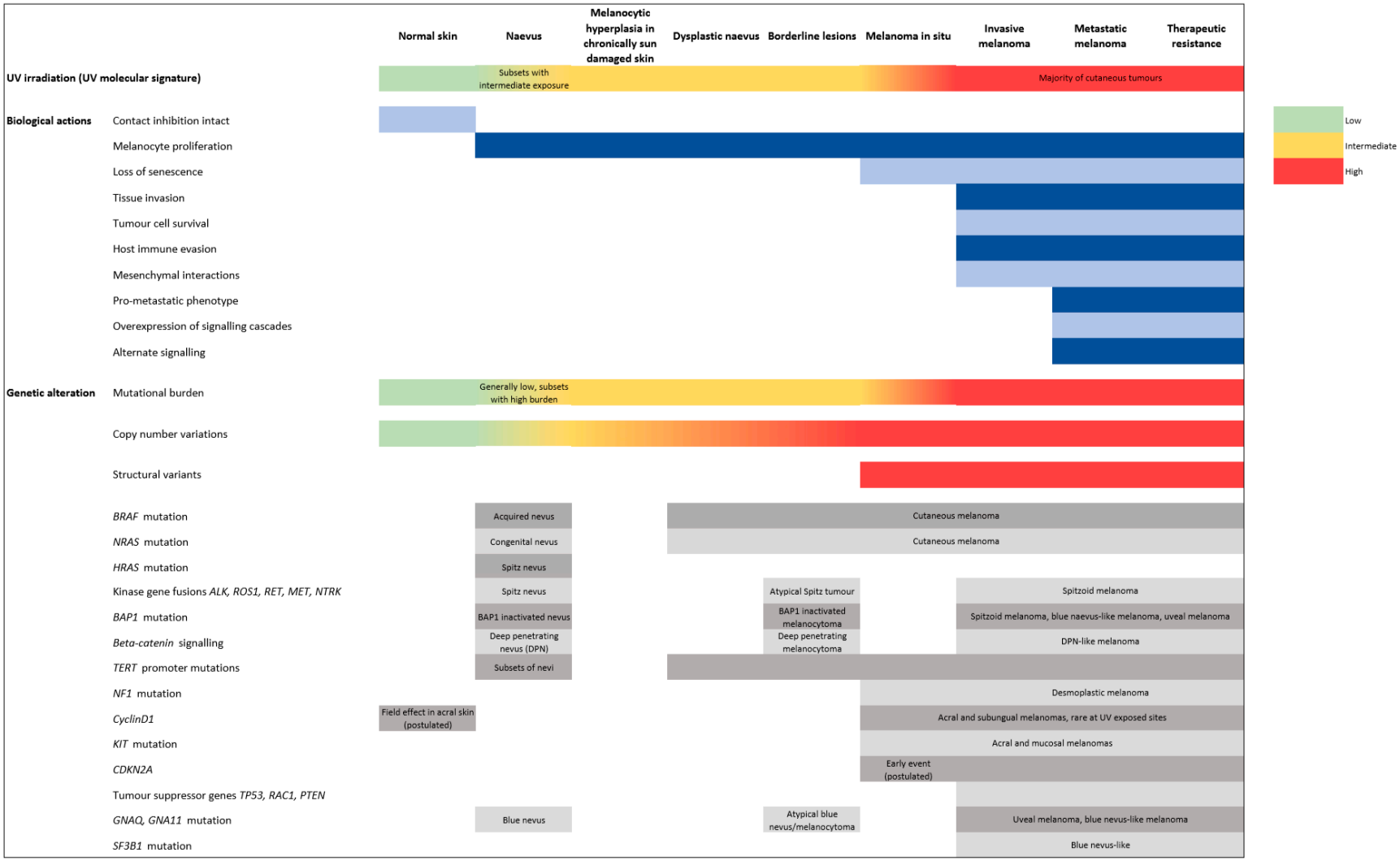

Figure 1. Key phenotypic and molecular events in melanoma pathogenesis and progression. Readers are referred to the text for in depth discussion of each event.

2. Melanocytes in Normal Skin and Early Melanocytic Proliferations

Normal cutaneous melanocytes reside as inconspicuous cells along the basal epidermis, the superficial layer of the skin. Melanocytes possess dendritic processes that provide points of contact with the cell membranes of neighboring keratinocytes, by which the transfer of photoprotective melanin pigment is facilitated [24]. Normal melanocytes maintain uniform cell density relative to other melanocytes and the alteration of this density-dependent regulation is a key developmental event that allows the clustering of proliferating melanocytes in benign nevi and the radial and vertical growth phases of melanoma [25,26].

Melanocytic hyperplasia in the epidermis at the edges of lentigo maligna (a type of melanoma in situ occurring on chronically sun damaged skin) is a commonly observed histological phenomenon that is a manifestation of a dysregulated single cell microenvironment and may account for the risk of local recurrence after incomplete wide local excision of melanoma (Figure 2A) [27,28]. However, little is known about the mutational burden of individual melanocytes in sun-damaged skin. Genomic studies have demonstrated an array of various mutations in chronically sun-exposed skin, most of which are likely to be localized to keratinocytes, but it has been postulated that individual native melanocytes may also acquire high mutation burdens [29,30]. In acral skin, multiple gene amplifications (particularly cyclinD1) have been detected among native basal melanocytes in the background skin adjacent to acral melanomas, suggesting that single melanocytes have the ability to accumulate an oncogenic 'field effect' independent of being part of a nevus or melanoma in situ (Figure 2B) [31].

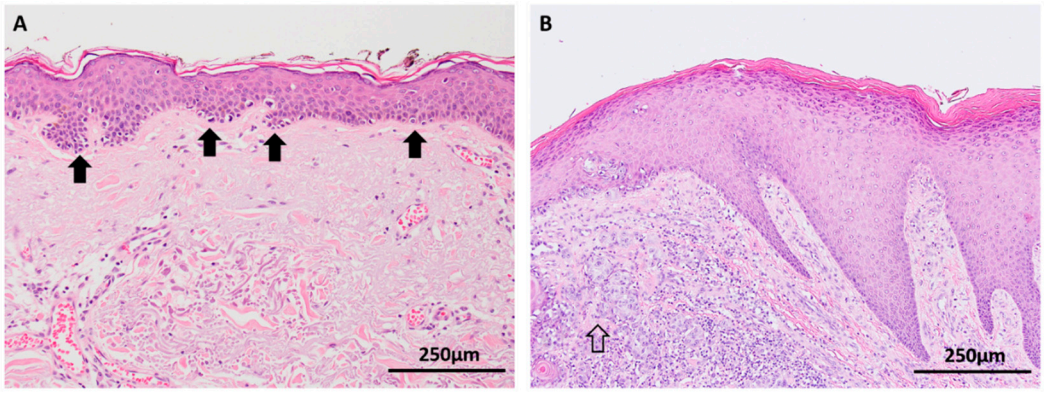

Figure 2. Background skin adjacent to melanomas (haematoxylin and eosin (H&E) images). (**A**) Melanocytic hyperplasia (arrows) in chronically sun damaged skin adjacent to lentigo maligna is a manifestation of a dysregulated single cell microenvironment. Various mutations have been identified in this background skin, many of which are attributed to keratinocytes, but native melanocytes are also postulated to acquire a high mutational burden. (**B**) CyclinD1 amplifications have been detected in melanocytes in epidermis adjacent to acral melanomas (open arrow).

3. Nevi

Nevi are benign clonal proliferations of melanocytes that rest in a state of senescence [32]. They are the most prevalent tumor among humans and are classified into several subtypes based on their clinical and pathological characteristics, the commonest being the common acquired nevus. Other commonly occurring subtypes include the congenital nevus, blue nevus and Spitz nevus (Figure 3). There are observed epidemiological and biological differences among the different subtypes of nevi but contemporary genomic data have shown that nevi are consistently characterized by a lower mutational load than melanomas [16,30,33].

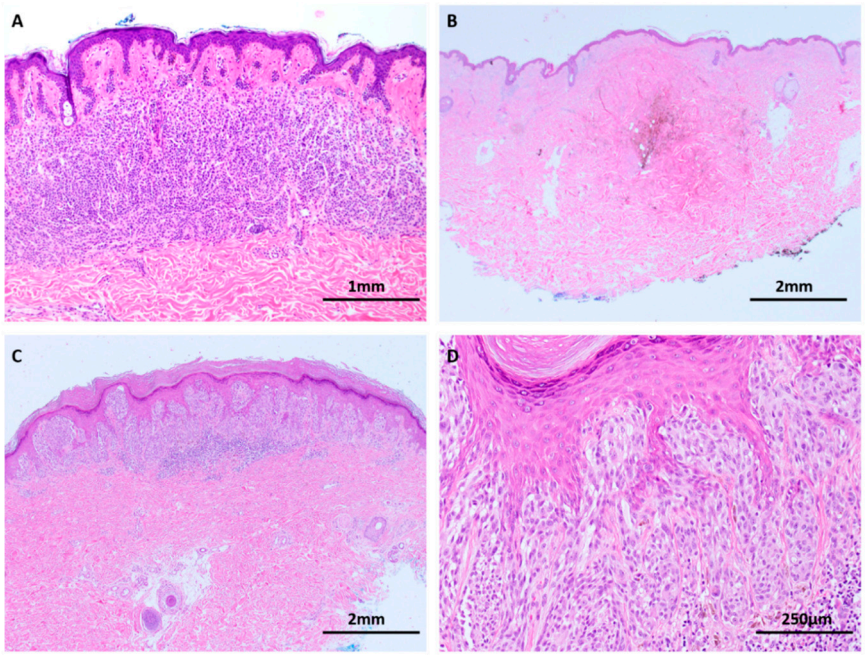

Figure 3. Subtypes of nevi (H&E images). (**A**) The common acquired nevus is a stable lesion resting in a state of senescence and the vast majority possess a low mutational burden. (**B**) Blue nevus. (**C**) Spitz nevus. Both blue nevi and Spitz nevi lack significant chromosomal aberrations by comparative genomic hybridization. (**D**) Some Spitz nevi are associated with isolated gain of chromosome 11p and HRAS mutations, and harbor translocations involving kinase gene fusions.

Several studies, including some utilizing whole genome sequencing techniques, have shown that the vast majority of acquired nevi possess single driver mutations of either *BRAF* V600E or *NRAS* Q61R/L without other somatic mutations (Figure 2) [9,34–36]. *NRAS* is most frequently observed in congenital melanocytic nevi and is believed to occur in utero [34,37]. In contrast, *BRAF* mutations are more commonly seen in acquired nevi, a characteristic they have in common with many melanomas [34]. Congenital nevi, blue nevi and most Spitz nevi lack chromosomal aberrations, as detected by comparative genomic hybridization [38]. Some Spitz nevi are associated with the isolated gain of chromosome 11p and *HRAS* mutation on that arm, a unique finding that appears to set them apart from melanomas [31]. Many Spitz nevi harbor translocations involving kinase gene fusions involving genes such as *ALK1*, *ROS1*, *RET*, *MET* and *NTRK*.

As noted above, *BRAF* and *NRAS* mutations are also key drivers in many melanomas. However, in contrast to melanomas, which acquire additional driver mutations, nevi enter a suppressive state of replicative senescence which is regulated by the tumor suppressor gene *CDKN2A* via its protein p16, and various transcriptional controls of the cell cycle [33].

Recent molecular studies have unveiled novel findings in benign nevi. Colebatch et al. profiled 14 congenital and acquired nevi by whole exome sequencing and identified subclonal *TERT* promoter mutations, a finding which has not been previously observed in benign nevi [34]. They also demonstrated a subset of nevi with high mutation loads and the UV signatures sbs 7a and 7b, in contrast to the ubiquitous signatures 1 and 5 seen among nevi with low mutation burdens [34]. This finding underscores earlier epidemiological observations that UV irradiation is a common risk factor for both nevi and melanomas, and which hinted at a shared pathway of tumorigenesis caused by the effects of solar UV [39].

The *CDKN2B-CDKN2A* gene cluster is commonly deleted in many types of cancer, and the tumor promoting effects of the *CDKN2A*-encoded p16 and p14 (ARF) tumor suppressors are well studied in melanocytic lesions [11,12,14,40]. The p16 (INK4A) protein binds proteins CDK4 and CDK6, and in

this bound state these proteins are unable to stimulate progression of the cell cycle [41]. In contrast, the p14 (ARF) protein reduces the breakdown of p53, a protein that is a key regulator of cell division, senescence and apoptosis [42]. Via these respective mechanisms, p16 and p14 (ARF) ultimately prevent cell division and therefore inhibit tumor progression. The *CDKN2B*-encoded protein p15 is less well studied but has been demonstrated to arrest cell proliferation in melanocytic nevi and its loss promotes melanomagenesis [43]. Changes affecting many other genes are also common, particularly in advanced melanoma, including ARID2, RAC1, and SF3B1, amongst many others [36].

4. Borderline Lesions

The concept of 'borderline' or 'intermediate' lesions was born out of epidemiological and histological observations of tumors with clinically unexpected behavior (such as the frequent lymph node involvement in atypical Spitz tumors despite excellent patient outcomes, including infrequent distant metastasis and mortality), or apparently histologically benign lesions which are associated with an increased risk of progression to melanoma [44,45]. Consequently, the risk profile of such a lesion lies intermediate between overtly benign nevi and unequivocally malignant melanomas. These lesions are unified by their significant diagnostic and management challenges but are biologically heterogeneous. Importantly, their grouping together as 'borderline' lesions serves as a theoretical framework for histologic evaluation and further study rather than for nosologic purposes. Examples include dysplastic nevi, Spitzoid neoplasms, atypical blue nevi and related neoplasms, and melanocytomas. Recent genetic investigation has led to significant advances in our understanding of these biologically diverse tumor groups.

The dysplastic nevus has long been recognized as a clinical marker of increased risk for cutaneous melanoma, particularly in patients with more than 10 dysplastic nevi or the dysplastic nevus syndrome [46]. These benign clonal proliferations of melanocytes are clinically, histologically and biologically distinct from other nevus subtypes and have been postulated to represent an intermediate phase between benign nevi and melanoma [16]. Indeed, the diagnostic distinction of severely dysplastic nevus and early melanoma in situ is an enduring problem in histopathology, with well-documented interobserver variability, even among experts (Figure 4) [47–49]. Like benign nevi, dysplastic nevi (DN) have been shown to rest in a state of senescence and possess a significantly lower mutational load compared to melanoma [33]. Malignant transformation is occasionally observed in DN but requires the presence of additional alterations in the critical tumor suppressor genes [33]. Recent studies of primary melanomas and their adjacent precursor 'intermediate' lesions (dysplastic nevi and melanoma in situ) using a multigene targeted sequencing platform have confirmed that DN lack any alterations in the tumor suppressor genes *CDKN2A, TP53, NF1, RAC1,* and *PTEN*, that they have UV signature patterns distinct from those of melanoma, and that their overall mutation burden appears to be intermediate between benign nevi and melanoma [16,33]. However, the observation that DN tend to harbor non-V600E *BRAF* and *NRAS* mutations suggests that there are likely to be alternative pathways to Clark's classically postulated model of a linear step-wise progression to melanoma [4,16].

TERT promoter mutations, which are present in up to 80% of cutaneous melanomas, have also been observed among dysplastic nevi [16]. This finding and that of Colebatch et al. (above) has put the concept of *TERT* promoter mutations as potential biomarkers for melanoma into question [34]. Nonetheless, the identification of differing mutational landscapes between nevi and melanomas does have the potential to assist with the diagnosis of dysplastic nevi, but validation studies are still required and this approach is currently not appropriate for the routine diagnostic work-up of ambiguous lesions.

The blue nevus spectrum of lesions includes cutaneous blue nevi, atypical blue nevi and blue nevus-like melanomas. All are characteristically pigment-synthesizing tumors that may be diagnostically challenging (Figure 5). Most blue nevi and related tumors harbor mutations of *GNAQ* and *QNA11*, a characteristic they have in common with uveal melanoma [50,51]. Recently, *BAP1* or *SF3B1* mutations have been found to occur alongside *GNAQ* and *GNA11* in approximately 20% of cases of unequivocally malignant blue nevus-like melanoma, suggesting that this is a late mutational

event [52]. *BAP1* loss of expression is a useful ancillary test to supplement histological diagnosis for these lesions.

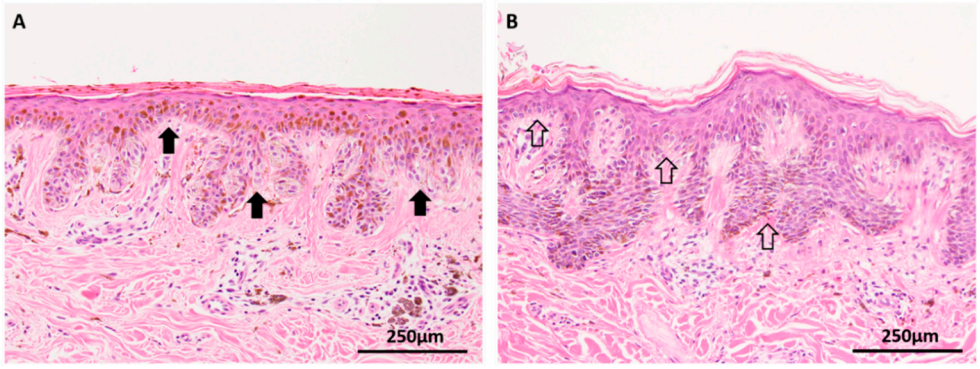

Figure 4. Dysplastic nevi have an overall mutational burden between that of nevi and melanoma (H&E images). (**A**) Multiple dysplastic nevi, such as this example, are clinical markers of increased risk for cutaneous melanoma and diagnosis of lesions with mild to moderate degrees of cytological and architectural atypia (solid arrows) is relatively reproducible. (**B**) Lesions with histological features between severely dysplastic nevus and melanoma in situ, such as this case with focal pagetoid spread and lentiginous architecture (open arrows) among an otherwise nested junctional component, are subject to interobserver and intraobserver variability, even among experts. Identification of differing mutational profiles between dysplastic nevi and melanomas has the potential to assist in diagnosis of these challenging lesions.

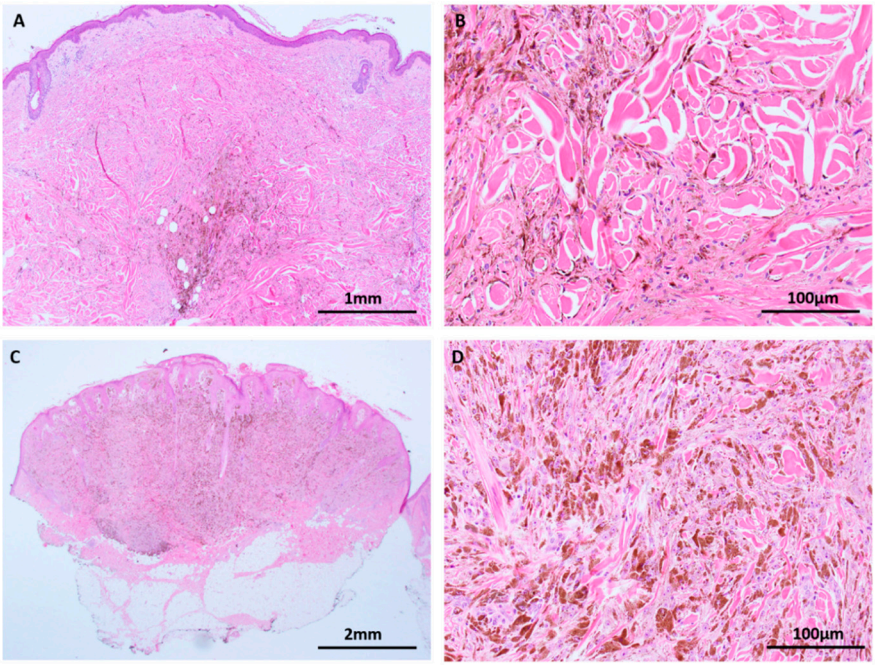

Figure 5. Blue nevi and atypical variants are characterized by *GNAQ* and *GNA11* mutations (H&E images). (**A**,**B**) Blue nevus. The pigmented spindle cells show minimal cytological atypia in benign lesions. (**C**,**D**) This atypical blue nevus shows nuclear atypia, raising suspicion for malignancy but other histological features fall short of melanoma. Recent investigations suggest that *BAP1* mutation is a late event on the pathway to malignancy among blue nevus-like lesions so *BAP1* loss may be a useful ancillary test to support a diagnosis of malignancy in ambiguous cases.

Atypical Spitzoid tumor, a tumor of uncertain malignant potential that lies between definitely benign Spitz nevus and melanoma, is another lesion that is molecularly complex. A characteristic of all Spitz lesions is the presence of pathways that are distinct from conventional nevi and the majority of cutaneous melanomas [53]. Early in development, Spitzoid tumors harbor *HRAS*, *BRAF* and *BAP1* mutations (Figure 6), as well as kinase gene fusions of *ALK*, *NTRK*, *RET*, *ROS1* and *MET*, and these abnormalities explain the rapid initial growth phase so clinically characteristic of benign Spitz nevi [54]. Atypical Spitz tumors develop aberrations in tumor suppressor genes, including activation of p53 via telomere shortening, and activation of p16 via epigenetic *CDKN2A* regulation. Further accumulation of mutations involving *PTEN*, *ARID2A* and *TERT* appear to promote transformation to a malignant phenotype [55].

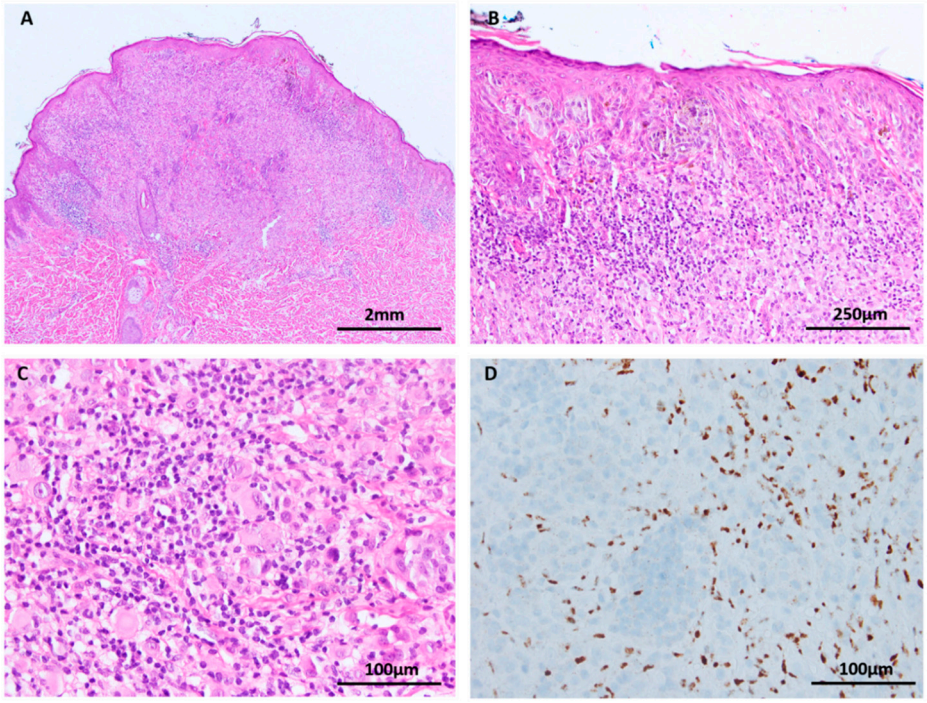

Figure 6. *BAP1* inactivated Spitz tumor (**A**–**C**: H&E images, **D**: *BAP1* immunohistochemistry). (**A**) Low power silhouette of a *BAP1* inactivated Spitz tumor (H&E × 12.5). (**B**,**C**) These lesions often have a characteristic voluminous cytoplasm and a prominent lymphocytic reaction, suggesting a peculiar. (**D**) Lesional melanocytes show loss of nuclear expression of *BAP1*. Lymphocytes serve as a positive internal control.

Pigmented epithelioid melanocytoma (PEM) is a unique deeply pigmented dermal melanocytic proliferation that overlaps with lesions previously described as epithelioid blue nevus of the Carney complex and many lesions previously termed as animal type melanoma and pigment-synthesizing melanoma [56]. Much like other borderline lesions, these tumors have a clinical behavior and risk stratification intermediate between benign nevi and melanoma. Most of these tumors (80%) show a loss of expression of protein kinase A regulatory subunit 1 alfa (R1alfa), an important cofactor in cyclic adenosine monophosphate (cAMP) signaling involved in melanocyte proliferation, and a mutation that is seen frequently among patients with Carney complex [57].

The deep penetrating nevus (DPN) and its spectrum of atypical variants are another group of heavily pigmented melanocytic lesions that may be confused for melanoma (Figure 7). Atypical DPNs possess some histological characteristics of melanoma but fall short of a diagnosis of overt malignancy. DPNs and atypical DPNs are typified by gain-of-function mutations of beta-catenin and loss of *APC*, both of which exert actions upon the WNT pathway [58,59]. These lesions also possess

concurrent MAPK pathway mutations and are often observed to arise in association with conventional nevi (combined nevi), findings which suggest that a beta-catenin mutation is an intermediate step that transforms a conventional nevus into a DPN [60]. This mechanism is likely responsible for the distinctive large cell size, pigmented cytoplasm and lack of maturation of DPN and atypical variants [60]. The vast majority of atypical DPNs are clinically stable; however, malignant behavior is occasionally seen. Indeed, the rare melanomas that share histological similarities to atypical DPNs (termed DPN-like melanoma) also possess the same WNT pathway mutations, suggesting a continuum of transformation [60].

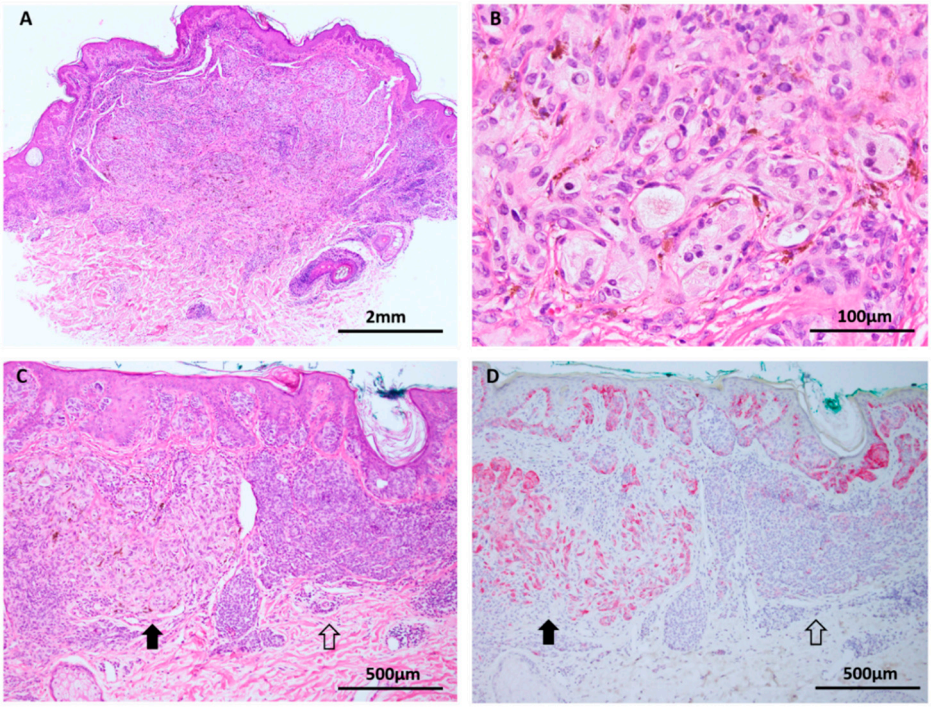

Figure 7. Deep penetrating nevus (**A**–**C**: H&E images, **D**: HMB45 immunohistochemistry). (**A**) Deep penetrating nevi are often seen in conjunction with a conventional nevus (combined nevus). (**B**) Both components harbor MAPK pathway mutations but activated WNT signaling appears to drive transformation to the deep penetrating nevus phenotype with its distinctive large cells, pigment synthesis and lack of maturation. (**C**,**D**) In addition to the distinct genetic differences, the components are delineated by morphology and differing HMB45 expression, with stronger labelling in the DPN component (solid arrows) compared to the conventional nevus component (open arrows).

Genetic studies have made significant advances in unravelling many aspects of intermediate lesions and the practical application of ancillary genetic studies has the potential to supplement histological diagnosis, but currently many intermediate or borderline lesions present significant diagnostic and management challenges. Appropriate communication between pathologists and clinicians of estimated risk of aggressive behavior for these ambiguous lesions is of utmost importance. To this end, the term 'melanocytoma' has been advocated by the World Health Organization to delineate intermediate lesions with uncertain malignant potential (e.g., atypical DPN and *BAP1* inactivated atypical Spitz tumors) from their clearly benign and overtly malignant counterparts [61].

5. Melanoma Arising in Chronically Sun-Damaged Skin

As discussed briefly above, multiple epidemiological and genomic studies have provided evidence for the tumorigenic role of UV irradiation in the development of cutaneous nevi and the vast majority of melanomas [39,62,63]. Early epidemiological observations showed that melanoma has significant

regional variation with the highest incidences in New Zealand and Australia, is associated with chronic sun exposure, particularly repeated intense childhood sun damage, and has a higher incidence in individuals who use artificial UV tanning beds [39,62,63]. Genomic studies have subsequently confirmed that melanoma is associated with a high mutational load and mutagenic UV signatures enriched with C > T mutations consistent with the effects of ultraviolet irradiation [13,36,64,65].

The development of melanoma can be divided into the "radial growth phase" (which includes melanoma in situ and the junctional components of invasive melanomas), the "vertical growth phase" and metastasis (local and distant). While this model may erroneously imply a linear progression of disease, it is still useful for conceptualizing the mechanisms of progression.

6. Radial Growth Phase Melanoma

The loss of contact inhibition has been shown to be an important event in vitro for controlling cell density and is postulated to be critical in vivo to facilitate the radial growth phase of the junctional components of melanocytic lesions, particularly early melanomas (Figure 8A) [25,66,67]. As previously noted, normal melanocytes have dendritic handles that regulate equidistant melanocyte distribution within the basal epidermis. Loss of contact inhibition manifests histologically as lentiginous ("back-to-back") growth and melanocyte nests. To varying degrees, these features are present in both benign nevi and melanoma in situ, however they are more pronounced in melanoma, manifesting histologically as confluent lentiginous growth, variably sized nests, pagetoid spread and subepidermal clefts [66,67].

Melanoma in situ has been conceptualized as an early melanoma confined by the epidermal basement membrane and, as such, does not have the biological potential to metastasize once it has been completely excised (Figure 8A) [46]. In their study of lentigo maligna cases with and without invasive components, Moreno et al. showed that early lesions had sparse inflammation, while advanced forms of lentigo maligna melanoma had dense lymphocytic infiltrates, suggesting the immunogenicity of melanoma in situ is lower than that of invasive forms of this melanoma subtype [67].

TERT promoter mutations have been frequently observed in studies of melanoma in situ but, as noted above, they have also been documented in a subset of nevi with high mutational burdens and dysplastic nevi. *TERT* promoter mutations may therefore be an earlier event in the pathway to melanoma than first thought [16,34]. In contrast, Shain et al. have shown that mutations of *PTEN* and *TP53* are confined to advanced melanomas, but it remains to be elucidated at which point on the spectrum of radial to vertical growth these mutations occur. [16] They have also shown that a minority of intermediate melanocytic lesions, and melanoma in situ, have heterozygous loss of *CDKN2A*, but homozygous loss of *CDKN2A* was only seen in invasive melanomas [16]. Furthermore, it is possible, and indeed likely, that such mutations do not always accumulate in a set order or sequence during malignant transformation.

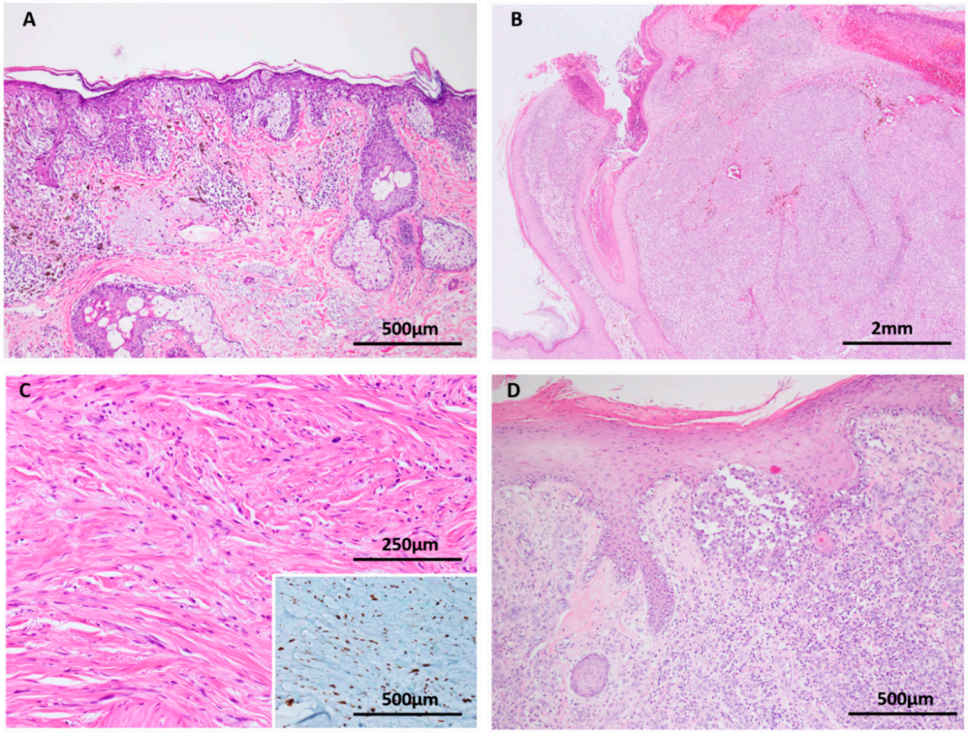

Figure 8. Melanoma (**A**–**D**: H&E images, **C**: inset Sox 10 immunohistochemistry). (**A**) Melanoma in situ is conceptualised as an early melanoma confined by the basement membrane. Loss of contact inhibition is a biological event that is thought to allow the radial growth of melanocytes through the epidermis. *TERT* promoter mutations are identified in melanoma in situ. (**B**) Nodular melanoma. The vertical growth phase of melanoma requires the accumulation of mutations that promote tissue invasion, tumor cell survival, mesenchymal interactions and host immune system evasion. (**C** and inset) Desmoplastic melanomas, shown here with Sox 10 immunohistochemistry, frequently harbor *NF1* mutations. (**D**) Melanomas at sun protected sites, such as this acral melanoma, are biologically distinct from their sun exposed counterparts due to higher frequencies of *KIT* mutations and multiple gene amplifications, commonly cyclinD1.

7. Vertical Growth Phase Melanoma

Progression to invasive melanoma requires the accumulation of mutations that continue to allow dysregulated tumor cell proliferation, promote the acquisition of tumor cell survival characteristics and activate advantageous mesenchymal interactions, all while tumor cells evade host immune control (Figure 8B,C) [67–76].

Mesenchymal interactions at the tumor stroma interface are key determinants of melanoma tumor progression. The malignant stromal phenotype is characterized by protein expression that enhances melanoma survival and progression. One of the earliest observations in melanoma was that of mast cell recruitment to the perilesional stroma by chemotactic factors, such as IL-3, produced by melanoma cells [68]. The subsequent release of growth factors such as the basic fibroblast growth factor (bFGF) and the vascular endothelial growth factor (VEGF) promotes the mitotic activity of tumor cells and tumor neovascularization [68,69]. These mechanisms are underscored by observations that the microvasculature is more concentrated in melanomas than benign nevi, being densest among aggressive melanomas, and that upregulation of VEGF expression, under the influence of the oncogene c-*MYC*, is associated with the progression of melanoma [69–74]. Targeting angiogenesis with VEGF-blocking agents has been an effective treatment strategy in several cancer types and its potential role in melanoma continues to be investigated in ongoing clinical trials [75].

Tumor-generated factors have also been shown to play a significant role in the downregulation of protective immune system interactions. In addition to its direct angiogenic effects described above, VEGF has been recognized as an important moderator of immune cell suppression [75]. Other tumor-generated immunosuppressive cytokines include interleukin 10 (IL-10), which reduces the action of melanoma-associated macrophages and lymphocytes [76]. The correlation of high IL-10 expression with melanoma progression provides further evidence that this chemokine is another important influencer in the vertical growth phase of melanoma [76]. Additional and more in-depth immunological interactions are discussed later.

A high expression of the proto-oncogene *c-MYC* has long been identified as a key factor in neoplastic transformation for many tumor types [77]. Well-studied in melanoma, *c-MYC* is implicated in more aggressive vertical growth phase melanomas and progression to metastasis [78,79]. In the appropriate genetic and epigenetic mileu, *MYC* activation leads to aberrations of normal cell proliferation, differentiation, apoptosis and senescence [80–82]. These mechanisms result in the hallmark features of tumorigenesis such as unrestrained tumor growth and aberrant protein synthesis, and interactions with the tumor microenvironment to influence vascular growth and dampen the normal response of the host immune system [80]. A necessary feature for sustained and progressive melanoma growth, *MYC* expression can be suppressed by treatment with BRAF/MEK inhibitors leading to tumor shrinkage, manifesting as clinical and/or pathological response; recovery of the overexpression of *MYC* has been shown to drive resistance to these treatments [18].

8. Metastatic Melanoma

Tumor metastasis is dependent on critical factors that drive tumor cell motility and dissemination into lymphatics and vascular channels, and that facilitate tumor cell survival and proliferation at distant sites (Figure 9A).

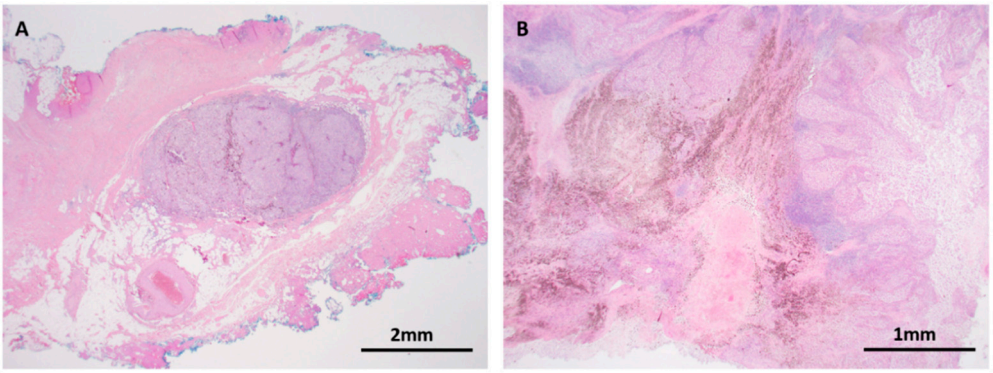

Figure 9. Metastatic melanoma. (**A**) H&E × 12.5. In transit metastasis. Tumor metastasis is dependent on critical factors that drive tumor cell motility, dissemination into angiolymphatics, and tumor cell proliferation away from the primary site. (**B**) H&E × 40. Targeted therapy and immunotherapy have heralded a revolutionary era in melanoma management. Pathological response manifests variably as tumor cell necrosis, melanosis, lymphocytic infiltration and fibrosis, as seen in this lymph node metastasis of melanoma after neoadjuvant therapy. Mechanisms of resistance and primary non-response are the focus of active research.

Some of these steps are linear and predictable, such as the reproducible patterns of metastasis to the first lymph nodes in the regional lymphatic basins which were identified in the seminal studies on sentinel node drainage patterns [83]. However, there is evidence that dissemination of melanocytes may also occur via alternative pathways, reflecting the temporally and spatially heterogeneous clinical patterns of metastatic disease [84–90]. Understanding these cellular processes and the genetic events that underpin them are essential for comprehensive knowledge of the pathogenesis of metastasis and may also provide targets for prognostic biomarkers and therapies.

Tumor-derived exosomes have emerged as important factors in tumor progression [15,84]. Melanoma-derived exosomes influence vascular permeability at potential metastatic sites and alter bone marrow progenitors towards a pro-metastatic phenotype [84]. Exosomes are regulated by the RAB family of proteins, which are highly expressed in melanoma tumor cells [84]. Guo et al. have shown that overexpression of GTPase RAB27A alters the composition of exosomes towards a pro-invasive phenotype, which in turn influences cancer cell movement; this finding correlates with poor survival in a subset of melanomas [15]. Indeed, blockage of exosome contents has been postulated as a potential therapeutic target [85].

Mouse models have shown that the microenvironment of distant sites has an important role in determining whether disseminated tumor cells are eliminated or progress from micrometastases to macrometastases [86]. An example of a chemokine in the microenvironment that influences implantation and growth of metastasis is IL-10, which appears to render lymph nodes susceptible to metastasis through its action on melanoma-associated macrophages and lymphocytes [76].

Peculiar patterns of metastasis sometimes occur that suggest there may be other parallel pathways of progression and inherent differing affinities of tumor cells for particular distant sites. For example, uveal melanomas, which harbor recurrent *BAP1* mutations when they metastasize, have an exceptional tendency to metastasize to the liver [87,88]. This event occurs in up to 90% of patients and may be the only site of metastatic disease [88]. Interestingly, kinetic modelling studies have suggested that melanoma metastasis to the liver may in fact precede the development of a detectable uveal primary by up to 5 years [89]. Clinical knowledge that cutaneous melanomas more commonly spreads to the lung, liver, brain and bone over other sites also hints at potential site specific factors that make certain organs more susceptible to metastasis [90].

9. Role of the Immune System

Melanoma is one of the most immunogenic tumors and immunogenicity has been observed to occur prior to the development of invasion [67]. In circumstances of optimal host immunosurveillance, melanoma encounters an early innate immune response that is mediated by interplay of macrophages, granulocytes, dendritic cells and natural killer cells [91,92]. Subsequent to this, the adaptive immune system plays a central role with effector CD4 + and CD8 + T-cells targeting melanoma cells through the actions of interferon-gamma (IFN-γ) or direct cytotoxic interactions [93]. When these mechanisms are appropriately activated, the elimination of the melanoma tumor occurs. However, many melanomas evade these checks and balances and continue to proliferate in an unregulated manner. Considerable effort is currently being dedicated to investigating these processes. Some of the most important mechanisms to have emerged relate to the actions that suppress T-cell function. For example, mutations in MHC class 1 may cause lymphocytes to be ineffective in recognizing melanoma cells [94]. Mutations in CTLA4, PD1 and LAG3 may upregulate immune checkpoints, thereby inhibiting T-cell function [95]. Similarly, the upregulation of ligands PDL1 and PDL2 or the overactivity of regulatory T cells are other key influences of effector T cells [96]. Importantly, these actions can be exploited by novel immunotherapies (discussed later).

The innate immune system also plays a key role in antitumoral immune surveillance and responses. It affects development and growth of melanomas through the release of pro- and anti-inflammatory cytokines and growth factors. Cross-talk between components of the innate and adaptive immune systems, and between immune cells and tumor cells, plays a critical role in tumor maintenance and progression [91,92].

There are important differences in the epidemiological profiles of melanomas arising on acral skin (the palms and soles) and at mucosal (internal body) sites, and these early observations provided important evidence in favor of alternative non-UV triggered pathways of melanomagenesis (Figure 1). Genomic studies have subsequently confirmed that acral and mucosal melanomas are biologically distinct from their cutaneous counterparts at sun-exposed sites (Figure 8D). Principally, acral and mucosal melanomas harbor a higher frequency of *KIT* gene mutations and multiple gene

amplifications, most frequently of the cyclinD1 gene (Figure 1) [97]. CyclinD1 amplifications, which are infrequent in melanomas arising on sun-exposed sites, occur early in the progression of acral and subungual melanomas [31]. They are also detected among native basal melanocytes in background skin immediately adjacent to acral melanomas, suggesting a 'field effect' that may account for an increased risk of recurrent melanomas [31]. Furthermore, acral and mucosal melanomas are characterized by frequent structural variants and lower numbers of point mutations compared with cutaneous melanomas.

10. Melanoma Heterogeneity and Implications for Therapeutic Strategies

Targeted therapy against aberrant MAPK pathway signaling has heralded a revolutionary era in melanoma resulting in significantly better anti-tumor activity and survival compared to traditional chemotherapy (Figure 9B). *BRAF-V600* mutation is a robust predictive biomarker for response to selective kinase inhibitors, which can arrest the cell cycle and lead to reduced tumor growth. However, patients almost universally develop resistance to *BRAF*-targeted therapy, usually in the first 12 months of treatment [98]. Approximately 50% of patients treated with dabrafenib or vemurafenib develop disease progression 6 to 7 months after starting treatment [99,100]. Multiple mechanisms of acquired resistance in vivo have been described, including elevated expression of the kinases *CRAF*, *COT1* or mutant *BRAF*, activating mutations in *N-RAS*, *MEK1* or *AKT1*, aberrant splicing of *BRAF* and persistent activation of receptor tyrosine kinases, including PDGFRβ and IGF-1R, and activation of alternate signaling pathways such as Notch1 [20,98,101–108]. Immunotherapy is an alternative treatment modality that harnesses the power of the host's adaptive immune system by enhancing its own immune surveillance capabilities. To achieve this, anti-PD-1 checkpoint therapies interrupt the PD-1/PD-L1 axis, which in turn releases its inhibitory grip on effector T cells. Disinhibited T cells are therefore able to function against melanoma cells to eliminate the tumor [109]. This therapy has translated into significant clinical outcomes with pathological regression and improved survival occurring in 30–40% of advanced melanoma patients [110,111]. Supplementation with antibody therapy to another immune checkpoint inhibitor, CTLA-4, appears to further enhance clinical outcomes [112]. Responders appear to have activated T-cell signatures with the expression of EOMES, CD69 and CD45RO [113]. Despite promising initial responses, resistance to immune therapies inevitably develops. Early research suggests that an abundance of tumor and host factors are responsible for the varied clinical outcomes achieved by these therapies, such as the interference of CTLA-4, by genetic defects in IFN-γ, and variations in gut microbiota [17,19,114]. Investigation into non-responders and those patients who develop resistance is the interest of considerable active research. Combining immunotherapy with targeted therapy may improve responses but may be associated with unacceptable toxicity [115].

11. Biomarker Assays

Knowledge of the molecular makeup of melanoma creates the potential for biomarker assays for a range of settings, such as supplementing histological diagnosis of difficult lesions and improving the early detection of recurrent or metastatic disease.

TERT promoter mutations have been shown to correlate with poorer outcomes in subsets of melanoma patients with *BRAF/NRAS* mutations, and this may serve as a potential future biomarker. As they are also an early event in the transformation to melanoma, they may also be a valuable diagnostic biomarker for melanoma [116,117].

The evaluation of circulating tumor DNA (ctDNA) assays is a valuable tool for monitoring the response to immunotherapy and appears to be more informative than radiological monitoring in patients with extracranial stage 4 disease [118,119]. Challenges in this area include the considerable heterogeneity among melanoma subtypes, for example the molecular differences between UV associated cutaneous melanomas, non-V600E *BRAF* mutant melanomas, and acral and mucosal melanomas. This implies that ctDNA assays may need to be personalized and targeted to the mutation profile of an individual's melanoma. Circulating tumor DNA does not appear to be as useful in prognostic

modelling in earlier stage disease with current assay sensitivities but may prove to be useful when more sensitive techniques for its detection are employed.

12. Conclusions

In summary, melanomagenesis results from the sequential accumulation of key genetic mutations. Alterations of the oncogenes *BRAF* and *NRAS*, which act through the MAP-kinase pathway, are the most frequent early events that drive melanocyte proliferation in both nevi and melanoma. After an initial phase of proliferation, however, nevi rest in a state of senescence, which is influenced by the p16 *Rb* pathway and transcriptional regulation of the cell cycle. Transformation to melanoma requires additional molecular events and characteristically leads to a high mutational burden. *TERT* promoter mutations appear to be an early event, while the inactivation of tumor suppressor genes, such as *PTEN*, *CDKN2A*, *NF1* or *TP53*, drives the later vertical growth phase of advanced melanoma. The key molecular abnormalities that drive progression to metastasis are subjects of ongoing scientific inquiry.

The genomic study of melanocytic lesions continues to prove a powerful tool that has already unveiled a wealth of information and has led to our current comprehensive understanding of melanomagenesis. Continued study of the mutational landscape will continue to unveil potential strategies for therapeutic agents. Furthermore, the observation that nevi and melanoma have low and high mutational burdens, respectively, as well as distinctive UV signatures, has the potential to enhance our diagnosis of histologically ambiguous lesions through the use of sequencing-based technologies.

Funding: The APC was funded by Australian Government National Health and Medical Research Council (NHMRC) grant number APP1093017.

Conflicts of Interest: R.A.S. has received fees for professional services from Merck Sharp & Dohme, GlaxoSmithKline Australia, Bristol-Myers Squibb, Dermpedia, Novartis Pharmaceuticals Australia Pty Ltd, Myriad, NeraCare and Amgen.

References

1. *Melanoma of the Skin*; Australian Institute of Health and Welfare: Canberra, Australia, 2016.
2. Thompson, J.F.; Scolyer, R.A.; Kefford, R.F. Cutaneous melanoma. *Lancet* **2005**, *365*, 687–701. [CrossRef]
3. Cohen, J.V.; Buchbinder, E.I. The Evolution of Adjuvant Therapy for Melanoma. *Curr. Oncol. Rep.* **2019**, *21*, 106. [CrossRef] [PubMed]
4. Clark, W.H., Jr.; Elder, D.E.; Guerry, D.T.; Epstein, M.N.; Greene, M.H.; Van Horn, M. A study of tumor progression: The precursor lesions of superficial spreading and nodular melanoma. *Hum. Pathol.* **1984**, *15*, 1147–1165. [CrossRef]
5. Curtin, J.A.; Fridlyand, J.; Kageshita, T.; Patel, H.N.; Busam, K.J.; Kutzner, H.; Cho, K.H.; Aiba, S.; Brocker, E.B.; LeBoit, P.E.; et al. Distinct sets of genetic alterations in melanoma. *N. Engl. J. Med.* **2005**, *353*, 2135–2147. [CrossRef] [PubMed]
6. Palmieri, G.; Colombino, M.; Casula, M.; Manca, A.; Mandala, M.; Cossu, A. Molecular Pathways in Melanomagenesis: What We Learned from Next-Generation Sequencing Approaches. *Curr. Oncol. Rep.* **2018**, *20*, 86. [CrossRef]
7. Gurzu, S.; Beleaua, M.A.; Jung, I. The role of tumor microenvironment in development and progression of malignant melanomas—A systematic review. *Rom. J. Morphol. Embryol.* **2018**, *59*, 23–28.
8. Alsina, J.; Gorsk, D.H.; Germino, F.J.; Shih, W.; Lu, S.-E.; Zhang, Z.-G.; Yang, J.-M.; Hait, W.N.; Goydos, J.S. Detection of Mutations in the Mitogen-Activated Protein Kinase Pathway in Human Melanoma. *Clin. Cancer Res.* **2003**, *9*, 6419–6425.
9. Davies, H.; Bignell, G.R.; Cox, C.; Stephens, P.; Edkins, S.; Clegg, S.; Teague, J.; Woffendin, H.; Garnett, M.J.; Bottomley, W.; et al. Mutations of the BRAF gene in human cancer. *Nature* **2002**, *417*, 949–954. [CrossRef]
10. Roh, M.R.; Eliades, P.; Gupta, S.; Tsao, H. Genetics of melanocytic nevi. *Pigment Cell Melanoma Res.* **2015**, *28*, 661–672. [CrossRef]
11. Harms, P.W.; Hocker, T.L.; Zhao, L.; Chan, M.P.; Andea, A.A.; Wang, M.; Harms, K.L.; Wang, M.L.; Carskadon, S.; Palanisamy, N.; et al. Loss of p16 expression and copy number changes of CDKN2A in a spectrum of spitzoid melanocytic lesions. *Hum. Pathol.* **2016**, *58*, 152–160. [CrossRef]

12. Rocco, J.W.; Sidransky, D. p16(MTS-1/CDKN2/INK4a) in cancer progression. *Exp. Cell Res.* **2001**, *264*, 42–55. [CrossRef] [PubMed]
13. Hodis, E.; Watson, I.R.; Kryukov, G.V.; Arold, S.T.; Imielinski, M.; Theurillat, J.P.; Nickerson, E.; Auclair, D.; Li, L.; Place, C.; et al. A landscape of driver mutations in melanoma. *Cell* **2012**, *150*, 251–263. [CrossRef] [PubMed]
14. Reed, J.A.; Loganzo, F., Jr.; Shea, C.R.; Walker, G.J.; Flores, J.F.; Glendening, J.M.; Bogdany, J.K.; Shiel, M.J.; Haluska, F.G.; Fountain, J.W.; et al. Loss of expression of the p16/cyclin-dependent kinase inhibitor 2 tumor suppressor gene in melanocytic lesions correlates with invasive stage of tumor progression. *Cancer Res.* **1995**, *55*, 2713–2718. [PubMed]
15. Guo, D.; Lui, G.Y.L.; Lai, S.L.; Wilmott, J.S.; Tikoo, S.; Jackett, L.A.; Quek, C.; Brown, D.L.; Sharp, D.M.; Kwan, R.Y.Q.; et al. RAB27A promotes melanoma cell invasion and metastasis via regulation of pro-invasive exosomes. *Int. J. Cancer* **2019**, *144*, 3070–3085. [CrossRef] [PubMed]
16. Shain, A.H.; Yeh, I.; Kovalyshyn, I.; Sriharan, A.; Talevich, E.; Gagnon, A.; Dummer, R.; North, J.; Pincus, L.; Ruben, B.; et al. The Genetic Evolution of Melanoma from Precursor Lesions. *N. Engl. J. Med.* **2015**, *373*, 1926–1936. [CrossRef] [PubMed]
17. Gao, J.; Shi, L.Z.; Zhao, H.; Chen, J.; Xiong, L.; He, Q.; Chen, T.; Roszik, J.; Bernatchez, C.; Woodman, S.E.; et al. Loss of IFN-gamma Pathway Genes in Tumor Cells as a Mechanism of Resistance to Anti-CTLA-4 Therapy. *Cell* **2016**, *167*, 397–404. [CrossRef]
18. Singleton, K.R.; Crawford, L.; Tsui, E.; Manchester, H.E.; Maertens, O.; Liu, X.; Liberti, M.V.; Magpusao, A.N.; Stein, E.M.; Tingley, J.P.; et al. Melanoma Therapeutic Strategies that Select against Resistance by Exploiting MYC-Driven Evolutionary Convergence. *Cell Rep.* **2017**, *21*, 2796–2812. [CrossRef]
19. Pitt, J.M.; Vetizou, M.; Daillere, R.; Roberti, M.P.; Yamazaki, T.; Routy, B.; Lepage, P.; Boneca, I.G.; Chamaillard, M.; Kroemer, G.; et al. Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors. *Immunity* **2016**, *44*, 1255–1269. [CrossRef]
20. Poulikakos, P.I.; Persaud, Y.; Janakiraman, M.; Kong, X.; Ng, C.; Moriceau, G.; Shi, H.; Atefi, M.; Titz, B.; Gabay, M.T. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature* **2011**, *480*, 387–390. [CrossRef]
21. Massi, D.; LeBoit, P.E. *Histological Diagnosis of Nevi and Melanoma*, 2nd ed.; Springer: Berlin/Heidelberg, Germany, 2014.
22. Busam, K.; Gerami, P.; Scolyer, R.A. *Pathology of Melanocytic Tumors*, 1st ed.; Elsevier: Philadelphia, PA, USA, 2018.
23. Barnhill, R.L.; Piepkorn, M.W.; Busam, K. *Pathology of Melanocytic Nevi and Melanoma*, 3rd ed.; Springer: Berlin/Heidelberg, Germany, 2014.
24. Cichorek, M.; Wachulska, M.; Stasiewicz, A.; Tymińska, A. Skin melanocytes: Biology and development. *Postepy Dermatol. Alergol.* **2013**, *30*, 30–41. [CrossRef]
25. Morais, M.C.C.; Stuhl, I.; Sabino, A.U.; Lautenschlager, W.W.; Queiroga, A.S.; Tortelli, T.C.; Chammas, R.; Suhov, Y.; Ramos, A.F. Stochastic model of contact inhibition and the proliferation of melanoma in situ. *Sci. Rep.* **2017**, *7*, 8026. [CrossRef]
26. Thingnes, J.; Lavelle, T.J.; Hovig, E.; Omholt, S.W. Understanding the Melanocyte Distribution in Human Epidermis: An Agent-Based Computational Model Approach. *PLoS ONE* **2012**, *7*, e40377. [CrossRef] [PubMed]
27. Shiau, C.J.; Thompson, J.F.; Scolyer, R.A. Controversies and evolving concepts in the diagnosis, classification and management of lentigo maligna. *Expert Rev. Dermatol.* **2013**, *8*, 195–214. [CrossRef]
28. Menzies, S.W.; Liyanarachchi, S.; Coates, E.; Smith, A.; Cooke-Yarborough, C.; Lo, S.; Armstrong, B.; Scolyer, R.A.; Guitera, P. Estimated risk of progression of lentigo maligna to lentigo maligna melanoma. *Melanoma. Res.* **2019**. [CrossRef] [PubMed]
29. Martincorena, I.; Roshan, A.; Gerstung, M.; Ellis, P.; Van Loo, P.; McLaren, S.; Wedge, D.C.; Fullam, A.; Alexandrov, L.B.; Tubio, J.M.; et al. Tumor evolution. High burden and pervasive positive selection of somatic mutations in normal human skin. *Science* **2015**, *348*, 880–886. [CrossRef]
30. Colebatch, A.J.; Scolyer, R.A. Trajectories of premalignancy during the journey from melanocyte to melanoma. *Pathology* **2018**, *50*, 16–23. [CrossRef]
31. Bastian, B.C. Understanding the progression of melanocytic neoplasia using genomic analysis: From fields to cancer. *Oncogene* **2003**, *22*, 3081–3086. [CrossRef]

32. Ross, A.L.; Sanchez, M.I.; Grichnik, J.M. Nevus senescence. *ISRN Derm.* **2011**, *2011*, 642157. [CrossRef]
33. Melamed, R.D.; Aydin, I.T.; Rajan, G.S.; Phelps, R.; Silvers, D.N.; Emmett, K.J.; Brunner, G.; Rabadan, R.; Celebi, J.T. Genomic Characterization of Dysplastic Nevi Unveils Implications for Diagnosis of Melanoma. *J. Invest Dermatol.* **2017**, *137*, 905–909. [CrossRef]
34. Colebatch, A.J.; Ferguson, P.; Newell, F.; Kazakoff, S.H.; Witkowski, T.; Dobrovic, A.; Johansson, P.A.; Saw, R.P.M.; Stretch, J.R.; McArthur, G.A.; et al. Molecular Genomic Profiling of Melanocytic Nevi. *J. Invest Dermatol.* **2019**, *139*, 1762–1768. [CrossRef]
35. Cancer Genome Atlas Network. Genomic classification of cutaneous melanoma. *Cell* **2015**, *161*, 1681–1696. [CrossRef] [PubMed]
36. Hayward, N.K.; Wilmott, J.S.; Waddell, N.; Johansson, P.A.; Field, M.A.; Nones, K.; Patch, A.M.; Kakavand, H.; Alexandrov, L.B.; Burke, H.; et al. Whole-genome landscapes of major melanoma subtypes. *Nature* **2017**, *545*, 175–180. [CrossRef] [PubMed]
37. Charbel, C.; Fontaine, R.H.; Malouf, G.G.; Picard, A.; Kadlub, N.; El-Murr, N. NRAS mutation is the sole recurrent somatic mutation in large congenital melanocytic nevi. *J. Invest Dermatol.* **2014**, *134*, 1067–1074. [CrossRef] [PubMed]
38. Bastian, B.C.; Xiong, J.; Frieden, I.J.; Williams, M.L.; Chou, P.; Busam, K.; Pinkel, D.; LeBoit, P.E. Genetic changes in neoplasms arising in congenital melanocytic nevi: Differences between nodular proliferations and melanomas. *Am. J. Pathol.* **2002**, *161*, 1163–1169. [CrossRef]
39. Armstrong, B.K.; Kricker, A. The epidemiology of UV induced skin cancer. *J. Photochem. Photobiol. B Biol.* **2001**, *63*, 8–18. [CrossRef]
40. Tate, J.G.; Bamford, S.; Jubb, H.C.; Sondka, Z.; Beare, D.M.; Bindal, N.; Boutselakis, H.; Cole, C.G.; Creatore, C.; Dawson, E.; et al. COSMIC: The Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res.* **2018**, *47*, D941–D947. [CrossRef]
41. Villacanas, O.; Perez, J.J.; Rubio-Martinez, J. Structural analysis of the inhibition of Cdk4 and Cdk6 by p16(INK4a) through molecular dynamics simulations. *J. Biomol. Struct. Dyn.* **2002**, *20*, 347–358. [CrossRef]
42. Bates, S.; Phillips, A.C.; Clark, P.A.; Stott, F.; Peters, G.; Ludwig, R.L.; Vousden, K.H. p14ARF links the tumour suppressors RB and p53. *Nature* **1998**, *395*, 124–125. [CrossRef]
43. McNeal, A.S.; Liu, K.; Nakhate, V.; Natale, C.A.; Duperret, E.K.; Capell, B.C.; Dentchev, T.; Berger, S.L.; Herlyn, M.; Seykora, J.T.; et al. CDKN2B Loss Promotes Progression from Benign Melanocytic Nevus to Melanoma. *Cancer Discov.* **2015**, *5*, 1072–1085. [CrossRef]
44. Murali, R.; Sharma, R.N.; Thompson, J.F.; Stretch, J.R.; Lee, C.S.; McCarthy, S.W.; Scolyer, R.A. Sentinel lymph node biopsy in histologically ambiguous melanocytic tumors with spitzoid features (so-called atypical spitzoid tumors). *Ann. Surg. Oncol.* **2008**, *15*, 302–309. [CrossRef]
45. Busam, K.J.; Murali, R.; Pulitzer, M.; McCarthy, S.W.; Thompson, J.F.; Shaw, H.M.; Brady, M.S.; Coit, D.G.; Dusza, S.; Wilmott, J.; et al. Atypical spitzoid melanocytic tumors with positive sentinel lymph nodes in children and teenagers, and comparison with histologically unambiguous and lethal melanomas. *Am. J. Surg. Pathol.* **2009**, *33*, 1386–1395. [CrossRef] [PubMed]
46. Elder, D.E. Melanoma progression. *Pathology* **2016**, *48*, 147–154. [CrossRef] [PubMed]
47. Elmore, J.G.; Barnhill, R.L.; Elder, D.E.; Longton, G.M.; Pepe, M.S.; Reisch, L.; Al, E. Pathologists' diagnosis of invasive melanoma and melanocytic proliferations: Observer accuracy and reproducibility study. *BMJ* **2017**, *357*, j2813. [CrossRef] [PubMed]
48. Cook, M.G.; Clarke, T.J.; Humphreys, S.; Fletcher, A.; McLaren, K.M.; Smith, N.P.; Stevens, A.; Theaker, J.M.; Melia, J. The evaluation of diagnostic and prognostic criteria and the terminology of thin cutaneous malignant melanoma by the CRC Melanoma Pathology Panel. *Histopathology* **1996**, *28*, 497–512. [CrossRef] [PubMed]
49. Brochez, L.; Verhaeghe, E.; Grosshans, E.; Haneke, E.; Pierard, G.; Ruiter, D.; Naeyaert, J.M. Inter-observer variation in the histopathological diagnosis of clinically suspicious pigmented skin lesions. *J. Pathol.* **2002**, *196*, 459–466. [CrossRef] [PubMed]
50. Perez-Alea, M.; Vivancos, A.; Caratu, G.; Matito, J.; Ferrer, B.; Hernandez-Losa, J.; Cortes, J.; Munoz, E.; Garcia-Patos, V.; Recio, J.A. Genetic profile of GNAQ-mutated blue melanocytic neoplasms reveals mutations in genes linked to genomic instability and the PI3K pathway. *Oncotarget* **2016**, *7*, 28086–28095. [CrossRef] [PubMed]

51. Afshar, A.R.; Damato, B.E.; Stewart, J.M.; Zablotska, L.B.; Roy, R.; Olshen, A.B.; Joseph, N.M.; Bastian, B.C. Next-Generation Sequencing of Uveal Melanoma for Detection of Genetic Alterations Predicting Metastasis. *Transl. Vis. Sci. Technol.* **2019**, *8*, 18. [CrossRef] [PubMed]
52. Griewank, K.G.; Muller, H.; Jackett, L.A.; Emberger, M.; Moller, I.; van de Nes, J.A.; Zimmer, L.; Livingstone, E.; Wiesner, T.; Scholz, S.L.; et al. SF3B1 and BAP1 mutations in blue nevus-like melanoma. *Mod. Pathol.* **2017**, *30*, 928–939. [CrossRef]
53. Dimonitsas, E.; Liakea, A.; Sakellariou, S.; Thymara, I.; Giannopoulos, A.; Stratigos, A.; Soura, E.; Saetta, A.; Korkolopoulou, P. An update on molecular alterations in melanocytic tumors with emphasis on Spitzoid lesions. *Ann. Transl. Med.* **2018**, *6*, 249. [CrossRef]
54. Wiesner, T.; Kutzner, H. [Morphological and genetic aspects of Spitz tumors]. *Pathologe* **2015**, *36*, 37–43. [CrossRef]
55. Lee, S.; Barnhill, R.L.; Dummer, R.; Dalton, J.; Wu, J.; Pappo, A.; Bahrami, A. TERT Promoter Mutations Are Predictive of Aggressive Clinical Behavior in Patients with Spitzoid Melanocytic Neoplasms. *Sci. Rep.* **2015**, *5*, 11200. [CrossRef] [PubMed]
56. Lim, C.; Murali, R.; McCarthy, S.W.; Krivanek, J.; Scolyer, R.A. Pigmented epithelioid melanocytoma: a recently described melanocytic tumour of low malignant potential. *Pathology* **2010**, *42*, 284–286. [CrossRef] [PubMed]
57. Zembowicz, A.; Knoepp, S.M.; Bei, T.; Stergiopoulos, S.; Eng, C.; Mihm, M.C.; Stratakis, C.A. Loss of expression of protein kinase a regulatory subunit 1alpha in pigmented epithelioid melanocytoma but not in melanoma or other melanocytic lesions. *Am. J. Surg. Pathol.* **2007**, *31*, 1764–1775. [CrossRef] [PubMed]
58. De la Fouchardiere, A.; Caillot, C.; Jacquemus, J.; Durieux, E.; Houlier, A.; Haddad, V.; Pissaloux, D. beta-Catenin nuclear expression discriminates deep penetrating nevi from other cutaneous melanocytic tumors. *Virchows. Arch.* **2019**, *474*, 539–550. [CrossRef] [PubMed]
59. Erstine, E.M.; Lazar, A.J. Toward an effective use of beta-catenin immunohistochemistry in the evaluation of challenging melanocytic lesions. *Virchows. Arch.* **2019**, *474*, 535–537. [CrossRef] [PubMed]
60. Yeh, I.; Lang, U.E.; Durieux, E.; Tee, M.K.; Jorapur, A.; Shain, A.H.; Haddad, V.; Pissaloux, D.; Chen, X.; Cerroni, L.; et al. Combined activation of MAP kinase pathway and beta-catenin signaling cause deep penetrating nevi. *Nat. Commun.* **2017**, *8*, 644. [CrossRef]
61. Elder, D.E.; Massi, D.; Scolyer, R.A.; Willemze, R. *WHO Classification of Tumours*, 4th ed.; International Agency for Research on Cancer: Lyon, France, 2018.
62. Lancaster, H.O. Some geographical aspects of the mortality from melanoma in Europeans. *Med. J. Aust.* **1956**, *43*, 1082–1087. [CrossRef]
63. International Agency for Research on Cancer Working Group on artificial ultraviolet (UV) light and skin cancer. The association of use of sunbeds with cutaneous malignant melanoma and other skin cancers: A systematic review. *Int. J. Cancer* **2007**, *120*, 1116–1122.
64. Berger, M.F.; Hodis, E.; Heffernan, T.P.; Deribe, Y.L.; Lawrence, M.S.; Protopopov, A.; Ivanova, E.; Watson, I.R.; Nickerson, E.; Ghosh, P.; et al. Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature* **2012**, *485*, 502–506. [CrossRef]
65. Alexandrov, L.B.; Nik-Zainal, S.; Wedge, D.C.; Aparicio, S.A.J.R.; Behjati, S.; Biankin, A.V.; Bignell, G.R.; Bolli, N.; Borg, A.; Børresen-Dale, A.-L.; et al. Signatures of mutational processes in human cancer. *Nature* **2013**, *500*, 415. [CrossRef]
66. LeBoit, P.E. The Morphology of Tumor Progression in Melanoma In Situ. *JAMA Derm.* **2019**, *155*, 775–776. [CrossRef] [PubMed]
67. Moreno, A.; Manrique-Silva, E.; Viros, A.; Requena, C.; Sanmartin, O.; Traves, V.; Nagore, E. Histologic Features Associated With an Invasive Component in Lentigo Maligna Lesions. *JAMA Derm.* **2019**, *155*, 782–788. [CrossRef] [PubMed]
68. Reed, J.A.; McNutt, N.S.; Bogdany, J.K.; Albino, A.P. Expression of the mast cell growth factor interleukin-3 in melanocytic lesions correlates with an increased number of mast cells in the perilesional stroma: Implications for melanoma progression. *J. Cutan. Pathol.* **1996**, *23*, 495–505. [CrossRef] [PubMed]
69. Toth-Jakatics, R.; Jimi, S.; Takebayashi, S.; Kawamoto, N. Cutaneous malignant melanoma: Correlating between neovascularization and peritumor accumulation of mast cells overexpressing vascular endothelial growth factor. *Hum. Pathol.* **2000**, *31*, 955–960. [PubMed]

70. Brychtova, S.; Bezdekova, M.; Brychta, T.; Tichy, M. The role of vascular endothelial growth factors and their receptors in malignant melanomas. *Neoplasma* **2008**, *55*, 273–279. [PubMed]
71. Mehnert, J.M.; McCarthy, M.M.; Jilaveanu, L.; Flaherty, K.T.; Aziz, S.; Camp, R.L.; Rimm, D.L.; Kluger, H.M. Quantitative expression of VEGF, VEGF-R1, VEGF-R2, and VEGF-R3 in melanoma tissue microarrays. *Hum. Pathol.* **2010**, *41*, 375–384. [CrossRef]
72. Tas, F.; Duranyildiz, D.; Oguz, H.; Camlica, H.; Yasasever, V.; Topuz, E. Circulating serum levels of angiogenic factors and vascular endothelial growth factor receptors 1 and 2 in melanoma patients. *Melanoma Res.* **2006**, *16*, 405–411. [CrossRef]
73. Rajabi, P.; Neshat, A.; Mokhtari, M.; Rajabi, M.A.; Eftekhari, M.; Tavakoli, P. The role of VEGF in melanoma progression. *J. Res. Med. Sci.* **2012**, *17*, 534–539.
74. Knies-Bamforth, U.E.; Fox, S.B.; Poulsom, R.; Evan, G.I.; Harris, A.L. c-Myc interacts with hypoxia to induce angiogenesis in vivo by a vascular endothelial growth factor-dependent mechanism. *Cancer Res.* **2004**, *64*, 6563–6570. [CrossRef]
75. Ott, P.A.; Hodi, F.S.; Buchbinder, E.I. Inhibition of immune checkpoints and vascular endothelial growth factor as combination therapy for metastatic melanoma: An overview of rationale, preclinical evidence, and initial clinical data. *Front Oncol.* **2015**, *5*, 202. [CrossRef]
76. Itakura, E.; Huang, R.R.; Wen, D.R.; Paul, E.; Wunsch, P.H.; Cochran, A.J. IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence. *Mod. Pathol.* **2011**, *24*, 801–809. [CrossRef] [PubMed]
77. Meyer, N.; Penn, L.Z. Reflecting on 25 years with MYC. *Nat. Rev. Cancer* **2008**, *8*, 976–990. [CrossRef] [PubMed]
78. Schlagbauer-Wadl, H.; Griffioen, M.; van Elsas, A.; Schrier, P.I.; Pustelnik, T.; Eichler, H.G.; Wolff, K.; Pehamberger, H.; Jansen, B. Influence of increased c-Myc expression on the growth characteristics of human melanoma. *J. Invest Dermatol.* **1999**, *112*, 332–336. [CrossRef] [PubMed]
79. Lin, X.; Sun, R.; Zhao, X.; Zhu, D.; Zhao, X.; Gu, Q.; Dong, X.; Zhang, D.; Zhang, Y.; Li, Y.; et al. C-myc overexpression drives melanoma metastasis by promoting vasculogenic mimicry via c-myc/snail/Bax signaling. *J. Mol. Med.* **2017**, *95*, 53–67. [CrossRef] [PubMed]
80. Gabay, M.; Li, Y.; Felsher, D.W. MYC activation is a hallmark of cancer initiation and maintenance. *Cold Spring Harb. Perspect. Med.* **2014**, *4*. [CrossRef]
81. D'Agnano, I.; Valentini, A.; Fornari, C.; Bucci, B.; Starace, G.; Felsani, A.; Citro, G. Myc down-regulation induces apoptosis in M14 melanoma cells by increasing p27kip1 levels. *Oncogene* **2001**, *20*, 2814–2825. [CrossRef]
82. Versteeg, R.; Peltenburg, L.T.; Plomp, A.C.; Schrier, P.I. High expression of the c-myc oncogene renders melanoma cells prone to lysis by natural killer cells. *J. Immunol.* **1989**, *143*, 4331–4337.
83. Reintgen, D.; Cruse, C.W.; Wells, K.; Berman, C.; Fenske, N.; Glass, F.; Schroer, K.; Heller, R.; Ross, M.; Lyman, G.; et al. The orderly progression of melanoma nodal metastases. *Ann. Surg.* **1994**, *220*, 759–767. [CrossRef]
84. Peinado, H.; Alečković, M.; Lavotshkin, S.; Matei, I.; Costa-Silva, B.; Moreno-Bueno, G.; Al, E. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* **2012**, *18*, 883–891. [CrossRef]
85. Li, J.; Chen, J.; Wang, S.; Li, P.; Zheng, C.; Zhou, X.; Tao, Y.; Chen, X.; Sun, L.; Wang, A.; et al. Blockage of transferred exosome-shuttled miR-494 inhibits melanoma growth and metastasis. *J. Cell Physiol.* **2019**. [CrossRef]
86. Luzzi, K.J.; MacDonald, I.C.; Schmidt, E.E.; Kerkvliet, N.; Morris, V.L.; Chambers, A.F.; Groom, A.C. Multistep nature of metastatic inefficiency: Dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am. J. Pathol.* **1998**, *153*, 865–873. [CrossRef]
87. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; Duan, S.; Cao, L.; Worley, L.A.; Council, M.L.; Matatall, K.A.; Helms, C.; Bowcock, A.M. Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science* **2010**, *330*, 1410–1413. [CrossRef] [PubMed]
88. Bakalian, S.; Marshall, J.C.; Logan, P.; Faingold, D.; Maloney, S.; Di Cesare, S.; Martins, C.; Fernandes, B.F.; Burnier, M.N., Jr. Molecular pathways mediating liver metastasis in patients with uveal melanoma. *Clin. Cancer Res.* **2008**, *14*, 951–956. [CrossRef] [PubMed]

89. Eskelin, S.; Pyrhonen, S.; Summanen, P.; Hahka-Kemppinen, M.; Kivela, T. Tumor doubling times in metastatic malignant melanoma of the uvea: Tumor progression before and after treatment. *Ophthalmology* **2000**, *107*, 1443–1449. [CrossRef]
90. Balch, C.M.; Houghton, A.N.; Sober, A.J.; Soong, S.J. *Cutaneous Melanoma*, 4th ed.; Quality Medical Publishing: St. Louis, MO, USA, 2003.
91. Mignogna, C.; Scali, E.; Camastra, C.; Presta, I.; Zeppa, P.; Barni, T.; Donato, G.; Bottoni, U.; Di Vito, A. Innate immunity in cutaneous melanoma. *Clin. Exp. Dermatol.* **2017**, *42*, 243–250. [CrossRef] [PubMed]
92. Gonzalez-Gugel, E.; Saxena, M.; Bhardwaj, N. Modulation of innate immunity in the tumor microenvironment. *Cancer Immunol. Immunother.* **2016**, *65*, 1261–1268. [CrossRef]
93. Ivashkiv, L.B.; Donlin, L.T. Regulation of type I interferon responses. *Nat. Rev. Immunol.* **2014**, *14*, 36–49. [CrossRef]
94. Bradley, S.D.; Chen, Z.; Melendez, B.; Talukder, A.; Khalili, J.S.; Rodriguez-Cruz, T.; Liu, S.; Whittington, M.; Deng, W.; Li, F.; et al. BRAFV600E Co-opts a Conserved MHC Class I Internalization Pathway to Diminish Antigen Presentation and CD8+ T-cell Recognition of Melanoma. *Cancer Immunol Res.* **2015**, *3*, 602–609. [CrossRef]
95. Pardoll, D.M. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* **2012**, *12*, 252–264. [CrossRef]
96. Togashi, Y.; Shitara, K.; Nishikawa, H. Regulatory T cells in cancer immunosuppression — implications for anticancer therapy. *Nat Rev Clin Oncol* **2019**, *16*, 356–371. [CrossRef]
97. Takata, M.; Murata, H.; Saida, T. Molecular pathogenesis of malignant melanoma: A different perspective from the studies of melanocytic nevus and acral melanoma. *Pigment Cell Melanoma Res.* **2010**, *23*, 64–71. [CrossRef] [PubMed]
98. Lim, S.Y.; Menzies, A.M.; Rizos, H. Mechanisms and strategies to overcome resistance to molecularly targeted therapy for melanoma. *Cancer* **2017**, *123*, 2118–2129. [CrossRef] [PubMed]
99. Hauschild, A.; Grob, J.J.; Demidov, L.V.; Jouary, T.; Gutzmer, R.; Millward, M.; Rutkowski, P.; Blank, C.U.; Miller, W.H.; Kaempgen, E.; et al. An update on BREAK-3, a phase III, randomized trial: Dabrafenib (DAB) versus dacarbazine (DTIC) in patients with BRAF V600E-positive mutation metastatic melanoma (MM). *J. Clin. Oncol.* **2013**, *31*, 9013. [CrossRef]
100. Chapman, P.B.; Hauschild, A.; Robert, C.; Larkin, J.M.G.; Haanen, J.B.A.G.; Ribas, A.; Hogg, D.; Hamid, O.; Ascierto, P.A.; Testori, A.; et al. Updated overall survival (OS) results for BRIM-3, a phase III randomized, open-label, multicenter trial comparing BRAF inhibitor vemurafenib (vem) with dacarbazine (DTIC) in previously untreated patients with BRAFV600E-mutated melanoma. *J. Clin. Oncol.* **2012**, *30*, 8502. [CrossRef]
101. Montagut, C.; Sharma, S.V.; Shioda, T.; McDermott, U.; Ulman, M.; Ulkus, L.E.; Dias-Santagata, D.; Stubbs, H.; Lee, D.Y.; Singh, A.; et al. Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. *Cancer Res.* **2008**, *68*, 4853–4861. [CrossRef] [PubMed]
102. Johannessen, C.M.; Boehm, J.S.; Kim, S.Y.; Thomas, S.R.; Wardwell, L.; Johnson, L.A.; Emery, C.M.; Stransky, N.; Cogdill, A.P.; Barretina, J.; et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* **2010**, *468*, 973–977. [CrossRef]
103. Shi, H.; Moriceau, G.; Kong, X.; Lee, M.K.; Lee, H.; Koya, R.C.; Ng, C.; Chodon, T.; Scolyer, R.A.; Dahlman, K.B.; et al. Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat. Commun.* **2012**, *3*, 724. [CrossRef]
104. Corcoran, R.B.; Dias-Santagata, D.; Bergethon, K.; Iafrate, A.J.; Settleman, J.; Engelman, J.A. BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation. *Sci. Signal.* **2010**, *3*, ra84. [CrossRef]
105. Nazarian, R.; Shi, H.; Wang, Q.; Kong, X.; Koya, R.C.; Lee, H.; Chen, Z.; Lee, M.K.; Attar, N.; Sazegar, H.; et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* **2010**, *468*, 973–977. [CrossRef]
106. Wagle, N.; Emery, C.; Berger, M.F.; Davis, M.J.; Sawyer, A.; Pochanard, P.; Kehoe, S.M.; Johannessen, C.M.; Macconaill, L.E.; Hahn, W.C.; et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J. Clin. Oncol.* **2011**, *29*, 3085–3096. [CrossRef]
107. Villanueva, J.; Vultur, A.; Lee, J.T.; Somasundaram, R.; Fukunaga-Kalabis, M.; Cipolla, A.K.; Wubbenhorst, B.; Xu, X.; Gimotty, P.A.; Kee, D.; et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase

switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* **2010**, *18*, 683–695. [CrossRef] [PubMed]
108. Martz, C.A.; Ottina, K.A.; Singleton, K.R.; Jasper, J.S.; Wardell, S.E.; Peraza-Penton, A.; Anderson, G.R.; Winter, P.S.; Wang, T.; Alley, H.M.; et al. Systematic identification of signaling pathways with potential to confer anticancer drug resistance. *Sci. Signal* **2014**, *7*, ra121. [CrossRef] [PubMed]
109. Paschen, A.; Schadendorf, D. The Era of Checkpoint Inhibition: Lessons Learned from Melanoma. *Recent Results Cancer Res.* **2020**, *214*, 169–187. [CrossRef] [PubMed]
110. Amaria, R.N.; Reddy, S.M.; Tawbi, H.A.; Davies, M.A.; Ross, M.I.; Glitza, I.C.; Cormier, J.N.; Lewis, C.; Hwu, W.J.; Hanna, E.; et al. Neoadjuvant immune checkpoint blockade in high-risk resectable melanoma. *Nat. Med.* **2018**, *24*, 1649–1654. [CrossRef] [PubMed]
111. Wolchok, J.D.; Chiarion-Sileni, V.; Gonzalez, R.; Rutkowski, P.; Grob, J.J.; Cowey, C.L.; Lao, C.D.; Wagstaff, J.; Schadendorf, D.; Ferrucci, P.F.; et al. Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N. Engl. J. Med.* **2017**, *377*, 1345–1356. [CrossRef]
112. Larkin, J.; Chiarion-Sileni, V.; Gonzalez, R.; Grob, J.J.; Cowey, C.L.; Lao, C.D.; Schadendorf, D.; Dummer, R.; Smylie, M.; Rutkowski, P.; et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N. Engl. J. Med.* **2015**, *373*, 23–34. [CrossRef]
113. Gide, T.N.; Quek, C.; Menzies, A.M.; Tasker, A.T.; Shang, P.; Holst, J.; Madore, J.; Lim, S.Y.; Velickovic, R.; Wongchenko, M.; et al. Distinct Immune Cell Populations Define Response to Anti-PD-1 Monotherapy and Anti-PD-1/Anti-CTLA-4 Combined Therapy. *Cancer Cell* **2019**, *35*, 238–255. [CrossRef]
114. IFNgamma Mutations Prompt CTLA-4 Inhibitor Resistance. *Cancer Discov.* **2017**, *7*, OF3. [CrossRef]
115. Yu, C.; Liu, X.; Yang, J.; Zhang, M.; Jin, H.; Ma, X.; Shi, H. Combination of Immunotherapy With Targeted Therapy: Theory and Practice in Metastatic Melanoma. *Front. Immunol.* **2019**, *10*. [CrossRef]
116. Nagore, E.; Rachakonda, S.; Kumar, R. TERT promoter mutations in melanoma survival. *Oncotarget* **2019**, *10*, 1546–1548. [CrossRef]
117. Heidenreich, B.; Kumar, R. TERT promoter mutations in telomere biology. *Mutat. Res.* **2017**, *771*, 15–31. [CrossRef] [PubMed]
118. Lee, J.H.; Long, G.V.; Boyd, S.; Lo, S.; Menzies, A.M.; Tembe, V.; Guminski, A.; Jakrot, V.; Scolyer, R.A.; Mann, G.J.; et al. Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. *Ann. Oncol.* **2017**, *28*, 1130–1136. [CrossRef] [PubMed]
119. Lee, J.H.; Saw, R.P.; Thompson, J.F.; Lo, S.; Spillane, A.J.; Shannon, K.F.; Stretch, J.R.; Howle, J.; Menzies, A.M.; Carlino, M.S.; et al. Pre-operative ctDNA predicts survival in high-risk stage III cutaneous melanoma patients. *Ann. Oncol.* **2019**, *30*, 815–822. [CrossRef] [PubMed]

CHAPTER 3: PROGNOSTIC FACTORS IN PRIMARY MELANOMA

Overview: Parameters such as Breslow thickness (BT), ulceration and microsatellitosis are key prognostic determinants for primary cutaneous melanoma, and they are subject to variation between tissue levels. There is, however, a paucity of well-designed studies looking at how these features vary between levels, and the consequent impact on staging parameters. The hypothesis driving the ensuing study is that there may be a subset of patients whose staging would be better optimised if multiple levels were examined, particularly if initial Breslow thickness sits close to the threshold of a higher stage. The performance of additional tissue levels is a simple test that is potentially impactful and highly cost-effective in improving the staging of, and management decisions for, patients with melanoma.

Specific aims: Our primary aim was to evaluate the variability in prognostic parameters that occur when increased numbers of tissue levels, taken at regular intervals, are examined histopathologically for the primary AJCC prognostic determinants. A secondary aim was to determine how many tissue levels would optimise assessment.

Contribution to literature: This is the first study to enquire about the effect of tissue levels, in a standardised manner, on multiple prognostic features in a single analysis.

Journal of Cutaneous Pathology

WILEY

JOURNAL OF JCP CUTANEOUS PATHOLOGY

ORIGINAL ARTICLE OPEN ACCESS

Evaluation of Multiple Tissue Levels Frequently Upstages Patients With Clinically Localized Thin Primary Cutaneous Melanoma

Louise A. Jackett[1,2] | James P. Gullifer[1] | Richard A. Scolyer[2,3,4,5]

[1]Department of Anatomical Pathology, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia | [2]Melanoma Institute Australia, The University of Sydney, Sydney, New South Wales, Australia | [3]Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital & NSW Health Pathology, Sydney, New South Wales, Australia | [4]Faculty of Medicine and Health, The University of Sydney, Sydney, New South Wales, Australia | [5]Charles Perkins Centre, The University of Sydney, Sydney, New South Wales, Australia

Correspondence: Richard A. Scolyer (richard.scolyer@health.nsw.gov.au)

Received: 14 May 2024 | **Revised:** 1 September 2024 | **Accepted:** 13 September 2024

Funding: This study was supported by the NHMRC Investigator Grant (2022/GNT2018514).

Keywords: Breslow | melanoma | pathology | staging | ulceration

ABSTRACT

Background: Breslow thickness (BT), ulceration, and microsatellitosis are critical prognostic parameters for cutaneous melanoma staging. These parameters can vary depending on the number of tissue levels examined from individual paraffin blocks. We sought to evaluate all prognostic histopathologic parameters in melanoma for their variations between levels, taken at regular intervals, in a single study.

Methods: We analyzed 40 consecutive cases of primary cutaneous (nonacral) melanoma through five hematoxylin and eosin sections, taken at 100 μm intervals, for staging and prognostic parameters.

Results: Examination of additional levels resulted in (a) an increase in BT in 47.5% (19 out of 40) of cases and (b) detection of ulceration in a further 5% (2/40). This resulted in upstaging for 20% (8 out of 40) of patients (15% because of BT, 2.5% because of ulceration, and 2.5% because of BT and ulceration). The upstaging effect was incremental, with approximately 5% of patients upstaged with each additional 100 μm interval (up to 400 μm). Incipient ulceration and epidermal consumption were infrequent (10% of cases); however, when present, ulceration was subsequently observed in half of cases. We encountered no cases where microsatellitosis was detected at deeper levels.

Conclusion: The performance of additional tissue levels is a simple and inexpensive procedure that can improve the accuracy of staging for patients with thin (pT1) primary cutaneous melanomas. It may be pertinent for pathologists to consider additional levels for thin melanomas when a BT measurement is close to a staging threshold (e.g., within 0.1–0.3 mm for pT1a vs. pT1b, or pT1b vs. pT2a), or when incipient ulceration is encountered.

1 | Introduction

Breslow thickness (BT) is the most important prognostic parameter in the assessment of primary cutaneous melanoma [1, 2]. Taken together, BT and ulceration determine the pathological T (pT) stage of melanoma in the eighth edition of the AJCC Cancer Staging Manual [2]. In addition, the presence of microsatellites has prognostic significance comparable to nodal basin involvement and is now incorporated into pathological N stage [2]; in its presence, melanoma is categorized as Stage II disease.

As originally emphasized by Breslow in 1977, melanoma tumor thickness can be spatially heterogeneous [3]. Most macroscopic handling protocols, therefore, advocate that primary melanomas are submitted for histopathologic examination in their entirety, a process that results in tissue slices of approximately 2–3 mm thickness [4, 5]. Depending on the number and depth of levels examined from such slices, a handful of studies have shown that BT and other prognostic factors can vary [6–11].

There is no standardized recommendation for the number or depth of levels a pathologist should examine microscopically when evaluating melanoma [4, 5]. It is the authors' experience working in several high-volume melanoma referral centers that the number of levels evaluated varies considerably across and within institutions. A large survey of US dermatopathology practices also found inconsistency [12]. Standardization of the number and depth of levels has not been addressed for the multi-institution data sets used by AJCC [2].

Although staging systems derived from large patient data sets can account for population variability, many of an individual patient's melanoma prognostic parameters are reported in a binary fashion and directly determine management. Given that vertical tumor thickness alters the primary T stage category in increments of 0.1 mm, foci of ulceration can appear "incipient" at the edges, and microsatellites are often submillimeter, there is value in understanding the degree of variability that may occur when a number of tissue levels are examined histopathologically [2, 13–15].

Of the few studies that have formally addressed this issue, most have been inconsistent in the distances of the intervals examined [6–11]. Most previous studies have evaluated only a single parameter [6–11].

We evaluated the variability in prognostic parameters that occurred when increased numbers of tissue levels taken at regular intervals were examined histopathologically for the primary prognostic determinants of the AJCC stage, namely BT, ulceration, and microsatellites. We also sought to determine whether the assessment of multiple tissue levels beyond the initial hematoxylin and eosin (H&E) staining is warranted, and if so, how many levels, and at what depth, would optimize assessment. To the best of our knowledge, this is the first study to consider variability when multiple histopathologic parameters are evaluated in multiple histopathological tissue sections taken at regular intervals in a single study.

2 | Methods

We studied 40 consecutive cases with an unequivocal diagnosis of primary cutaneous (nonacral) melanoma treated at a major melanoma referral center (Peter McCallum Cancer Center). Only patients with invasive melanomas were included in this study. Melanomas that were already maximally staged as pT4b with microsatellites and those with clinically evident lymph node metastases were excluded.

Specimens included all complete excision biopsies, punch biopsies (excisional or partial), shave biopsies (excisional or partial), and incisional biopsies, for which the primary purpose of diagnosing and staging melanoma. Wide local excisions of previously biopsied invasive melanomas were excluded. Very small biopsies with a significant risk of tissue cutting out on levels were also excluded.

All melanomas were sliced macroscopically at 2–3 mm intervals, submitted in their entirety for pathological examination, and routinely processed into formalin-fixed, paraffin-embedded blocks. After a diagnosis of unequivocal melanoma was made on an initial H&E-stained section, four additional sections at 100 μm intervals were cut and stained.

In cases where invasive melanoma was present in multiple blocks, additional levels were performed and evaluated on all relevant blocks, and the highest parameters were recorded.

Age, sex, site, melanoma subtype, and biopsy modality were recorded for each patient. The following histological parameters were assessed for each H&E slide: measurement of BT (to the nearest 0.1 mm), ulceration (present, absent, or incipient), AJCC pT stage, microsatellites, lymphovascular invasion (LVI), tumor-infiltrating lymphocytes (TILs, examined according to ICCR recommendations), and intermediate or late regression. The mitotic rate was performed on only one level in an area deemed to be the tumor hotspot. BT was measured using an ocular graticule, calibrated for 0.1 mm increments. Microsatellites were defined according to the AJCC staging manual (eighth edition), where consensus pathological opinion was that the tumor represented a spatially distinct tumor not separated by fibrosis or other abnormal tissue reactions. The AJCC stage was compared between the first level and any subsequent upgrade.

To account for intra- and inter-observer variability, cases were evaluated with initial and repeat evaluations at least 3 months apart (L.J.), with a second pathologist validation assessment (J.G.).

3 | Results

Forty patients were analyzed. The clinicopathological parameters of the patients are outlined in Table 1. The majority of patients were male (65%, 26 out of 40), and the age range was 44–92 years (mean, 72 years; median, 75 years). Most cases were diagnostic excision biopsies (34 out of 40, 85%) and most were located on the head and neck (17 out of 40, 42.5%). Superficial spreading melanoma was the most common subtype (20 out of 40; 50%).

3.1 | Breslow Thickness

On the initial level, the mean BT of all cases was 1.3 mm (0.3–4.8 mm, median 0.9, Figure 1a,b). After review of additional levels, there was a change in maximum BT in 47.5% (19

TABLE 1 | Clinicopathological characteristics of the studied 40 cases.

Characteristic	No. of cases ($n = 40$)	%
Age (mean = 72, median = 75)		
<40	0	0%
40–49	1	2.5%
50–59	5	12.5%
60–69	10	25%
70–79	10	25%
80+	14	35%
Gender		
M	26	65%
F	14	35%
Biopsy type		
Excision biopsy	34	85%
Shave	2	5%
Punch biopsy	2	5%
Incisional	2	5%
Subtype		
SSM	20	50%
LMM	12	30%
NM	4	10%
DM	2	5%
Not able to be determined	1	2.5%
LMM with DM	1	2.5%
Site group		
Head and neck	17	42.5%
Upper limb	10	25%
Trunk	8	20%
Lower limb	5	12.5%
Mitotic rate (per mm^2)		
Mean	2.8	
Std. deviation	4.39	
Range	0–21	
Tumor infiltrating lymphocytes		
Non-brisk	14	35%
Absent	9	22.5%
Brisk—ICCR pattern 1	10	25%
Brisk—ICCR pattern 2	7	18%
Intermediate or late regression		
Absent	26	65%
Present—associated	9	22.5%
Present—un-associated	5	12.5%

out of 40) of the cases (Table 2, Figures 2 and 3a,b). When there was an increase in BT from the initial levels, the change was an average of 0.11 mm (range 0.1–0.5 mm).

Of these cases, the final BT was achieved at Level 2 in five cases (5 out of 19, 26%), Level 3 in six cases (6 out of 19, 32%), Level 4 in three cases (3 out of 19, 16%), and Level 5 in five cases (5 out of 19, 26%).

3.2 | Ulceration

Ulceration was present in 7.5% (3 out of 40) of cases in the initial slide review. In addition, four cases (10%) had features of incipient ulceration, and the additional levels confirmed true ulceration in two of these cases (5%, Table 2, Figure 2). In one case, the final ulceration status was observed on Level 2 (Figure 3c,d), and the other on Level 3.

3.3 | pT Stage

In the majority of cases (32 out of 40, 80%), the pathological stage did not change with levels beyond Level 1. However, eight cases (8 out of 40, 20%) were upstaged after evaluating additional levels (Tables 3 and 4). This included five cases in which pT1a was changed to pT1b, one case in which pT1b was changed to pT2a, one case in which pT1b was changed to pT2b, and one case in which pT2a was changed to pT2b. The changes in parameters, and the respective levels, are outlined in Table 4.

Changes in BT, ulceration, and final pT status were not significantly associated with sex, melanoma subtype, site, TILs, or intermediate and late regression.

3.4 | Microsatellitosis

Microsatellitosis was identified in two cases (2 out of 40, 5%), one for T2b melanoma and one for T4a melanoma, but this was present at all levels examined. There were no cases in which convincing microsatellitosis was identified by studying the additional levels.

We noted that for one case (2.5%), a pT4b melanoma, we found a small deposit of melanoma associated with a lymphoid aggregate in the subcutis at Level 2, which appeared to be physically separated from the main melanoma by a thrombosed blood vessel, the nature and significance of which were difficult to classify, but we were not convinced that this constituted a true microsatellite.

3.5 | Lymphovascular Invasion

No cases of LVI were identified in the first-level evaluation. While it did not affect the pathological staging, in one case (2.5%), a pT2a melanoma, we identified a small lymphovascular channel tumor deposit that turned up on Level 4 (Figure 3e,f).

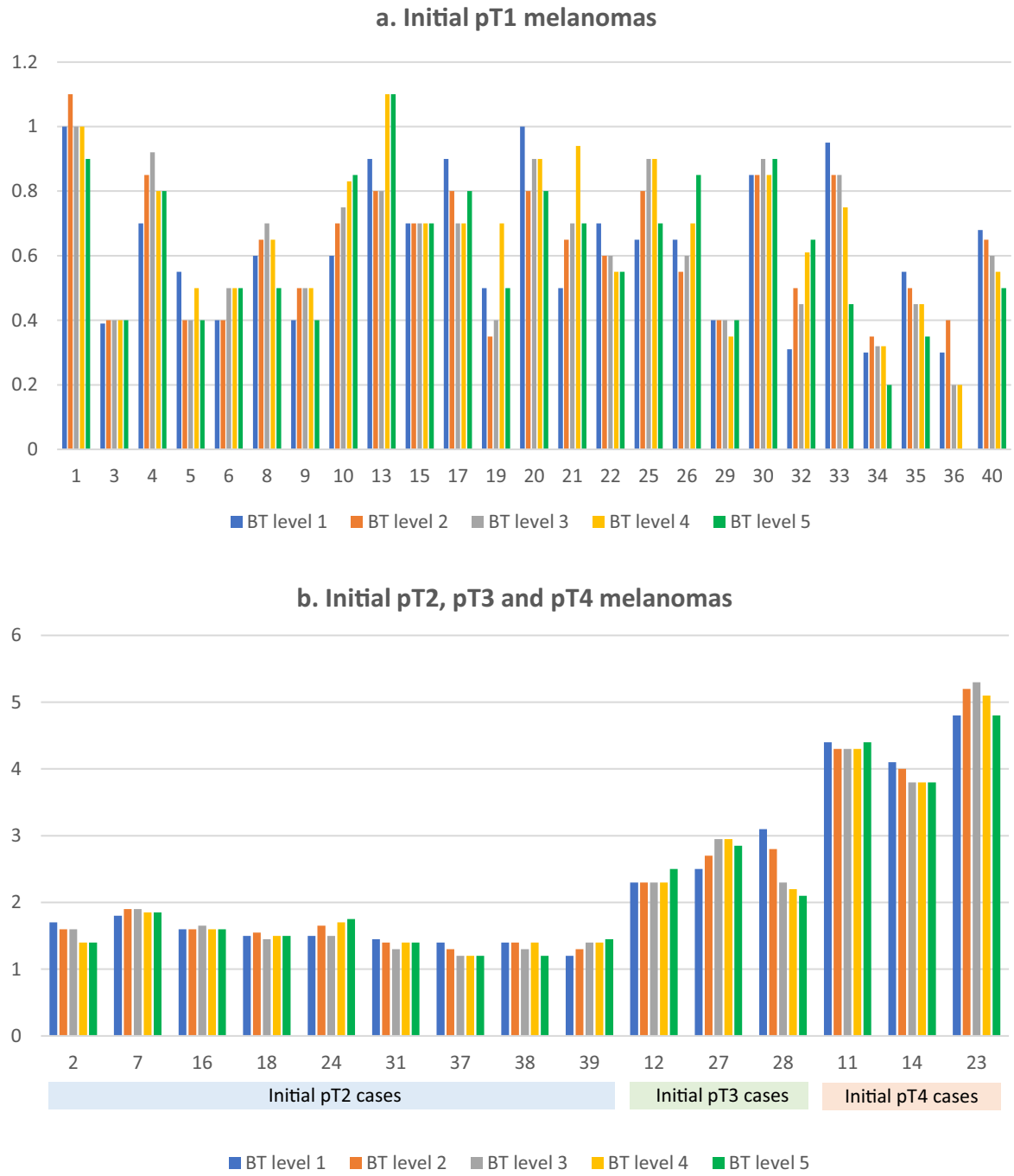

FIGURE 1 | Variability of Breslow thicknesses (BT) across five sequential levels at 100 μm depth intervals, for each of the 40 cases, grouped as (a) initial pT1 melanomas and (b) initial pT2, pT3 and pT4 melanomas (*y* axis = BT in mm; *x* axis = case numbers, batches).

4 | Discussion

In an era where nonanatomic factors are increasingly relevant to precision medicine, the assessment of key histopathologic parameters of primary melanoma remains one of the most powerful tests to determine a patient's prognosis and guide management decisions.

The 8th edition of the AJCC Cancer Staging Manual incorporates three key anatomical factors of primary melanoma (BT, ulceration, and microsatellites) into pathological staging [2]. Mitotic rate and LVI remain important independent prognostic markers and are utilized in prognostic nomograms, but are not currently incorporated into the formal AJCC stage categories [16, 17].

Several studies have shown that many features of melanoma are subject to variation based on the profile of the tumor that is examined in a particular plane of section [6–11]. This phenomenon is not unique to melanoma and has been documented in a range of tissue types and tumor pathologies [18–20].

TABLE 2 | Changes in Breslow thickness (BT), ulceration, and pathological T stage.

Characteristic	No. of cases ($n = 40$)	%
Change in BT		
No	21	52.5%
Yes	19	47.5%
Maximum BT achieved at which level?		
Level 1	21	52.5%
Level 2	5	12.5%
Level 3	6	15%
Level 4	3	7.5%
Level 5	5	12.5%
Change in ulceration status		
No	38	95%
Yes	2	5%
Final ulceration status achieved at which level?		
Level 1	38	95%
Level 2	1	2.5%
Level 3	1	2.5%
Level 4	0	0%
Level 5	0	0%
Change in pathological T (pT) stage?		
No	32	80%
Yes	8	20%
Final pT achieved at which level?		
Level 1	32	80%
Level 2	2	5%
Level 3	2	5%
Level 4	3	7.5%
Level 5	1	2.5%

Being a highly prevalent disease with major global health and economic burdens, maximizing the information from H&E assessment of melanoma is desirable because H&E slides are cheaper and easier to produce than ancillary techniques, such as immunohistochemistry (IHC) and molecular analysis. Melanoma research that considers equity for underresourced and pressured health systems is valuable.

4.1 | Breslow Thickness

We have shown that the BT is influenced by the number of tissue levels examined. We found additional levels resulted in an increase in BT of at least 0.1 mm for 47.5% (19 out of 40) of cases, with an average increase of 0.1 mm (range 0.1–0.5 mm). This resulted in upstaging in seven cases (17.5%), all of which were initially staged as pT1.

Our findings are in line with a limited number of H&E-only studies that have encountered a similar upstaging effect in 9%–18.5% of cases [6, 7, 10, 11]. In one series of 54 melanomas, this effect was significant when the first four levels were examined, with no additional significant benefit between Levels 5 and 10 [6]. This is also concordant with our findings, where we found that 80%, 85%, 90%, and 98% of cases will be maximally staged at Levels 1, 2, 3, and 4 (of a total of five levels), respectively (Table 2).

Two studies compared the measurement of BT between H&E and IHC stains (S100, melanA, HMB45 ± Sox10) [8, 9]. In one study, 29 out of 41 invasive melanomas (70%) showed an increase in BT of at least 0.1 mm after quad-panel IHC stains compared to the H&E slide, which led to a change in pT stage in 11 cases (11 out of 41, 26%) [8]. In another study, greater tumor thickness was observed in 27 lesions (27 out of 36, 75%) after a combination of three IHC stains (S100, melanA, HMB45) compared to the initial H&E staining, resulting in a one-step increase in the pT score for 22% of lesions [9]. In both studies, most cases with significant changes were thin (pT1) melanomas. Both groups concluded that IHC staining led to a superior evaluation of BT compared to H&E staining. However, since their findings are similar to ours, a clinically significant change in BT is likely to be, at least in part, a function of the additional levels being cut and examined rather than the IHC stain per se. Considering the significant cost and resources required to generate IHC stains compared with H&E staining, this is a judicious point to note. However, we recognize that IHC stains serve other functions in the workup of melanoma cases.

4.2 | Ulceration

The importance of ulceration as a poor prognostic factor has been well documented [21–23]. In our study, we found that in the absence of deeper levels, ulceration would have been missed in two cases (5%). This is concordant with Dyson et al. who found that ulceration was detected only by examining deeper levels in 3% of the cases [7].

Several investigators have also shown that epidermal consumption and incipient ulceration have similar prognostic significance to true ulceration [7, 14]. Dyson et al. and Paver et al. showed that incipient ulceration lay adjacent to true ulcerations in additional sections. These observations suggest that epidermal consumption, incipient ulceration, and true ulceration are on a spectrum and that patients may be under-staged if only the former tissue patterns are profiled in tissue sections [14, 15]. We identified only four patients whose melanomas had epidermal changes consistent with consumption or incipient ulceration, two of which revealed true ulceration at the subsequent level. This suggests that epidermal consumption and incipient

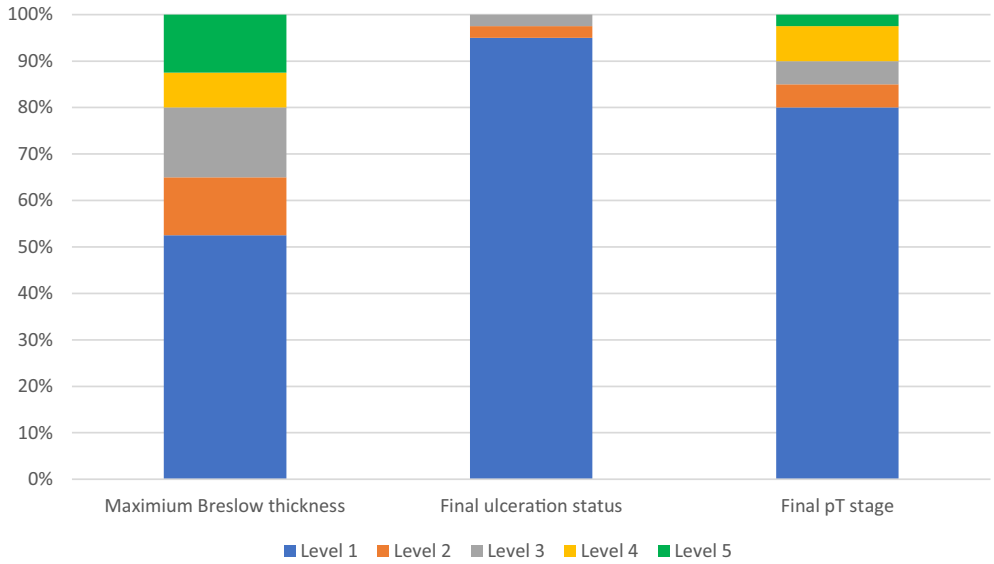

FIGURE 2 | Level at which maximal Breslow thickness, final ulceration status, and final pathological T (pT) stage are determined, as a proportion of all cases ($n = 40$).

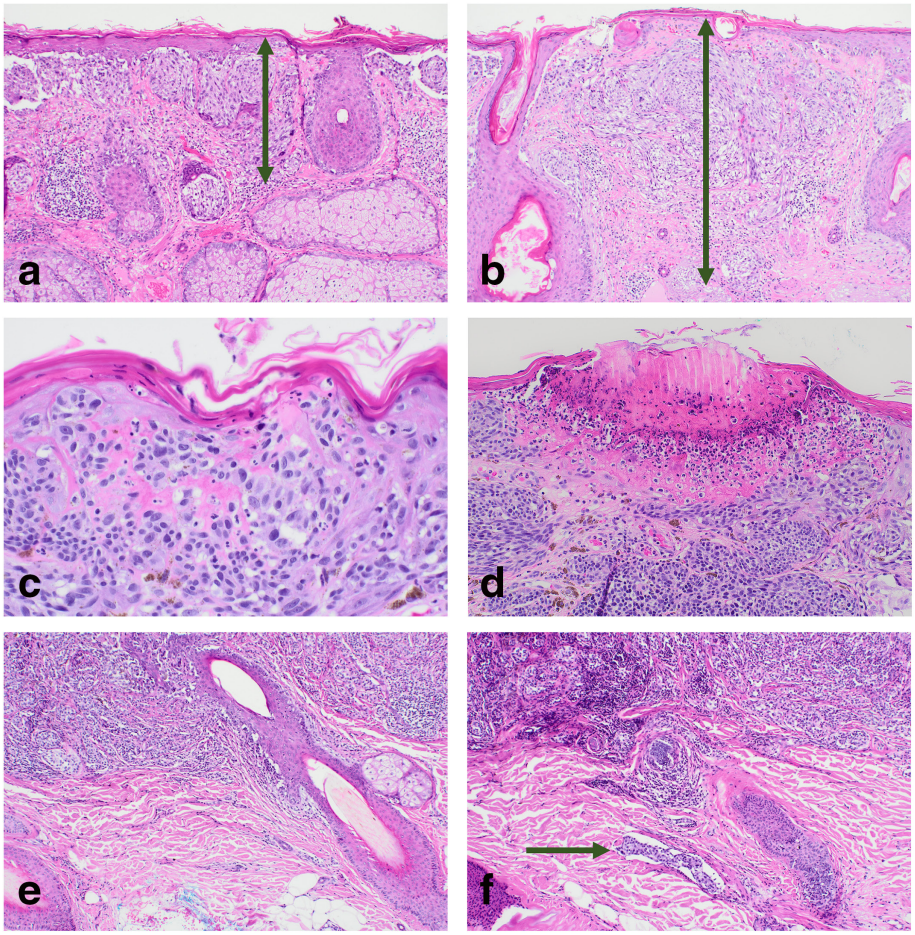

FIGURE 3 | Microscopic images of three case examples. (a H&E ×100) and (b H&E ×100) Case 21:82M forehead lentigo maligna melanoma (LMM), Breslow thickness (BT) 0.5 mm on Level 1 (a) and 0.9 mm on Level 4 (b), resulting in upstaging from pT1a to pT1b. (c H&E ×400) and (d H&E ×200) Case 39:82M, LMM on arm, BT 1.5 mm, showing incipient ulceration with fibrin and epidermal consumption on Level 2 (c), and ulceration at the next 100 μm interval on Level 3 (d). This resulted in the upstaging from pT2a to pT2b. (e H&E ×100) and (f H&E ×100) Case 31:79M, LMM on scalp, BT 1.4 mm, showing no LVI on the first three levels (g), and LVI on level 4 (h). (This did not result in a change in pT.)

TABLE 3 | Distribution of pathological T (pT) stage observation at respective batch levels, No. of cases (%).

Level	pT1a	pT1b	pT2a	pT2b	pT3a	pT3b	pT4a	pT4b
1	19 (47.5)	6 (15)	9 (22.5)	0 (0)	2 (5)	1 (2.5)	1 (2.5)	2 (5)
2	18 (45)	6 (15)	10 (25)	0 (0)	2 (5)	1 (2.5)	1 (2.5)	2 (5)
3	17 (42.5)	7 (17.5)	9 (22.5)	1 (2.5)	2 (5)	1 (2.5)	1 (2.5)	2 (5)
4	15 (37.5)	8 (20)	9 (22.5)	2 (5)	2 (5)	1 (2.5)	1 (2.5)	2 (5)
5	14 (35)	9 (22.5)	9 (22.5)	2 (5)	2 (5)	1 (2.5)	1 (2.5)	2 (5)

ulceration are uncommon findings; however, their recognition should trigger a pathologist to examine further.

4.3 | Microsatellitosis

To the best of our knowledge, no study has specifically evaluated how tissue levels influence the detection of microsatellites and LVI. We did not find any cases of microsatellitosis picked up at deeper levels, suggesting that the performance of levels solely for this purpose is likely to be of low yield.

5 | Limitations

Our study had several limitations. By including partial biopsies (punch, shave, and incisional biopsies, which accounted for 15% of our cohort), prognostic parameters cannot always be fully assessed. However, we included them to be reflective of wider clinical practice because it is important for pathologists to report all parameters regardless of the specimen type.

We did not include wide local excisions and recognized that these specimens will often determine a patient's final BT. However, we specifically excluded these cases so that biopsy site changes did not contaminate the measurement of the prognostic parameters.

We tried to address a limitation of prior literature, in which most studies did not specify the depth at which tissue levels were cut [6, 8, 9]. The lack of standardization in prior studies means that it is difficult to determine optimum sampling standards. In the only available study that attempted to standardize interval distance, Patrick et al. produced 10 slides at 40 μm intervals and found a ceiling effect at around five to seven levels, equating to approximately 200 μm of tissue examined [11]. In contrast, Dyson et al. evaluated more variable amounts of tissue, using intervals of 50–100 μm between the initial two slides (depending on specimen size), and 300–500 μm between five additional slides, to examine through an entire block [7]. By their own admission, they did not set out to establish a standard for optimal tissue sampling. However, sampling through an entire block presents practical and ethical problems, particularly in an era when primary tissue may be an invaluable resource for molecular testing to guide targeted and other modern treatments.

To address this gap in the literature and improve its application to wider laboratory practice, we chose five batches at 100 μm intervals (a total of 400 μm between Levels 1 and 5), which represents approximately 20% of a 2-mm tissue slice. This is an interval that is relatively common across routine laboratory practice, but also balances the need to ensure that lesional tissue is not exhausted from the block for potential ancillary testing. We recognize that intervals of 50 μm may be more appropriate for very small specimens, and the information garnered from our study should assist pathologists in determining how much tissue should be examined in such cases.

6 | Clinical and Staging Value of Examining Multiple Tissue Levels

Understanding the variability of information that can be obtained from additional tissue levels is useful for pathologists who have (a) an obligation to use laboratory resources judiciously and (b) a responsibility to balance tissue evaluation with the need to preserve tissue for ancillary studies, now and in the future.

Our study demonstrates that when compared to the initial H&E section, examination of additional tissue levels in 100 μm increments resulted in (a) an increase in the BT in 47.5% of cases, and (b) detection of ulceration in a further 5% of cases. This correlated with upstaging of patients' pT stage in 20% of cases (15% because of BT alone, 2.5% because of ulceration alone, and 2.5% because of BT and ulceration). We found no relationship between changes in parameters and melanoma subtype, site, age, mitotic rate, initial BT, TIL, or regression.

There is an incremental effect, such that 80% of cases of melanoma were accurately pathologically staged at the initial level, with roughly another 5% of patients being upstaged with each additional 100 μm interval examined (up to 400 μm). We found that only cases initially staged as pT1 were eventually upstaged because of increased BT; the average increase in BT was 0.11 mm. This suggests that it is pertinent for pathologists to consider performing additional levels on thin (pT1) melanomas when a BT assessment is within 0.1–0.3 mm of a threshold measurement (i.e., pT1a vs. pT1b or pT1b vs. pT2a).

Incipient ulceration and epidermal consumption are rare findings; however, when present, ulceration was subsequently observed in half of the cases. Incipient ulceration may, therefore, serve as a clue for true ulceration, and it is worth a pathologist pursuing additional levels.

Additional levels only rarely identify other prognostic parameters such as LVI and microsatellitosis.

TABLE 4 | Features of the eight cases where there was a change in pathological T (pT) stage after evaluation of levels.

Case number	pT stage at Level 1	Breslow thickness at Level 1	Ulceration status at Level 1	Final pT status	Final BT (level identified)	Final ulceration status (level identified)
21	pT1a	0.5 mm	Absent	pT1b	0.9 mm (Level 4)	Absent
10	pT1a	0.6 mm	Absent	pT1b	0.9 mm (Level 4)	Absent
4	pT1a	0.7 mm	Absent	pT1b	0.9 mm (Level 2)	Absent
25	pT1a	0.7 mm	Absent	pT1b	0.9 mm (Level 3)	Absent
26	pT1a	0.7 mm	Absent	pT1b	0.9 mm (Level 5)	Absent
1	pT1b	1 mm	Absent	pT2a	1.1 mm (Level 2)	Absent
13	pT1b	0.9 mm	Incipient	pT2b	1.2 mm (Level 4)	Present (Level 2)
39	pT2a	1.2 mm	Incipient	pT2b	1.5 mm (Level 3)	Present (Level 3)

Our study adds to several other studies that have shown changes in BT and staging using immunohistochemical staining. However, our observations suggest that the improved evaluation may be a function of leveling the tissue rather than the stain per se. IHC does have other roles, and pathologists should incorporate immunohistochemical studies based on their judgment.

Importantly, the upstaging effect observed in our study may help explain why a small percentage of patients with thin lesions ($\leq$1.0 mm) have unexpectedly poor prognoses [24, 25]. Given this potential clinical impact, our findings will undoubtedly raise questions for many pathologists and laboratories regarding how and if they should change their practice.

In answering this question, we emphasize the importance of the pathologist's judgment and discretion. While we recommend levels for thin pT1 lesions where the measurement is within 0.1–0.3 mm of the next T category, or when "incipient" ulceration is identified, we recognize that a pathologist's decision to examine additional tissue must consider multiple factors. These include the degree of diagnostic certainty, biopsy type and size, overall size of the lesion, tissue patterns such as regression, need for IHC, and rationalization of the tissue for molecular testing. In our study, we specifically kept our cohort to unequivocal melanomas and cases where there was no significant risk of tumor cutting out; however, this does not account for all real-life encounters with melanomas.

It is hoped that our findings will alert pathologists to instances where additional tissue-level examination may be judged as most beneficial, rather than mandate a radical change in practice. Importantly, we caution laboratories performing multiple levels upfront in all cases of melanocytic lesions. Doing so could risk the loss of precious tissue and is likely to lead to injudicious use of resources for many patients. However, we hope that our findings will provide evidence for the degree of variability in prognostic parameters, of which many pathologists will already be aware anecdotally, and allow them to make informed decisions regarding the benefits and potential costs of cutting through further tissue.

In conclusion, the performance of additional tissue levels is a simple and inexpensive test that can provide valuable prognostic information for patients with thin (pT1) primary cutaneous melanomas. Its value lies mostly in the evaluation of pT1 melanomas with an initial BT that is within 0.1–0.3 mm of the threshold for the next T category, and in the rare instance that incipient ulceration is identified. Pathologists should use discretion and judge cases on an individualized basis.

Author Contributions

L.A.J. conceived of the project. L.A.J. and R.A.S. designed the study and methodology. L.A.J. identified the patient cohort, collected all data, and analyzed the data. J.P.G. performed validation analysis on the collected data. All authors discussed the results and contributed to the final manuscript.

Acknowledgments

Richard A. Scolyer is supported by an NHMRC Investigator Grant (2022/GNT2018514). The support from The Cameron Family

Foundation as well as from colleagues at Melanoma Institute Australia and the Royal Prince Alfred Hospital is also gratefully acknowledged.

Disclosure

The authors have nothing to report.

Ethics Statement

Ethics approval and waiver use of medical information has been granted through independent ethics committees and research boards (reference Peter Mac No: 24/73R, HREC Reference: HREC/108528/PMCC).

Conflicts of Interest

Richard A. Scolyer has received fees for professional services from SkylineDx BV, IO Biotech ApS, MetaOptima Technology Inc., F. Hoffmann-La Roche Ltd., Evaxion, Provectus Biopharmaceuticals Australia, Qbiotics, Novartis, Merck Sharp & Dohme, NeraCare, AMGEN Inc., Bristol-Myers Squibb, Myriad Genetics, and GlaxoSmithKline. The other authors declare no conflicts of interest.

Data Availability Statement

Research data supporting this publication are available on request from the author.

References

1. M. F. Azzola, H. M. Shaw, J. F. Thompson, et al., "Tumor Mitotic Rate Is a More Powerful Prognostic Indicator Than Ulceration in Patients With Primary Cutaneous Melanoma. Analysis of 3661 Patients From a Single Center," *Cancer* 97, no. 6 (2003): 1488–1498.

2. AJCC (American Joint Committee on Cancer), *AJCC Cancer Staging Manual*, 8th ed. (Cham, Switzerland: Springer, 2017).

3. A. Breslow, "Problems in the Measurement of Tumor Thickness and Level of Invasion in Cutaneous Melanoma," *Human Pathology* 8 (1977): 1–2.

4. RCPA (Royal College of Pathologists of Australasia), *Primary Cutaneous Melanoma Structured Reporting Protocol*, 3rd ed. (Surry Hills, NSW: RCPA, 2023).

5. R. A. Scolyer, M. J. Judge, A. Evans, et al., "Data Set for Pathology Reporting of Cutaneous Invasive Melanoma: Recommendations From the International Collaboration on Cancer Reporting (ICCR)," *American Journal of Surgical Pathology* 37, no. 12 (2013): 1797–1814.

6. J. Bishop and B. Tallon, "Number of Levels Required to Assess Breslow Thickness in Cutaneous Invasive Melanoma: An Observational Study," *Journal of Cutaneous Pathology* 46 (2019): 819–822, https://doi.org/10.1111/cup.13541.

7. S. W. Dyson, J. Bass, J. Pomeranz, C. Jaworsky, J. Sigel, and S. Somach, "Impact of Thorough Block Sampling in the Histologic Evaluation of Melanomas," *Archives of Dermatology* 141, no. 6 (2005): 734–736, https://doi.org/10.1001/archderm.141.6.734.

8. A. M. Pop, M. Monea, P. Olah, R. Moraru, and O. S. Cotoi, "The Importance of Immunohistochemistry in the Evaluation of Tumor Depth of Primary Cutaneous Melanoma," *Diagnostics (Basel)* 13, no. 6 (2023): 1020, https://doi.org/10.3390/diagnostics13061020.

9. K. Kamyab-Hesary, A. Ghanadan, K. Balighi, S. F. Mousavinia, and M. Nasimi, "Immunohistochemical Staining in the Assessment of Melanoma Tumor Thickness," *Pathology Oncology Research* 26 (2020): 885–891, https://doi.org/10.1007/s12253-019-00635-y.

10. A. R. Solomon, C. N. Ellis, and J. T. Headington, "An Evaluation of Vertical Growth in Thin Superficial Spreading Melanomas by Sequential Serial Microscopic Sections," *Cancer* 52 (1983): 2338–2341, https://doi.org/10.1002/1097-0142(19831215)52:12<2338::AID-CNCR2820521229>3.0.CO;2-T.

11. R. J. Patrick, S. Corey, and L. F. Glass, "The Use of Sequential Serial Sectioning of Thin Melanomas in Determining Maximum Breslow Depth," *Journal of the American Academy of Dermatology* 57, no. 5 (2007): S127–S128.

12. A. D. Rabinowitz and D. N. Silvers, "Dermatopathology Standards," *Journal of Cutaneous Pathology* 23, no. 2 (1996): 194–196, https://doi.org/10.1111/j.1600-0560.1996.tb01295.x.

13. R. A. Scolyer, H. M. Shaw, J. F. Thompson, et al., "Interobserver Reproducibility of Histopathologic Prognostic Variables in Primary Cutaneous Melanomas," *American Journal of Surgical Pathology* 27 (2003): 1571–1576.

14. E. C. Paver, T. Ahmed, H. Burke, et al., "Prognostic Significance of Incipient Ulceration in Primary Cutaneous Melanoma," *JAMA Dermatology* 159, no. 12 (2023): 1359–1367, https://doi.org/10.1001/jamadermatol.2023.4193.

15. R. F. Walters, P. A. Groben, K. Busam, et al., "Consumption of the Epidermis: A Criterion in the Differential Diagnosis of Melanoma and Dysplastic Nevi That Is Associated With Increasing Breslow Depth and Ulceration," *American Journal of Dermatopathology* 29, no. 6 (2007): 527–533, https://doi.org/10.1097/DAD.0b013e318156e0a7.

16. S. N. Lo, J. Ma, R. A. Scolyer, et al., "Improved Risk Prediction Calculator for Sentinel Node Positivity in Patients With Melanoma: The Melanoma Institute Australia Nomogram," *Journal of Clinical Oncology* 38 (2020): 2719–2727, https://doi.org/10.1200/JCO.19.02362.

17. M. A. El Sharouni, A. H. R. Varey, A. J. Witkamp, et al., "Predicting Sentinel Node Positivity in Patients With Melanoma: External Validation of a Risk-Prediction Calculator (The Melanoma Institute Australia Nomogram) Using a Large European Population-Based Patient Cohort," *British Journal of Dermatology* 185, no. 2 (2021): 412–418, https://doi.org/10.1111/bjd.19895.

18. C. P. Maingi and K. F. Helm, "Utility of Deeper Sections and Special Stains for Dermatopathology Specimens," *Journal of Cutaneous Pathology* 25, no. 3 (1998): 171–175.

19. R. Murali, C. DeSilva, S. W. McCarthy, J. F. Thompson, and R. A. Scolyer, "Sentinel Lymph Nodes Containing Very Small (<0.1 mm) Deposits of Metastatic Melanoma Cannot be Safely Regarded as Tumor-Negative," *Annals of Surgical Oncology* 19, no. 4 (2012): 1089–1099, https://doi.org/10.1245/s10434-011-2208-z.

20. Y. K. Chitkara and C. L. Eyre, "Evaluation of Initial and Deeper Sections of Esophageal Biopsy Specimens for Detection of Intestinal Metaplasia," *American Journal of Clinical Pathology* 123, no. 6 (2005): 886–888, https://doi.org/10.1309/MGRX-LQG9-QVRV-VMCG.

21. H. Grande Sarpa, K. Reinke, L. Shaikh, et al., "Prognostic Significance of Extent of Ulceration in Primary Cutaneous Melanoma," *American Journal of Surgical Pathology* 30, no. 11 (2006): 1396–1400, https://doi.org/10.1097/01.pas.0000213262.61855.7d.

22. C. M. Balch, J. A. Wilkerson, T. M. Murad, S. J. Soong, A. L. Ingalls, and W. A. Maddox, "The Prognostic Significance of Ulceration of Cutaneous Melanoma," *Cancer* 45, no. 12 (1980): 3012–3017, https://doi.org/10.1002/1097-0142(19800615)45:12<3012::aid-cncr2820451223>3.0.co;2-o.

23. F. E. In 't Hout, L. E. Haydu, R. Murali, J. J. Bonenkamp, J. F. Thompson, and R. A. Scolyer, "Prognostic Importance of the Extent of Ulceration in Patients With Clinically Localized Cutaneous Melanoma," *Annals of Surgery* 255, no. 6 (2012): 1165–1170, https://doi.org/10.1097/SLA.0b013e31824c4b0b.

24. R. Murali, L. E. Haydu, G. V. Long, et al., "Clinical and Pathologic Factors Associated With Distant Metastasis and Survival in Patients With Thin Primary Cutaneous Melanoma," *Annals of Surgical Oncology* 19, no. 6 (2012): 1782–1789, https://doi.org/10.1245/s10434-012-2265-y.

25. Z. Hothem, A. Bayci, B. J. Thibodeau, et al., "Using Global Gene Expression to Discriminate Thin Melanomas With Poor Outcomes," *Molecular & Cellular Oncology* 4, no. 1 (2016): e1253527, https://doi.org/10.1080/23723556.2016.1253527.

HISTOPATHOLOGICAL ASSESSMENT OF MELANOMA

CHAPTER 4: MICROSCOPIC EVALUATION OF PRIMARY MELANOMA TUMOUR BEDS

Overview: Wide local excision (WLE) of primary melanoma tumour beds after biopsy-proven melanoma is the current standard in initial definitive treatment for patients with primary cutaneous melanoma. Rates of residual melanoma in the tumour bed after a complete excision biopsy (CEB) are reported to occur in 0-6.3% of cases. Many features that convey high risk to a patient with melanoma are assumed to increase the chance that residual disease is found in the primary tumour bed, however there is a paucity of literature methodically evaluating the clinicopathological features in such cases.

Specific aims: Our aim was to determine the frequency of and clinicopathological characteristics associated with residual melanoma in WLE specimens performed after a CEB of primary cutaneous or acral melanoma, and assess its relevance in the context of clinical practice.

Contribution to literature: This work provides evidence to help inform pathology practices and guidelines when they are balancing the importance of tissue evaluation, patient safety, and cost-effectiveness in daily practice.

Pathology (February 2022) **54(1)**, pp. 71–78

ANATOMICAL PATHOLOGY

Residual melanoma in wide local excision specimens after 'complete' excision of primary cutaneous *in situ* and invasive melanomas

Louise A. Jackett[1,2,3,4], Laveniya Satgunaseelan[3,4], Edward Roper[3,4], Serigne N. Lo[1,2,5], John F. Thompson[1,2], Richard A. Scolyer[1,2,3,4]

[1]*Melanoma Institute Australia, The University of Sydney, Sydney, NSW, Australia;* [2]*Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia;* [3]*Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Sydney, NSW, Australia;* [4]*NSW Health Pathology, Sydney, NSW, Australia;* [5]*Department of Melanoma and Surgical Oncology, Royal Prince Alfred Hospital, Sydney, NSW, Australia*

Summary

Wide local excision (WLE) to achieve adequate clearance margins is the standard initial definitive treatment for patients with biopsy-proven primary cutaneous melanoma. Residual melanoma in WLE specimens after prior complete excision-biopsy (CEB) is reported in 0–6.3% of cases. However, studies evaluating the prevalence, clinicopathological features and relevance of persistent disease in WLE specimens are limited.

This study sought to determine the frequency of and clinicopathological characteristics associated with residual melanoma in WLE specimens performed after a CEB of primary cutaneous or acral melanoma (*in situ* or invasive) with clinically and histologically tumour-free margins, and assess its relevance.

A review of the research database and pathology archives of a large Australian tertiary referral melanoma treatment centre was performed. Eligible patients were those for whom a definitive WLE was performed after CEB of a primary melanoma (*in situ* or invasive) with negative clinical and histological margins, between May 2013 and May 2015. All partial biopsies were excluded.

Of 640 eligible patients, 510 (79.7%) had invasive melanoma and 130 (20.3%) had melanoma *in situ*. Residual disease was identified in 20 cases (20/640, 3.1%), of which three (15%) were melanoma *in situ* on CEB and 17 (85%) were invasive melanoma. On univariate analysis, the presence of residual disease in WLE specimens was associated with lentigo maligna (LM)/LM melanoma (LMM) subtype [odds ratio (OR) 10.33; 95% confidence interval (CI) 2.84–37.54; p=0.004], nodular melanoma (NM) subtype (OR 4.92; 95% CI 1.53–15.85; p=0.0076) and, for invasive tumours, higher tumour mitotic rate (mean 7.7, SD 7.51 vs 3.4, SD 4.83; OR 1.11; 95% CI 1.04–1.18; p=0.0014). Breslow thickness >4 mm was associated with a higher risk of residual disease (OR 7.30; 95% CI 1.88, 28.26; p=0.004). Cases with residual disease had primary tumours with a significantly larger diameter (median 14 mm, range 4–25) than those without residual disease (median 9 mm, range 2–60), (OR 1.07; 95% CI 1.03–1.11; $p \leq 0.001$) and were also more likely to be amelanotic (38% vs 14%), (OR 3.69; 95% CI 1.17, 11.60; p=0.026). Residual disease was associated with assessment of >3 slides of tissue (OR 6.98; 95% CI 1.54–31.62; p=0.0118) and complete blocking of the scar (OR 31.69; 95% CI 3.98–252.21; p=0.0011).

Residual melanoma in WLE specimens is an infrequent occurrence. Risk factors for residual disease are LM/LMM and NM melanoma subtypes, higher mitotic rate, larger lesion diameter and amelanosis. Tumours with these features warrant more extensive pathological sampling. WLE after CEB for melanoma remains an important procedure to reduce local recurrence; however, limited pathological sampling of the WLE scar is probably appropriate for cases lacking high risk features.

Key words: Diagnosis; melanoma; pathology; recurrence; treatment; management.

Received 29 December 2020, revised 6 May, accepted 17 May 2021
Available online 13 August 2021

INTRODUCTION

Wide local excision (WLE) is the standard initial definitive treatment for patients with primary cutaneous melanomas. The objective is to achieve adequate clearance margins.[1–4] Although WLE is thought to have a therapeutic benefit by controlling local disease, it may not impact overall survival.[2,5–7] It is a relatively infrequent occurrence to find residual melanoma in the WLE specimen after a prior excision-biopsy that has shown complete clinical and histological excision of a primary cutaneous melanoma. Prior limited studies have reported rates of up to 6.3%.[2,8–11] The presence of microsatellites in WLE specimens is an adverse prognostic factor in melanoma patients and results in upstaging to stage III melanoma in the American Joint Committee on Cancer (AJCC) staging system.[12] The detection of such metastases is important because it warrants more intensive follow-up and possibly even consideration of adjuvant systemic drug therapy.

A contemporary retrospective study of consecutive WLE specimens from 807 melanoma patients in whom the histological margins were reported to be clear in a prior diagnostic excision-biopsy, managed at a specialised Australian

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2021.05.094

melanoma unit, found that residual or locally metastatic disease was present in 4.2% of cases.[2] Residual disease was most likely when the primary lesion was either of lentigo maligna (LM) or lentigo maligna melanoma (LMM) subtype.[2] Other studies have reported lower rates of residual disease in WLE specimens, and as a consequence, several investigators have questioned both the clinical value and the cost-effectiveness of the pathological examination of WLE specimens, and whether limited or more extensive sampling for pathological examination is advisable.[8–11,13–15] Indeed, given the paucity of evidence, some consensus pathology reporting guidelines have commented that if no suspicious areas are identified after careful macroscopic examination, microscopic examination of a single paraffin block of tissue may be sufficient.[9,16,17]

Given the limited data addressing the prevalence of persistent disease in WLE specimens and the clinicopathological features associated with its occurrence in patients with a prior diagnostic excision-biopsy with clear margins, we analysed our own experience at a large Australian tertiary referral melanoma treatment centre. We sought to determine the frequency and clinicopathological characteristics associated with the presence of residual melanoma in WLE specimens performed after a complete excision-biopsy (CEB) of primary cutaneous melanoma with clinically and histologically tumour-free margins.

METHODS

This study was undertaken at Melanoma Institute Australia (MIA) and its affiliated diagnostic pathology service at the Royal Prince Alfred Hospital (RPAH), Sydney, Australia. It was conducted in accordance with the ethical approval given by the Sydney Local Health District Human Research Ethics Committee (Protocol No. X15-0454 and HREC/11/RPAH/444).

The MIA research database and RPAH pathology archives were reviewed to identify patients with *in situ* or invasive melanomas who had CEBs and WLEs and were managed between May 2013 to May 2015. Eligible patients were those in whom a definitive WLE was performed after CEB of primary cutaneous or acral melanoma (*in situ* or invasive) with negative clinical and histological margins. All patients who had partial biopsies were excluded, including punch biopsies, incision biopsies and shave biopsies, as they had positive margins.

The Australian Melanoma Clinical Practice Guidelines recommend CEB with 2 mm clinical margins as the preferred method for initial diagnosis of cutaneous melanoma.[1] This method facilitates histological assessment of the entire lesion and reduces the risk of a false-negative diagnosis associated with partial biopsies.[17] If a pathological diagnosis of melanoma is confirmed, optimal management subsequently involves definitive WLE of the biopsy site to achieve a wider clearance margin of tumour-free skin, the extent of which is determined by the Breslow thickness of the primary tumour.[18] The Australian guidelines recommend 5–10 mm margins for melanoma *in situ*; 1 cm margins for melanoma <1.0 mm Breslow thickness; 1–2 cm margins for melanomas 1.01–2.00 mm and 2.01–4.00 mm without other adverse prognostic factors; and 2 cm margins for melanomas >4 mm or those 2.01–4.00 mm with adverse prognostic features.[1] For patients included in our study, the WLE procedures were performed by MIA surgeons according to these guidelines.[1]

All specimens were reviewed by MIA-affiliated pathologists at a single specialist pathology service (RPAH). WLE specimens were macroscopically handled according to guidelines of the Royal College of Pathologists of Australasia.[16] WLE specimens were serially sliced across the long axis into 2–3 mm thick slices and examined for macroscopic disease including masses or pigmentation. If no gross disease was identified, the primary melanoma had been completely excised on the prior specimen, and the primary lesion showed no high risk features such as a desmoplastic melanoma component, neurotropism, microsatellites or lymphovascular invasion, then limited, representative sampling was performed (generally up to four transverse slices through the centre of the scar submitted in one paraffin block). The scar was completely submitted if any of the adverse features mentioned above were present. Tissue samples were then processed for histological examination by routine paraffin embedding, microtome sectioning and staining with haematoxylin and eosin.

Immunohistochemistry was not routinely performed in the evaluation of WLE specimens as part of this study; it was employed where necessary at the discretion of the reporting pathologist. When immunohistochemistry was utilised to assist in the evaluation of junctional melanocytic proliferations, generally a melanocytic nuclear marker such as Sox10 was employed. Sox10 and S100 were also sometimes utilised to differentiate scar from residual desmoplastic melanoma.[19] Occasionally, melanA and HMB45 were used to differentiate cellular constituents such as inflammatory cells from residual melanoma cells.

Sentinel lymph node biopsy was performed in accordance with Australian guidelines, usually in those cases where Breslow thickness was either ≥1 mm, or 0.75–1 mm thickness with adverse pathological features.[1]

Clinical parameters recorded were age, sex, and anatomical site (head and neck, chest, abdomen, back, upper extremity, lower extremity). Macroscopic parameters recorded for the primary lesion in the CEB specimen included an assessment of amelanosis and the diameter of the melanoma. Microscopic pathological parameters of the primary melanoma were recorded as outlined in the Royal College of Pathologists of Australasia (RCPA) guidelines for the reporting of cutaneous melanoma.[16] These were melanoma subtype [superficial spreading (SSM), nodular (NM), desmoplastic (DM), lentigo maligna/lentigo maligna melanoma (LM/LMM) acral lentiginous melanoma (ALM)], Breslow thickness, Clark level, mitotic rate, ulceration, associated naevus, neurotropism, lymphovascular invasion, microsatellites, desmoplastic melanoma component, regression and presence of solar elastosis. Margin status was recorded for both the *in situ* component (peripheral margin) and the invasive component (peripheral and deep margins).

Gross parameters recorded for the WLE specimen included length of scar, width of specimen, depth of specimen, number of slices submitted and whether the scar was submitted in entirety or as representative sections. Microscopic parameters for the WLE specimen were presence or absence of residual melanoma, size of the residual disease, distance from the scar and margin status.

Patients and clinicopathological factors were summarised using frequency (proportion) for categorical variable and mean (standard deviation) for continuous variables. Univariate logistic regression was performed to assess the association between presence of residual disease in the WLE specimen and the clincopathological features of the primary melanoma. Due to the small number of cases with presence of residual disease, multivariate analysis was not performed.

RESULTS

Clinicopathological characteristics of all cases (Table 1)

A total of 640 eligible patients who had WLE following CEB for either melanoma *in situ* or invasive melanoma between May 2013 and May 2015 were identified. The median age at diagnosis was 62 years (range 19–94, mean 61); 61% of patients were male; 510 cases (79.7%) were invasive melanoma while 130 (20.3%) were melanoma *in situ*.

Melanoma *in situ* subtypes included LM type (26/130, 20%), non-LM type (10, 8%), or unclassified pattern (94, 72%). Invasive melanoma subtypes were SSM (346/510, 68%), NM (104, 20%), DM (13, 2.5%), LMM (12, 2.4%), ALM (2, 0.4%), or unclassified (33, 6.5%).

In decreasing frequency, cases of melanoma *in situ* were located on the upper extremity (38/130, 29%), back (36, 28%), lower extremity (23, 18%), head and neck (22, 17%), chest (10, 8%), or abdomen (1, 0.8%). Invasive melanomas were located on the back (175/510, 34%), upper extremity (145, 28%), lower extremity (102, 20%), head and neck (51, 10%), chest (27, 5.3%), or abdomen (10, 2%).

The median Breslow thickness for all invasive melanomas was 1.0 mm (range 0.2–14 mm, mean 2.0 mm).

Residual melanoma in wide excision specimens

The characteristics of the CEB and WLE specimens for all cases with residual disease in WLE specimens are documented in Fig. 1.

Residual invasive melanoma or melanoma *in situ* were identified in 20 cases (20/640, 3.1%). Of all these cases with residual disease, three (15%) were originally diagnosed as melanoma *in situ* on CEB, representing 2.3% of all melanoma *in situ* cases (3/130). Two cases were LM subtype and one case was unclassified. Residual disease in all three cases was in the form of melanoma *in situ*, and there were no unexpected instances of invasive melanoma, microsatellites, lymphovascular invasion or perineural invasion.

The remaining 17 cases with residual disease (17/20, 85%) were originally diagnosed as invasive melanomas on CEB, representing 3.3% of all cases of invasive melanoma (17/510). Of these patients, there were seven cases of NM (41%), three cases of LMM (18%), five cases of SSM (25%), one case of DM (6%) and one case of ALM (6%). In this group, the majority of cases of residual disease were in the form of melanoma *in situ* (12/17, 71%). The remaining five cases showed invasive disease, of which three cases (15%) were in the form of tumour within or adjacent to the scar (3/20, 15%), one case (5%) was a clinically detected in-transit metastasis, and one case (5%) harboured microsatellites, lymphovascular invasion and tumour in the scar (Fig. 2).

The latter case (Case T) was a SSM (Breslow thickness 6 mm, tumour mitotic rate 18/mm^2, ulcerated) that had both microsatellites and lymphovascular invasion in the CEB specimen. The WLE harboured intra-lymphatic tumour plugs, a 1 mm metastatic deposit in the scar and a 0.3 mm microsatellite in the subcutis located 2 mm away from the scar.

Case N was a NM (Breslow thickness 2.1 mm, tumour mitotic rate 12/mm^2, not ulcerated) that showed neurotropism in the CEB. The WLE contained a 5.5 mm deposit of melanoma that corresponded to a clinically detected in-transit metastasis located 7 mm from the scar; this deposit did not appear to be associated with a nerve.

Case R was an ALM (Breslow thickness 4.9 mm, tumour mitotic rate 19/mm^2, not ulcerated) that showed lymphovascular invasion in the primary tumour. A clearance margin of 0.2 mm was reported for *in situ* melanoma and 0.8 mm clearance for the invasive component to the peripheral margin; *in situ* disease was present in the WLE specimen.

The primary tumour in Case K was an invasive DM (Breslow thickness 1.7 mm, no dermal mitoses, not ulcerated) with a junctional LM component and the WLE specimen contained residual LM only.

Case Q was notable for being a thick NM with a very high mitotic rate (21/mm^2, Breslow thickness 4.3 mm, not ulcerated) and the WLE specimen contained residual spindle cell melanoma.

Most WLE procedures (19/20, 95%) were performed within 6 weeks of the original diagnosis, except for one case that was performed 8 weeks after CEB (Case 16, data not shown).

Predictors of residual melanoma in wide excision specimens

The univariate logistic regression results are provided in Table 1. The presence of residual melanoma in WLE specimens was associated with LM/LMM subtype [odds ratio (OR) 10.33; 95% confidence interval (CI) 2.84–37.54; p=0.004), NM subtype (OR 4.92; 95% CI 1.53–15.85; p=0.0076) and, for invasive tumours, higher tumour mitotic rate (mean 7.7, SD 7.51 vs 3.4, SD 4.83; OR 1.11; 95% CI 1.04–1.18; p=0.0014).

For invasive melanomas, there was no significant difference in mean Breslow thickness (mean 1.7 mm; OR 1.19; 95% CI 0.98–1.45; p=0.075) between those with and without residual disease. However, when stratified according to thickness ($\leq$1 mm, >1 to $\leq$2 mm, >2 to $\leq$4 mm, >4 mm), Breslow thickness >4 mm was associated with higher risk of residual disease (OR 7.30; 95% CI 1.88, 28.26; p=0.004). Cases with residual disease showed a primary tumour with a significantly greater diameter (median 14 mm, range 4–25) than those without residual disease (median 9 mm, range 2–60), (OR 1.07; 95% CI 1.03–1.11; $p\leq$0.001), and were also more likely to be amelanotic (38% vs 14%), (OR 3.69; 95% CI 1.17, 11.60; p=0.026). WLE specimens with a width >25 mm were more likely to contain residual melanoma (OR 4.53; 95% CI 1.24–16.50; p=0.0221), probably reflecting the increased Breslow thickness of the primary melanoma. Residual disease was associated with sampling of >3 blocks of tissue (OR 6.98; 95% CI 1.54–31.62; p=0.0118) and complete blocking of the scar (OR 31.69; 95% CI 3.98–252.21; p=0.0011). For both *in situ* and invasive melanomas, there was no statistically significant association with sex, age, site or associated naevus. For invasive melanomas, there was no statistically significant association with the presence of ulceration, Clark level, lymphovascular invasion, neurotropism, microsatellites, presence of a desmoplastic melanoma component or regression. There was no significant difference in the frequency of residual melanoma in WLE specimens when margins of the CEB were stratified as above or below 1 mm for *in situ* disease, or above or below 2 mm for invasive melanoma.

DISCUSSION

Our study confirms that residual melanoma in WLE specimens after CEB is an infrequent occurrence. The rate of residual disease, both *in situ* and invasive melanomas, in our study (3.1%) is within the range previously found in the limited number of other relevant studies (0–4.2%).[2,8–11]

We found that LM/LMM subtype was significantly associated with a higher risk of residual melanoma, as did Bolshinsky *et al.*[2] This finding may reflect the recognised difficulty in delineating the horizontal growth phase and peripheral extent of LM/LMM both clinically and histologically.[20] Furthermore, since melanocytes may be activated after surgery (demonstrating an increase in number, pagetoid spread and/or cytological atypia), the association with LM/LMM may partially reflect interobserver variability in assessing this phenomenon.[21] We sought to mitigate overinterpretation of melanocyte induction by submitting cases of residual disease to an additional independent pathology review prior to inclusion in the study. NM was also significantly associated with the presence of residual melanoma in

Table 1 Logistic regression of univariate analysis of clinicopathological features and the presence of disease remaining in wide local excision specimens after complete excision-biopsy of melanoma or melanoma *in situ*

Variable	No melanoma remaining (N=620) *n* (%)	Melanoma remaining (N=20) *n* (%)	Total	OR (95% CI)	*p* value
Sex					
Female	241 (39)	9 (45)	250	1.00	
Male	379 (61)	11 (55)	390	0.78 (0.32, 1.90)	0.5811
Age mean (SD)	60.9 (14.92)	67.2 (10.25)		1.03 (1.00, 1.07)	0.0629
Macroscopic tumour diameter, mm, median (range)	9 (2–60)	14 (4–25)		1.07 (1.03, 1.11)	**0.0001**
Site					
Lower extremity	121 (20)	4 (20)	125	1.00	
Upper extremity	177 (29)	6 (30)	183	1.03 (0.28, 3.71)	0.9695
Chest/Abdomen/Back/Trunk	255 (41)	4 (20)	259	0.47 (0.12, 1.93)	0.2976
Head and neck	67 (11)	6 (30)	73	2.71 (0.74, 9.94)	0.1329
Pigmentation					
Pigmented	401 (86)	8 (62)	409	1.00	
Amelanosis	68 (14)	5 (38)	73	3.69 (1.17, 11.60)	**0.0258**
Melanoma subtype					
Superficial spreading	341 (55)	5 (25)	346	1.00	
Lentigo maligna	33 (5)	5 (25)	38	10.33 (2.84, 37.54)	**0.0004**
Nodular	97 (16)	7 (35)	104	4.92 (1.53, 15.85)	**0.0076**
Desmoplastic	12 (2)	1 (5)	13	5.68 (0.62, 52.48)	0.1255
Other	137 (22)	2 (10)	139	1.00 (0.19, 5.19)	0.9958
Ulceration					
Ulcerated	70 (11)	4 (20)	74	1.00	
Non-ulcerated	549 (89)	16 (80)	565	0.51 (0.17, 1.57)	0.2401
Breslow thickness, mm, mean (SD)	1.7 (1.73)	2.4 (1.88)		1.19 (0.98, 1.45)	0.0748
Breslow thickness, mm					
≤1	251 (51)	4 (24)	255	1.00	
>1 to ≤2	117 (24)	5 (29)	122	2.68 (0.71, 10.17)	0.1469
>2 to ≤4	80 (16)	3 (18)	83	2.35 (0.52, 10.74)	0.2692
>4	43 (9)	5 (29)	48	7.30 (1.88, 28.26)	**0.0040**
Clark Level (missing data = 3)					
1	127 (20)	3 (15)	131	1.00	
2	98 (16)	2 (10)	99	0.88 (0.14, 5.37)	0.8895
3	144 (23)	4 (20)	148	1.19 (0.26, 5.40)	0.8261
4	230 (37)	9 (45)	239	1.67 (0.44, 6.28)	0.4481
5	18 (3)	2 (10)	20	4.74 (0.74, 30.34)	0.1002
Diagnosis					
In situ	127 (20)	3 (15)	130	1.00	
Invasive	493 (80)	17 (85)	510	1.46 (0.42, 5.06)	0.5508
Mitotic rate mean (SD)	3.4 (4.83)	7.7 (7.51)		1.11 (1.04, 1.18)	**0.0014**
Mitotic rate					
0	148 (24)	4 (20)	152	1.00	
≥1	472 (76)	16 (80)	488	1.25 (0.41, 3.81)	0.6896
Lymphovascular invasion					
Absent	472 (96)	15 (88)	487	1.00	
Present	19 (4)	2 (12)	21	3.31 (0.71, 15.53)	0.1287
Neutrotropism					
Absent	482 (98)	16 (94)	498	1.00	
Present	9 (2)	1 (6)	10	3.35 (0.40, 28.03)	0.2651
Microsatellites					
Absent	487 (99)	16 (94)	503	1.00	
Present	4 (1)	1 (6)	5	7.61 (0.80, 71.99)	0.0767
Desmoplasia					
Absent	472 (96)	16 (94)	488	1.00	
Present	19 (4)	1 (6)	20	1.55 (0.20, 12.33)	0.6772
Regression					
Absent	167 (28)	5 (25)	172	1.00	
Early/Intermediate/Late	426 (72)	15 (75)	441	1.18 (0.42, 3.29)	0.7572
Associated naevus					
Absent	322 (52)	14 (70)	336	1.00	
Present	297 (48)	6 (30)	303	0.46 (0.18, 1.22)	0.1212
Solar elastosis					
Absent	137 (27)	3 (19)	140	1.00	
Present	374 (73)	13 (81)	387	1.59 (0.45, 5.66)	0.4760
In-situ peripheral margin					
≤1	163 (28)	8 (44)	171	1.00	
>1	409 (72)	10 (56)	419	0.50 (0.19, 1.28)	0.1494
Invasive peripheral margin					
≤2	197 (40)	11 (65)	208	1.00	
>2	293 (60)	6 (35)	299	0.37 (0.13, 1.01)	0.0518
Invasive deep margin					
≤2	148 (30)	8 (47)	156	1.00	
>2	343 (70)	9 (53)	352	0.49 (0.18, 1.28)	0.1449

Table 1 (*continued*)

Variable	No melanoma remaining (N=620) *n* (%)	Melanoma remaining (N=20) *n* (%)	Total	OR (95% CI)	*p* value
Scar length					
≤20	263 (43)	11 (55)	274	1.00	
>20 to ≤30	164 (27)	5 (25)	169	0.73 (0.25, 2.14)	0.5643
>30	185 (30)	4 (20)	189	0.52 (0.16, 1.65)	0.2648
Specimen width					
≤15	200 (32)	3 (15)	203	1.00	
>15 to ≤25	258 (42)	6 (30)	264	1.55 (0.38, 6.28)	0.5387
>25	162 (26)	11 (55)	173	4.53 (1.24, 16.50)	**0.0221**
Specimen depth					
≤10	234 (38)	6 (33)	240	1.00	
>10 to ≤20	291 (47)	8 (44)	299	1.07 (0.37, 3.13)	0.8987
>20	91 (15)	4 (22)	95	1.71 (0.47, 6.22)	0.4121
Number of slices submitted					
1	193 (32)	2 (11)	195	1.00	
2–3	237 (40)	4 (22)	241	1.63 (0.30, 8.99)	0.5757
>3	166 (28)	12 (67)	178	6.98 (1.54, 31.62)	**0.0118**
Sampling					
Representative slices	206 (76)	1 (9)	207	1.00	
All embedded	65 (24)	10 (91)	75	31.69 (3.98, 252.27)	**0.0011**
Local metastatic disease					
Absent	194 (87)	8 (67)	202	1.00	
Present	28 (13)	4 (33)	32	3.46 (0.98, 12.26)	0.0540

Continuous variables are summarised as mean (standard deviation) and categorical variables by their frequency (percentage).
Bold *p* values are statistically significant.
CI, confidence interval; OR, odds ratio.

WLE specimens. This may reflect the fact that these tumours were thicker (mean Breslow thickness 2.9 mm) when compared to cases overall (mean Breslow thickness 1.7 mm) and the residual disease group as a whole (mean Breslow thickness = 2.4 mm). Although there was no significant difference in mean Breslow thickness between the two groups

Demographics			Characteristics of primary melanoma in complete excision biopsy													Characteristics of residual disease in wide local excision specimen							
Case reference	Age at diagnosis	Gender	Site	Subtype	Breslow thickness (mm)	Mitotic rate (/mm2)	LVI	Neurotropism	Microsatellites	Desmoplasia	In-situ peripheral margin	Invasive peripheral margin	Invasive deep margin	Macroscopic diameter (mm)	Amelanosis	Scar length (mm)	Number of slices submitted	Representative or all sampled	Size of residual disease (mm)	Location relating to scar	Margins (positive; negative)	In situ or invasive disease	Comments
Melanoma in situ																							
A	69	M	H&N	LM	n/a	n/a	n/a	n/a	n/a	n/a	1	n/a	n/a	14	No	14	4	Rep	2	Over scar	Negative	In situ	
B	48	F	H&N	LM	n/a	n/a	n/a	n/a	n/a	n/a	0.6	n/a	n/a	MD	MD	10	7	MD	2	Over and adjacent to scar	Negative	In situ	
C	66	M	UE	MIS unclassified	n/a	n/a	n/a	n/a	n/a	n/a	1.3	n/a	n/a	15	No	50	MD	MD	4	Adjacent to scar	Negative	In situ	
Invasive melanoma																							
D	68	M	UE	SSM	0.44	0	A	A	A	A	0.4	1.25	2.25	MD	MD	10	3	Rep	6	Over and adjacent to scar	Negative	In situ	
E	86	F	Back	LMM	0.6	1	A	A	A	A	2	4	4	10	No	37	2	MD	4	Over and adjacent to scar	Negative	In situ	
F	68	F	H&N	SSM	0.67	0	A	A	A	A	0.5	5.5	4	17	No	6	8	Rep	3	Over and adjacent to scar	Positive (peripheral margin)	In situ	
G	69	F	UE	SSM	0.85	4	A	A	A	A	1	1.2	1.6	6	Yes	30	7	MD	4	1mm from scar	Negative	In situ	
H	81	M	H&N	SSM	1.1	0	A	A	A	A	1.5	1.5	2	MD	MD	10	9	Rep	6	Over and adjacent to scar	Negative	In situ	
I	68	M	UE	LMM	1.15	5	A	A	A	A	0.3	2.5	3	MD	MD	15	13	Rep	8	Over and adjacent to scar	Negative	In situ	
J	67	M	H&N	NM	1.4	4	A	A	A	A	1	1.2	1.8	4	No	12	10	MD	2.5	1mm away from scar	Negative	In situ	
K	59	F	UE	DM	1.7	0	A	A	A	P	n/a	0.15	1.5	4	No	22	8	Rep	1	At edge of scar	Negative	In situ	
L	50	M	Back	NM	1.75	3	A	A	A	A	1.1	1.3	3	MD	MD	15	5	Rep	5	Immediately deep to scar in subcutis	Negative	Invasive	
M	83	F	UE	NM	2.05	4	A	A	A	A	1.2	1.4	2.1	MD	MD	20	MD	Rep	50	Over and adjacent to scar	Negative	In situ	
N	60	M	LE	NM	2.1	12	A	A	A	A	n/a	3	3.5	9	Yes	20	4	MD	5.5	7mm away from scar	Negative	Invasive	Clinically detected in transit deposit
O	61	M	H&N	NM	2.4	13	A	P	A	A	1.4	2.3	2	7	No	30	1	MD	22	Over and adjacent to scar	Negative	In situ	
P	76	F	Back	LMM	4.2	10	A	A	A	A	2.5	0.1	6	15	Yes	55	11	Rep	4	At edge of scar	Negative	In situ	
Q	65	M	LE	NM	4.3	21	A	A	A	A	2	2	2	15	Yes	22	1	MD	2.8	Within scar	Negative	Invasive	Residual spindle cell and desmoplastic component
R	65	F	LE	AL	4.9	19	P	A	A	A	0.2	0.8	1.5	15	No	30	5	All	0.3	Over scar	Negative	In situ	
S	79	M	LE	NM	6	17	A	A	A	A	2	1	0.8	MD	MD	15	2	Rep	15	Immediately deep to scar in subcutis	Positive (deep margin)	Invasive	
T	55	F	Back	SSM	6	18	P	A	P	A	1.5	2.4	2.5	25	Yes	65	3	MD	0.3, 1	Within scar and satellite	Negative	Invasive	Intra-lymphatic plugs; 0.3mm satellite in subcutis 2mm from scar; 1mm deposit within scar
	Mean 71	M = 11			Mean 2.4mm	Mean 7.7	P = 2 A = 18	P = 1 A = 19	P = 1 A = 19	P = 1 A = 19	Mean 1.2	Mean 1.9	Mean 2.6	Mean 12	Yes = 8 No = 5 MD = 7	Mean 24.4	Mean 5.7	Rep = 10 All = 1 MD = 8	Mean 7.8		Negative = 18 Positive = 2	In situ = 15 Invasive = 5	

Fig. 1 Select clinicopathological characteristics of the complete excision-biopsies (CEB) and wide local excisions (WLE) for patients whose WLE contained residual melanoma after CEB. A, absent; AL, acral lentiginous; DM, desmoplastic melanoma; H&N, head and neck; LE, lower extremity; LM, lentigo maligna; LMM, lentigo maligna melanoma; MD, missing data; MIS, melanoma *in situ*; NM, nodular melanoma; P, present; SSM, superficial spreading melanoma; UE, upper extremity.

overall, tumours that were greater than 4 mm thick were more likely to harbour residual disease in WLE specimens (OR 7.30; 95% CI 1.88, 28.26; p=0.004). Similarly, amelanotic melanomas were more frequently associated with residual disease in WLE likely not only reflecting difficulty in detecting this subtype clinically but also potentially signifying a more aggressive tumour biology.

The most common form of residual disease was melanoma *in situ* and only a few cases showed lymphovascular invasion or microsatellites. This reflects the previously proposed theory that a WLE procedure is likely to be most useful in control of residual disease rather than influencing local metastasis, since few cases showed residual disease with metastatic potential.[2]

Interestingly, the interval between primary melanoma CEB and WLE does not appear to affect survival.[22] While we did not determine the interval between CEB and WLE for all cases in our study, all cases that harboured residual melanoma had an inter-procedure duration concordant with national guidelines (less than 6 weeks) except for just one outlier, where there was an interval of 8 weeks.

We acknowledge several limitations to our study. Although our sampling processes are in line with current Australian guidelines,[16] we did not submit every WLE scar in entirety for histological examination or assess blocks using step sections. Indeed, residual disease was associated with >3 blocks of tissue and complete blocking of the scar. It is unclear from our data whether greater sampling of WLE

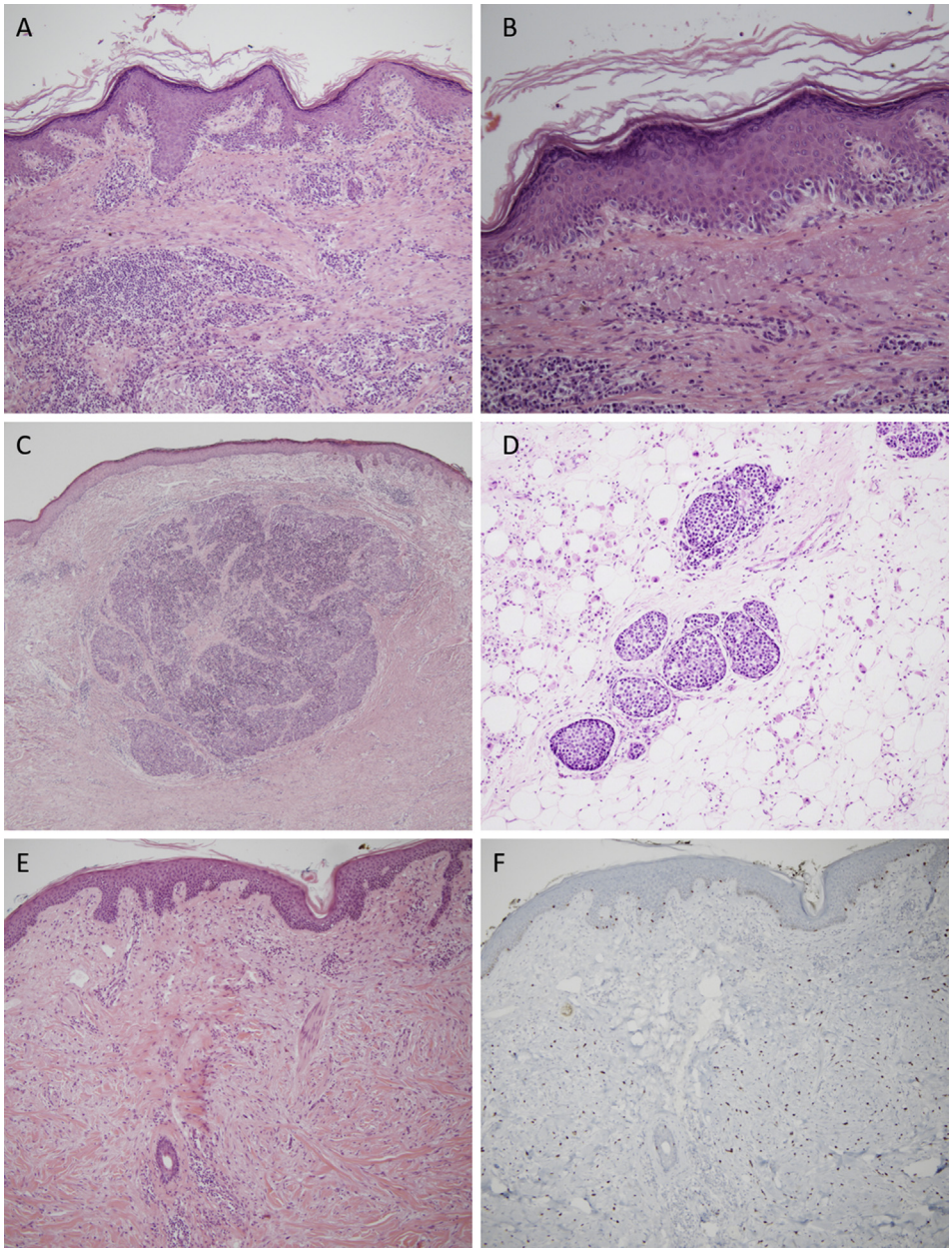

Fig. 2 Examples of residual melanoma in wide excision specimens. (A,B) Residual lentigo maligna overlying and adjacent to the scar related to prior excision biopsy (Case I, H&E). (C) Clinically detected intransit deposit (Case N, H&E). (D) Intralymphatic tumour cells (Case T, H&E). (E,F) Residual desmoplastic melanoma within scar related to the prior excision biopsy (Case Q, H&E, Sox10).

specimens was performed in those cases more likely to contain residual melanoma (i.e., whether this represents selection bias). While our rates of residual disease are similar to those of other studies that have employed variable sampling techniques,[2,8–11] we recognise the possibility that sampling error led to underestimation of the true incidence of residual disease.

It has previously been suggested that extensive random blocking of the scar in the absence of a macroscopic abnormality is unlikely to change results in a clinically significant way.[9,11] Fives *et al.* examined two separate cohorts of melanoma WLEs with different grossing protocols at their institution.[11] Consecutive cases managed in 2010 underwent extensive random sampling of the scar regardless of margins or macroscopically recognised lesions. In contrast, consecutive cases in 2012 had only three blocks of the scar submitted if there was no macroscopic lesion and CEB margins had been >1 mm. For cases with no macroscopic lesion and CEB margins >1 mm, the incidence of residual melanocytic lesions was comparable for the two groups, 4.3% and 6.3%, respectively.

In most cases in our study, residual disease was in the form of melanoma *in situ* that was completely excised, and it is unlikely that detecting or not detecting this form of residual disease will have significant clinical impact, as previously suggested by some authors.[9,11] However, we also observed cases with residual invasive disease, albeit rarely (5/640, <1% of all cases). Nevertheless, in the modern era of effective systemic drug therapies where adjuvant drug therapy is often considered for patients with stage III melanoma, detection of microsatellite metastases (or satellite or in transit metastasis) in WLE specimens could have important clinical implications regarding eligibility for systemic therapies. Of the five (of 640) cases with residual invasive disease in their WLE specimens, two had >3 slices submitted for microscopic examination and the other three cases had $\leq$3 slices sampled. These five included: one case of residual desmoplastic melanoma deep to the scar (5 blocks, Case L); a case of clinically detected satellite/in-transit located 7 mm away from the scar (4 blocks, Case N); intralymphatic plugs, a microsatellite and melanoma in the scar (3 blocks, Case T); residual nodular melanoma at deep aspect of scar (2 blocks, Case S); and residual spindle cell/desmoplastic melanoma within scar (1 block, Case Q). Hence the detection of residual disease in only one of these patients was likely to have affected their eligibility for adjuvant drug therapy.

Our study also identified the following pathological features to be associated with a higher rate of detection of residual disease in WLE specimens: LM/LMM and NM subtypes, higher tumour mitotic rate, Breslow thickness >4 mm, larger primary tumour diameter and amelanosis. In contrast, there was no statistically significant association with the presence of ulceration, Clark level, lymphovascular invasion, neurotropism, microsatellites, presence of a desmoplastic melanoma component or regression.

These findings have important implications for laboratory resources with several studies now suggesting that exhaustive histological examination of WLEs after an original excisional biopsy with negative margins is of limited value.[8–11,13,23] It is noteworthy that our study's findings do not support current guidelines for handling of WLE specimens, which encourage more extensive sampling of WLE specimens when microsatellitosis, neurotropism, extensive regression and lymphovascular invasion are detected in the initial biopsy specimen.[16] Therefore, review of these guidelines is warranted. On the basis of our study's findings, melanomas of LM/LMM and NM subtypes, higher mitotic rate, Breslow thickness >4 mm, larger lesion diameter and amelanosis probably warrant additional sampling of WLE specimens, although it must be borne in mind that Molenkamp *et al.* showed no significant relationship between the presence of residual melanoma in WLE specimens and disease-free survival or overall survival after 5 years follow-up.[24]

Another limitation of our study is the fact that many CEBs were originally processed and reported at external pathology laboratories. We addressed this limitation by only including cases for which the original biopsy had been formally reviewed by pathologists at our institution. For all these cases, complete histological information was documented in a standardised synoptic report, including histopathological parameters and margin status. As discussed above for WLE examination, step sectioning was not exhaustively employed for CEBs; therefore, there is a risk that margins originally reported as negative were in fact positive.

The WLE procedure is likely to function, in part, by capturing histologically or clinically unrecognised disease in the original CEB. This is supported by our finding that residual disease was significantly associated with amelanosis and larger tumour diameter, both of which are factors that may influence completeness of excision and recognition at gross sampling.

CONCLUSIONS

This study confirms that the finding of residual disease in WLE specimens performed within standard timeframes is an infrequent occurrence (3.1% in our study). WLE after CEB remains an important procedure to reduce local recurrence. Risk factors for residual disease appear to be LM/LMM and NM subtypes, higher mitotic rate, Breslow thickness >4 mm, larger lesion diameter and amelanosis. While most residual disease occurs in the form of melanoma *in situ* and is unlikely to be clinically significant, rare cases of residual invasive disease (<1% in our study) are detected and occasionally these could have management implications for individual patients (one of 640 patients in our study). More extensive sampling of WLE specimens may be appropriate when the above risk factors are present.

Conflicts of interest and sources of funding: RAS has received fees for professional services from Evaxion, Provectus Biopharmaceuticals Australia, Qbiotics, Novartis, MSD Sharp & Dohme, NeraCare, AMGEN Inc, Bristol-Myers Squibb, Myriad Genetics, GlaxoSmithKline. JFT has received honoraria for advisory board participation from Merck Sharpe Dohme Australia and Bristol Myers Squibb Australia, and honoraria, travel and conference expenses from GlaxoSmithKline, Provectus Inc, and Novartis. LJ was supported by the Melanoma Institute Australia Jani Haenke Pathology Fellowship. RAS is supported by an NHMRC Practitioner Fellowship (APP1141295). This work was supported by a National Health and Medical Research Council of Australia (NHMRC) Program Grant (APP1093017) (to RAS and JFT). The other authors declare no conflicts of interest.

Address for correspondence: Prof Richard Scolyer, Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Camperdown, NSW 2050, Australia. E-mail: richard.scolyer@health.nsw.gov.au

References

1. Cancer Council Australia. Clinical practice guidelines for the diagnosis and management of melanoma. 2020; cited Dec 2020. http://wiki.cancer.org.au/australia/Guidelines:Melanoma
2. Bolshinsky V, Lin MJ, Serpell J, *et al*. Frequency of residual melanoma in wide local excision (WLE) specimens after complete excisional biopsy. *J Am Acad Dermatol* 2016; 74: 102–7.
3. McKenna DB, Lee RJ, Prescott RJ, Doherty VR. A retrospective observational study of primary cutaneous malignant melanoma patients treated with excision only compared with excision biopsy followed by wider local excision. *Br J Dermatol* 2004; 150: 523–30.
4. Ethun CG, Delman KA. The importance of surgical margins in melanoma. *J Surg Oncol* 2016; 113: 339–45.
5. Lens MB, Dawes M, Goodacre T, Bishop JA. Excision margins in the treatment of primary cutaneous melanoma: a systematic review of randomized controlled trials comparing narrow vs wide excision. *Arch Surg* 2002; 137: 1101–5.
6. Sladden MJ, Balch C, Barzilai DA, *et al*. Surgical excision margins for primary cutaneous melanoma. *Cochrane Database Syst Rev* 2009; 4: CD004835.
7. Kelly JW, Sagebiel RW, Calderon W, *et al*. The frequency of local recurrence and microsatellites as a guide to re-excision margins for cutaneous malignant melanoma. *Ann Surg* 1984; 200: 759–63.
8. Hocevar M, Dragonja Z, Pilko G, Gazic B, Zgajnar J. Residual melanoma after an excisional biopsy is an independent prognostic factor for local recurrence and overall survival. *Eur J Surg Oncol* 2014; 40: 1271–5.
9. Martin HM, Birkin AJ, Theaker JM. Malignant melanoma re-excision specimens-how many blocks? *Histopathology* 1998; 32: 362–7.
10. de Waal AC, Vossen R, Aben KK, Kiemeney LA, van Rossum MM, Blokx WA. Limited role for histopathological examination of re-excision specimens of completely excised melanomas. *Virchows Arch* 2014; 465: 225–31.
11. Fives C, Heffron CCBB. Sampling of wide local excision specimens in cutaneous malignant melanoma. *J Cutan Pathol* 2018; 45: 198–203.
12. Gershenwald JE, Scolyer RA, Hess KR, *et al.* Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition Cancer Staging Manual. *CA Cancer J Clin* 2017; 67: 472–92.
13. McGoldrick RB, Ng D, Sawyer A, Mackey S, Vadodaria S, Powell BWEM. Malignant melanoma re-excision specimens: is there a need for histopathological analysis? *J Plast Reconstr Aesthet Surg* 2008; 61: 983–4.
14. Kirkham N. What is there to find in malignant melanoma reexcision specimens? *Histopathology* 1998; 32: 566–7.
15. Johnson R, Sviland L. Is extensive histological examination of wide excision specimens necessary following a diagnosis of melanoma? *Histopathology* 1998; 32: 379–80.
16. Royal College of Pathologists of Australasia (RCPA). *Primary Cutaneous Melanoma Structured Reporting Protcol (2nd Edition 2014)*. Sydney: RCPA, 2014. https://www.rcpa.edu.au/Library/Practising-Pathology/Structured-Pathology-Reporting-of-Cancer/Cancer-Protocols
17. Ng JC, Swain S, Dowling JP, Wolfe R, Simpson P, Kelly JW. The impact of partial biopsy on histopathologic diagnosis of cutaneous melanoma: experience of an Australian tertiary referral service. *Arch Dermatol* 2010; 146: 234–9.
18. Hayes AJ, Maynard L, Coombes G, *et al*. Wide versus narrow excision margins for high-risk, primary cutaneous melanomas: long-term follow-up of survival in a randomised trial. *Lancet Oncol* 2016; 17: 184–92.
19. Jackett LA, McCarthy SW, Scolyer RA. SOX10 expression in cutaneous scars: a potential diagnostic pitfall in the evaluation of melanoma re-excision specimens. *Pathology* 2016; 48: 626–8.
20. Florell SR, Boucher KM, Leachman SA, *et al*. Histopathologic recognition of involved margins of lentigo maligna excised by staged excision: an interobserver comparison study. *Arch Dermatol* 2003; 139: 595–604.
21. Botella-Estrada R, Sanmartin O, Sevila A, Escudero A, Guillen C. Melanotic pigmentation in excision scars of melanocytic and non-melanocytic skin tumors. *J Cutan Pathol* 1999; 26: 137–44.
22. McKenna DB, Lee RJ, Prescott RJ, Doherty VR. The time from diagnostic excision biopsy to wide local excision for primary cutaneous malignant melanoma may not affect patient survival. *Br J Dermatol* 2002; 147: 48–54.
23. Johnson R, Sviland L. Is extensive histological examination of wide local excision specimens necessary following a diagnosis of melanoma? *Histopathology* 1998; 32: 379–80.
24. Molenkamp BG, Sluitjer BJ, Oosterhof B, Meijer S, Van Leeuwen PA. Non-radical diagnostic biopsies do not negatively influence melanoma patient's survival. *Ann Surg Oncol* 2007; 14: 1424–30.

CHAPTER 5: UTILITY OF MOLECULAR ANALYSIS FOR CUTANEOUS UNDIFFERENTIATED MELANOMA AND ITS MIMICS

Overview: Some sarcomatoid tumours of the skin can defy definitive classification by histological and immunohistochemical means alone, and not infrequently present a diagnostic dilemma in dermatopathology practice. These heterogeneous tumours types, such as undifferentiated melanoma (UM), atypical fibroxanthoma (AFX) and pleomorphic dermal sarcoma (PDS), can be difficult to diagnose prospectively without the integration of molecular information. In our clinical experience working at large melanoma and non-melanoma skin cancer referral centres, some tumours initially diagnosed as AFX or PDS are occasionally later re-diagnosed as UM, usually based on unexpected clinical behaviour and the subsequent finding of a MAPK pathway variant that is typically associated with melanoma.

There is a limited understanding of the utility of molecular testing at initial diagnosis, in a prospective manner, when considering these tumours as a group. The hypothesis of the following study is that there may be a subset of patients with immunohistologically unclassifiable cutaneous sarcomatoid neoplasms for whom the finding of a MAPK pathway variant can improve the recognition of UM upfront.

Specific aims: Our aim was to examine the frequency of typical melanoma-associated MAPK-pathway related variants (*BRAF, NRAS, KIT, GNAQ and GNA11*) among a cohort of primary cutaneous sarcomatoid neoplasms.

Contribution to literature: Given that the treatment options for patients with UM vary from patients with AFX and PDS, there is value in improving the recognition of UM at initial diagnosis. As far as we aware, this study is the first of its kind.

Molecular analysis of cutaneous sarcomatoid neoplasms frequently identifies melanoma driver variants

Louise A. Jackett, MBBS, FRCPA[1,2,3,4,5]; Catherine Mitchell, MBBS, FRCPA [1,5,6]; Cameron Snell, MBBS, DPhil, FRCPA[1]; Chelsee Hewitt, PhD[6]; Shravan Yellenki, MLabMed[6]; Hayden Snow, MBBS, FRACS[3,5,7]; David Speakman, MBBS, FRACS [5,7]; Chris Angel, MD, FRCPA[1]; Christine Khoo, MBBS, FRCPA[1]; Jia-Min Pang, MBBS, FRCPA, PhD[1]; Serigne N. Lo, PhD[2,4,8]; Richard A. Scolyer, MBBS, FRCPA, FRCPath[2,4, 8, 9]; Stephen Fox, MBBS, DPhil, FRCPA[1,5,6,7]; David Gyorki, MBBS, MD, FRACS[3,5,7]

[1.] Department of Anatomical Pathology, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

[2.] Melanoma Institute Australia, The University of Sydney, Sydney, NSW, Australia.

[3.] Melanoma Research Victoria, Melbourne, Victoria, Australia.

[4.] Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia

[5.] Sir Peter MacCallum Department of Oncology, University of Melbourne

[6.] Department of Molecular Pathology, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

[7.] Department of Surgery, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

[8.] Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia

[9.] Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital & NSW Health Pathology, Sydney, NSW, 2050, Australia.

Address for Correspondence:

Louise A. Jackett

Department of Anatomical Pathology
Peter MacCallum Cancer Centre,
Grattan Street, Parkville, VIC, 3052, Australia
Email: louise.jackett@petermac.org

Accepted for publication: 22 February 2025

Journal: American Journal of Surgical Pathology

Publisher's reference: AJSP-D-24-00590R1

Editor-in-Chief: Jason L. Hornick, M.D., Ph.D.

Running title: Molecular analysis of cutaneous sarcomatoid neoplasms

Key words: melanoma, pathology, undifferentiated tumour, dedifferentiated tumour, molecular

Word count: main text 3709 (after revisions); abstract 250.

E**thical statement:** Ethics approval and waiver use of medical information has been granted through independent ethics committee and research boards (Peter Mac No: 24/77R; HREC Reference: HREC/108601/PMCC).

Conflict of interest statement: RAS funding and disclosures are detailed below. Other authors do not have any conflicts of interests to declare.

Author contribution statement: LJ, CM, DG, and RS performed the study concept and design; LJ developed the methodology, performed statistical analysis, writing, review, and revision of the paper; CH and SY performed molecular data analysis, interpretation of data, and technical and material support; HS, DS, and DG provided clinical revision of the paper; CS, CA, CK, JMP, CVV, and SF provided pathological revision of the paper; SL provided expert statistical analysis review. All the authors have read and approved the final manuscript.

Funding: RAS is supported by an NHMRC Investigator Grant (2022/GNT2018514). Support from The Cameron Family as well as from colleagues at Melanoma Institute Australia and the Royal Prince Alfred Hospital is also gratefully acknowledged.

Disclosures: RAS has received fees for professional services from SkylineDx BV, IO Biotech ApS, MetaOptima Technology Inc., F. Hoffmann-La Roche Ltd., Evaxion, Provectus Biopharmaceuticals Australia, Qbiotics, Novartis, Merck Sharp & Dohme, NeraCare, AMGEN Inc., Bristol-Myers Squibb, Myriad Genetics, and GlaxoSmithKline.

Abstract

Primary cutaneous neoplasms that lack definitive histological and immunophenotypic evidence of differentiation are a heterogeneous group of tumors with diverse prognoses and management options. These include undifferentiated and dedifferentiated melanoma (UM/DM), atypical fibroxanthoma (AFX), pleomorphic dermal sarcoma (PDS), and sarcomatoid squamous cell carcinoma. Diagnosis requires careful correlation between the clinicopathological and molecular features. The finding of a MAPK pathway variant commonly associated with melanoma may support the diagnosis of melanoma over other tumors in this group.

To examine the frequency of typical melanoma-associated MAPK pathway-related variants (*BRAF, NRAS, KIT, GNAQ, GNA11*) among a cohort of primary cutaneous sarcomatoid neoplasms, we conducted a retrospective analysis of 37 cases of immunohistologically unclassifiable primary cutaneous neoplasms, submitted for targeted NGS analysis. All cases lacked a history of a prior relevant tumor, were negative for melanocytic markers (S100, SOX10, HMB45, and Melan-A), or showed less than 5% staining with one or two of these markers. Other lineage markers were negative.

We identified typical melanoma driver variants in seven cases (7/37, 19%), including *NRAS* (5/37, 14%), *KIT* (1/37, 3%), and *GNAQ* (1/37, 3%). There were no significant differences in age, sex, tumor site, or mitotic rate between patients with and without a melanoma driver variant. Melanoma cases were thicker (16.3 mm versus 9.25 mm, $p = 0.041$) and more likely to show epithelioid cell phenotype ($p = 0.008$).

In our cohort, nearly 20% of patients with immunohistologically unclassifiable cutaneous tumors could be re-classified as having primary UM/DM after molecular testing, thereby opening alternative management pathways.

Introduction

Primary cutaneous neoplasms that lack definitive histological and immunophenotypic evidence of differentiation are a heterogeneous group of tumors with diverse prognoses and management options. This mixed bag includes atypical fibroxanthoma (AFX), pleomorphic dermal sarcoma (PDS), sarcomatoid squamous cell carcinoma (SCC), and dedifferentiated or undifferentiated melanoma (DM, UM). During pathological work-up, such tumors are often provisionally referred to as 'sarcomatoid,' 'spindle cell, ' 'undifferentiated' or 'unclassifiable' neoplasms.

Traditionally, assessment includes comprehensive clinical, morphological, and immunohistochemical features. When lineage-specific markers are absent, a pathologist will often diagnose either AFX or PDS, depending on features such as size, involvement of subcutis, presence of lymphovascular invasion, perineural invasion, and necrosis.

However, knowledge of the plasticity of melanoma and its molecular profile over recent decades has led to the recognition of dedifferentiated and undifferentiated forms of melanoma, which are defined by the partial or complete loss of identifiable melanocytic features, respectively.(1)

Differentiating DM and UM from AFX or PDS (and others) is valuable because their prognoses and management strategies differ. AFX is typically treated with complete excision, has recurrence rates of 5% if incompletely excised, and has a low metastatic potential.(2,3) PDS is often managed by wide local excision, has recurrence rates of 20% when incompletely excised, and shows locoregional or distant metastasis in 10-12% of cases, particularly in immunosuppressed patients (2,4). DM and UM are often managed with wide local excision, and depending on the patient's stage, other management options may be considered, such as sentinel node biopsy.(5) Furthermore, recent evidence supports the consideration of adjuvant systemic therapy for patients with high-risk primary and node-positive melanoma.(6)

It is not possible to diagnose primary cutaneous UM prospectively and definitively using conventional light microscopic tools alone. Without molecular analysis, the UMs (and some DMs) may be missed. Indeed, DMs and UMs have been found to be significantly under-recognized among soft tissue tumors when genomic analysis is not employed.(7)

However, there is no literature addressing the clinical impact of molecular testing of such tumors for the primary purpose of excluding UM/DM before a diagnosis of AFX or PDS is accepted. Specifically, there is a lack of studies examining clinicopathological and molecular features in a manner that is reflective of a pathologist's prospective work-up of these heterogeneous tumors in daily practice.

Therefore, we aimed to determine the frequency of typical melanoma driver variants and the consequent rate of reclassification to melanoma among primary cutaneous sarcomatoid neoplasms seen in our practice. The secondary aim was to analyze the clinicopathological features of these tumors.

Understanding the rate of melanoma driver variants and identifying any associated features of the primary tumor may provide pathologists and clinicians with a better understanding of how and when molecular testing can be employed in AFX- and PDS-like tumors.

Materials and Methods

This study was a retrospective review of prospectively collected data from a major referral center for melanoma and complex skin cancer (Peter MacCallum Cancer Center).

Inclusion and exclusion criteria

We searched archives for all cases of cutaneous epithelioid and/or spindle cell tumors between January 2019 and May 2024 that were regarded as AFX-like, PDS-like, or otherwise unclassifiable after morphological and immunohistochemical assessment (including an extensive panel of keratins, melanocytic stains, and mesenchymal markers), that had also undergone next-generation sequencing (NGS) (n=37). In all cases, NGS was pathologist-initiated for the specific purpose of

excluding melanoma. We included cases with provisional diagnostic terms of either AFX, PDS, undifferentiated carcinoma, DM or UM, as well any other cases with descriptive terms relating to an 'undifferentiated,' 'sarcomatoid,' 'spindle cell,' 'pleomorphic' or 'unclassifiable' cutaneous tumor.

We excluded cases that were ultimately diagnosed as secondary melanoma, carcinoma, or another tumor based on a history of a relevant prior tumor, either by clinical history alone or by comparable morphology, immunohistochemistry, and/or molecular phenotype. We also excluded DMs with a recognizable conventional component, such as melanoma in situ, melanin pigment, or any areas of convincing (strong) expression of Sox10, S100, melanA, or HMB45. We limited our cohort to cutaneous (dermosubcutaneous) tumors and excluded deep soft tissue tumors.

Clinicopathological data collection

We recorded clinical and demographic data for all patients, including age, sex, body site, presence and severity of chronic sun damage (CSD), and biopsy type. We noted provisional diagnoses prior to the molecular workup. Where data were available, we recorded the tumor cell type, tumor (Breslow) thickness, and mitotic rate (per mm2).

Immunohistochemical features

We recorded the expression patterns of immunohistochemical stains, including the four principle melanocytic markers (S100, SOX10, HMB45, and Melan-A), as well as other lineage markers used in diagnosis, such as PRAME, epithelial stains, mesenchymal markers, and CD10. Specifically, there was no convincing staining with melanocytic markers, or only very focal (up to 5%) and weak positivity with one or two of these markers. We included only those cases in which convincing markers of another lineage were negative.

Molecular Analysis

Molecular analysis of three MAPK pathway driver genes frequently seen in melanoma (*BRAF*, *NRAS*, and *KIT*) was performed in all cases, as well as *GNAQ* and *GNA11* in all but two cases. *NF1* was not included in the panel.

DNA was extracted from macro-dissected FFPE tumor tissues using a QIAamp® DNA FFPE Tissue Kit (Qiagen®).

In 35 cases, targeted sequencing of clinically relevant biomarkers was performed using the Oncomine™ Precision Assay (OPA) on an Ion Torrent™ Genexus™ System (Thermo Fisher Scientific Inc.). Primary sequencing analysis, read alignment, and variant calling were performed using Ion Torrent Genexus Software v6.2.1. The gene panel included clinically relevant small DNA variants in 45 genes (*AKT1, AKT2, AKT3, ALK, AR, ARAF, BRAF, CDK4, CDKN2A, CHEK2, CTNNB1, EGFR, ERBB2, ERBB3, ERBB4, ESR1, FGFR1, FGFR2, FGFR3, FGFR4, FLT3, GNA11, GNAQ, GNAS, HRAS, IDH1, IDH2, KIT, KRAS, MAP2K1, MAP2K2, MET, NRAS, NTRK1, NTRK2, NTRK3, PDGFRA, PIK3CA, PTEN, RAF1, RET, ROS1, SMO, TP53*) and CNVs in 14 genes (*ALK, AR, CD274, CDKN2A, EGFR, ERBB2, ERBB3, FGFR1, FGFR2, FGFR3, KRAS, MET, PIK3CA, PTEN*).

In two cases, amplicon-based NGS of *BRAF* (exons 11 and 15), *NRAS* (exons 2, 3, and 4), *KIT* (exons 11, 13, and 17), and *TP53* (exon 6) genes was performed, followed by targeted sequencing on an Illumina MiSeq™, 2x150bp. Aligned reads and called variants were analyzed using PathOS v1.5.

Statistical Analysis

Statistical analysis was performed independently by two authors (LJ and SL), using Fisher Exact test for categorial variables and Mann-Whitney test for continuous variables.

Ethics

All procedures were performed in compliance with relevant laws and institutional guidelines, and the project was approved by the institutional ethics review committee (ethics approval reference number 24/77R, approval granted May 22, 2024).

Results

Cohort characteristics

We identified 110 cases that matched our diagnostic terminology inclusion criteria, of which 44 had undergone molecular sequencing. Seven cases were excluded from this group after their tumors were confirmed to be either recurrent or metastatic (melanoma, carcinoma, or leiomyosarcoma) on clinical history. Of the 37 final cases, 54% (20/37) were externally referred and 46% (17/37) were in-house referred.

Clinicopathological features of whole cohort

The clinicopathological features of all cases are presented in Table 1. The mean age of all patients was 77 years (median, 78, range 46-89) and 89% (33/37) were male.

The specimen types were mostly complete excisions (28/37; 76%). All punch and shave biopsies (9/37, 25%) ultimately underwent complete resection, in which no lineage-specific features were identified.

The tumor site was most frequently the head and neck (31/37, 84%), with occasional cases on the trunk (3/37, 8%), lower limb (2/37, 5%), and upper limb (1/37, 3%). Almost all tumors showed moderate-to-severe chronic sun damage (CSD) (34/37, 92%).

Prior to molecular work-up, cases were classified in one of five broad categories, including ‘undifferentiated spindle cell tumor’ (10/37, 27%), ‘PDS or favor PDS’ (10/37, 27%), ‘undifferentiated sarcomatoid tumor’ (9/37, 24%), ‘AFX or favor AFX’ (5/37, 14%) or ‘undifferentiated epithelioid tumor’ (3/37, 8%).

Immunohistochemistry of whole cohort

Most cases (78%, 29/37) were evaluated using four melanocytic stains (Sox10, S100, melanA, and HMB45); nine cases (24%) were evaluated using only three (S100, Sox10, melanA); two (5%) were evaluated using only two markers (S100 and Sox10).

Of all cases, six (16%) showed weak staining of a melanocytic marker in up to 5% of cells, which was initially considered insufficient for a confident diagnosis of DM (Figure 1). These were: three cases with S100, one case with S100 and HMB45, one case with Sox10 and S100, and one case with Sox10.

PRAME staining was performed in 19 patients (51%), of whom 13 showed staining of either weak or moderate intensity (11 focal, 2 diffuse). PRAME positivity was not considered sufficient for the definitive diagnosis of melanoma.

Molecular profiling and melanoma reclassification rate

Information on the molecular analysis is presented in Table 2. We identified a MAPK pathway-related driver variant in seven cases (7/37, 19%), including *NRAS* (5/37, 14%), *KIT* G827R (1/37, 3%), and *GNAQ* R183G (1/37, 3%). *NRAS* variants included one case each of G12S, G12D, Q61R, Q61K, and Q61L.

As a result, seven cases (19%) were ultimately reclassified and clinically managed as melanoma.

Because of the absence of any melanoma MAPK driver variants, two cases were ultimately regarded as sarcomatoid SCC based on very focal epithelial marker expression (which, in the absence of keratin expression, had been considered potentially aberrant on initial examination), and one was ultimately regarded as myxofibrosarcoma instead of PDS owing to its prominent myxoid stroma.

TP53 variants were detected in 11 cases (21/37; 57%). Three of these variants were observed alongside melanoma MAPK variants (two cases with concurrent *NRAS* and one case with *GNAQ*). In most other *TP53* positive cases, they were ultimately classified as either AFX or PDS, except in one case that was reclassified as dedifferentiated SCC. When considering the final diagnostic groups, the

frequency of *TP53* variants was 57% (4/7) among AFX cases, 55% (11/20) among PDS cases, and 43% (3/7) among melanoma cases.

We noted two cases of concurrent *TP53* and *ATK1* variants. We found only one instance of an *HRAS* variant in one of the cases ultimately classified as a dedifferentiated carcinoma. *BRAF* and *GNA11* variants were not identified in this study.

Clinical characteristics of melanoma versus non-melanoma groups

The mean age of the patients with tumors ultimately classified as melanoma was 77 years (median, 84 years; range, 55–89 years). Most patients were male (6/7, 85%), and the tumors were mostly located on the head and neck (6/7, 86%). These characteristics were similar to those of the non-melanoma group, with no statistically significant differences in age, sex, tumor site, or presence of CSD (Table 3).

Pathological findings of melanoma versus non-melanoma groups

Compared to cases without a melanoma driver variant, reclassified melanomas were significantly more likely to show epithelioid, or mixed epithelioid and spindle, cell types (86% vs. 34%, p = 0.008). Melanoma cases as a cohort were significantly thicker than the rest of the cohort (16.3 mm versus 9.25 mm, p = 0.041 Mann-Whitney), however there was no significant difference in mean mitotic rate (11.8/mm2 versus 20.2/mm2, p = 0.105).

Immunohistochemistry features of melanoma versus non-melanoma groups

Reclassified tumors were regarded as DM (3 cases) if they showed any expression of Sox10, S100, melanA, or HMB45, and otherwise as UM (four cases) with no positivity. Reclassified melanoma cases were more likely to show at least focal expression of Sox10, S100, or HMB45 (43% versus 7%, p = 0.037). In 2 cases with sparse S100 staining, a melanoma driver variant was not identified.

When the five cases with *any* expression of Sox10, S100, HMB45, and melanA were excluded from the analysis, the rate of reclassification to UM was 12.5% (4/32).

There was no statistically significant difference in PRAME expression between the two groups, with 57% (4/7) of melanoma-variant cases and 30% (9/30) of non-melanoma-variant cases showing at least some level of PRAME staining (p = 0.113).

Discussion

Although the role of molecular testing in the differential diagnosis between primary sarcoma and metastatic melanoma has been investigated in deep soft tissue tumors, there is a paucity of data on the role of molecular analysis in differentiating difficult-to-classify primary cutaneous tumors.(7-15) Many of these studies are single-case studies.(13-15) Only one larger series of 11 cutaneous cases has been reported, all of which were DMs (not UMs) that included both conventional melanoma and either dedifferentiated or divergent areas.(12)

Therefore, this study is the first substantial and systematic study to specifically consider the utility of molecular analysis for superficial cutaneous tumors, where the differential diagnosis is between UM/DM, AFX, PDS, and/or other rare sarcomatoid tumors. Based on the concept of defining melanoma by the presence of characteristic MAPK driver variants, which is underpinned by our knowledge of melanoma plasticity and its ability to dedifferentiate(1,16,17), we re-assigned 19.4% of cases as melanoma. The variants identified were *NRAS*, *KIT* and *GNAQ*, with a notable absence of any *BRAF* variants. This spectrum is consistent with high-CSD melanomas, which rarely harbor *BRAF* p.V600E variants and more commonly show activating variants of *NRAS* or *KIT*.(18) One case showed a *GNAQ* variant, which was a clinically amelanotic tumor with a predominantly epithelioid cell phenotype. *GNAQ* variants are hallmarks of uveal melanomas and the blue nevus-like melanoma group, and both groups show a spectrum of epithelioid and spindled morphologies.(19,20)

The validity of defining undifferentiated melanoma by the presence of a MAPK variant, and the corollary of using this genomic information to exclude AFX or PDS, is an important question. Large cohort studies of conventional melanoma have established the mutational landscape of melanoma, which is enriched for MAPK variants in BRAF, NRAS, KIT, GNAQ and GNA11.(21) Similar genomic

results have been found in studies where differentiated melanoma has been observed alongside either immunohistochemically-negative tumour or heterologous elements (8,12,14). In contrast, studies of confidently diagnosed AFX and PDS typically lack these variants, with PDS more commonly harboring TP53 and NOTCH variants, and occasionally HRAS and KRAS.(22-24). Other genomic features, such as high tumor mutational burden, UV signature, TERT promoter mutations and CDKN2A variants, are not discriminatory as they are frequently reported in each of AFX, PDS and melanoma.(22,23,25) The above epidemiological and biological data have helped to define BRAF, NRAS, KIT, GNAQ and GNA11 variants as the characteristic genotype of melanoma, and this is the convention we have followed in this study.

Similar to our findings among cases of the skin, Cipriani *et al* reported that 21% of soft tissue 'sarcomas' and patients with a history of melanoma harbored a *BRAF* variant, which contrasted with the absence of *BRAF* variants in their control group of 90 sarcoma patients without a history of melanoma.(11) Kasago *et al* studied 19 cases of deep (soft tissue-located) UM/DM initially reported as unclassified or undifferentiated malignant neoplasms or sarcomas that were submitted for targeted next-generation sequencing (NGS).(9) These were re-assigned as UM/DM due to the finding of melanoma driver variants (*BRAF* in 26%, *NRAS* in 32%, *NF1* in 42%), in the presence of a UV signature and high tumor mutation burden (TMB). In contrast, their control cohort of 15 UPS of soft tissue lacked evidence of a UV signature and showed a dominant aging signature in 47% of cases (7/15). Although a UV signature and clinical history can be helpful in distinguishing DM/UM from UPS in soft tissue tumors, these features are of limited value when considering the differential diagnoses of cutaneous melanoma, AFX, and PDS, where UV damage is common to all and they are all potential primaries.(25) As discussed above, these tumours are also genetically similar in other ways, sharing high rates of variants in *TERT* promoter and *CDKN2A*.(25)

NF1 alterations occur in approximately 20% of cutaneous (non-acral) major melanoma subtypes and are enriched (40–90%) in sarcomatoid, dedifferentiated, or undifferentiated melanomas, and in half

of desmoplastic melanomas.(9,12,21,26,27) Thus, it is possible that we failed to identify at least two *NF1* melanomas that had focal S100 staining without a variant, as supported by similar PRAME staining in the two groups. However, as with Kasago et al., we are of the opinion that the presence of *NF1* variant is unlikely to be sufficient evidence to support a diagnosis of melanoma, since *NF1* alterations are also frequently present in UM/DM, AFX, and UPS.(9)

Consistent with the known frequent *TP53* variants in AFX and PDS(23), we identified *TP53* variants in just over half of our AFX and PDS cases. This rate is lower than that reported by other investigators and likely reflects the limited coverage of *TP53* by the OPA molecular panel used in our study.(23) The finding of only a single *HRAS* variant in our cohort (in a case ultimately classified as sarcomatoid SCC) supports the assertions of other investigators that *RAS* variants among AFX and PDS are rare.(23).

Similar to other studies on UV-driven tumors, most of our cases occurred in elderly male patients and on the head and neck (6/7, 86%), with only one case being from the trunk of a female patient.(22) We observed that patient age, sex, tumor location, and mitotic rate were not significantly different between melanoma and non-melanoma cases; therefore, these features are unlikely to be discriminatory in elderly patients. Our findings also suggest that dedifferentiated and undifferentiated forms of melanoma are more likely to occur in older age groups.

Tumors in the melanoma group were significantly thicker (mean 16.3 mm) than those in the non-melanoma cohort (mean 9.25 mm). Our melanoma cases were also more likely to show epithelioid cell morphology, suggesting that there should be a low threshold for molecular testing to exclude melanoma when there is an epithelioid cell component in a very thick tumor.

Although at the time of initial diagnosis, we considered the amount of S100 and Sox10 staining in our cases to be too weak and focal to confidently support a diagnosis of melanoma, our findings suggest that *any* level of melanocytic marker staining is indicative of melanoma or that this finding should at least prompt molecular testing. Thus we acknowledge that the two cases in our cohort

with sparse Sox10 +/- S100 that lacked *BRAF*/*NRAS*/*KIT*/*GNAQ*/*GNA11* variants could still be DMs, along with an unknown number of potential UMs in the 'non-melanoma' group that might be identified with more comprehensive molecular testing.

Our findings do not imply that the rate of melanoma-associated variants among all AFX-like tumors seen in routine practice is as high as that seen in our study. This is because our cohort was enriched for difficult-to-classify tumors, and we specifically excluded cases with a 'classical' AFX-like appearance (eg. a circumscribed dermal tumor with no involvement of subcutis and no necrosis). When we consider the subset of AFX-candidate tumors in our cohort (which included 5 initially classified as 'favour AFX'; 2 as 'undifferentiated spindle cell tumor'; and 1 as 'undifferentiated sarcomatoid tumor') the frequency of finding a MAPK variant was lower (12.5%, 1/8) than the whole cohort rate (20%). We recognise, however, that this is still likely be an overestimation of the rates among all AFX-like tumors when strict diagnostic criteria are adhered to. A lower rate is certainly more consistent with the historically low metastatic rates for AFX in routine practice. However, our findings could explain the rare occurrence of metastasis in AFX. Rather than suggesting that molecular testing should be performed in all AFX-like tumors, our findings support pathologists having a low threshold for molecular testing of dermal-based sarcomatoid tumours that do not meet strict AFX morphological criteria.

We acknowledge the effect of potential selection bias on our findings. Access to molecular testing was improved during the latter part of the study period. Molecular testing was also not always performed because of logistical or resourcing constraints, perceived lack of benefit, or clinician/patient preference. Any bias was mitigated by the exclusion of unequivocal DM cases with a known history of melanoma and cases with a pattern of disease thought likely to be melanoma.

We recognize that it may not always be appropriate for patients to undergo molecular sequencing, particularly in partial biopsies. Partial biopsies may often suggest a diagnosis of AFX or PDS; however, resection of the tumor can result in the identification of lineage-specific features, such as

melanoma in situ, areas of invasive tumor that are reactive to melanocytic stains, or the presence of keratinization and epithelial markers (to render a diagnosis of carcinoma). However, this study supports the recommendation that a diagnosis of AFX or PDS should not be definitively rendered on a partial biopsy since there is an inability to assess the entire tumor and adjacent skin.

In our cohort, all patients underwent either upfront excision or biopsy followed by excision. We recognize that in many resource settings, re-assessment of the tumor on complete excision after a partial biopsy may be a more judicious diagnostic approach than upfront molecular testing. However, since excision margins and the role of sentinel node biopsy vary depending on the tumor type, molecular testing may be considered even in partial biopsies depending on local resources.

The prevalence of AFX, PDS, and UM/DM is highest in countries with predominantly Caucasian populations and high cumulative sun exposure. Therefore, these tumors frequently present a diagnostic dilemma for clinicians and pathologists across North America, Australia and New Zealand. Our findings support increased funding for molecular testing to improve the diagnosis and management of patients with sarcomatoid tumors.

Conclusion

The diagnosis of a primary cutaneous sarcomatoid neoplasm is a frequent problem in clinical and pathological practice, particularly in populations with a high tumor burden related to chronic UV damage. In the absence of molecular testing, there is a risk that UM and DM will be underrecognized and misclassified as AFX or PDS.

In our cohort, nearly 20% of patients with an immunohistologically unclassifiable cutaneous tumor could be re-classified as having UM or DM after testing for MAPK pathway melanoma driver variants, thereby introducing alternative management pathways. These variants were most commonly *NRAS* and less commonly *KIT* and *GNAQ*. No cases of *BRAF* variants were found in our cohort.

Tumors with and without melanoma MAPK variants all had similar demographics, occurring in older males on the head and neck. Therefore, the patient age, sex, and body site were not discriminatory. Epithelioid cell type, thicker tumor, and equivocal Sox10 or S100 staining were the only indicators of the presence of a melanoma driver variant.

Molecular testing of primary cutaneous sarcomatoid neoplasms, which are otherwise unclassifiable by light microscopy and immunohistochemistry alone, has the potential to improve the detection of UM and DM by identifying MAPK pathway-driver variants.

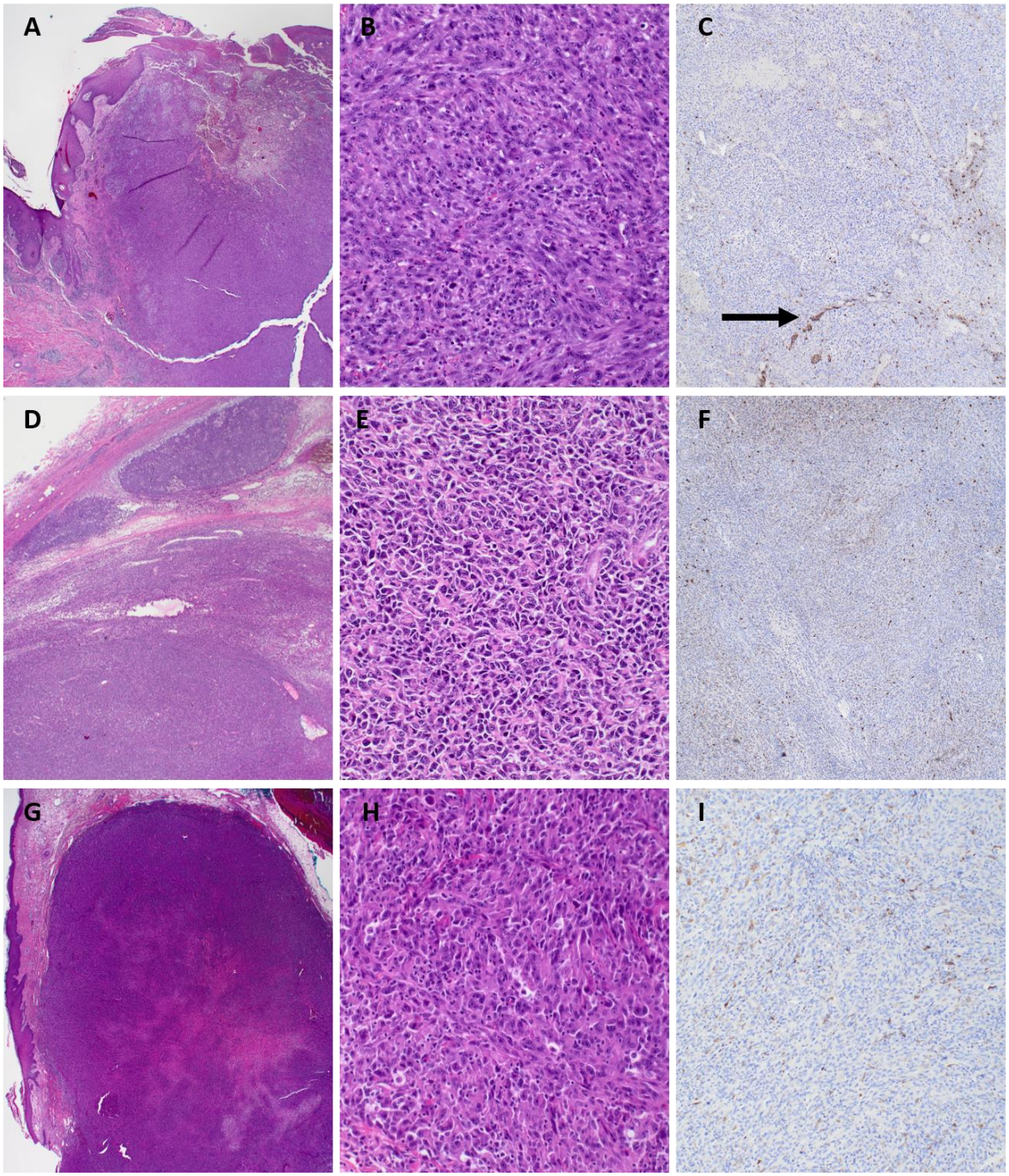

Figure 1. Examples of tumours with similar mixed epithelioid and spindle cell phenotypes and equivocal melanocytic marker expression patterns.

(A to C) Case 30. 74M skin tumour from lower neck. This ulcerated dermal tumour (A, H&E x12.5) showed a mixture of epithelioid and spindle cells (B, H&E x200). Immunohistochemistry showed sparse and weak staining of both S100 (C, S100 x40) and Sox10, and slightly stronger staining in a

cluster of approximately 20 cells (arrow). This was considered insufficient alone to support a diagnosis of melanoma, however the finding of *NRAS* Q61K variant by NGS ultimately supported this tumour being dedifferentiated melanoma.

(D to F) Case 22. 84F skin tumour on buttock. This dermal-based tumour (D, H&E x20) showed mixed epithelioid and spindle morphology (E, H&E x200) and patchy, weak S100 expression in tumour cells, which contrasted with strong staining of dendritic cells (F, S100 x40). *NRAS* Q61R variant was identified, and the tumour was ultimately classified as dedifferentiated melanoma.

(G to I) Case 14. 87F skin tumour clavicular region. An ulcerated deep dermal tumour (G, H&E x12.5) showed mixed epithelioid and spindle morphology (H, H&E x200), and equivocal (weak and sparse) S100 expression (I, S100 x100). *TP53* variant was identified in the absence of any targeted MAPK pathway variants, and this case was finally classified as pleomorphic dermal sarcoma.

Table 1. Clinicopathological characteristics of all cases

Characteristic	No. of cases (n=37)	%
Age (mean = 77, median = 78, range 46-89)		
<40	0	0%
40-49	1	3%
50-59	1	3%
60-69	6	16%
70-79	11	30%
80+	18	49%
Gender		
M	33	89%
F	4	11%
Mitotic rate per mm2	Mean 13.4 (range 2 - 50)	
Breslow thickness mm	Mean 10.6 (range 1 - 42)	
Biopsy type		
Excision upfront	28	76%
Punch biopsy then excision	8	22%
Shave biopsy then excision	1	3%
Site group		
Head and neck	31	84%
Trunk	3	8%
Lower limb	2	5%
Upper limb	1	3%
Chronic sun damage		
Absent	3	8%
Present	34	92%
Initial diagnosis		
Undifferentiated spindle cell tumour	10	27%
Favour pleomorphic dermal sarcoma	10	27%
Undifferentiated sarcomatoid tumour	9	24%
Favour atypical fibroxanthoma	5	14%
Undifferentiated epithelioid tumour	3	8%
Cell type		
Predominantly spindle	21	57%
Mixed epithelioid and spindle	11	30%
Predominantly epithelioid	5	14%
No. of melanoma markers performed (S100, Sox10, MelanA, HMB45)		
4	26	70%
3	9	24%
2	2	5%
No. of cases with any PRAME staining		
Not done	18	49%
Any staining	13	35%
Negative	6	16%
PRAME staining - diffuse versus focal		
Not done	18	49%
Focal	11	30%
Negative	6	16%
Diffuse	2	5%

Table 2. Molecular results of all cases

Characteristic	No. of cases (n=37)	%
Variant		
No variant detected	10	27%
TP53	9	24%
TP53 x2	4	11%
TP53, CDKN2A	2	5%
*NRAS**	2	5%
*NRAS, IDH1**	2	5%
AKT1, TP53	1	3%
HRAS	1	3%
TP53, AKT1	1	3%
*GNAQ, TP53, CDKN2A**	1	3%
*KIT, FGFR2, TP53**	1	3%
TP53 x2*, CDKN2A*	1	3%
NRAS, PIK3CA, TP53 x2**	1	3%
CDKN2A	1	3%
Melanoma driver variant (*BRAF, NRAS, KIT, GNAQ, GNA11*)		
No variant detected	30	81%
Yes	7	19%
Final diagnosis		
PDS	20	54%
AFX	7	19%
Melanoma	7	19%
Dedifferentiated SCC	2	5%
Myxofibrosarcoma	1	3%

*Indicates cases ultimately re-classified as melanoma based on molecular findings.

Table 3. Comparison of clinicopathological characteristics of melanoma patients for discordant PM and LNM samples

Characteristic	Melanoma driver mutation found (n=7)	%	Melanoma driver mutation not found (n=30)	%	p-value
Age (years)					
Mean	77		76		0.928
Sex					
Male	6	86%	27	90%	0.674
Female	1	14%	3	10%	
Biopsy type					
Excision	7	100%	21	70%	0.440
Punch biopsy	0	0%	8	27%	
Shave biopsy	0	0%	1	3%	
Site group					
Head and neck	6	86%	25	83%	0.745
Trunk	1	14%	2	7%	
Lower limb	0	0%	2	7%	
Upper limb	0	0%	1	3%	
Breslow thickness					
Mean (range)	16.3 mm (3 - 40)		9.25 mm (1 - 42)		**0.041**
Mitotic rate (per mm2)					
Mean (range)	11.8 (2- 45)		20.2 (2 - 50)		0.105
Cell type					
Spindle	1	14%	20	67%	**0.008**
Epithelioid and spindle	3	43%	8	27%	
Epithelioid	3	43%	2	7%	
Any expression of S100, Sox10, HMB45, melanA					
Yes	3	43%	2	7%	0.037
No	4	57%	28	93%	
PRAME expression (any)					
Yes	4	57%	9	30%	0.089
No	2	29%	4	13%	
Not done	1	14%	17	57%	
PRAME expression (any)					
Diffuse	0	0%	2	7%	0.113
Focal	4	57%	7	23%	
Negative	2	29%	4	13%	
Not done	1	14%	17	57%	
Chronic sun damage					
Present	6	86%	28	93%	0.478
Absent	1	14%	2	7%	

References (27)

1. Baraban E., Cooper K. Dedifferentiated and undifferentiated neoplasms: a conceptual approach. Semin. Diagn. Pathol. 2020;5:50682. doi: 10.1053/j.semdp.2020.09.002.

2. Helbig D, Ziemer M, Dippel E, et al. S1-guideline atypical fibroxanthoma (AFX) and pleomorphic dermal sarcoma (PDS). J Dtsch Dermatol Ges. 2022 Feb;20(2):235-243. doi: 10.1111/ddg.14700.

3. Wang WL, Torres-Cabala C, Curry JL, et al. Metastatic atypical fibroxanthoma: A series of 11 cases with minimal or no subcutaneous involvement. Am J Dermatopathol. 2015 Jun;37(6):455-61. doi: 10.1097/DAD.0000000000000237.

4. Logan IT, Vroobel KM, le Grange F, et al. Pleomorphic dermal sarcoma: Clinicopathological features and outcomes from a 5-year tertiary referral center experience. Cancer Rep (Hoboken). 2022 Nov;5(11):e1583. doi: 10.1002/cnr2.1583.

5. Seth R, Agarwala SS, Messersmith H, et al. Systemic Therapy for Melanoma: ASCO Guideline Update. J Clin Oncol. 2023 Oct 20;41(30):4794-4820. doi: 10.1200/JCO.23.01136.

6. Cancer Council Australia. Clinical practice guidelines for the diagnosis and management of melanoma v1.1. Published on 4/5/2024. Available from: https://app.magicapp.org/#/guideline/Lkk3pL

7. Agaimy A, Stoehr R., Hornung A, et al. Dedifferentiated and undifferentiated melanomas: report of 35 new cases with literature review and proposal of diagnostic criteria. Am. J. Surg. Pathol. 2021;45:240–254. doi: 10.1097/PAS.0000000000001645.

8. Agaimy A, Specht K, Stoehr R, et al. Metastatic malignant melanoma with complete loss of differentiation markers (undifferentiated/dedifferentiated melanoma): analysis of 14 patients emphasizing phenotypic plasticity and the value of molecular testing as surrogate diagnostic marker. Am. J. Surg. Pathol. 2016;40:181–191. doi: 10.1097/PAS.0000000000000527.

9. Kasago IS, Chatila WK, Lezcano CM, et al. Undifferentiated and dedifferentiated metastatic melanomas masquerading as soft tissue sarcomas: mutational signature analysis and immunotherapy response. Mod Pathol. 2023 Aug;36(8):100165. doi: 10.1016/j.modpat.2023.100165. Epub 2023 Mar 27. PMID: 36990277; PMCID: PMC10698871.

10. Kavuncuoglu A, Durmaz CD, Gokoz O, et al. Undifferentiated melanoma resembling undifferentiated round cell sarcoma: The diagnostic power of molecular melanoma signature. Int J Surg Pathol. 2022 May;30(3):346-349. doi: 10.1177/10668969211052238.

11. Cipriani NA, Letovanec I, Hornicek FJ, et al. BRAF mutation in 'sarcomas': a possible method to detect de-differentiated melanomas. Histopathology. 2014 Apr;64(5):639-46. doi: 10.1111/his.12305.

12. Ferreira I, Droop A, Edwards O, et al. Clinicopathological spectrum and genomic landscape of de-/trans-differentiated melanomas. Mod Pathol. 2021 Nov;34(11):2009-2019. doi: 10.1038/s41379-021-00857-z.

13. Lefferts JA, Loehrer AP, Yan S, et al. CD10 and p63 expression in a sarcomatoid undifferentiated melanoma: A cautionary (and molecularly annotated) tale. J. Cutan. Pathol. 2020;47:541–547. doi: 10.1111/cup.13646.

14. Cazzato G, Lospalluti L, Colagrande A, et al. Dedifferentiated melanoma: a diagnostic histological pitfall-review of the literature with case presentation. Dermatopathology (Basel). 2021 Oct 15;8(4):494-501. doi: 10.3390/dermatopathology8040051.

15. Valiga A, Fuller CG, Doyle JA, et al. Sarcomatoid dedifferentiated melanoma: The diagnostic role of next-generation sequencing. Am. J. Dermatopathol. 2022;44:282–286. doi: 10.1097/DAD.0000000000002092.

16. Banerjee SS, Eyden B. Divergent differentiation in malignant melanomas: A review. Histopathology. 2008 Jan;52(2):119-29. doi: 10.1111/j.1365-2559.2007.02823.

17. Massi D, Mihic-Probst D, Schadendorf D, et al. Dedifferentiated melanomas: morpho-phenotypic profile, genetic reprogramming, and clinical implications. Cancer Treat. Rev. 2020;88:102060. doi: 10.1016/j.ctrv.2020.102060.

18. Maldonado JL, Fridlyand J, Patel H, et al. Determination of BRAF mutations in primary melanoma. J Natl Cancer Inst. 2003 Dec 17;95(24):1878-90. doi: 10.1093/jnci/djg123.

19. Johansson PA, Brooks K, Newell F, et al. Whole-genome landscapes of uveal melanoma show an ultraviolet radiation signature in iris tumors. Nat Commun. 2020 May 15;11(1):2408. doi: 10.1038/s41467-020-16276-8.

20. Pérez-Alea M, Vivancos A, Caratú G, et al. The genetic profile of GNAQ-mutated blue melanocytic neoplasms revealed mutations in genes associated with genomic instability and the PI3K pathway. Oncotarget. 2016 May 10;7(19):28086-95. doi: 10.18632/oncotarget.8578.

21. Hayward N, Wilmott J, Waddell N, et al. Whole-genome landscapes of major melanoma subtypes. Nature 545, 175–180 (2017).

22. Ak M, Kahraman A, Arnold FM, et al. Clinicopathological and genomic profiles of atypical fibroxanthoma and pleomorphic dermal sarcoma identify overlapping signatures with a high mutational burden. Gene (Basel). 2021 Jun 25;12(7):974. doi: 10.3390/genes12070974.

23. Griewank KG, Wiesner T, Murali R, et al. Atypical fibroxanthoma and pleomorphic dermal sarcoma harbor frequent NOTCH1/2 and FAT1 mutations and similar DNA copy number alteration profiles. Mod Pathol. 2018 Mar;31(3):418-428. doi: 10.1038/modpathol.2017.146.

24. Sakamoto A, Oda Y, Itakura E, Oshiro Y, Tamiya S, Honda Y, Ishihara A, Iwamoto Y, Tsuneyoshi M. H-, K-, and N-ras gene mutation in atypical fibroxanthoma and malignant fibrous histiocytoma. Hum Pathol. 2001 Nov;32(11):1225-31. doi: 10.1053/hupa.2001.28953. PMID: 11727262.

25. Griewank KG, Schilling B, Murali R, et al. TERT promoter mutations are common in atypical fibroxanthomas and pleomorphic dermal sarcomas. Mod Pathol 2013;27:502–508.

26. Shain AH, Garrido M, Botton T, et al. Exome sequencing of desmoplastic melanoma identifies recurrent NFKBIE promoter mutations and diverse activating mutations in the MAPK pathway. Nat Genet. 2015 Oct;47(10):1194-9. doi: 10.1038/ng.3382.

27. Wiesner T, Kiuru M, Scott SN, et al. NF1 mutations are common in desmoplastic melanoma. Am J Surg Pathol. 2015 Oct;39(10):1357-62. doi: 10.1097/PAS.0000000000000451.

CHAPTER 6: MOLECULAR TESTING IN CHALLENGING MELANOCYTIC LESIONS OF THE SKIN

Overview: Naevoid melanomas (NM) are subtle melanomas that morphologically simulate naevi and are prone to under-diagnosis in both clinical and histopathological practice. Conversely, certain types of naevi, such as mitotically active naevi of pregnancy (MANP), are sometimes confused for NMs and therefore over-diagnosed. There is value in directly comparing the molecular features of these challenging tumours to determine if ancillary molecular information can improve their diagnosis.

Specific aims: To compare the variety and differences of hotspot variants and noncoding mutations harboured in a cohort of NMs and MANPs.

Contribution to literature: This is a forerunner study that considers the role of molecular testing for an oft-recurring diagnostic dilemma related to over- and under-diagnosis in dermatopathology.

Molecular Profiling of Noncoding Mutations Distinguishes Nevoid Melanomas From Mitotically Active Nevi in Pregnancy

Louise A. Jackett, MBBS, FRCPA,†‡ Andrew J. Colebatch, PhD, FRCPA,*†‡ Robert V. Rawson, MBBS, FRCPA,*†‡ Peter M. Ferguson, MBChB, PhD, FRCPA,*† John F. Thompson, MD, FRACS, FACS,*†‡ Stanley W. McCarthy, MBBS, FRCPA,*†‡ James S. Wilmott, PhD,†‡ and Richard A. Scolyer, MD, FRCPA, FRCPath*†‡*

Abstract: The accurate recognition of subtle melanomas and their distinction from benign mimics is an oft-recurring diagnostic problem, critical for patient management. Melanomas that bear resemblance to benign nevi (so-called nevoid melanomas, NMs) and benign mitotically active nevi in pregnancy (MANP) are 2 lesions particularly prone to error. Molecular data, including analysis of noncoding regions, in MANP and NM are very limited. This study sought to identify differences in clinical, pathologic, and molecular characteristics between MANP and NMs to facilitate correct diagnosis and reduce the risk of overtreatment or undertreatment. Clinicopathologic characteristics of NM (n = 18) and MANP (n = 30) were evaluated, and mutation data were analyzed using next-generation sequencing for available cases in each group (NM, n = 8; MANP, n = 12). All MANP showed innocent histopathologic characteristics apart from increased mitotic activity, frequently in both superficial and deep parts of the lesion (median dermal mitotic rate: 2/mm^2, range: 1 to 7/mm^2). All cases of NM demonstrated a characteristic nevoid silhouette, subtle atypical architectural and cytologic features, and variable mitoses (median mitotic rate: 3/mm^2, range: 1 to 5/mm^2). Median NM tumor thickness was 1.4 mm. Four of 10 NM patients with follow-up had metastatic disease, including 3 patients who developed widespread metastases, with 1 disease-related death. No other recurrences have been identified (follow-up period: 24 to 60 mo). None of the 15 MANP patients with available follow-up had a recurrence. Most NMs harbored hotspot mutations in *NRAS* (6/8, 75%). Noncoding mutations were significantly more common in NMs than in MANP (median: 4 vs. 0, $P = 0.0014$). Copy number alterations were infrequent but, when present, were seen in NMs (3/8 NMs vs. 0/12 MANP). All NMs but only 1 of 12 MANP had >1 abnormality in the noncoding regions. Similar to conventional common acquired nevi, MANP mostly harbored driver *BRAF* mutations, while activating *NRAS* mutations, noncoding mutations, and copy number alterations were rare. NM and MANP have subtle but recognizable distinguishing histopathologic characteristics that are underpinned by molecular differences. Mutation analysis of targeted noncoding mutations may assist in the diagnosis of difficult lesions.

Key Words: melanoma, nevus, noncoding mutations, pregnancy, molecular profile, pathology, diagnosis, NRAS, BRAF, prognosis, treatment

(*Am J Surg Pathol* 2020;44:357–367)

The accurate diagnosis of subtle melanomas and benign mimics of melanoma is a difficult area in both clinical and histopathologic practice. Melanomas that bear resemblance to benign nevi (so-called nevoid melanomas, NM) and benign mitotically active nevi in pregnancy (MANP) are 2 such groups of lesions that frequently cause diagnostic difficulty.

NMs are bona fide melanomas that morphologically simulate nevi and are associated with a risk of underdiagnosis. To this end, the entity has also been referred to as "minimal deviation melanoma," "lawyer's melanoma," or "a diagnosis of a nevus that you later regret," as they are often not diagnosed prospectively and come to attention only after metastasis has occurred. Consequently, they are a frequent source of medicolegal action.[1,2] The histopathologic features of NM have been documented in prior studies and 2 major subtypes have been recognized: the maturing nevoid phenotype and the papillomatous phenotype.[1–8] Few studies of their molecular features have been undertaken; however, and as far as we are aware, no prior published study has systematically evaluated noncoding mutations in them.[9–11]

From the *Royal Prince Alfred Hospital; †Melanoma Institute Australia; and ‡Central Clinical School, The University of Sydney, Sydney, NSW, Australia.

Conflicts of Interest and Source of Funding: Supported by the Melanoma Institute Australia (MIA), the New South Wales Department of Health, NSW Health Pathology, the National Health and Medical Research Council of Australia (NHMRC) and Cancer Institute NSW. This research was also supported through funding by Ray and Penni Jones and The Ainsworth Foundation. R.A.S. and J.S.W. are supported by NHMRC Fellowships. J.F.T. is supported by the Melanoma Foundation of The University of Sydney. L.A.J. and A.J.C. are supported by a Jani Haenke Melanoma Pathology Fellowship through the BB & A Miller Foundation and MIA. R.V.R. was supported by a Cameron Melanoma Pathology Fellowship and MIA and is currently supported by a Clinical Researcher Scholarship from Sydney Research. P.M.F. was supported by a Deborah and John McMurtrie Pathology Fellowship and MIA. R.A.S. reports receiving fees for professional services from Merck Sharp & Dohme, GlaxoSmithKline Australia, Bristol-Myers Squibb, Dermpedia, Novartis Pharmaceuticals Australia Pty Ltd, Myriad, NeraCare GmbH, and Amgen. S.W.M. disclosed that there is no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

Correspondence: Richard A. Scolyer, MD, FRCPA, FRCPath, Melanoma Institute Australia, 40 Rocklands Road, North Sydney, NSW 2065, Australia (e-mail: richard.scolyer@health.nsw.gov.au).

In contrast, it is recognized that, in certain circumstances, benign melanocytic nevi may have atypical clinical and/or histologic features that risk overinterpretation as melanoma. Examples of this phenomenon include nevi occurring on special sites such as acral skin or along the embryological milk-line, and nevi influenced by pathologic, environmental, or physiological changes such as trauma, recent intense UV exposure, and pregnancy.[12,13] Changes in nevi during pregnancy have been addressed in several studies, most of which have focussed on their clinical features. Characteristically, there is an increase in size or change in pigmentation.[13–20] Histopathologic studies analyzing such lesions are few, and these have documented the occasional presence of mitotic activity. In cases referred to us in consultation, we have seen otherwise typical compound nevi with frequent dermal mitoses, a feature that has raised concern for NM. In some cases, a history of concurrent pregnancy was not provided to the pathologist until this information was specifically sought from the clinician by the pathologist. The few pathological studies of MANP individuals that have been undertaken have generally focussed on histopathological features only, as well as immunohistochemical markers[21,22]; very limited research has evaluated the differences between mitotically active nevi in the general population and melanomas.[9,14,18,20,23–25] Although fluorescent in situ hybridization (FISH) differences between MANP have been described[9] in a few cases, there have been no dedicated molecular studies of mitotically active nevi in the setting of pregnancy published to date, as far as we are aware.

Recent studies have shown that nevi can acquire mutations in a stepwise fashion that results in their progression to melanoma.[26,27] Dominant driver mutations such as *BRAF* and *NRAS* occur early in nevi and, in most, are their only oncogenic mutation.[28,29] Interestingly, some of the early to intermediate mutation events in the transformation of nevi to melanomas occur in noncoding, promoter regions of the telomerase reverse transcriptase (*TERT*) gene, which maintains telomere length.[26] This is followed by the acquisition of additional loss of function mutations affecting the critical tumor suppressor proteins *PTEN*, *TP53*, and *CDK2NA*.[26,27,30] Our research recently described the protein-coding and non–protein-coding mutations that are frequently present in a range of melanoma subtypes.[31] The findings suggested that the presence or absence of coding and noncoding mutations could be used to aid the pathologic diagnosis of difficult lesions.

Accurate diagnosis of NM and MANP is of great practical importance not only to reduce the risk of overtreatment or undertreatment, but also because it is essential if optimal patient outcomes are to be achieved. This study sought to identify differences in clinical, pathologic, and molecular characteristics between NM and MANP, which may assist in their accurate pathologic diagnosis.

MATERIALS AND METHODS

This study was conducted with Human Research Ethics Committee approval (The Sydney Local Health District Human Research Ethics Committee, Protocol

TABLE 1. Key Clinicopathologic Characteristics of Nevoid Melanomas That Underwent Mutational Analysis

Case No.	Age/Sex	Site	Associated Nevus	Breslow Thickness (mm)	Clark Level	Dermal Mitotic Rate	Location of Mitoses	Symmetry	Regression	Pagetoid Spread	Lentiginous Proliferation	Inflammatory Infiltrate	Cytologic Atypia	Maturation
1	55/female	Left shoulder	Incomplete	1.5	4	5	Superficial and deep	Mild asymmetry	Absent	Minimal	Minimal	Absent	Mild	Absent
2	34/male	Right cheek	Absent	1.8	4	2	Superficial and deep	Asymmetrical	Absent	Absent	Absent	Absent	Mild	Present
3	47/female	Left leg	Absent	1.5	4	3	Superficial and deep	Symmetrical	Absent	Absent	Absent	Absent	Moderate	Absent
4	64/female	Right arm	Dysplastic compound nevus	1.4	4	3	Superficial and deep	Symmetrical	Absent	Minimal	Minimal	Minimal	Mild	Absent
5	19/male	Right lower leg	Dermal nevus	1.4	4	4	Superficial and deep	Mild asymmetry	Absent	Minimal	Minimal	Absent	Moderate	Present
6	62/male	Left upper back	Compound nevus	1.5	3	2	Superficial	Symmetrical	Absent	Absent	Absent	Absent	Mild	Incomplete
7	83/female	Scalp	Absent	1.5	4	3	Superficial	Symmetrical	Absent	Absent	Minimal	Absent	Mild	Incomplete
8	47/male	Right anterior shoulder	Absent	0.7	3	1	Superficial and deep	Mild asymmetry	Absent	Absent	Minimal	Absent	Mild	Present

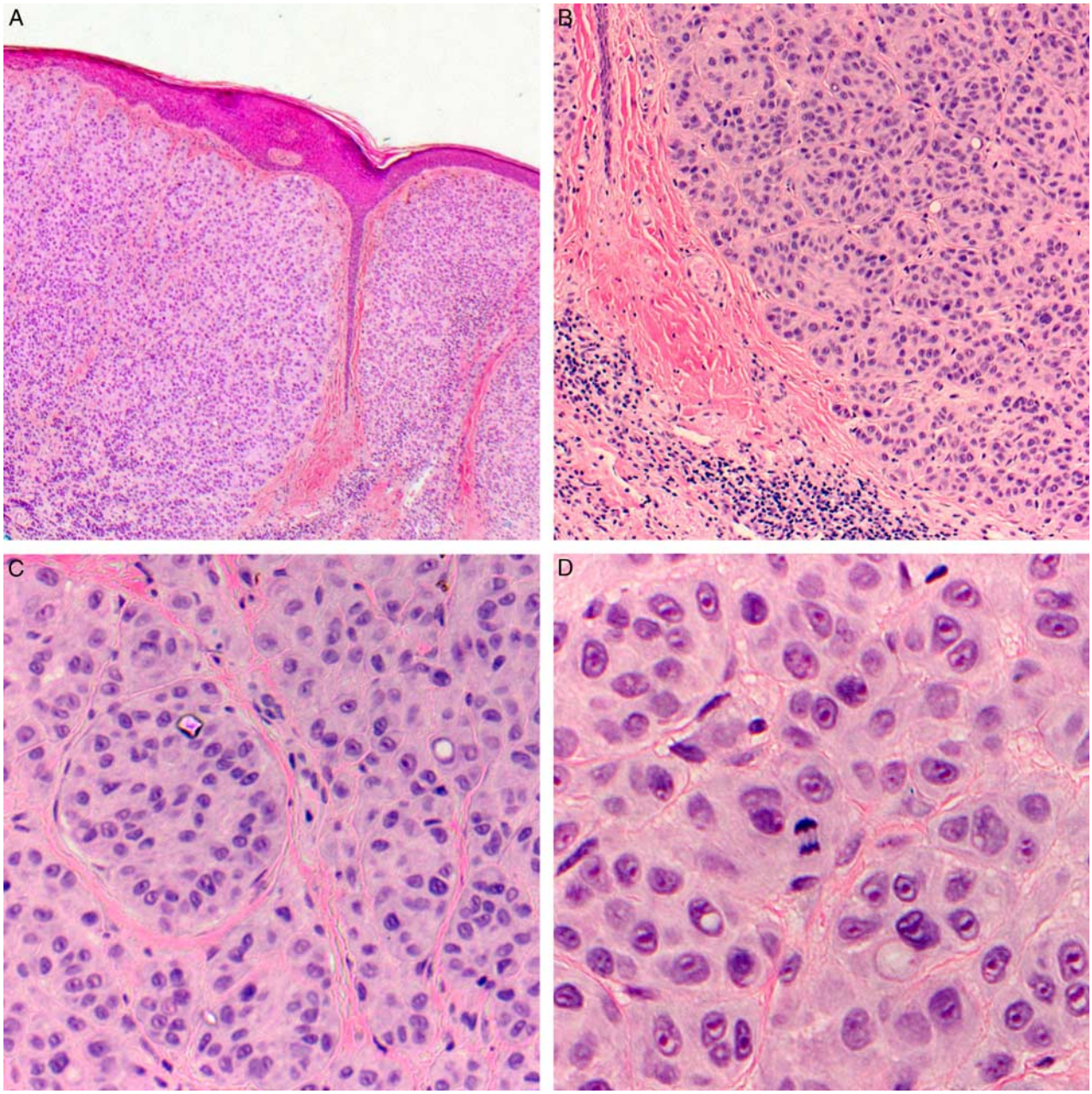

FIGURE 1. A–D, 55 year old female; left shoulder (patient case 1—NM). This NM shows apparent maturation with depth. However, the presence of expansile, sheet-like growth of the dermal component, elongated rete ridges, deep mitotic figures, asymmetry, and cytologic atypia at high power indicate a diagnosis of NM (hematoxylin and eosin stain).

No. X15-0454 and HREC/11/RPAH/444). The archives of the Department of Tissue Pathology and Diagnostic Oncology at Royal Prince Alfred Hospital, Sydney, and consultation files of 2 of the authors (S.W.M. and R.A.S.) were searched for cases of NM and melanocytic lesions from women who were either pregnant or up to 6 weeks postpartum, accessioned between 2003 and 2016. All cases were reviewed by at least 2 of the authors to confirm the diagnosis. Confirmed NMs and nevi from pregnant women that demonstrated mitotic activity were selected for further study, and follow-up data were sought. A detailed assessment of the clinical and pathologic features of each case was performed, including the evaluation of any available immunohistochemical stains. Cases for which paraffin-embedded tissue was available underwent molecular analysis.

DNA Extraction

Tumor-enriched DNA was extracted from archival formalin-fixed biopsies using the High Pure FFPET DNA Isolation Kit following the manufacturer's protocols (Roche). DNA quality was assessed using a quantitative

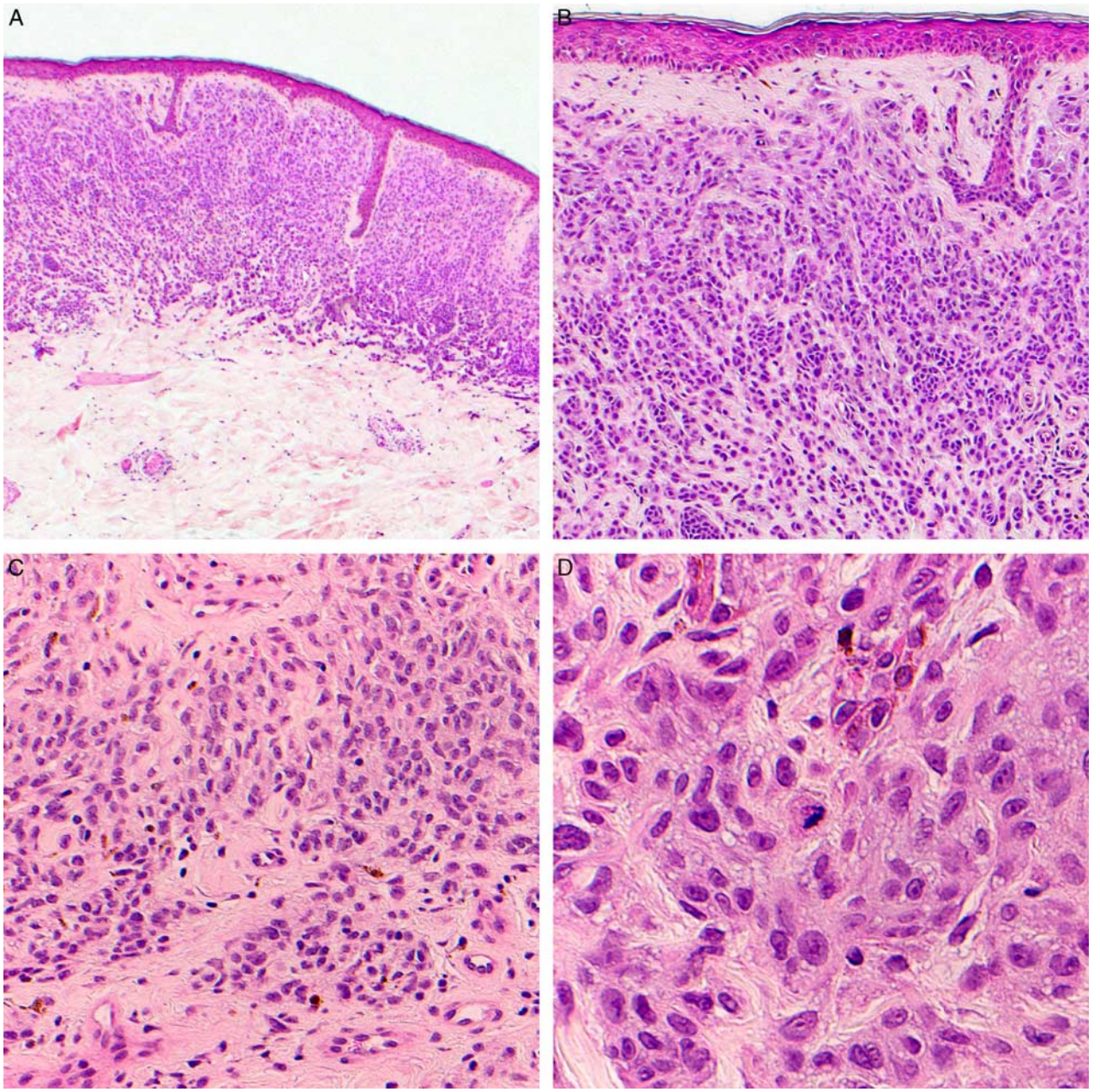

FIGURE 2. A–D, 19 year old male; right lower leg (patient case 5—NM). This NM shows both apparent symmetry and maturation with depth. However, the dermal component shows sheet-like growth, and, at higher power, there is significant cytologic atypia, and mitotic activity is present (hematoxylin and eosin stain).

polymerase chain reaction, presequencing quality control assay (PreSeq DNA QC assay; ArcherDX).

Next-generation Custom Amplicon Sequencing

All recurrently mutated coding and noncoding genes discovered in our previous whole-genome sequencing of a large cohort of melanomas were designed into a custom next-generation sequencing (NGS) panel in the Archer Assay Designer (ArcherDX).[31] The panel covers coding mutations in *ARID2*, *BAP1*, *BRAF*, *CDKN2A*, *EZH2*, *GNA11*, *GNAQ*, *HRAS*, *KIT*, *KRAS*, *MAP2K1*, *MAP2K2*, *NF1*, *NRAS*, *PPP6C*, *PTEN*, *RAC1*, *RAF1*, *RB1*, *SF3B1*, *TERT*, and *TP53*, along with noncoding hotspots in *AP3D1*, *ARHGEF18*, *BLCAP*, *C16ORF59*, *CDC20*, *CHCHD2*, *DHX16*, *DPH3*, *ERGIC3*, *FTH1*, *HSBP1*, *KBTBD8*, *MRPS31*, *MRPS33*, *NFKBIE*, *NSUN6*, *PES1*, *RALY*, *RNF185*, *RPL13A*, *RPL18A*, *RPL29*, *RPL34*, *RPS14*, *RPS27*, *SLC30A6*, *SMUG1*, *SWI5*, *SYF2*, *TERT*, *UBXN8*, *YAE1D1*, and *ZNF778*. Subsequent sequencing libraries were prepared following the Archer VariantPlex Somatic Protocol for Illumina and barcoded using the molecule-level barcoding (or unique molecule identifier tagging, ArcherDX).

Libraries were pooled and loaded, and sequencing was performed using the Miniseq Illumina sequencer using high-output reagents.

Sequence Alignment and Variant Calling

The Illumina Local Run Manager Analysis Service 1.3.0.1 was used to generate FASTQ files, and they were processed using the VariantPlex Archer Analysis Pipeline v5.1.8. Sequences were aligned to the hg19 reference genome, and somatic variants were called using Freebayes and Lofreq and the duplicate calls merged in the Archer Analysis 5 software. The quality of the sequencing data was assessed as the number of unique start sites (from DNA and ambiguous reads) calculated per GSP2 (across the entire panel). Samples with a sequence or variant score of <50 for average unique DNA were excluded from further analysis as per the manufacturer's guidelines. Subsequently, variants were filtered using the sequencing metrics of the annotated variant call file for a total unique start sites and reads supporting the variant (unique alternative observations and alternative observations) of >5, allele frequency of >0.2, read depth of >30 and call that strand bias was not detected. Remaining variants were annotated using the Cancer Genome Interpreter to predict the functional significance of the variants and produce the mutation annotated file.

RESULTS

Clinicopathologic Features of NMs

Eighteen cases of NM were identified. The mean age was 46 years (range: 17 to 83 y). Lesions were located on the upper extremity (4/18, 22%), head and neck region (3/18, 17%), lower extremity (3/18, 17%), and trunk (4/18, 22%). The anatomic site was unknown in 4 cases (27%). Median Breslow thickness was 1.4 mm (range: 0.6 to 2 mm). Mitotic activity was present in all cases (median dermal mitotic rate: 3/mm^2, range: 1 to 5/mm^2). The clinicopathologic features of the 8 NM cases that underwent molecular testing are presented in Table 1. All cases demonstrated a nevoid silhouette showing resemblance at low-power magnification to a benign nevus. Despite the appearance of a fair degree of symmetry and no or very limited pagetoid epidermal invasion, all cases included ≥ 3 of the following features: subtle asymmetry, expansile or sheet-like growth of the dermal component, subtle but significant cytologic atypia (usually mild but occasionally moderate) in the dermal component, elongated long thin rete ridges, incomplete or absent maturation of the dermal component, and multiple dermal mitoses (Figs. 1, 2). Prominent pagetoid spread or confluent junctional lentiginous architecture was absent. There was a mild lymphocytic infiltrate associated with the dermal component in 1 case; an accompanying inflammatory cell infiltrate was lacking in all other cases. All patients were treated with wide local excision. Of the 10 patients with available follow-up, 1 patient had involvement of a sentinel lymph node, and 3 other patients developed widespread metastatic disease, including 1 patient who died of melanoma. No recurrent disease was identified in any other patient (follow-up: 24 to 60 mo).

Clinicopathologic Features of MANP

We identified 30 cases of MANP that had >1 mitotic figure in the dermal component of the lesion (median dermal mitotic rate: 2/mm^2, range: 1 to 7/mm^2). These cases accounted for an overall 48% of all benign nevi from pregnant women during the search period (30/62). The mean age of the MANP patients was 31 years (range: 18 to 45 y), and this was comparable to the mean age for the entire cohort of pregnant patients with biopsied melanocytic lesions (32 y, range: 18 to 45 y). Where known, the median gestational age for patients with a MANP was 26 weeks (range: 6 to 38 wk). Three patients were up to 6 weeks postpartum. In 15 patients with available follow-up, there have been no reports of recurrence or metastatic disease (follow-up period: 2 to 14 y). The clinicopathologic features of the 12 MANP cases that underwent molecular testing are presented in Table 2.

The anatomic sites of the lesions were the trunk (16/30, 53%), lower extremity (4/30, 13%), upper extremity (4/30, 13%), head and neck (3/30, 10%), vulva (2/30, 7%), or unknown (1/30, 3%). The clinical indication for excision was most commonly an increase in size of the lesion or change in pigmentation, usually darkening. The most

TABLE 2. Key Clinicopathologic Characteristics of MANP That Underwent Mutational Analysis

Case No.	Age/Sex	Site	Gestation (wk)	Clinical Information	Diagnosis	Dermal Mitotic Rate (/mm^2)
9	19/female	Vulva	Postpartum	Atypical vulval polyp	Combined nevus	3
10	27/female	Right knee	Unknown	Darkening	Combined nevus	2
11	27/female	Mid sternum chest	30	Increasing size	Dermal nevus	3
12	27/female	Lower abdomen	Unknown	Pigmented lesion	Compound nevus	2
13	31/female	Abdomen	Unknown	Nevus with keratosis	Compound nevus	2
14	33/female	Right breast	18	Increasing size	Compound nevus	1
15	31/female	Back	15	Enlarging	Compound nevus	2
16	33/female	Right fourth toe	17	Atypical lesion	Compound nevus	1
17	27/female	Right breast	32	Enlarging	Compound nevus	3
18	30/female	Abdomen	38	Mole at site of pfannenstiel incision removed at caesarian section	Compound nevus	1
19	39/female	Left forearm	28	Pink papule	Compound nevus	1
20	32/female	Right forearm	Postpartum, lactation	Dermatofibroma	Dermal nevus	2

common histologic subtypes were conventional compound or dermal nevi (22/30, 70%) while the other lesions were combined nevi (4/30, 13%), genital nevi (2/30, 7%), a deep penetrating nevus (1/30, 3%), and a dysplastic compound nevus (1/30, 3%). In all cases, apart from low-grade mitotic activity (median dermal mitotic rate: 2/mm^2, range: 1 to 7/mm^2), the lesions showed benign characteristics including symmetry, maturation of the nevus cells with depth in the dermis, at most mild to moderate cytologic atypia in the dysplastic nevus and absent severe cytologic atypia, expansile and sheet-like growth of the dermal component, and absent confluent lentiginous growth and pagetoid invasion of the epidermal component. Mitotic figures were frequently located in both superficial and deep portions of the nevus (Figs. 3, 4).

Molecular Features of MANP and NM

A total of 8 cases of NM (Table 1) and 12 cases of MANP (Table 2) had adequate tumor tissue available for DNA extraction and passed sequencing data quality controls. Mutation data are provided as an oncoplot in Figure 5A.

Six of 8 NM cases (75%) showed a known driver mutation of *NRAS* (3 cases with *Q61R*, 2 cases with *Q61K*, 1 case with *G13R*). One of the cases with *NRAS Q61K* carried a concurrent *BRAF* mutation; however, this was a non-*V600* passenger mutation (*G466E*). Three copy number alterations were detected, all loss of copy number, and all occurred in *CDK2NA* (n = 2, Fig. 5B) or *TERT* (n = 1) in NM. Mutations in select noncoding regions were present in all NM samples, with the most common events occurring in the *TERT* promoter mutation (n = 5/8, 63%), as expected for cutaneous melanoma.[31]

Ten (of 12) cases of MANP (83%) showed a *BRAF* V600E driver mutation. A single case (patient 19; Fig. 4) of a compound nevus harbored an *NRAS Q61R* mutation, a passenger mutation of *NFKBIE* and promoter mutations of *FLT3LG* and *ARHGEF18*. Copy number alterations were absent in the MANP group. Likewise, mutations in noncoding regions of the genes of interest were infrequent, with only *FLT3LG* being mutated in >1 (n = 2) MANP. *TERT* promoter was wild-type for all MANP cases.

The overall tumor mutation burden was significantly higher in the NM compared with the MANP group ($P = 0.0182$, Fig. 5C). There were no significant differences in the number of passenger or known melanoma driver mutations (as detected by the Cancer Genome Interpreter) between the 2 groups (Figs. 5D, E). However, there was a significantly higher mutation count in the noncoding region of the targeted genes (selected on the basis of their known occurrence in melanoma) in the NM group compared with the MANP group ($P < 0.0001$, Fig. 5F).

DISCUSSION

The diagnosis of NMs and MANP remains challenging in both clinical and histopathologic practice. It is the authors' experience that the presence of mitotic activity in nevi that are biopsied during pregnancy (and nonpregnant states) is occasionally a reason to seek a second opinion, and reliance on this feature as the sole marker of malignancy leads to the overdiagnosis of melanoma. In contrast, failure to recognize subtle cytologic and architectural features can lead to a failure to diagnose NM and adverse patient outcomes. In our study, 89% (16/18) of the NM and 87% (26/30) of the MANP were cases referred from outside pathologists for a second opinion, highlighting the fact that these cases remain difficult to diagnose in everyday practice and are at risk of misdiagnosis. This selection bias probably underscores why mitotically active nevi accounted for 48% of all benign nevi from pregnant women in our institutional database during the study period.

In our cohort of MANP lesions, the most common reason for biopsy was a change in the size or pigmentation of a preexisting nevus. This is in line with other studies that have long concluded that pregnancy can induce a range of skin changes, including color variation, growth, or altered dermoscopic features of nevi.[13,14,18,20,23,32–34] These changes are possibly the result of melanocyte sensitivity to the altered hormonal milieu of pregnancy or to the increased tyrosinase from human placental lipids.[17,35–37] Recently, the degree to which pregnancy induces changes in nevi has been challenged.[38] Nonetheless, several investigators report that 6% to 30% of pregnant women perceive a change in at least

TABLE 2. (*continued*)

Location of Mitoses	Symmetry	Regression	Pagetoid Spread	Lentiginous Proliferation	Inflammatory Infiltrate	Cytologic Atypia
Superficial and deep	Symmetrical	Present	Absent	Present	Absent	Absent
Superficial only	Mild asymmetry	Focal fibrosis	Focal	Absent	Patchy	Spitzoid population
Superficial and deep	Symmetrical	Absent	Absent	Absent	Absent	Minimal
Superficial and deep	Symmetrical	Absent	Absent	Absent	Absent	Mild
Superficial and deep	Symmetrical	Absent	Absent	Absent	Absent	Absent
Superficial	Symmetrical	Absent	Absent	Absent	Absent	Absent
Superficial and deep	Symmetrical	Absent	Absent	Absent	Absent	Absent
Superficial and deep	Symmetrical	Focal	Focal	Absent	Absent	Absent
Superficial and deep	Symmetrical	Absent	Absent	Minimal	Absent	Absent
Superficial	Symmetrical	Absent	Absent	Absent	Absent	Absent
Superficial	Symmetrical	Absent	Absent	Absent	Absent	Absent
Superficial and deep	Symmetrical	Absent	Absent	Absent	Absent	Absent

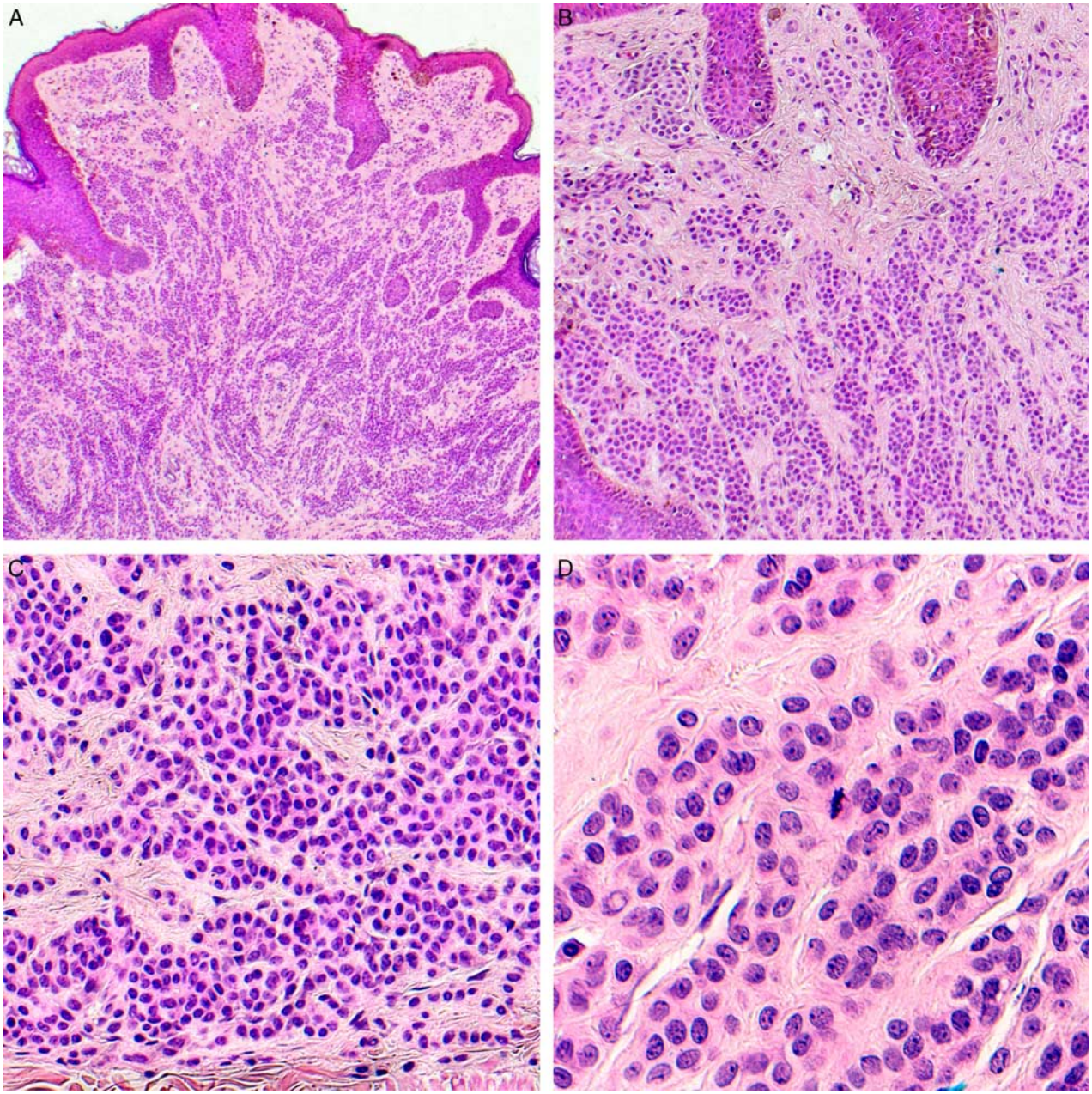

FIGURE 3. A–D, 27 year old female; right breast (patient case 17—benign mitotically active nevus of pregnancy). The sections show a compound melanocytic nevus with a polypoid appearance at low power. The nevus is composed of nests and sheets of bland nevocytes showing maturation with depth and symmetry. There are occasional superficial and deep mitoses (hematoxylin and eosin stain).

1 mole, which is occasionally concerning enough to seek medical attention.[14,18,20,23] Physiological changes in nevi appear to be transient; however, there is consensus in the literature that any lesion causing genuine concern for melanoma should undergo prompt biopsy.[13,34,35] Not all (and possibly a minority of) clinically altered nevi occurring during pregnancy show atypical features from a histologic standpoint; however, an increase in mitotic activity is, in our experience, an occasional finding.[24,38] Our cohort of MANP lesions was selected on the basis of the presence of mitotic activity; similar to prior studies, mitotic activity in our cohort was usually of a low rate but occasionally high (up to 7/mm^2), and the nevi otherwise maintained innocent morphologic characteristics.[9,23,24]

We identified activating *BRAF V600E* mutations in 83% of our nevus cases, which is similar to other studies of conventional nevi.[28,29] Our study, however, is the first to document this finding in a group of mitotically active nevi. Until now, only 2 published studies have examined the molecular characteristics of mitotically active nevi using FISH and/or comparative genomic hybridization.[9,11] One of these studies undertook FISH analysis of 10 mitotically

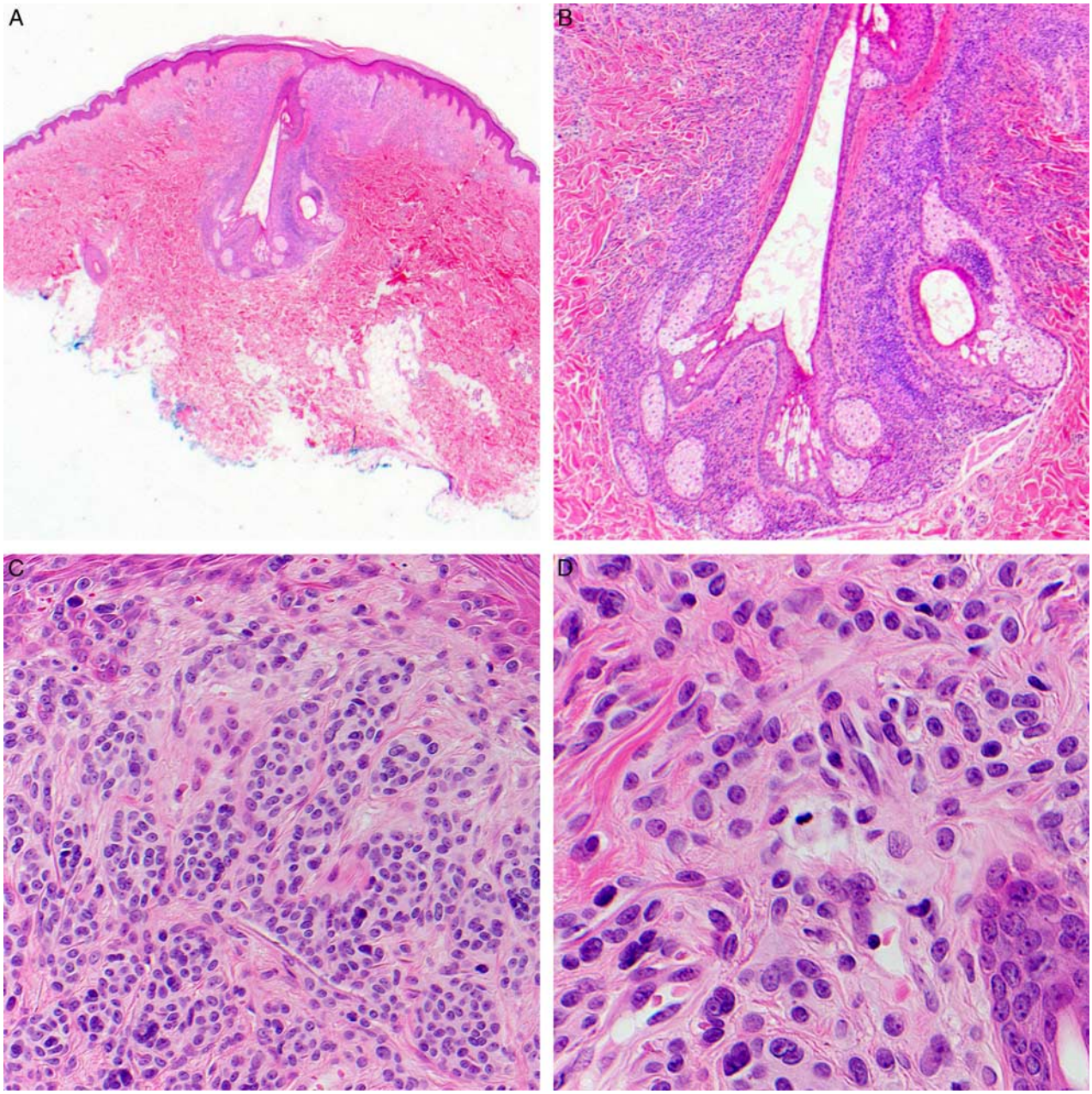

FIGURE 4. A–D, 39 year old female; left forearm (patient case 19—benign mitotically active nevus of pregnancy). The sections show a predominantly dermal nevus composed of nests and sheets of bland nevocytes showing maturation with depth and symmetry. There are occasional superficial and deep mitoses (hematoxylin and eosin stain).

active banal nevi from both men and women (including 3 pregnant patients) and 10 NMs.[9] The investigators found no chromosomal copy number changes in any of the mitotically active nevi, but there were consistent aberrations of chromosome 6 and/or 11 in the NM group.[9] Likewise, our study only found copy number alterations in the NM cases; however, the sensitivity of the targeted NGS panel to detect copy number variations (CNVs) is much lower than FISH. Indeed, most of the NMs in our study lacked CNVs, and relying on molecular adjunctive testing based solely on CNV may underdiagnose some NMs. This could be due to the single cell analysis of FISH detecting minor clones compared with the NGS. Furthermore, our NGS panel did not cover the centromere region *CEP6*, which could be included to further improve the copy number detection rate of the NGS panel in NM.

One MANP case showed *NRAS* Q61R mutation with passenger mutations of *NFKBIE* and *BAP1* and promoter mutations of *FLT3LG* and *ARHGEF18* (patient 19). This case was a compound nevus (Fig. 4). The finding of a single

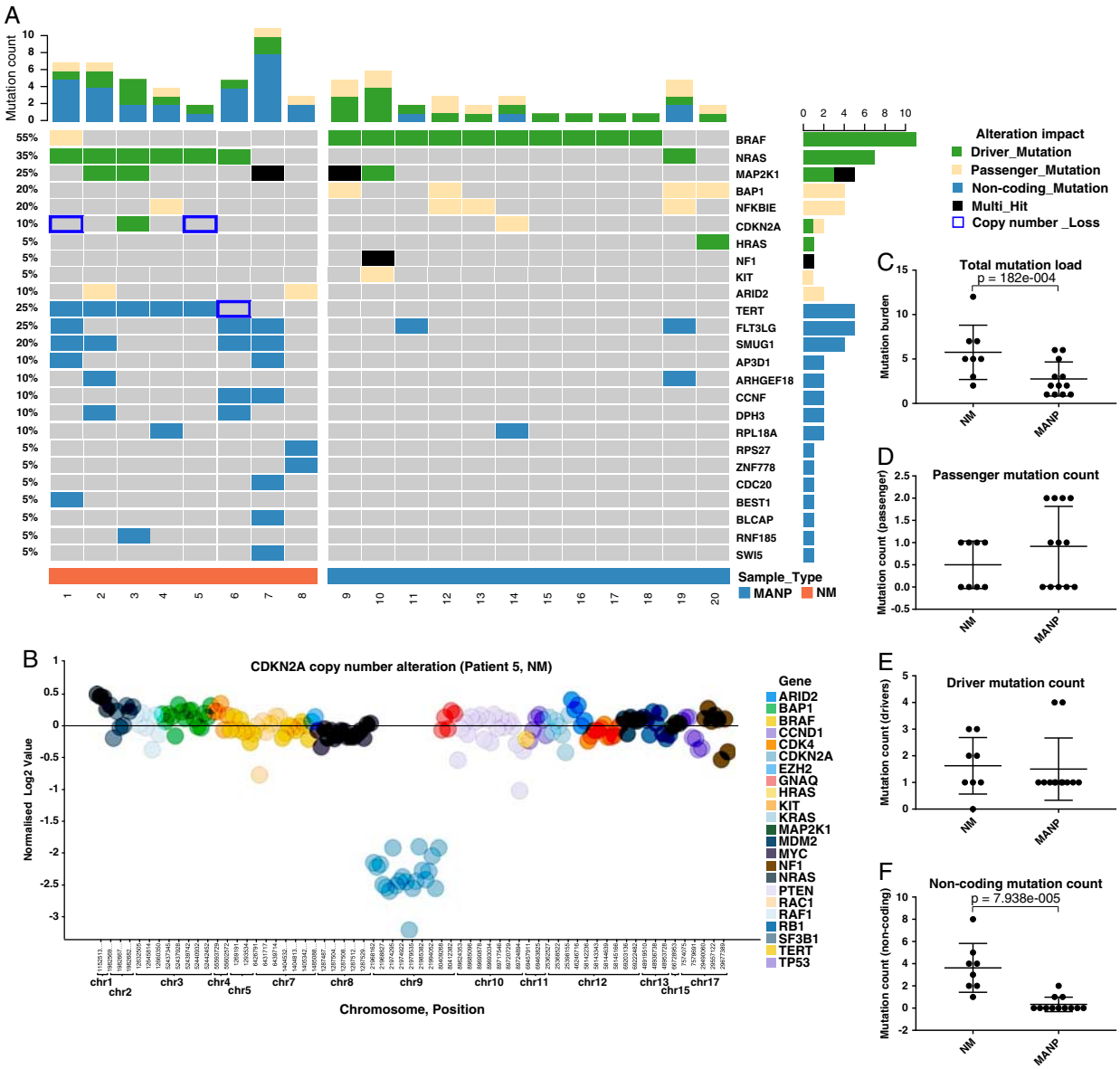

FIGURE 5. A–F, Oncoplot and mutational counts for 8 cases of NM (cases 1 to 8) and 12 cases of MANP (cases 9 to 20). A, Oncoplot showing somatic mutations present in known melanoma oncogenic drivers and noncoding promoters. B, An example of copy number loss at the *CDKN2A* locus in a patient with NM (case 5). Comparison of somatic mutation counts between NM cases and MANP, for total mutation burden (C), passenger mutation (D), driver mutation (E), and noncoding mutation (F) counts.

acquired nevus case with an *NRAS* mutation (1/12; 8.3%) is consistent with other studies that have found *NRAS* mutations in up to 20% of conventional acquired nevi.[39,40] The presence of 2 promoter mutations in benign acquired nevi, as seen in this case, has been demonstrated previously by our group using whole-genome sequencing.[29]

There was also only 1 case of MANP with an *HRAS* mutation and a passenger mutation in *BAP1* (case 20). This case was a dermal nevus with banal congenital-type features and no atypia apart from mitotic activity (2/mm^2). In contrast to our findings, *HRAS* mutations are not commonly reported in conventional nevi but are noted in up to 25% of Spitz nevi.[39,40]

Our study identified mutations in the noncoding regions of the genome as the most discriminating molecular feature discriminating NM from MANP. Melanomas have been shown to be enriched for mutations in noncoding transcription factor binding sites, at least partially due to reduced ability to complete DNA repair in these regions.[41] Cutaneous malignancies such as melanoma are further enriched for noncoding mutations in ETS transcription factor binding sites as a result of UV mutagenesis,[42,43] in turn due to binding of ETS transcription factors, irrespective of DNA repair status.[44,45] Therefore, the accumulation of mutations in certain noncoding regions may identify lesions that have undergone

mutagenic exposure and which have reduced rates of successful DNA repair that potentially underlie their pathogenesis as malignant lesions.

However, noncoding mutations in the promoter region of *TERT* have been shown to be an early event in the oncogenic transformation of nevi to melanoma, and many recent studies have assessed the utility of *TERT* mutation detection to aid in the diagnostic and prognostic assessment of melanocytic lesions.[26,29] Our study only detected *TERT* mutations in the NM cohort, but mutations were not present in all NMs, and we have previously shown that some melanoma subtypes (particularly mucosal and acral) have a lower *TERT* mutation frequency.[31] We found that the presence of ≥ 3 noncoding mutations alone can diagnose NM from MANP with 100% specificity and 50% sensitivity. Therefore, mutation testing with an expanded panel of frequently mutated noncoding regions may have diagnostic utility for melanocytic lesions that are difficult to classify as benign or malignant.

We recognize several limitations to our study. Ours was a small cohort and quality tissue was only available for a limited number of histologically suitable cases for molecular analysis. Follow-up was limited. Our study also used a custom amplicon NGS panel that is not commercially available to other laboratories. The average age of the MANP cases was younger than for cases of NM. This is a potential confounder when considering the number of noncoding mutations within the lesions, given the correlation between stigmata of chronic UV exposure (such as age, anatomical site, presence of solar elastosis) and noncoding promoter mutation burden.[42]

CONCLUSIONS

In our study of NMs and MANP, we showed a dichotomy of molecular findings. *NRAS* was the most common mutation in NM and was present in the majority of cases (6/8, 75%). In contrast, NRAS mutations are much less frequent in cutaneous melanomas of other types.[31,46] Noncoding mutations were largely restricted to NM, including *TERT* mutations that were present in the majority of NMs and absent in MANP. Copy number alterations were infrequent but, when present, were only seen in NM. Similar to conventional nevi, MANP cases mostly harbored driver *BRAF* mutations and activating *NRAS* mutations were rare. Noncoding mutations and copy number alterations were also rare in MANP. Accurate recognition of NM and MANP requires close attention to histologic clues that may be deceptively subtle. Our molecular findings confirm that these groups of lesions are biologically separate and, when present, noncoding mutations and copy number alterations are largely restricted to NMs. Detailed pathologic examination remains the gold-standard method for diagnosis; however, NGS analysis may have a potential ancillary role.

REFERENCES

1. McCarthy SW, Scolyer RA. Melanocytic lesions of the face: diagnostic pitfalls. *Ann Acad Med Singapore*. 2004;33:3–14.
2. Theodosiou G, Johansson I, Hamnerius N, et al. Naevoid malignant melanoma: a diagnosis of a naevus that you later regret. *Acta Derm Venereol*. 2017;97:745–746.
3. McNutt NS, Urmacher C, Hakimian J, et al. Nevoid malignant melanoma: morphologic patterns and immunohistochemical reactivity. *J Cutan Pathol*. 1995;22:502–517.
4. Kossard S, Wilkinson B. Small cell (naevoid) melanoma: a clinicopathologic study of 131 cases. *Australas J Dermatol*. 1997;38:54–58.
5. McCarthy SW, Scolyer RA. Pitfalls and important issues in the pathologic diagnosis of melanocytic tumors. *Ochsner J*. 2010;10:66–74.
6. Cook MG, Massi D, Blokx WAM, et al. New insights into naevoid melanomas: a clinicopathological reassessment. *Histopathology*. 2017;71:943–950.
7. Idriss MH, Rizwan L, Sferuzza A, et al. Nevoid melanoma: a study of 43 cases with emphasis on growth pattern. *J Am Acad Dermatol*. 2015;73:836–842.
8. Massi G, LeBoit PE. *Histological Diagnosis of Nevi and Melanoma*. Berlin, Germany: Springer-Verlag; 2014.
9. Gerami P, Wass A, Mafee M, et al. Fluorescence in situ hybridization for distinguishing nevoid melanomas from mitotically active nevi. *Am J Surg Pathol*. 2009;33:1783–1788.
10. Yelamos O, Busam KJ, Lee C, et al. Morphologic clues and utility of fluorescence in situ hybridization for the diagnosis of nevoid melanoma. *J Cutan Pathol*. 2015;42:796–806.
11. Yélamos O, Arva NC, Obregon R, et al. A comparative study of proliferative nodules and lethal melanomas in congenital nevi from children. *Am J Surg Pathol*. 2015;39:405–415.
12. Tronnier M, Wolff HH. UV-irradiated melanocytic nevi simulating melanoma in situ. *Am J Dermatopathol*. 1995;17:1–6.
13. Zampinio MR, Corazza M, Costantino D, et al. Are melanocytic nevi influenced by pregnancy? A dermoscopic evaluation. *Dermatol Surg*. 2006;32:1497–1504.
14. Grin CM, Rojas AI, Grant-Kels JM. Does pregnancy alter melanocytic nevi? *J Cutan Pathol*. 2001;28:389–392.
15. Driscoll MS, Grant-Kels JM. Nevi and melanoma in pregnancy. *Dermatol Clin*. 2006;24:199–204.
16. Driscoll MS, Grant-Kels JM. Hormones, nevi, and melanoma: an approach to the patient. *J Am Acad Dermatol*. 2007;57:919–931.
17. Geraghty LN, Pomeranz MK. Physiologic changes and dermatoses of pregnancy. *Int J Dermatol*. 2011;50:771–782.
18. Sanchez JL, Figueroa LD, Rodriguez E. Behavior of melanocytic nevi during pregnancy. *Am J Dermatopathol*. 1984;6:89–91.
19. MacKelfresh J, Chen SC, Monthrope YM. Pregnancy and changes in melanocytic nevi. *Obstet Gynecol*. 2005;106:857–860.
20. Pennoyer JW, Grin CM, Driscoll MS, et al. Changes in size of melanocytic nevi during pregnancy. *J Am Acad Dermatol*. 1997;36:378–382.
21. Nading MA, Nanney LB, Boyd AS, et al. Estrogen receptor β expression in nevi during pregnancy. *Exp Dermatol*. 2008;17:489–497.
22. Koh SS, Roehmholdt BF, Cassarino DS. Immunohistochemistry of p16 in nevi of pregnancy and nevoid melanomas. *J Cutan Pathol*. 2018;45:891–896.
23. Foucar E, Bentley TJ, Laube DW, et al. A histopathologic evaluation of nevocellular nevi in pregnancy. *Arch Dermatol*. 1985;121:350–354.
24. Chan MP, Chan MM, Tahan SR. Melanocytic nevi in pregnancy: histologic features and Ki-67 proliferation index. *J Cutan Pathol*. 2010;37:843–851.
25. Colebatch AJ, Scolyer RA. Trajectories of premalignancy during the journey from melanocyte to melanoma. *Pathology*. 2018;50:16–23.
26. Chiba K, Lorbeer FK, Shain AH, et al. Mutations in the promoter of the telomerase gene TERT contribute to tumorigenesis by a two-step mechanism. *Science*. 2017;357:1416–1420.
27. Shain AH, Yeh I, Kovalyshyn I, et al. The genetic evolution of melanoma from precursor lesions. *N Engl J Med*. 2015;373:1926–1936.
28. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33:19–20.
29. Colebatch AJ, Ferguson P, Newell F, et al. Molecular genomic profiling of melanocytic nevi. *J Invest Dermatol*. 2019;139:1762–1768.

30. Shain AH, Joseph NM, Yu R. Genomic and transcriptomic analysis reveals incremental disruption of key signaling pathways during melanoma evolution. *Cancer Cell*. 2018;34:45–55.
31. Hayward NK, Wilmott JS, Waddell N, et al. Whole-genome landscapes of major melanoma subtypes. *Nature*. 2017;545:175–180.
32. Elling SV, Powell FC. Physiological changes in the skin during pregnancy. *Clin Dermatol*. 1997;15:35–43.
33. Akturk AS, Bilen N, Bayramgurler D, et al. Dermoscopy is a suitable method for the observation of the pregnancy-related changes in melanocytic nevi. *J Eur Acad Dermatol Venereol*. 2007;21: 1086–1090.
34. Goldberg D, Maloney M. Dermatologic surgery and cosmetic procedures during pregnancy and the post-partum period. *Dermatol Ther*. 2013;26:321–330.
35. Motosko CM, Bieber AK, Pomeranz MK, et al. Physiologic changes of pregnancy: a review of the literature. *Int J Womens Dermatol*. 2017;3:219–224.
36. Bieber AK, Martires KJ, Stein JA, et al. Pigmentation and pregnancy: knowing what is normal. *Obstet Gynecol*. 2017;129: 168–173.
37. Tyler KH. Physiological skin changes during pregnancy. *Clin Obstet Gynecol*. 2015;58:119–124.
38. Bieber AK, Martires KJ, Driscoll MS, et al. Nevi and pregnancy. *J Am Acad Dermatol*. 2016;75:661–666.
39. Lazova R, Pornputtapong N, Halaban R, et al. Spitz nevi and spitzoid melanomas: exome sequencing and comparison with conventional melanocytic nevi and melanomas. *Mod Pathol*. 2017;30:640–649.
40. Roh MR, Eliades P, Gupta S, et al. Genetics of melanocytic nevi. *Pigment Cell Melanoma Res*. 2015;28:661–672.
41. Sabarinathan R, Mularoni L, Deu-Pons J, et al. Nucleotide excision repair is impaired by binding of transcription factors to DNA. *Nature*. 2016;532:264–267.
42. Colebatch AJ, Di Stefano L, Wong SQ, et al. Clustered somatic mutations are frequent in transcription factor binding motifs within proximal promoter regions in melanoma and other cutaneous malignancies. *Oncotarget*. 2016;7:66569–66585.
43. Fredriksson NJ, Elliott K, Filges S, et al. Recurrent promoter mutations in melanoma are defined by an extended context-specific mutational signature. *PLoS Genet*. 2017;13:e1006773.
44. Elliott K, Boström M, Filges S, et al. Elevated pyrimidine dimer formation at distinct genomic bases underlies promoter mutation hotspots in UV-exposed cancers. *PLoS Genet*. 2018;14:e1007849.
45. Mao P, Brown AJ, Esaki S, et al. ETS transcription factors induce a unique UV damage signature that drives recurrent mutagenesis in melanoma. *Nat Commun*. 2018;9:2626.
46. Long GV, Menzies AM, Nagrial AM, et al. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol*. 2011;29:1239–1246.

MOLECULAR TESTING FOR MELANOMA AND ITS MIMICS

CHAPTER 7: MELANOMA MIMICRY BEYOND THE SKIN

This chapter presents the clinical case example of a tumour initially thought to be primary mucosal melanoma of the bronchus, where additional investigation by our team led to recognition of an alternative diagnosis and changed the patient's course of treatment. This case is supplemented by a review of the literature on primary melanoma of the lung, the occurrence of which is rare and debated.

The literature review was published as a book chapter prior to recognition of our case, and we update contemporary knowledge in our final discussion.

Extraenteric Gastrointestinal Neuroectodermal Tumour Masquerading as Mucosal Melanoma of the Bronchus

Louise A Jackett[1,2], Catherine Mitchell[1,2], Julie Chu[3]

1. Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, Australia

2. Sir Peter MacCallum Department of Oncology, University of Melbourne, Melbourne, Australia

3. Department of Radiation Oncology, Peter MacCallum Cancer Centre, Melbourne, Australia

Address for Correspondence:

Louise A. Jackett

Department of Anatomical Pathology
Peter MacCallum Cancer Centre,
Grattan Street, Parkville, VIC, 3052, Australia
Email: louise.jackett@petermac.org

Submitted for publication: 24 February 2025

Journal: Pathology

Editor-in-chief: Clin Prof John Burnett

Publisher's reference: PAT-S-25-00128

Key words: melanoma, pathology, GNET, CREB, molecular analysis

Word count: main text 1269

Ethical statement: Patient consent was obtained to publish this case.

Conflict of interest statement: All authors do not have any conflicts of interests to declare.

Author contribution statement: LJ oversaw the pathological work-up of this case and described the immunomorphological and molecular findings in the paper, drafted the manuscript and coordinated author review; CM assisted with pathological review and review of manuscript; JC provided clinical revision of the paper. All authors have read and approved the final draft.

Funding: No sources of funding were received for this work.

Disclosures: There are no relevant disclosures.

Correspondence to *Pathology* journal

We present a 37-year-old female with a novel case of endobronchial extraenteric gastrointestinal neuroectodermal tumour (GNET) with *EWSR1*::*CREB1* fusion that mimicked melanoma. We document this case with a description of the clinical course, histopathological and immunohistochemical findings, and 6 years follow-up. We also highlight potential diagnostic pitfalls, particularly with melanoma, and briefly discuss other relevant tumours associated with *EWSR1* and *CREB* family fusions. Patient consent was obtained to publish this case.

The patient presented with several months' history of a non-productive cough shortly after moving to Australia from Sudan. Her past medical history was notable for latent and treated tuberculosis infection. The patient had no known personal or family history of malignancy and she denied use of tobacco-related products. Physical examination was unremarkable.

A positron emission tomography (PET)-computed tomography (CT) scan showed a 35 x 19 mm FDG-avid mass in the left main bronchus, without any other sites of avidity (Figure 1a). Bronchoscopy demonstrated a multilobulated polypoid tumour apparently arising from the left upper lobe bronchus and occluding the distal left main bronchus. The tumour was partly resected by laser bronchoscopic surgery.

Macroscopically, the resected tumour was received as multiple fragments of tan tissue with homogeneous firm texture, measuring 30 x 25 x 4 mm in aggregate.

Histological examination showed a highly cellular neoplasm, formed by solid nodules and sheets of predominantly plump spindled cells, merging into areas of epithelioid cells (Figure 1b-d). There was minimal fibrous stroma without any myxoid areas or a characteristic vascular pattern. The tumour cells showed mild pleomorphism and even vesicular chromatin pattern with small nucleoli. Rare mitoses were identified (1/mm^2). There were areas of acute haemorrhage but no necrosis. The tumour exclusively involved subepithelial tissues without an intraepithelial component.

Immunohistochemical stains (Figure 1e-h) showed the tumour to have diffuse and strong nuclear expression of Sox 10, and patchy moderate to strong staining with S100. There was focal moderate to strong cytoplasmic expression of synaptophysin and CD56. The following stains were negative: HMB45, Melan-A, AE1/AE3, EMA, Cam 5.2, TTF-1, chromogranin, CD99, SMA, desmin, CD31, CD34. The Ki-67 proliferation index was 10%. A diagnosis of melanoma was made.

The patient underwent work-up to consider metastatic melanoma versus a primary bronchial melanoma. The patient had no personal history of prior melanoma. On examination there were no

suspicious pigmented lesions on either acral or non-acral skin, nor at visible mucosal sites. Eye examination revealed no evidence of uveal melanoma. CT and PET imaging showed no other sites of disease. Considering the patient's skin-type (Fitzpatrick type V), she was considered at low risk for cutaneous UV driven melanoma, which made a regressed primary cutaneous melanoma unlikely. Given the discordance, the diagnosis was questioned.

Subsequently, dual-colour, break-apart rearrangement FISH probe for *EWSR1* (22q12) (Vysis, USA) evaluated on paraffin embedded tissue sections demonstrated rearrangement involving the *EWSR1* gene. Further evaluation by targeted next generation sequencing (TruSight RNA Fusion Assay, evaluating 507 genes associated with oncogenic fusions) detected a fusion involving *EWSR1* and *CREB1* (Figure 2), whilst no *BRAF*, *NRAS* or *KIT* variants were detected (Illumina TruSight Next Generation Sequencing targeted panel). The diagnosis was revised to endobronchial extraenteric gastrointestinal neuroectodermal tumour (GNET) with *EWSR1::CREB1* fusion.

Restaging PET scan four months after presentation demonstrated a residual, but stable, intensely avid left bronchial tumour, with associated left upper lobe collapse and cystic change in the left lower lobe. No nodal or distant metastases were seen.

The patient then underwent left pneumonectomy, which showed a 29 mm residual tumour with the same macroscopic and immunohistological appearance as the endobronchial biopsy. Bronchial and soft tissue hilar resections margins were close (0.5mm) but clear. The patient received adjuvant radiotherapy (50Gy/25#) followed by 3 monthly clinical surveillance.

At 5 years and 4 months after initial presentation, CT CAP demonstrated a L5 vertebral body lesion with cortical breach posteriorly and associated pathological superior endplate fracture. PET scan confirmed lucency in posterior L5 and no other potential sites of metastatic disease. L5 bone biopsy showed a cellular tumour with positive SOX10 immunostaining and *EWSR1* disruption detected by FISH, confirming metastatic tumour with the same features as the endobronchial primary tumour. The patient underwent stereotactic ablative body radiotherapy (SABR) of 20Gy/1# to the L5 metastasis and continues on 3 monthly surveillance (total 6 years 3 months follow-up).

Our case highlights the rare occurrence of extraenteric GNET in the lung and the challenging differential diagnosis with melanoma, both primary and metastatic. Since melanoma has a great propensity to metastasise to the lung, the vast majority of S100 and Sox10 positive tumours in lung are metastatic melanomas.(1) There are currently several highly effective targeted and immuno-

based systemic therapies for advanced melanoma patients (stage III and stage IV disease), and it is crucial that pathologists accurately recognise melanoma in lung tissue samples and raise the possibility of metastasis.(2) In such cases, patients require comprehensive screening for another potential primary site. Molecular testing also has an important role, not only for predicting response to targeted therapy, but also potentially for diagnosis, where the finding of certain MAPK pathway variants or a UV signature may support metastatic melanoma.(3)

Upon exclusion of metastatic melanoma, a melanoma in lung may be considered to be primary, but this remains incredibly rare.(4) The true existence of primary lung melanoma has been questioned, partly because melanocytes are not normal constituents of the bronchial mucosa (making an explanation of histogenesis difficult), and partly because cutaneous melanomas may go undetected (for example, due to regression).(5) The relatively recent recognition of tumours with *EWSR1* rearrangements may mean that the true incidence of primary lung melanoma is even lower, if occurring at all.

Collectively, tumours harbouring fusions between *EWSR1* or *FUS* and a member of the cyclic AMP response element (CRE)-binding protein (CREB) family, including *CREB1*, *CREM* and *ATF1*, are a rare and heterogeneous group of neoplasms that occur in diverse primary locations and have varied histological appearances.(6,7) In the thoracic cavity, *EWSR1*::*CREB* tumours are incredibly rare and include primary pulmonary myxoid sarcoma, mesothelioma, and the recently proposed 'malignant epithelioid neoplasm with predilection for mesothelial-lined cavities'.(8,9) In contrast to our case, all of these entities are S100 and Sox10 negative.

Another consideration among tumours with *EWSR1::CREB* family fusions is with clear cell sarcoma (CCS), which is S100 and Sox10 positive. However, our case differs from CCS (metastatic or otherwise) in the following ways: CCS cells have more abundant eosinophilic to cleared cytoplasm and prominent macronucleoli; there is often a delicate meshwork of fibrocollagenous tissue; CCS may have Touton giant cells; pagetoid spread into adjacent mucosa may be seen; and Melan-A and HMB45 are typically positive.(10)

Our case's profile of S100+, Sox10-, Melan-A-, HMB45- and *EWSR1*::*CREB1* fusion is ultimately most akin to GNET/CCSLT, which is a rare and aggressive sarcoma characterised by *EWSR1*/*FUS*::*ATF1*/*CREB1* fusions. Most commonly occurring in the small intestine of adult patients, this tumour is being increasingly recognised at extraenteric sites and in children (10). Extraenteric locations have included the soft tissues of the trunk, the retroperitoneum, orbit, oral cavity, urinary bladder and falciform ligament/liver.(10,11) To the best of our knowledge, only one case of GNET/CCSLT has ever been reported in lung, involving the bronchus of a 40 year old male.(12) While

the endobronchial location and age of that patient are similar to our case, that tumour harboured *EWSR1::ATF1* fusion, which contrasts with the *EWSR1::CREB1* fusion found in ours.

We propose this case as the first report of GNET/CCSLT of the bronchial tree with *EWSR1*::*CREB1* fusion, which broadens the spectrum of extraenteric locations at which this tumour is described. Six years of follow-up in our case contributes to a better understanding of the clinical behaviour of these rare but increasingly recognised tumours.

Acknowledgement: We thank Dr Simon Knight and colleagues in the Molecular Pathology Department at Austin Health, and colleagues from the FISH service at St Vincent's Pathology, Melbourne. We also thank Chris McEvoy and David Choong from the Department of Molecular Pathology at Peter MacCallum Cancer Centre.

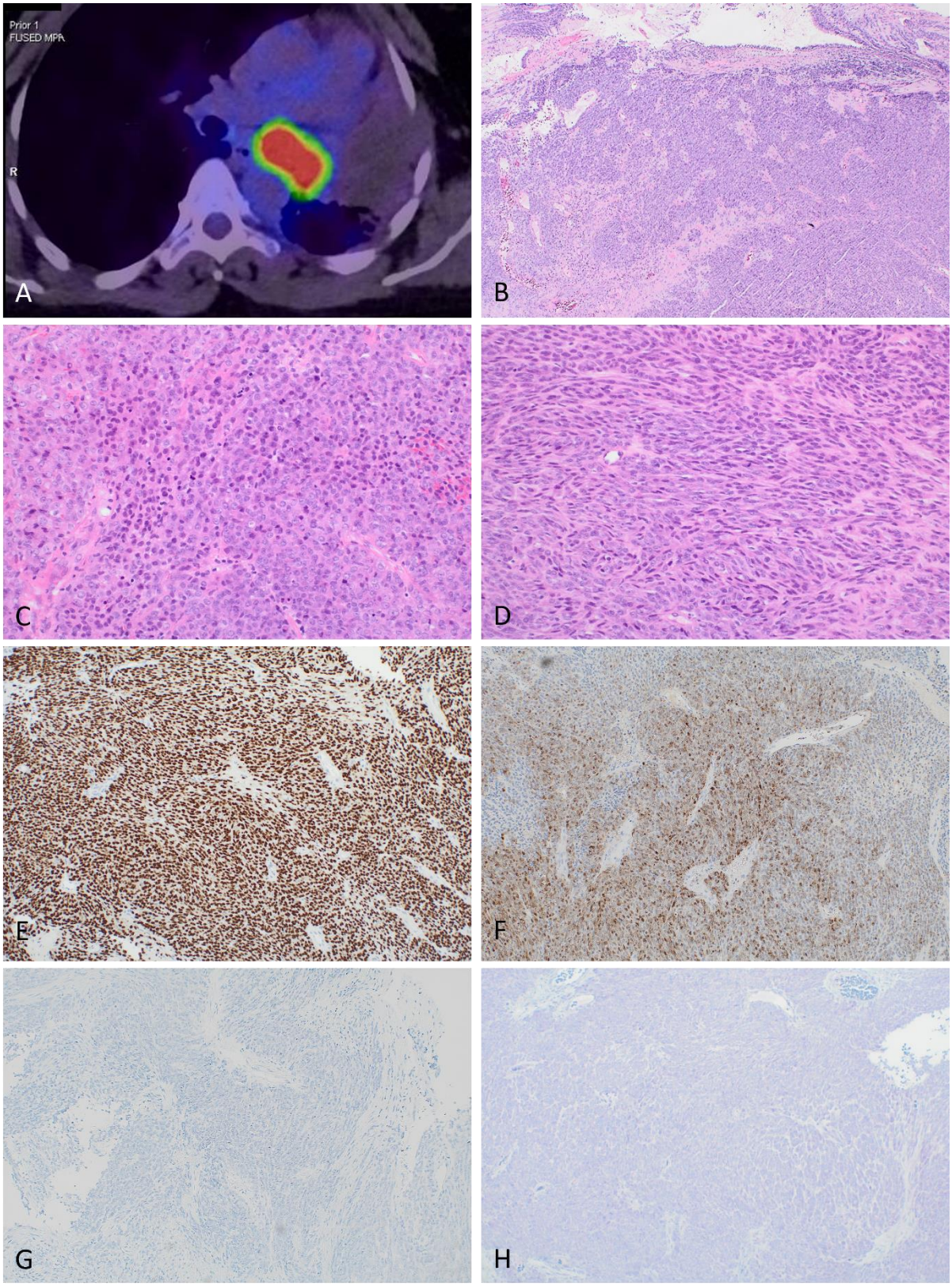

Figure 1. Extraenteric gastrointestinal neuroectodermal tumour of the bronchus with *EWSR1*::*CREB1* fusion. (A) PET showed an avid mass in left main bronchus 35x19mm. (B) The tumour was a poorly circumscribed lobulated mass occupying subepithelial tissues without an intra-epithelial component. (C) Most of the tumour cells were epithelioid with mild pleomorphism, vesicular chromatin pattern

and small nucleoli. (D) Other areas of the tumour comprised spindle cells with mild pleomorphism. The tumour showed (E) diffuse, strong SOX10 expression, (F) patchy S100 protein expression, and no staining with (G) HMB45 or (H) melanA. (A. PET-CT image, B. H&E x 40, C and D. H&E x 200, E. Sox10 x100, F. S100 x100, G. HMB45 x100, H. MelanA x100)

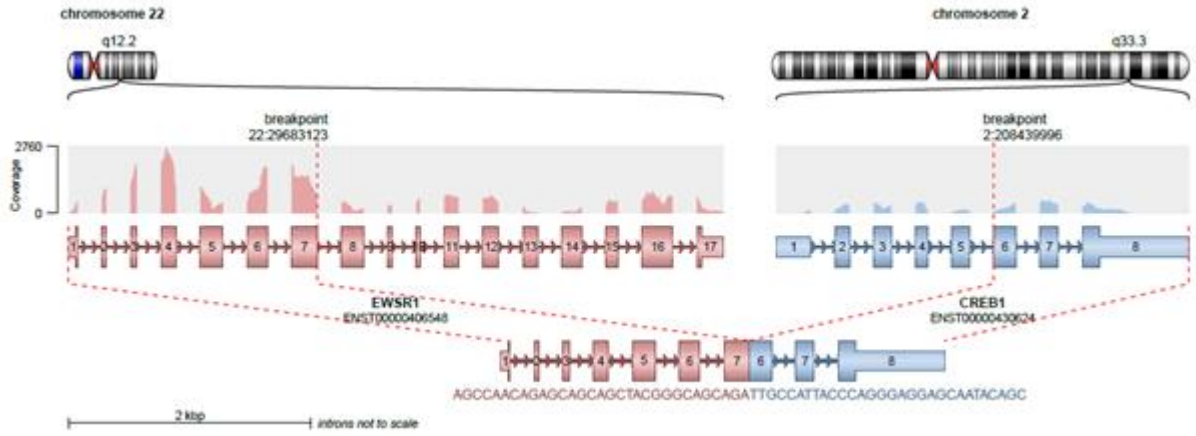

Figure 2. Targeted RNA sequencing detected an *EWSR1*(exon 7)::*CREB1* (exon 6) fusion.

References

1. Keung EZ, Gershenwald JE. The eighth edition American Joint Committee on Cancer (AJCC) melanoma staging system: implications for melanoma treatment and care. Expert Rev Anticancer Ther. 2018 Aug;18(8):775-784. doi: 10.1080/14737140.2018.1489246. PMID: 29923435; PMCID: PMC7652033.

2. Seth R, Agarwala SS, Messersmith H, Alluri KC, Ascierto PA, Atkins MB, Bollin K, Chacon M, Davis N, Faries MB, Funchain P, Gold JS, Guild S, Gyorki DE, Kaur V, Khushalani NI, Kirkwood JM, McQuade JL, Meyers MO, Provenzano A, Robert C, Santinami M, Sehdev A, Sondak VK, Spurrier G, Swami U, Truong TG, Tsai KK, van Akkooi A, Weber J. Systemic Therapy for Melanoma: ASCO Guideline Update. J Clin Oncol. 2023 Oct 20;41(30):4794-4820. doi: 10.1200/JCO.23.01136. Epub 2023 Aug 14. PMID: 37579248.

3. Agaimy A., Specht K., Stoehr R.,Lorey T., Märkl B., Niedobitek G., Straub M., Hager T., Reis A.C.,Schilling B., et al. Metastatic Malignant Melanoma With Complete Loss of Differentiation Markers(Undifferentiated/Dedifferentiated Melanoma): Analysis of 14 Patients Emphasizing Phenotypic Plasticity and the Value of Molecular Testing as Surrogate Diagnostic Marker.Am. J. Surg.Pathol.2016;40:181–191. doi:10.1097/PAS.0000000000000527

4. de Wilt JH, Farmer SE, Scolyer RA, McCaughan BC, Thompson JF. Isolated melanoma in the lung where there is no known primary site: metastatic disease or primary lung tumour? Melanoma Res. 2005 Dec;15(6):531-7. doi: 10.1097/00008390-200512000-00008. PMID: 16314739.

5. Del Fiore P, Rastrelli M, Dall'Olmo L, Cavallin F, Cappellesso R, Vecchiato A, Buja A, Spina R, Parisi A, Mazzarotto R, Ferrazzi B, Grego A, Rotondi A, Benna C, Tropea S, Russano F, Filoni A, Bassetto F, Tos APD, Alaibac M, Rossi CR, Pigozzo J, Sileni VC, Mocellin S. Melanoma of Unknown Primary: Evaluation of the Characteristics, Treatment Strategies, Prognostic Factors in a Monocentric Retrospective Study. Front Oncol. 2021 Mar 5;11:627527. doi: 10.3389/fonc.2021.627527. Erratum in: Front Oncol. 2021 Apr 14;11:686051. doi: 10.3389/fonc.2021.686051. PMID: 33747946; PMCID: PMC7977284.

6. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol. 2001 Aug;2(8):599-609. doi: 10.1038/35085068. PMID: 11483993.

7. Dermawan, J.K., Vanoli, F., Herviou, L. *et al.* Comprehensive genomic profiling of *EWSR1/FUS::CREB* translocation-associated tumors uncovers prognostically significant recurrent genetic alterations and methylation-transcriptional correlates. *Mod Pathol* 35, 1055–1065 (2022). https://doi.org/10.1038/s41379-022-01023-9

8. Thway K, Nicholson AG, Lawson K, Gonzalez D, Rice A, Balzer B, Swansbury J, Min T, Thompson L, Adu-Poku K, Campbell A, Fisher C. Primary pulmonary myxoid sarcoma with EWSR1-CREB1 fusion: a new tumor entity. Am J Surg Pathol. 2011 Nov;35(11):1722-32. doi: 10.1097/PAS.0b013e318227e4d2. PMID: 21997693.

9. Argani P, Harvey I, Nielsen GP, Takano A, Suurmeijer AJH, Voltaggio L, Zhang L, Sung YS, Stenzinger A, Mechtersheimer G, Dickson BC, Antonescu CR. EWSR1/FUS-CREB fusions define a distinctive malignant epithelioid neoplasm with predilection for mesothelial-lined cavities. Mod Pathol. 2020 Nov;33(11):2233-2243. doi: 10.1038/s41379-020-0646-5. Epub 2020 Aug 7. PMID: 32770123; PMCID: PMC7584759.

10. Ulici V, Hornick JL, Davis JL, Mehrotra S, Meis JM, Halling KC, Fletcher CDM, Kao E, Folpe AL. Extraenteric Malignant Gastrointestinal Neuroectodermal Tumor-A Clinicopathologic and Molecular Genetic Study of 11 Cases. Mod Pathol. 2023 Jul;36(7):100160. doi: 10.1016/j.modpat.2023.100160. Epub 2023 Mar 17. PMID: 36934861.

11. Allanson BM, Weber MA, Jackett LA, Chan C, Lau L, Ziegler DS, Warby M, Mayoh C, Cowley MJ, Tucker KM, Long GV, Maher A, Anazodo A, Scolyer RA. Oral malignant gastrointestinal neuroectodermal tumour with junctional component mimicking mucosal melanoma. Pathology. 2018 Oct;50(6):648-653. doi: 10.1016/j.pathol.2018.07.002. Epub 2018 Aug 31. PMID: 30177220.

12. Zheng Q, Chen H, Li Y. Primary Gastrointestinal-Type Clear Cell Sarcoma-like Tumor of the Bronchus: A Hitherto Unreported Bronchial Tumor. J Thorac Oncol. 2019 Sep;14(9):e202-e205. doi: 10.1016/j.jtho.2019.04.030. PMID: 31445737.

Section 4: Thoracic Tumors

17 Primary Melanoma of the Lung

Louise A. Jackett,[1,2,3] Richard A. Scolyer,[1,2,3] James F. Bishop,[4,5] and John F. Thompson[1,2,3]

[1] Melanoma Institute Australia, Sydney, New South Wales, Australia
[2] Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia
[3] Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia
[4] Victorian Comprehensive Cancer Centre, Parkville, Victoria, Australia
[5] University of Melbourne, Melbourne, Victoria, Australia

Introduction

Although most primary melanomas develop as cutaneous or ocular tumors and a few develop in recognized mucosal sites, the very occasional occurrence of primary melanomas in unusual noncutaneous sites, including the lung, is well documented.[1] Primary melanoma of the lung is undoubtedly a very rare tumor but its precise incidence is the subject of continuing debate. Some have even questioned whether the condition actually exists. The difficulty arises because an unrecognized primary cutaneous melanoma can occasionally undergo complete regression and disappear without trace, while a lung metastasis which has arisen from it continues to grow and may eventually present as an apparently isolated focus of melanoma.[2, 3] Melanocytes are not normally found in the tracheobronchial tree and consequently it is also difficult to explain the histogenesis of a primary lung melanoma. In patients who do have a history of primary cutaneous melanoma and who subsequently present with metastatic disease, the lungs are the only clinically detectable site of metastasis in 7–9% of patients.[4] Melanoma has a predilection to metastasize to the lungs,[5, 6] and approximately 70% of patients who die of melanoma are found to have pulmonary metastases at autopsy.[7, 8]

The literature contains numerous reports of patients with melanomas that seem to be of primary lung origin. In many of these patients, their history and subsequent follow-up appear to exclude the possibility of metastasis to the lung from a primary site elsewhere.[7, 9–51] Supporting evidence for a diagnosis of primary lung melanoma may be obtained from histopathological features of the tumor, from a pattern of subsequent involvement of regional lymph nodes which is consistent with that of other tumors of primary bronchial origin, and from a failure to find a primary melanoma elsewhere in the body either during life or at the time of autopsy.

The occurrence of primary melanomas in other noncutaneous sites has long been recognized[1, 12, 52] and is much more comprehensively documented in the literature. Ocular and conjunctival melanomas are the most common, but noncutaneous melanomas are also well recognized to arise in the urethra, vulva, vagina, leptomeninges, adrenal gland, nasopharynx, oropharynx, esophagus, gall bladder, and occasionally in other parts of the gastrointestinal tract including the stomach, small bowel, large bowel, and anal canal.[53] An important difference, however, is that melanocytes have been identified in most of these sites in normal individuals, providing a plausible histogenetic basis for the origin of the melanomas.

Historical background

Although there have been numerous case reports claiming a diagnosis of primary lung melanoma, the patients described in many of the early publications did not fulfill the criteria subsequently accepted as being necessary to definitively establish the diagnosis. The first two recorded cases, reported by Todd in 1888,[9] were certainly based on insufficient evidence to warrant the diagnosis. Similarly, other early reports by Kunkel and Torrey in 1916[10] and by Carlucci and Schleussner in 1942[11] were deficient because full autopsies were not performed to exclude the possibility of a primary melanoma elsewhere. It was not until 1963 that a patient fulfilling subsequently accepted criteria for the diagnosis of primary lung melanoma was reported.[7] In a 1987 publication, Alghanem *et al.* considered it appropriate to accept only seven previously reported cases as true primary lung melanomas, on the basis of stringent criteria for diagnosis.[31] Reporting a series of eight new cases in 1997, Wilson and Moran suggested that there were fewer than 25 previous cases acceptable as primary pulmonary

Textbook of Uncommon Cancer, Fifth Edition. Edited by Derek Raghavan, Manmeet S. Ahluwalia, Charles D. Blanke, Jubilee Brown, Edward S. Kim, Gregory H. Reaman, and Mikkael A. Sekeres.

Table 17.1 Published reports of 75 patients with primary melanomas of the trachea, bronchi, lungs, and pleura fulfilling most or all of the currently accepted diagnostic criteria.

References	Sex/age (years)	Site
Salm[7]	M/45	LL
Reed and Kent[14]	M/71	LL
Reid and Mehta[15]	F/60	Trachea
	M/35	LL
Jensen and Egedorf[16]	F/61	UL
Allen and Drash[17]	F/40	LL
Taboada *et al.*[18]	M/56	LL
	M/40	UL
Walter *et al.*[19]	M/33	LL
Mori *et al.*[20]	F/47	Trachea
Smith and Opipari[21]	M/49	Pleura
Adebonojo *et al.*[22]	F/55	UL
Robertson *et al.*[23]	F/70	Carina
Gephardt[25]	M/47	MSB
Verweij *et al.*[26]	M/46	Trachea, MSB
Angel and Prados[28]	F/41	ML
Cagle *et al.*[29]	M/80	ML
Carstens *et al.*[30]	F/29	UL
Alghanem *et al.*[31]	F/42	LL
Demeter *et al.*[32]	M/56	UL
Santos *et al.*[33]	M/58	LL
Bagwell *et al.*[35]	M/62	UL
Bertola *et al.*[36]	F/30	LL
Jennings *et al.*[38]	F/34	UK
Sanchez Navarro *et al.*[39]	M/75	MSB
Miller and Allen[40]	M/56	LL
	M/67	ML
	F/77	LL
Barzó *et al.*[41]	F/43	UL
	F/81	LL
Pasquini *et al.*[42]	F/66	LL
Farrell *et al.*[43]	F/66	LL
Wilson and Moran[46]	M/71	LL
	M/45	UL
	M/UK	ML
	F/55	UL
	M/52	UL
	M/64	UL
	M/48	UL
	M/50	UL
Sekine *et al.*[45]	UK	UK
Ost *et al.*[47]	M/90	UL
Ozdemir *et al.*[48]	M/41	LL
Testini *et al.*[49]	M/44	LL
Dountsis *et al.*[50]	F/41	UL
Lie *et al.*[51]	F/44	LL
de Wilt *et al.*[44]	M/71	ML
	F/49	LL
	M/61	LL
	M/62	LL
	M/66	LL
	M/60	LL
	F/49	UL
Kundranda *et al.*[97]	M/60	UK
Reddy *et al.*[98]	M/74	LL
Kotoulas *et al.*[99]	M/67	UK
Saint-Blancard *et al.*[100]	M/82	UL
Shikuma *et al.*[101]	M/71	LL
Maeda *et al.*[102]	M/68	UL
Pan *et al.*[103]	M/81	LL
Neri *et al.*[104]	M/58	LL
Mochizuki *et al.*[105]	M/84	LL
Seitelman *et al.*[106]	M/89	LL
Zuckermann *et al.*[107]	M/68	UL
Gong *et al.*[108]	F/52	UL, LL
	F/65	LL
Ouarssani *et al.*[109]	M/68	Bilateral LL
Kamaleshwaran *et al.*[110]	M/56	UK
Gupta *et al.*[111]	F/58	UL
Hwang *et al.*[112]	F/82	LL
Mahowald *et al.*[113]	M/55	UL
Postrzech-Adamczyk *et al.*[114]	F/69	UL
Watanabe *et al.*[115]	M/66	ML
	F/46	LL
Zhang *et al.*[116]	M/60	LL

F, female; LL, lower lobe; M, male; ML, middle lobe; MSB, main stem bronchus; UK, unknown; UL, upper lobe.

melanomas.[46] As far as could be determined from a comprehensive review of the literature by the present authors, the total number of reported cases for which the diagnosis had been established by appropriate criteria stood at 75 in 2016 (Table 17.1).

Biology

A histogenetic basis for the development of primary lung melanomas is not obvious, but several explanations have been proposed. Although melanocytes have not been demonstrated in the lower respiratory tract, some investigators hypothesize that there can be aberrant migration of these potential precursor cells to the lung, and suggest that this could explain the development of primary melanomas at this site.[16] This seems a plausible explanation because embryologically the respiratory tract develops from an outgrowth of the primitive foregut between the pharynx and esophagus, in the region of the larynx, and melanocytes have been identified in the mucosa of these sites.[54–56]

Because squamous metaplasia is occasionally observed in melanoma-affected epithelium, a proposed alternative explanation is that epithelial cells undergo metaplastic transformation into melanocytes.[14] However, it appears more likely that the squamous metaplasia is a consequence of bronchial involvement by melanoma. A condition described as "melanogenic metaplasia" of mucous glands in the oral cavity has also been reported,[57] and it is possible that a similar process might occur in the mucous glands of the tracheobronchial tree. Another proposal, perhaps more plausible, is that neuroendocrine (Kulchitsky) precursor cells have the potential to undergo melanocytic differentiation; both cell types are histogenetically related, being of neural crest origin.[38] This theory is supported by the occasional occurrence of melanocytic differentiation in carcinoid tumors[58] and reports of malignant tumors displaying neuroendocrine and melanocytic differentiation.[59,60] A similar theory has been proposed to explain the origin of

primary adrenal melanomas,[30, 61–63] since melanocytes have not been identified in this location either.

The occurrence of melanocytic differentiation within peripheral nerve sheath tumors, such as melanotic schwannomas (termed malignant melanotic schwannian tumors),[64] raises the possibility that primary pulmonary melanomas may also arise from native pulmonary neural elements.

Epidemiology

In a review from Melanoma Institute Australia (formerly the Sydney Melanoma Unit) in Sydney, Australia, de Wilt and colleagues identified 27 patients who presented with pulmonary melanoma as their first sign of disease and in whom no foci of melanoma at other sites were found.[44] This was from a total of 19,000 patients with melanoma treated over a 50-year period. Following detailed analysis of clinical, pathological, and follow-up data, the authors concluded that seven of these patients were likely to have had primary pulmonary melanomas. Reviewing 10,134 patients with primary lung tumors treated at the Mayo Clinic over a 10-year period, Miller and Allen[40] reported three patients with primary lung melanomas. In a report from Japan published in 1998, Sekine *et al.* identified one case of primary pulmonary melanoma from 3481 primary lung tumors resected over a 20-year period.[45] Each of these reports emphasized the rarity of primary lung melanoma, but cannot be used to quantify its incidence since the patients seen at each of the institutions were referred for treatment and the data were therefore not population based.

Molecular biology and genetics

During the past decade, critical molecular alterations in melanomas have been identified that are important for its development and progression. Mutations in genes coding for proteins in the mitogen-activated protein kinase (MAPK) signal transduction pathway have been found in about 70% of melanomas. This pathway involves signaling through RAS-RAF-MEK-ERK and controls cellular proliferation, apoptosis, and migration. Mutant BRAF, one of three types of RAF proteins, is present in approximately 40% of cutaneous melanomas, and mutant N-RAS occurs in approximately 25% of cases.[65] Mutant N-RAS also induces the phosphatidylinositol 3′ kinase (PI3K) cascade, which is another important pathway for control of cellular proliferation, apoptosis, and invasion. BRAF mutant primary melanoma typically occurs in younger individuals at axial sites where there is a low degree of chronic sun damage. In contrast, N-RAS mutant melanoma shows no age or anatomical site predilection. Activating mutations or gene amplification of the tyrosine kinase receptor C-KIT are present in 10–15% of acral and mucosal melanomas and occasionally in melanomas occurring at chronically sun-damaged anatomical sites. Uveal melanomas typically show mutations in *GNAQ* or *GNA11*. Many other less common mutation events implicated in the pathogenesis of melanoma have also been identified including those effecting

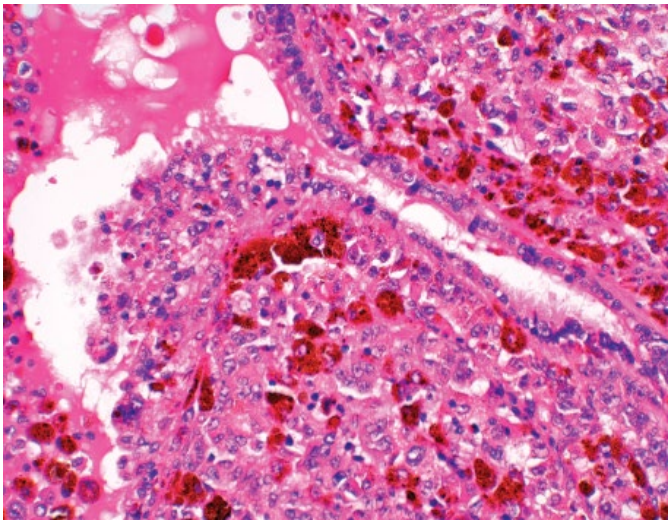

Figure 17.1 Primary pulmonary melanoma occurring in a male aged 61 years. Pleomorphic epithelioid melanoma cells are present, some of which are pigmented, beneath bronchial respiratory-type mucosa. There is focal mucosal surface erosion by the tumor (H&E, original magnification 100×).

PIK3CA (*PI3K*), *FLT3*, *PDGFR*, *MET*, and *ERBB4*. However, the molecular pathogenesis of a significant proportion of cutaneous melanomas remains unknown and, to the best of our knowledge, the mutation profile of primary melanoma of the lung has not been reported to date. Because mutations in *BRAF* and *C-KIT* are now being successfully exploited by new targeted therapies, as discussed in more detail below, it would appear appropriate to perform relevant mutation testing of primary pulmonary melanoma in patients in whom systemic treatment is being considered.

Pathology

In 1968, Allen and Drash suggested that the surgical pathologist must always consider the possibility of melanoma when examining a lung tumor if the diagnosis is not to be missed.[17] More than 40 years later this advice may still be appropriate. As melanoma remains "the great imitator" and unless the possibility is considered, primary lung melanoma may be misdiagnosed as a large cell carcinoma of the lung or as a sarcoma of the lung. Having considered the possibility of a melanoma, confirmation or exclusion of this possibility should then be achievable by careful examination for the classic features of melanoma, with support from immunohistochemistry.

A number of histological features (Fig. 17.1) have been suggested as being indicative of primary lung melanoma,[17, 28, 36] including:

- obvious melanoma cells, confirmed by immunohistochemical staining for S-100 and HMB-45 and possibly electron microscopy
- evidence of atypical melanocytes involving the bronchial epithelium
- "nesting" of melanocytic cells beneath the bronchial epithelium

• "pagetoid" invasion of the intact (i.e. nonulcerated) bronchial epithelium by melanoma cells.

However, these features were not demonstrated in many of the previously reported cases of primary pulmonary melanoma[46] and, furthermore, are probably not as specific as has been suggested. "*In situ* melanoma" changes may be absent in primary melanomas occurring at other sites, particularly ulcerated primary cutaneous melanomas. In addition, similar changes are sometimes observed in epidermotropic melanomas metastasizing to the skin,[66] and an intraepithelial growth of melanoma metastatic to the lung has been documented previously.[67] Indeed, the results of one recent series of 15 patients suggested that distinguishing primary from metastatic melanoma is best performed on the basis of clinical behavior, particularly the pattern of metastatic spread, rather than on histopathological criteria.[44]

Clinical aspects

Presentation

The cases of lung melanoma reported in the literature are divided approximately equally into those that presented as a polypoid obstructing lesion within the tracheobronchial tree and those that presented as a mass within the lung parenchyma. The tumor is almost always unifocal, but multifocal primary lung melanomas have been described.[26, 61] Primary pleural melanoma has also been reported.[21]

As expected, the presenting symptoms of a patient with a primary lung melanoma are determined by the site and size of the tumor. Initial recognition of an abnormality is sometimes made on a chest x-ray performed for an unrelated reason.[18, 36] A definitive histological diagnosis of melanoma can usually be obtained for endobronchial lesions by bronchoscopy and biopsy, and for lesions in the peripheral lung parenchyma by fine needle aspiration or core biopsy. However, a diagnosis of primary (rather than metastatic) lung melanoma is unlikely to be made at this time, and may not be reached until after full staging investigations, pathological examination of the resected tumor, an appropriate period of follow-up, and possibly at autopsy.

Patterns of metastasis

Primary melanoma of the lung metastasizes in a pattern consistent with that of other primary lung tumors – indeed, this is one of the points of evidence raised in support of the existence of lung melanoma as an entity. Thus involvement of regional lymph nodes in the lung hilum and mediastinum is likely to be observed. As with primary cutaneous melanomas, there is the additional possibility of systemic dissemination via the bloodstream to such sites as the brain, liver, adrenal gland, and so on. Metastatic disease involving the pleura, pericardium, and heart also occurs.[35, 36]

Criteria for diagnosis

To define cases of primary lung melanomas with greater certainty, minimal criteria on which the diagnosis should be based have been proposed. The first such proposal was by Jensen and Egedorf in 1967,[16] who suggested that a diagnosis of primary lung melanoma should be made in the following circumstances:

• no history suggestive of a previous melanoma (cutaneous or ocular)
• no demonstrable melanoma in any other organ at the time of operation
• a solitary tumor in the surgical specimen from the lung
• tumor morphology compatible with a primary tumor
• no evidence at autopsy of a primary melanoma elsewhere.

Other authors have since endorsed these criteria, with minor variations and additions.[17, 26, 31, 43, 68] As well, however, detailed staging investigations and prolonged follow-up are probably necessary before accepting a definitive diagnosis of primary pulmonary melanoma because, as recently reported by de Wilt *et al.*,[44] some patients presenting with apparently isolated pulmonary melanoma may subsequently develop cutaneous or nodal recurrences suggesting that the tumor originated from a regressed primary cutaneous melanoma. Although the criteria proposed by Jensen and Egedorf are undoubtedly appropriate and desirable, it is nevertheless clear that there are instances in which they cannot all be satisfied yet in which the likelihood is high that a lung tumor is a primary melanoma.[38]

Treatment

Surgery

Based on experience with primary melanomas arising in other sites, the treatment of choice for primary lung melanoma is radical surgical excision. This will usually involve formal lobectomy or pneumonectomy. For primary cutaneous melanoma, elective regional lymph node dissection is no longer performed having been superseded by sentinel lymph node biopsy. Now, in patients with clinical localized primary cutaneous melanoma, regional lymph node clearance is generally only performed in patients with a positive sentinel lymph node. Lymphatic mapping and sentinel lymphadenectomy have been performed in patients with primary and secondary lung tumors, including melanomas, and may provide more accurate pathological staging.[69] The major difficulty with this theoretically attractive approach is that the diagnosis of primary rather than secondary lung melanoma may not be established with reasonable certainty until after detailed pathological examination of the resected tumor. Furthermore, there is little possibility of performing satisfactory delayed regional lymph node clearance in the lung hilum and mediastinum following lobectomy and pneumonectomy. It therefore seems logical to clear these nodes at the time of the initial definitive lung surgery whenever possible.

In the absence of any evidence to indicate otherwise, the treatment of locally recurrent melanoma from a lung

primary must be based on the standard treatment principles established for other forms of melanoma. If radical surgical excision is possible, it provides the best form of palliation, and may even achieve cure. If whole-body imaging by computed tomography (CT) scanning, or more reliably by positron emission tomography (PET) scanning with ^{18}F-fluorodeoxyglucose,[62] does not reveal any evidence of metastatic disease elsewhere, radical surgery is certainly indicated. Because melanoma tissue almost always has a very high glucose uptake, whole-body PET/CT scans [62, 63, 70–75] have largely replaced CT and magnetic resonance imaging (MRI) scans as the staging investigation of choice in most major melanoma treatment centers. PET/CT is therefore likely to assist in determining whether a focus of melanoma in the lung is a primary or a secondary tumor. If a diagnosis of primary lung melanoma is confirmed, PET/CT scanning should also demonstrate whether distant metastasis has occurred and, if it has, should prevent inappropriate surgery as treatment for primary lung melanoma from being performed.

Radiotherapy and systemic therapies (including targeted therapies and immunotherapy)

If surgical clearance of locally recurrent disease following resection of a primary lung melanoma is not feasible, systemic therapy may be considered, or radiotherapy if the tumor mass in the chest is causing troublesome symptoms. Foci of metastatic disease outside the chest must similarly be treated on their merits, according to general principles for the treatment of metastatic melanoma, since there are no data to indicate that any different form of treatment is likely to be more effective for metastases from primary lung melanomas. Recently, the identification of oncogenic mutations in melanoma and both the rapid development and application of active targeted therapies and immunotherapies is revolutionizing the treatment of patients with metastatic melanoma.[76–78] It is likely that these therapies will be as effective in metastatic pulmonary melanoma as they are in melanomas arising in other locations.

Highly potent inhibitors of oncogenic mutated BRAF (usually administered in combination with an inhibitor of the downstream protein MEK) and C-KIT proteins have shown remarkable clinical efficacy in the treatment of metastatic melanoma patients.[79–81] Clinical trials of the orally administered inhibitors of V600 mutant BRAF showed shrinkage of tumor in the majority of BRAF mutant metastatic melanoma patients.[79, 81, 82] Furthermore, BRAF inhibitors have demonstrated a clear survival benefit over standard chemotherapy with dacarbazine in advanced stage metastatic melanoma patients.[81] However, drug resistance is a limiting factor and all but a few patients relapse, resulting in a progression-free survival of only 5–7 months. Most of the documented toxicities of BRAF inhibitors in humans appear mild and well tolerated and are less frequent (apart from fevers) when an inhibitor of MEK – another protein in the MAPK signaling pathway that is downstream of BRAF – is administered in combination with a BRAF inhibitor. Similarly, patients with C-KIT mutant metastatic melanoma have been treated with C-KIT inhibitors with some clinical responses.[83, 84]

Systemic treatment may also be provided by immunotherapies that target immune check points. Ipilimumab is a potent targeted T cell antibody directed against the cytotoxic T lymphocyte antigen 4 (CTLA-4) and is administered intravenously. Activation of CTLA-4 normally suppresses immune responses and hence CTLA-4 blockage by ipilimumab enhances the tumoral immunoactivity. Ipilimumab was shown to extend survival in clinical trials in patients with advanced stage melanoma, with a 2-year survival of over 30%.[85, 86] Serious immune-related systemic adverse reactions, some life threatening, occur in up to 15% of patients receiving ipilimumab and require specialized multidisciplinary care.[85, 86] Pembrolizumab and nivolumab disrupt the melanoma PD-L1/cytotoxic T cell PD-1 signaling axis and thereby obstruct one mechanism by which a tumor may suppress cytotoxic T cell activity. These agents have generated durable clinical responses in approximately 30–40% of patients with advanced stage melanoma and improved survival.[87]

Prognosis

The very limited information that can be gleaned from published reports of patients with primary lung melanomas indicates that the prognosis is generally very poor, with the principal determinant of outcome being the presence or absence of local (peribronchial and hilar) lymph node metastases. However, in one series of 15 patients who presented with isolated pulmonary melanoma with no known primary tumor, the overall actuarial survival was 42%.[44] This is remarkably high when compared with other studies reporting resection of pulmonary metastatic melanomas in which actuarial 5-year survival rates of about 20% were observed.[88–90] In addition, a number of long-term survivors after treatment of apparently primary pulmonary melanoma by radical surgery have been reported.[14, 15, 18, 44] Long-term survival after treatment of primary lung melanoma by chemotherapy, immunotherapy, or radiotherapy alone, however, has not been documented.

It is possible that some tumors diagnosed as primary lung melanomas may actually be metastases from totally regressed primary cutaneous melanomas. Thus the results of treating foci of metastatic melanoma from an occult primary site warrant review. Several recent large studies have suggested that the survival outcome for patients with metastatic disease from an occult primary tumor is somewhat more favorable compared with patients with known sites of primary disease.[91–96]

Recommendations

In patients presenting with apparently isolated pulmonary melanoma, if no other focus of primary or secondary melanoma in the body can be demonstrated by the best available imaging techniques, radical surgical treatment for

a suspected primary lung melanoma is entirely logical. Such treatment offers the patient the best chance of cure, even if the melanoma is subsequently shown to be a deposit of metastatic melanoma. The treatment will usually involve formal lobectomy or pneumonectomy and hilar and mediastinal lymphadenectomy. The treatment of locally recurrent and metastatic melanoma from a lung primary should be based on the standard treatment principles established for other forms of melanoma.

References

1. Dasgupta TK, Brasfield RD, Paglia MA. Primary melanomas in unusual sites. *Surg Gynecol Obstet* 1969;**128**:841–8.
2. Smith JL Jr, Stehlin JS Jr. Spontaneous regression of primary malignant melanomas with regional metastases. *Cancer* 1965;**18**:1399–415.
3. Milton GW, Lane Brown MM, Gilder M. Malignant melanoma with an occult primary lesion. *Br J Surg* 1967;**54**:651–8.
4. Gromet MA, Ominsky SH, Epstein WL, *et al.* The thorax as the initial site for systemic relapse in malignant melanoma: a prospective survey of 324 patients. *Cancer* 1979;**44**:776–84.
5. Sutton FD Jr, Vestal RE, Creagh CE. Varied presentations of metastatic pulmonary melanoma. *Chest* 1974;**65**:415–19.
6. Webb WR, Gamsu G. Thoracic metastasis in malignant melanoma. A radiographic survey of 65 patients. *Chest* 1977;**71**:176–81.
7. Salm R. A primary malignant melanoma of the bronchus. *J Pathol Bacteriol* 1963;**85**:121–6.
8. Dasgupta T, Brasfield R. Metastatic melanoma. A clinicopathological study. *Cancer* 1964;**17**:1323–39.
9. Todd FW. Two cases of melanotic tumors in the lungs. *J Am Med Assoc* 1888;**11**:53–4.
10. Kunkel OF, Torrey E. Report of a case of primary melanotic sarcoma of the lung presenting difficulties in differentiating from tuberculosis. *NY State J Med* 1916;**16**:198–201.
11. Carlucci GA, Schleussner RC. Primary melanoma of the lung: case report. *J Thorac Surg* 1942;**11**:643–9.
12. Allen AC, Spitz S. Malignant melanoma; a clinicopathological analysis of the criteria for diagnosis and prognosis. *Cancer* 1953;**6**:1–45.
13. Hsu CW, Wu SC, Ch'En CS. Melanoma of lung. *Chin Med J* 1962;**81**:263–6.
14. Reed RJ, Kent EM. Solitary pulmonary melanomas: two case reports. *J Thorac Cardiovasc Surg* 1964;**48**:226–31.
15. Reid JD, Mehta VT. Melanoma of the lower respiratory tract. *Cancer* 1966;**19**:627–31.
16. Jensen OA, Egedorf J. Primary malignant melanoma of the lung. *Scand J Respir Dis* 1967;**48**:127–35.
17. Allen MS Jr, Drash EC. Primary melanoma of the lung. *Cancer* 1968;**21**:154–9.
18. Taboada CF, McMurray JD, Jordan RA, *et al.* Primary melanoma of the lung. *Chest* 1972;**62**:629–31.
19. Walter P, Fernandes C, Florange W. Melanome malin primitif pulmonaire. *Ann Anat Pathol (Paris)* 1972;**17**:91–9.
20. Mori K, Cho H, Som M. Primary "flat" melanoma of the trachea. *J Pathol* 1977;**121**:101–5.
21. Smith S, Opipari MI. Primary pleural melanoma. A first reported case and literature reivew. *J Thorac Cardiovasc Surg* 1978;**75**:827–31.
22. Adebonojo SA, Grillo IA, Durodola JI. Primary malignant melanoma of the bronchus. *J Natl Med Assoc* 1979;**71**:579–81.
23. Robertson AJ, Sinclair DJ, Sutton PP, *et al.* Primary melanocarcinoma of the lower respiratory tract. *Thorax* 1980;**35**:158–9.
24. Weshler Z, Sulkes A, Kopolovitch J, *et al*. Bronchial malignant melanoma. *J Surg Oncol* 1980;**15**:243–8.
25. Gephardt GN. Malignant melanoma of the bronchus. *Hum Pathol* 1981;**12**:671–3.
26. Verweij J, Breed WP, Jansveld CA. Primary tracheo-bronchial melanoma. *Neth J Med* 1982;**25**:163–6.
27. de Gracia Roldan J, Vidal Pla R, Capdevila Morell J. Nodulo puylmonar soitario en el curso de melanoma maligno. A proposito de cuatro observaciones. *Arch Bronchoneumol* 1983;**19**:128–31.
28. Angel R, Prados M. Primary bronchial melanoma. *J La State Med Soc* 1984;**136**:13–15.
29. Cagle P, Mace ML, Judge DM, *et al.* Pulmonary melanoma. *Primary vs metastatic. Chest* 1984;**85**:125–6.
30. Carstens PH, Kuhns JG, Ghazi C. Primary malignant melanomas of the lung and adrenal. *Hum Pathol* 1984;**15**:910–14.
31. Alghanem AA, Mehan J, Hassan AA. Primary malignant melanoma of the lung. *J Surg Oncol* 1987;**34**:109–12.
32. Demeter SL, Fuenning C, Miller JB. Primary malignant melanoma of the lower respiratory tract: endoscopic identification. *Cleve Clin J Med* 1987;**54**:305–8.
33. Santos F, Entrenas LM, Sebastian F, *et al.* Primary bronchopulmonary malignant melanoma. Case report. *Scand J Thorac Cardiovasc Surg* 1987;**21**:187–9.
34. Andre N, Leroyer C, Proust A, *et al.* [Malignant endobronchial melanoma, apparently primary. Apropos of a case]. *Rev Pneumol Clin* 1988;**44**:143–5.
35. Bagwell SP, Flynn SD, Cox PM, *et al.* Primary malignant melanoma of the lung. *Am Rev Respir Dis* 1989;**139**:1543–7.
36. Bertola G, Pasquotti B, Morassut S, *et al.* Primary lung melanoma. *Ital J Surg Sci* 1989;**19**:187–9.
37. Cohen RE, Weaver MG, Montenegro HD, *et al.* Pulmonary blastoma with malignant melanoma component. *Arch Pathol Lab Med* 1990;**114**:1076–8.
38. Jennings TA, Axiotis CA, Kress Y, *et al.* Primary malignant melanoma of the lower respiratory tract. Report of a case and literature review. *Am J Clin Pathol* 1990;**94**:649–55.
39. Sanchez Navarro JJ, Galindo Jimeno M, Perez Sicilia M, *et al.* Melanoma maligno primitivo de pulmon. *Rev Clin Esp* 1991;**188**:68–9.
40. Miller DL, Allen MS. Rare pulmonary neoplasms. *Mayo Clin Proc* 1993;**68**:492–8.
41. Barzó P, Tuka P, Minik K, *et al.* [Primary malignant melanoma of the lung and lower respiratory tract]. *Orv Hetil* 1994;**135**:245–9.
42. Pasquini E, Rastelli E, Muretto P, *et al.* Primary bronchial malignant melanoma. A case report. *Pathologica* 1994;**86**:546–8.
43. Farrell DJ, Kashyap AP, Ashcroft T, *et al.* Primary malignant melanoma of the bronchus. *Thorax* 1996;**51**:223–4.
44. de Wilt JHW, Farmer SEJ, Scolyer RA, *et al.* Isolated melanoma in the lung when there is no known primary site: metastatic disease or primary lung tumour? *Melanoma Res* 2005;**15**:531–7.
45. Sekine I, Kodama T, Yokose T, *et al.* Rare pulmonary tumors – a review of 32 cases. *Oncology* 1998;**55**:431–4.
46. Wilson RW, Moran CA. Primary melanoma of the lung: a clinicopathologic and immunohistochemical study of eight cases. *Am J Surg Pathol* 1997;**21**:1196–202.
47. Ost D, Joseph C, Sogoloff H, *et al.* Primary pulmonary melanoma: case report and literature review. *Mayo Clin Proc* 1999;**74**:62–6.
48. Ozdemir N, Cangir AK, Kutlay H, *et al.* Primary malignant melanoma of the lung in oculocutaneous albino patient. *Eur J Cardiothorac Surg* 2001;**20**:864–7.
49. Testini M, Trabucco S, Di Venere B, *et al.* Ileal intussusception due to intestinal metastases from primary malignant melanoma of the lung. *Am Surg* 2002;**68**:377–9.
50. Dountsis A, Zisis C, Karagianni E, *et al.* Primary malignant melanoma of the lung: a case report. *World J Surg Oncol* 2003;**1**:26.
51. Lie CH, Chao TY, Chung YH, *et al.* Primary pulmonary malignant melanoma presenting with haemoptysis. *Melanoma Res* 2005;**15**: 219–21.
52. Scotto J, Fraumeni JF Jr, Lee JA. Melanomas of the eye and other noncutaneous sites: epidemiologic aspects. *J Natl Cancer Inst* 1976;**56**:489–91.

53. Ross MI, Henderson MA. Mucosal melanoma. In: Balch CM, Houghton AN, Sober AJ, *et al.* (eds) *Cutaneous Melanoma*. St Louis, MO: Quality Medical Publishing, 2009:337–50.
54. Fowler M, Sutherland HDA. Malignant melanoma of the oesophagus. *J Pathol Bacteriol* 1952;**64**:473–7.
55. Goldman JL, Lawson W, Zak FG, *et al.* The presence of melanocytes in the human larynx. *Laryngoscope* 1972;**82**:824–35.
56. Busuttil A. Dendritic pigmented cells within human laryngeal mucosa. *Arch Otolaryngol* 1976;**102**:43–4.
57. Batsakis JG, Regezi JA, Solomon AR, *et al.* The pathology of head and neck tumors: mucosal melanomas, part 13. *Head Neck Surg* 1982;**4**:404–18.
58. Grazer R, Cohen SM, Jacobs JB, *et al.* Melanin-containing peripheral carcinoid of the lung. *Am J Surg Pathol* 1982;**6**:73–8.
59. Rajaratnam R, Marsden JR, Marzouk J, *et al.* Pulmonary carcinoid associated with melanoma: two cases and a review of the literature. *Br J Dermatol* 2007;**156**:738–41.
60. Pilozzi E, Cacchi C, Di Napoli A, *et al.* Primary malignant tumour of the lung with neuroendocrine and melanoma differentiation. *Virchows Arch* 2011;**459**:239–43.
61. Rosenberg LM, Polanco GB, Blank S. Multiple tracheobronchial melanomas with ten-year survival. *J Am Med Assoc* 1965;**192**:717–19.
62. Damian DL, Fulham MJ, Thompson E, *et al.* Positron emission tomography in the detection and management of metastatic melanoma. *Melanoma Res* 1996;**6**:325–9.
63. Gritters LS, Francis IR, Zasadny KR, *et al.* Initial assessment of positron emission tomography using 2-fluorine-18-fluoro-2-deoxy-D-glucose in the imaging of malignant melanoma. *J Nucl Med* 1993;**34**:1420–7.
64. Torres-Mora J, Dry S, Li X, *et al.* Malignant melanotic schwannian tumor: a clinicopathologic, immunohistochemical, and gene expression profiling study of 40 cases, with a proposal for the reclassification of "melanotic schwannoma". *Am J Surg Pathol* 2014;**38**:94–105.
65. Lyle M, Haydu LE, Menzies AM, *et al.* The molecular profile of metastatic melanoma in Australia. *Pathology* 2016;**48**:188–93.
66. Heenan PJ. Local recurrence of melanoma. *Pathology* 2004;**36**:491–5.
67. Littman CD. Metastatic melanoma mimicking primary bronchial melanoma. *Histopathology* 1991;**18**:561–3.
68. Carter D, Eggleston J. Tumors of the lower respiratory tract. In: *Atlas of Tumor Pathology*, series 2, fascile 17. Washington DC: Armed Forces Institute of Pathology, 1979:220.
69. Faries MB, Bleicher RJ, Ye X, *et al.* Lymphatic mapping and sentinel lymphadenectomy for primary and metastatic pulmonary malignant neoplasms. *Arch Surg* 2004;**139**:870–6; discussion 876–7.
70. Boni R, Boni RA, Steinert H, *et al.* Staging of metastatic melanoma by whole-body positron emission tomography using 2-fluorine-18-fluoro-2-deoxy-D-glucose. *Br J Dermatol* 1995;**132**:556–62.
71. Rigo P, Paulus P, Kaschten BJ, *et al*. Oncological applications of positron emission tomography with fluorine-18 fluorodeoxyglucose. *Eur J Nucl Med* 1996;**23**:1641–74.
72. Valk PE, Pounds TR, Tesar RD, *et al.* Cost-effectiveness of PET imaging in clinical oncology. *Nucl Med Biol* 1996;**23**:737–43.
73. Wagner JD, Schauwecker D, Hutchins G, *et al.* Initial assessment of positron emission tomography for detection of nonpalpable regional lymphatic metastases in melanoma. *J Surg Oncol* 1997;**64**:181–9.
74. Gulec SA, Faries MB, Lee CC, *et al.* The role of fluorine-18 deoxyglucose positron emission tomography in the management of patients with metastatic melanoma: impact on surgical decision making. *Clin Nucl Med* 2003;**28**:961–5.
75. Dalrymple-Hay MJ, Rome PD, Kennedy C, *et al.* Pulmonary metastatic melanoma –- the survival benefit associated with positron emission tomography scanning. *Eur J Cardiothorac Surg* 2002;**21**:611–14; discussion 614–15.
76. Curtin JA, Fridlyand J, Kageshita T, *et al.* Distinct sets of genetic alterations in melanoma. *N Engl J Med* 2005;**353**:2135–47.
77. Scolyer RA, Long GV, Thompson JF. Evolving concepts in melanoma classification and their relevance to multidisciplinary melanoma patient care. *Mol Oncol* 2011;**5**:124–36.
78. Long GV, Menzies AM, Nagrial AM, *et al.* Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* 2011;**29**:1239–46.
79. Flaherty KT, Puzanov I, Kim KB, *et al.* Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* 2010;**363**:809–19.
80. Curtin JA, Busam K, Pinkel D, *et al.* Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol* 2006;**24**:4340–6.
81. Chapman PB, Hauschild A, Robert C, *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 2011;**364**:2507–16.
82. Kefford R, Arkenau HT, Brown MP, *et al.* Phase I/II study of GSK2118436, a selective inhibitor of oncogenic mutant BRAF kinase, in patients with metastatic melanoma and other solid tumors. *J Clin Oncol* 2010; **28**: abstract 8503.
83. Carvajal RD, Antonescu CR, Wolchok JD, *et al.* KIT as a therapeutic target in metastatic melanoma. *J Am Med Assoc* 2011;**305**:2327–34.
84. Handolias D, Hamilton AL, Salemi R, *et al.* Clinical responses observed with imatinib or sorafenib in melanoma patients expressing mutations in KIT. *Br J Cancer* 2010;**102**:1219–23.
85. Hodi FS, O'Day SJ, McDermott DF, *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;**363**:711–23.
86. Robert C, Thomas L, Bondarenko I, *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011;**364**:2517–26.
87. Robert C, Long GV, Brady B, *et al.* Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* 2015;**372**:320–30.
88. La Hei ER, Thompson JF, McCaughan BC, *et al.* Surgical resection of pulmonary metastatic melanoma: a review of 83 thoracotomies. *Asia Pacific Heart* 1996;**5**:111–14.
89. Harpole DH Jr, Johnson CM, Wolfe WG, *et al.* Analysis of 945 cases of pulmonary metastatic melanoma. *J Thorac Cardiovasc Surg* 1992;**103**:743–8; discussion 748–50.
90. Gorenstein LA, Putnam JB, Natarajan G, *et al.* Improved survival after resection of pulmonary metastases from malignant melanoma. *Ann Thorac Surg* 1991;**52**:204–10.
91. Norman J, Cruse CW, Wells KE, *et al.* Metastatic melanoma with an unknown primary. *Ann Plast Surg* 1992;**28**:81–4.
92. Cormier JN, Xing Y, Feng L, *et al.* Metastatic melanoma to lymph nodes in patients with unknown primary sites. *Cancer* 2006;**106**:2012–20.
93. Vijuk G, Coates AS. Survival of patients with visceral metastatic melanoma from an occult primary lesion: a retrospective matched cohort study. *Ann Oncol* 1998;**9**:419–22.
94. Lee CC, Faries MB, Wanek LA, *et al.* Improved survival after lymphadenectomy for nodal metastasis from an unknown primary melanoma. *J Clin Oncol* 2008;**26**:535–41.
95. Prens SP, van der Ploeg AP, van Akkooi AC, *et al.* Outcome after therapeutic lymph node dissection in patients with unknown primary melanoma site. *Ann Surg Oncol* 2011;**18**(13):3586–92.
96. Rutkowski P, Nowecki ZI, Dziewirski W, *et al.* Melanoma without a detectable primary site with metastases to lymph nodes. *Dermatol Surg* 2010;**36**:868–76.
97. Kundranda MN, Clark CT, Chaudhry AA, *et al.* Primary malignant melanoma of the lung: a case report and review of the literature. *Clin Lung Cancer* 2006;**7**:279–81.
98. Reddy VS, Mykytenko J, Giltman LI, *et al.* Primary malignant melanoma of the lung: review of literature and report of a case. *Am Surg* 2007;**73**:287–9.
99. Kotoulas C, Skagias L, Konstantinou G, *et al.* Primary pulmonary melanoma diagnosis: the role of immunohistochemistry and immunocytochemistry. *J Balkan Union Oncol* 2007;**12**:543–5.

100. Saint-Blancard P, Vaylet F, Jancovici R. [A rare pulmonary tumour, primary malignant melanoma]. *Rev Mal Respir* 2009;**26**:57–61.
101. Shikuma K, Omasa M, Yutaka Y, *et al.* Treatment of primary melanoma of the lung monitored by 5-S-cysteinyldopa levels. *Ann Thorac Surg* 2009;**87**:1264–6.
102. Maeda R, Isowa N, Onuma H, *et al.* Primary malignant melanoma of the lung with rapid progression. *Gen Thorac Cardiovasc Surg* 2009;**57**:671–4.
103. Pan XD, Zhang B, Guo LC, *et al.* Primary malignant melanoma of the lung in the elderly: case report and literature review. *Chin Med J (Engl)* 2010;**123**:1815–17.
104. Neri S, Komatsu T, Kitamura J, *et al.* Malignant melanoma of the lung: report of two cases. *Ann Thorac Cardiovasc Surg* 2011;**17**:170–3.
105. Mochizuki H, Chikui E, Tokumaru A, *et al.* [A case of pulmonary malignant melanoma mimicking lung abscess]. *Nihon Kokyuki Gakkai Zasshi* 2011;**49**:472–7.
106. Seitelman E, Donenfeld P, Kay K, *et al.* Successful treatment of primary pulmonary melanoma. *J Thorac Dis* 2011;**3**:207–8.
107. Zuckermann B, Papiashvilli M, Bar I. Primary pulmonary malignant melanoma of right upper lobe of lung. *Isr Med Assoc J* 2011;**13**:440–1.
108. Gong L, Liu XY, Zhang WD, *et al.* Primary pulmonary malignant melanoma: a clinicopathologic study of two cases. *Diagn Pathol* 2012;**7**:123.
109. Ouarssani A, Atoini F, Reda R, *et al.* Malignant melanoma of the lung: a case report. *Pan Afr Med J* 2012;**11**:68.
110. Kamaleshwaran KK, Natarajan S, Parthiban J, *et al.* Rare case of extradural spinal metastasis from primary lung malignant melanoma detected with fluorine-18 fluorodeoxyglucose-positron emission tomography/computed tomography. *Indian J Nucl Med* 2014;**29**:57–8.
111. Gupta A, Bhattacharya D, Jain S, *et al.* Primary malignant melanoma of the lung: case report and literature review. *Indian J Chest Dis Allied Sci* 2015;**57**:181–4.
112. Hwang KB, Hwang KE, Jung JW, *et al.* Primary pulmonary malignant melanoma: an unexpected tumor. *Tuberc Respir Dis (Seoul)* 2015;**78**:272–5.
113. Mahowald MK, Aswad BI, Okereke IC, *et al.* Long-term survival after pneumonectomy for primary pulmonary malignant melanoma. *Ann Thorac Surg* 2015;**99**:1428–30.
114. Postrzech-Adamczyk K, Chabowski M, Gluszczyk-Ferenc B, *et al.* Malignant melanoma of the lung: case series. *Kardiochir Torakochirurgia Pol* 2015;**12**:72–6.
115. Watanabe M, Yamamoto H, Hashida S, *et al.* Primary pulmonary melanoma: a report of two cases. *World J Surg Oncol* 2015;**13**:274.
116. Zhang X, Wang Y, Du J. Primary malignant melanoma of left lower lobe of lung: a case report and review of the literature. *Oncol Lett* 2015;**10**:528–30.

CHAPTER 8: TARGETED THERAPEUTICS IN MELANOMA

Overview: The transcriptional protein PRAME has recently shown promise as a therapeutic target in melanoma, and it is likely that investigators will need to develop an understanding of any biological co-dependency between response to PRAME-targeted therapies and PRAME expression by immunohistochemistry (IHC). As a first step, there is a requirement to understand the pattern of PRAME IHC expression between a primary melanoma, its locoregional metastases and/or distant metastases in individual patients. Heterogeneity in PRAME expression could have implications for clinical practice and trial design.

Specific aims: To examine the intra-patient inter-tumoral heterogeneity of PRAME expression by immunohistochemistry among a cohort of patients with multiple matched melanoma tumour samples.

Contribution to literature: Studies on concordance of PRAME expression between primary tumours and their metastases in individual melanoma patients are lacking. Ours is the first study to address this issue.

Assessment of Intrapatient Intertumoral Heterogeneity of PRAME Immunohistochemistry Expression in Primary and Metastatic Melanoma

Louise Jackett[1,2,3], Jordan Conway[2,3,4], John F Thompson[3,4,5,6], Richard A. Scolyer[2,3,4,7,8]

[1.] Department of Anatomical Pathology, Peter MacCallum Cancer Centre, Melbourne, Vic, Australia
[2.] Charles Perkins Centre, Melanoma Institute Australia, Sydney, NSW, Australia
[3.] Faculty of Medicine and Health, The University of Sydney, Sydney, NSW, Australia
[4.] Melanoma Institute Australia, The University of Sydney, Sydney, NSW, Australia

[5.] Department of Melanoma and Surgical Oncology, Royal Prince Alfred Hospital, Sydney, NSW, Australia

[6] Faculty of Health and Medical Sciences, The University of Western Australia, Perth, WA, Australia

[7.] Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Sydney, NSW, Australia

[8.] NSW Health Pathology, Sydney, NSW, Australia

Short Running Title: Heterogeneity of PRAME expression in melanoma

Key words: Melanoma, PRAME, biomarker, heterogeneity, immunohistochemistry, theragnostics

Submitted for publication: 19 February 2025

Journal: Modern Pathology

Editor-in-chief: Rhonda K. Yantiss, MD

Publisher's reference: MODPATH-D-25-00179

Abstract

Background: The transcriptional protein PRAME has been used as a diagnostic marker of melanoma in recent years but recently it has also shown promise as a therapeutic target in melanoma. There is a need to understand its expression profile between a primary melanoma, its locoregional metastases and/or distant metastases in individual patients. Heterogeneity in PRAME expression would have implications for clinical practice and trial design. Studies on concordance of PRAME expression between primary tumors and/or metastases in individual melanoma patients are lacking.

Aim: We sought to examine the intra-patient inter-tumoral heterogeneity of PRAME expression by immunohistochemistry (IHC) among a cohort of patients with multiple matched melanoma tumor samples.

Methods: Patients with advanced melanoma for whom multiple tumor specimens were available were identified from the database of Melanoma Institute Australia. We assessed PRAME IHC for matched double or triple samples of primary melanoma (PM), regional lymph node metastases (LNM), in-transit metastases (ITM) and/or distant metastases (DM) in individual patients.

Results: We reviewed 94 melanoma specimens from 38 patients. 87% (33/38) of PM specimens showed any level (score 1-4+) of PRAME staining. PRAME score was concordant between PM and LNM in 70% of patients (16/23, *kappa* = 0.45, *p value* = 0.02); between PM and ITM in 70% (7/10, *kappa* = 0.32, *p value* = 0.227); between PM and DM in 48% (10/21, *kappa* = 0.19, *p value* = 0.3); between LNM and ITM in 86% (6/8, *kappa* = 0.67, *p value* = 0.227); and between LNM and DM in 75% (6/8, *kappa* = 0.68, *p value* = 0.03). Only 62.5% (10/16) of matched triplet specimens were concordant across all three specimens. We observed bi-directional heterogeneity of PRAME expression.

Conclusion: We identified moderate intra-patient inter-tumoral concordance of expression for PRAME IHC. Heterogeneity in PRAME expression is potentially a function of inherent biological changes, but specimen quality may also play a part. Caution is recommended in relying on PRAME immunostaining to select patients with metastatic melanoma for anti-PRAME systemic therapies.

(Word count 327)

INTRODUCTION

PRAME (PReferentially expressed Antigen in MElanoma) is a transcriptional regulator protein with important actions in cell differentiation and proliferation that is enriched in melanomas and some other malignancies.(1,2,3,4,5,6,7) PRAME has shown promise as a therapeutic target for several immune-based treatments in multiple tumor types, including melanoma.(8,9,10)

Examples of these treatments include T cell receptor (TCR) bispecific therapy, for which a PRAME x CD3 bispecific has demonstrated good tolerance and effect in an ongoing phase 1 trial for unresectable metastatic cutaneous melanomas previously treated with immune checkpoint inhibitors.(10) This treatment is now being investigated in a phase 3 clinical trial (PRISM-MEL-301) for patients with previously untreated non-uveal melanoma.(11) Furthermore, PRAME has already shown promise as a target for CAR-T cell therapy in preclinical *in vitro* and *in vivo* studies for treatment of acute myelogenous leukaemia and this may play a role in melanoma treatment in the future.(9,12)

As research continues into PRAME-targeted treatments, there is a need to understand the expression profile of PRAME in melanoma tumor samples as it may provide important information in selecting patients who may benefit from this treatment. PRAME can be detected with cytogenetic and molecular methods however its detection by immunohistochemical (IHC) staining is more readily accessible in day-to-day practice (13,14,15). Melanoma occasionally shows immunomorphological plasticity, as illustrated by partial or complete loss of expression of S100, Sox10, melanA and HMB45 IHC stains in dedifferentiated and undifferentiated melanomas.(16). Furthermore, inter-tumoral heterogeneity is well documented for other predictive biomarkers, such as PDL1, in both melanoma and other tumor types.(17) It is therefore important to consider the potential for heterogeneity of expression between melanoma samples taken from different sites in the same patient as this may provide information regarding the usefulness of expression as a biomarker.

It is currently unknown whether the success of a PRAME-targeted therapy is contingent on PRAME being uniformly expressed by the tumor, either spatially or temporally. Discordance in expression patterns between a primary melanoma, its locoregional metastases (in lymph nodes or as in-transit metastases) and/or distant metastases may have implications for the effectiveness of therapy and are likely to have importance for future clinical practice and clinical trial design.

Thus, this study sought to examine the intra-patient inter-tumoral heterogeneity of PRAME protein expression by IHC from individual patients with multiple melanoma tumor samples (combinations of either primary tumors, regional lymph node metastases, in-transit metastases or distant metastases).

MATERIALS AND METHODS

Patients and Specimens

This study was performed with the approval of the Human Ethics Review Committee of the Sydney Local Health District (Protocol No X15-0454 & 2019/ETH06874) and with informed patient consent. Patients with advanced melanoma, for whom multiple tumor specimens at different disease stages were available, were identified from the database and archives of Melanoma Institute Australia and the Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Sydney. Only the algorithmically-determined culprit primary melanoma was included where there was a history of more than one primary melanomas. Specimen types included the primary melanoma (PM), lymph node metastases (LNM), in-transit metastases (ITM) and distant metastases (DM).

PRAME IHC Antibody

For each formalin-fixed paraffin-embedded (FFPE) melanoma specimen, two 4µm thick sections were cut, one of which was stained with haematoxylin and eosin (H&E) and the other used for PRAME IHC staining. PRAME IHC staining was performed using an automated IHC system (Roche Ventana Benchmark Ultra) using the Optiview DAB IHC Detection Kit. After deparaffinisation of FFPE sections, heat-induced epitope retrieval was applied using Cell Conditioning 1 (Antigen Unmasking) for 48 minutes. The PRAME (EPR20330) antibody was applied for 40 minutes at 36^{o}C (96.8^{o}F) and counterstained using Mayer's haematoxylin solution. A positive control was included in each IHC round.

IHC Staining Evaluation

H&E and IHC slides were evaluated with blinding to clinical and histopathological information. Cases were scored in quartiles according to the method described by Lezcano et al.(18) No staining was scored as zero; staining of 1 to 25% of tumor cells was scored as 1+; 26 to 50% as 2+; 51% to 75% as 3+; 76% or more as 4+. Staining of at least weak intensity was included in the score.

As a secondary data point, the intensity of staining was also recorded as either uniformly weak, moderate or strong, or as heterogeneous (eg. weak to moderate, weak to strong).

Statistical analysis

The concordance of the PRAME scores between pairings of PM and LNM, PM and ITM, PM and DM, LNM and ITM, and LNM and DM was assessed using Cohen's Kappa coefficient. Cohen's Kappa coefficient was interpreted according to the Landis and Koch criteria, classified as almost perfect (>0.8), substantial (>0.6), moderate (>0.4), fair (>0.2), slight (>0) or no agreement (<0).(19) Clinicopathological associations were evaluated using Fisher's Exact test for categorical variables and the Mann-Whitney test for continuous variables.

RESULTS

Patient cohort

We identified 38 eligible patients with matched double or triple melanoma samples (combinations of PM, LNM, ITM or DM), who also had sufficient tissue for additional testing. Clinicopathological features of the cohort are presented in Table 1. Briefly, 53% of patients were male (20 of 38), and the age range was 16-93 years (mean, 56 years; median, 54 years). Primary melanomas were mostly non-acral melanomas located on the lower limb (14, 37%), trunk (12, 32%), head and neck (4, 10%) and upper limb (3, 8%). Four (10%) cases were from acral or subungual locations, and for one patient the primary site was not recorded. Melanoma subtypes included superficial spreading (13, 34%), nodular (10, 26%), acral lentiginous (6, 16%), desmoplastic (3, 8%) and malignant blue naevus (1, 3%). Five (13%) cases were unclassified.

Melanoma tumor specimens

Types of melanoma tumor samples evaluated are detailed in Table 2. In total, 94 melanoma specimens from the 38 patients were evaluated. These included 38 PM samples (40%), 23 LNM samples (24%), 11 ITM samples (12%) and 22 DM samples (23%).

A primary melanoma was available for all patients. Seven (18%) patients had a matched triplet of PM, LNM and ITM samples, 8 (21%) had a triplet of PM, LNM and DM, and 1 (3%) had a triplet of PM, ITM and DM. There were 8 (21%) doublets of matched PM and LNM, 1 (3%) PM and ITM, and 11 (29%) PM and DM samples. One patient (3%) had one PM and two ITM samples, and another (3%) had one PM and two DM samples. The maximum number of specimens tested in a single patient was 3.

Specimen types were varied. Among the 38 PM specimens, there were 37 excisions and 1 partial (shave) biopsy. All LNM, ITM and DM specimens were resected specimens, apart from 1 core biopsy of a DM.

Distant metastatic sites included brain (11, 38%), non-in-transit skin/subcutaneous tissues (9, 31%), viscera (5, 17%), soft tissue or bone (3, 10%), and a non-regional lymph node (1, 3%).

PRAME immunohistochemistry characteristics

All melanoma specimens contained adequate volumes of tumor on matched H&E slides and PRAME IHC slides.

PRAME scores in PM specimens

PRAME scoring of the 38 PM specimens is presented in Table 3. 87% (33/38) of PM specimens showed any level (score 1-4+) of PRAME staining, and 13% (5/38) of PM samples were completely negative (score 0). Of PM specimens with any level of staining, this was assessed as 4+ in 26 (69%) cases, 3+ in 5 (13%) cases, and 1+ in 2 (5%) of cases. There were no cases of 2+ in the PM samples. Overall, 81% (31/38) of tested PM samples showed at least weak PRAME staining in 50% or more of the tumor (score 3+ or 4+).

PRAME intensity in PM specimens

We observed heterogeneity in intensity of PRAME staining in the PM samples (Table 3). This included strong staining in 37% (14 cases), moderate to strong staining in 18% (7 cases), moderate staining in 8% (3 cases), weak to moderate staining in 21% (8 cases), and weak staining in 3% (1 case). The latter case also showed a low percentage of staining (5%, score 1).

Intra-patient inter-tumoral concordance

The distributions of PRAME scores in each group are presented in Figure 1, and PRAME scores with concordance data are presented in Tables 4 and 5.

PM versus LNM

PRAME score was concordant between PM and matched LNM in 70% (16/23) of patients (*kappa* = 0.45, moderate concordance, *p value* = 0.02, Tables 4 and 5). The LNM samples more frequently scored lower (22%, 5/23) than higher (9%, 2/23) when compared to the PM (Table 5).

PM versus ITM

PRAME score was concordant between PM and ITM in 70% (7/10) of patients (*kappa* = 0.32, fair concordance, *p value* = 0.227, Tables 4 and 5). The ITM samples more frequently higher (20%, 2/10) than lower (10%, 1/10) when compared to the PM (Table 5).

PM versus DM

Between PM and DM, the PRAME score was only concordant in 48% (10/21) of patients (*kappa* = 0.19, slight concordance, *p value* = 0.3, Tables 4 and 5). When compared to the PM, DM samples scored lower in 29% (6/21) of patients and higher in 19% (4/21, Table 5).

LNM versus IT

PRAME was concordant between LNM and ITM in 86% (6/7) of cases (*kappa* = 0.67, substantial concordance, *p value* = 0.064, Table 5). The one discordant case was higher in the ITM compared to the LNM (1/7, Table 5).

LNM versus DM

PRAME was concordant between LNM and DM in 75% (6/8) of patients (*kappa* = 0.68, substantial concordance, *p value* = 0.03, Tables 4 and 5). In the two cases of discordance (2/8, 25%), PRAME score was lower in the DM (Table 5).

Triplet samples

PRAME scores were concordant in 71% (5/7) and 63% (5/8) of patients across all three matched triplet specimens for PM+LNM+ITM and PM+LNM+DM, respectively. The single patient with matched PM+ITM+DM samples, showed score 3+ in the PM and LNM samples, and score 1 in the DM sample. When considering patients with triplet samples as a single group, only 62.5% (10/16) patients showed concordance across all three of melanoma samples. Examples of concordant and discordant matched triplet specimens are provided in Figure 2.

PRAME score 1-4 versus PRAME score 0

When PRAME of any score (1-4+) was considered against score 0 (Table 6), the binary concordance rose to 96% (22/23) for PM versus LNM samples (*kappa* = 0.65, substantial concordance, *p-value* = 0.062); 90% (9/10) for PM versus ITM samples (*kappa* = 0.62, substantial concordance, *p-value* = 0.092); 84% (16/19) for PM versus DM samples (*kappa* = 0.58, moderate concordance, *p-value* =

1);100% (7/7) for LNM versus ITM samples (*kappa* = 1, *p-value* <0.001). and 100% (8/8) for LNM versus DM samples (*kappa* not assessable).

Clinicopathological associations

There were no significant differences in clinicopathological features between patients with concordant PM and LNM samples and those showing discordance (Table 7).

PRAME-negative PM cases

The features of the 5 PM cases that showed 0 PRAME staining are outlined in Table 8. Of these, one case was concordant with score 0 across each of the matched PM, LNM and DM specimens. Of the discordant cases, three were notable for showing score 3+ or 4+ in the more advanced specimen.

In two of the discordant cases (case 3 and 29), there were no sebaceous glands to confirm an internal positive control, however positive external controls were present. PRAME staining was repeated in these cases, yielding the same result. All other PRAME-negative PM cases had positive internal and external controls.

DISCUSSION

Tumor heterogeneity, both within individual tumors but also between primary and metastatic tumors, is an important issue in oncology, particularly as it relates to biomarker testing for treatment prediction (theragnostic) purposes.(20) Heterogeneity is a consequence of complex factors, including those related to the tumor itself (e.g. spatial variability or progression-related genetic alterations); those related to the tumor microenvironment; and those related to pre-analytical and analytical histopathological processes (e.g. biopsy type, sampling error, tissue fixation adequacy and degradation artefacts).(20)

Given the current clinical interest in PRAME-directed therapies and the need for the tumor to express PRAME before enrolment in some clinical trials of anti-PRAME treatment (e.g. the PRISM-MEL-301 trial)(11), there is a need to understand the IHC expression pattern of PRAME in patient tumor samples from different sites in the same patient. This is especially important if cut-off levels of PRAME expression are to be considered for selection of patients for treatment. In addition, understanding any heterogeneity in PRAME status may prove insightful if subsets of patients with varying responses or side effects emerge from such trials.

The importance of understanding inter-tumoral heterogeneity early on in PRAME theragnostic research is underscored by lessons learned from non-PRAME biomarkers in other tumor types. Several studies of biomarkers other than PRAME have shown that IHC expression is not always uniform between a patient's primary and metastatic tumor(s).(17,21,22) While some markers show remarkable homogeneity across a patient's tumor samples, such as BRAF IHC in melanoma, intra-patient inter-tumoral heterogeneity has been demonstrated for PDL1 IHC expression in melanoma, non-small cell lung, esophageal, gastric/gastro-esophageal, urothelial, triple negative breast, and head and neck squamous cell carcinomas.(17,23,24) Furthermore, the validity of determining HER2 status on the basis of a single tumor biopsy has been questioned after Geukins *et al* found HER2-low and HER2-zero breast cancer metastases co-existed in the same patient.(21)

To date, only very limited investigation has been conducted into PRAME IHC expression across tumor samples within an individual patient.(18) As part of their study of 155 cutaneous melanomas and 100 metastatic melanomas, Lezcano et al found that 83.2% of primary melanomas (129/155) and 87% of metastatic melanoma samples (87/100) showed 4+ labelling with PRAME.(18). In the small number of patients with matched primary and metastatic tumors, they found that 85% (12/14) showed concordant (4+) PRAME staining.(18) In the two patients with discordant expression. The PRAME staining score was higher in the metastasis than the primary tumor. One case was a primary mixed desmoplastic melanoma (1+ PRAME staining) and the other a primary lentigo maligna melanoma (2+ PRAME staining); both corresponding metastases showed strong, diffuse (4+) PRAME expression.

As far as we aware, this is the first substantial study to examine heterogeneity of PRAME IHC in intra-patient melanoma samples. Based on the limited findings of Lezcano et al, and the knowledge of melanoma's immunomorphological plasticity, we theorised that PRAME IHC status would show some degree of intra-patient, inter-tumoral non-uniformity across various stages of melanoma progression.

Our findings support this hypothesis. We observed heterogeneity of PRAME scores between PM and locoregional (LNM and ITM) samples (70% concordance), and between PM and DM samples (48%). In all these groups, concordance improved when PRAME score was considered in a binary (positive versus negative) fashion. LNM and ITM samples showed very high levels of concordance, which is compatible with the knowledge that these locoregional metastases have comparable effects on AJCC staging and prognostic estimates, and likely share biological similarities.

Our rate of concordance for PM and DM samples (48%, 10/21) is lower than the concordance rate (86%, 12/14) found by Lezcano *et al* among their small group of matched primaries and metastases.(18) In contrast to Lezcano *et al*, we observed that the PRAME score was more often lower in the distant/more advanced melanoma tumor sample of matched pairings, but not always. Given the small size of their cohort, it is difficult to directly compare these results. We note that our PRAME antibodies were the same (MAb EPR20330), but deployed on different stainer platforms. Further studies with larger cohorts are warranted.

Several reasons may explain the dynamic bi-directional heterogeneity of PRAME expression between tumor samples. Firstly, PRAME is a complex protein that is understood to drive tumorigenesis by its oncogenic function of upregulating pathways involved in meiosis and DNA repair, which leads to genomic instability.(25) Secondly, PRAME expression has been found to be associated with higher tumor stage and a poorer prognosis in several malignancies, including melanoma.(26) Given the immunomorphological plasticity of melanoma in other attributes, it is possible that PRAME biology may alter with progression of melanoma.

Sampling error may also contribute to observed differences in PRAME staining, however the high proportion of excisions among our cohort is likely to have reduced this effect in our study. Lezcano *et al* did not publish information about specimen types in their study for us to enable a comparison to be made.(18) Degradation of the PRAME protein is another possible reason for heterogeneity; we did observe two PM cases with score 0 for which there was an absence of an internal PRAME control (sebaceous glands; PRAME stains repeated), so we cannot say with complete certainty that our PRAME stain worked. Both archival tissue samples were more than 17 years old, so a degradation effect over time is certainly possible. However, we did encounter strong intensity score 4+ PRAME staining in several older samples so this does not appear to be a ubiquitous factor.

In our study, when we considered PRAME score 1-4+ (ie. any percentage of staining) in a binary analysis versus PRAME score 0, there was a trend to substantial concordance between the PM and LNM and PM and ITM samples, although this was not statistically significant. Awareness of this trend may be helpful for future trial designs if cut-off levels of PRAME staining are being considered to predict the response to PRAME-specific treatments.

We note that the frequency of PRAME score 4+ in our primary melanoma cohort (69%) is slightly lower than the frequency of the same staining pattern in two separate studies by Lezcano *et al* (78.5%, 104/129 and 83.2%, 129/155, respectively).(15,18) This could reflect the mix of melanoma subtypes, as we note that these investigators observed lower levels of staining in desmoplastic melanomas (35%) versus non-desmoplastic subtypes (88.2% to 90.9%)(15). Desmoplastic melanomas accounted for 8% of our cases, however information about the number of desmoplastic melanomas in the cohort by Lezcano *et al* was not published. (15)

Since we found no significant differences in clinicopathological features between PRAME-concordant and PRAME-discordant matched PM and LNM samples, it is unlikely that clinical factors other than melanoma subtype will be able to predict heterogeneity in PRAME staining.

Our study had some limitations. The melanoma tumor samples were sometimes heavily pigmented which made IHC assessment difficult; we mitigated this effect by using a red chromogen for melanotic tumors. We assessed archival biobank melanoma tumor samples rather than recent tumor samples and, as discussed above, degradation of the PRAME antigen may have potentially affected at least two cases. However, we did note that PRAME staining was generally higher in the PM specimens than in the matched metastases for other tissue blocks of a similar age, which suggests that heterogeneity is likely to be, at least in part, a function of inherent biological changes rather than simply specimen quality.

Finally, we emphasise that our findings do not indicate whether PRAME IHC should necessarily be used as a surrogate biomarker for administration of PRAME-targeted therapies. Melanoma is a good example of a tumor type in which PDL1 expression is a poor predictor of response to checkpoint inhibitor therapy, which contrasts with the utility of PDL1 in a range of other tumors.(27-29) Rather, our findings should alert future investigators to potential heterogeneity in PRAME IHC if it is to be used as a clinical trial enrolment criterion and/or studied as a predictive biomarker of treatment response.

CONCLUSION

In this study we found moderate concordance of PRAME expression across matched primary melanoma, lymph node metastasis, in-transit metastasis and distant metastasis tumor samples. When discordance was noted, PRAME IHC staining varied in a bi-directional manner. This

heterogeneity is likely to be a function of inherent biological changes, but specimen quality may also play a part.

Whether PRAME expression can predict treatment response and patient outcomes remains to be determined, and this is an area requiring further study. For investigators considering the predictive value of PRAME IHC for PRAME-targeted therapies, we recommend that PRAME heterogeneity be considered in trial designs, to reduce the risk that future patient management is negatively impacted by confounding results due to varying expression profiles.

(Word count 3317)

References

1. Ikeda H, Lethé B, Lehmann F, van Baren N, Baurain JF, de Smet C, Chambost H, Vitale M, Moretta A, Boon T, Coulie PG. Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. Immunity. 1997 Feb;6(2):199-208. doi: 10.1016/s1074-7613(00)80426-4. PMID: 9047241.

2. Simpson A.J.G., Caballero O.L., Jungbluth A., Chen Y.-T., Old L.J. Cancer/Testis Antigens, Gametogenesis and Cancer. Nat. Rev. Cancer. 2005;5:615–625. doi: 10.1038/nrc1669.

3. Thongprasert S, Yang PC, Lee JS, Soo R, Gruselle O, Myo A, Louahed J, Lehmann FF, Brichard VG, Coche T. The prevalence of expression of MAGE-A3 and PRAME tumor antigens in East and South East Asian non-small cell lung cancer patients. Lung Cancer. 2016 Nov;101:137-144. doi: 10.1016/j.lungcan.2016.09.006. Epub 2016 Oct 5. PMID: 27794402.

4. Oberthuer A, Hero B, Spitz R, Berthold F, Fischer M. The tumor-associated antigen PRAME is universally expressed in high-stage neuroblastoma and associated with poor outcome. Clin Cancer Res. (2004) 10:4307–13. doi: 10.1158/1078-0432.Ccr-03-0813.

5. Epping MT, Wang L, Edel MJ, Carlée L, Hernandez M, Bernards R. The human tumor antigen PRAME is a dominant repressor of retinoic acid receptor signaling. Cell. 2005 Sep 23;122(6):835-47. doi: 10.1016/j.cell.2005.07.003. PMID: 16179254.

6. Epping MT, Hart AA, Glas AM, Krijgsman O, Bernards R. PRAME expression and clinical outcome of breast cancer. Br J Cancer. (2008) 99:398–403. doi: 10.1038/sj.bjc.6604494

7. Tajeddine N, Gala JL, Louis M, Van Schoor M, Tombal B, Gailly P. Tumor-associated antigen preferentially expressed antigen of melanoma (PRAME) induces caspase-independent cell death in vitro and reduces tumorigenicity in vivo . Cancer Res. (2005) 65:7348–55. doi: 10.1158/0008-5472.Can-04-4011

8. Xu, Y.; Zou, R.; Wang, J.; Wang, Z.W.; Zhu, X. The role of the cancer testis antigen PRAME in tumorigenesis and immunotherapy in human cancer. Cell Prolif. 2020, 53, e12770.

9. Kirkey DC, Loeb AM, Castro S, McKay CN, Perkins L, Pardo L, Leonti AR, Tang TT, Loken MR, Brodersen LE, Loeb KR, Scheinberg DA, Le Q, Meshinchi S. Therapeutic targeting of PRAME with mTCRCAR T cells in acute myeloid leukemia. Blood Adv. 2023 Apr 11;7(7):1178-1189. doi: 10.1182/bloodadvances.2022008304. PMID: 35984639; PMCID: PMC10111362.

10. Hamid O, Williams A, Lopez JS, Olson D, Sato T, Shaw HM, Friedman CF, Thistlethwaite F, Middleton MR, Lebbe C, Ma VT, Izar B, Kar Han Lau P, Bechter OE, Kirk P, Yuan Y, Marshall S, Davar

D. Phase 1 safety and efficacy of IMC-F106C, a PRAME × CD3 ImmTAC bispecific, in post-checkpoint cutaneous melanoma (CM).. JCO 42, 9507- 9507(2024).DOI:10.1200/JCO.2024.42.16_suppl.9507

11. Long GV, Atkinson V, Ascierto PA, Davar D, Hamid O, Robert C, Butler MO, Dummer R, Hafner C, Khattak MA, Larkin J, Lorigan P, McKean M, Seedor RS, Shaw HM, Stephenson Jr. J, Yuan Y, Patel R, Thiyagarajah P, Schadendorf D. A phase 3 trial of IMC-F106C (PRAME x CD3) plus nivolumab versus standard nivolumab regimens in HLA-A*02:01+ patients with previously untreated advanced melanoma (PRISM-MEL-301).. JCO 42, TPS9602-TPS9602(2024). DOI:10.1200/JCO.2024.42.16_suppl.TPS9602

12. Yang J, Chen M, Ye J, Ma H. Targeting PRAME for acute myeloid leukemia therapy. Front Immunol. 2024 Mar 26;15:1378277. doi: 10.3389/fimmu.2024.1378277. PMID: 38596687; PMCID: PMC11002138.

13. Clarke LE, Flake DD 2nd, Busam K, Cockerell C, Helm K, McNiff J, Reed J, Tschen J, Kim J, Barnhill R, Elenitsas R, Prieto VG, Nelson J, Kimbrell H, Kolquist KA, Brown KL, Warf MB, Roa BB, Wenstrup RJ. An independent validation of a gene expression signature to differentiate malignant melanoma from benign melanocytic nevi. Cancer. 2017 Feb 15;123(4):617-628. doi: 10.1002/cncr.30385. Epub 2016 Oct 21. PMID: 27768230; PMCID: PMC5324582.

14. Ko JS, Matharoo-Ball B, Billings SD, Thomson BJ, Tang JY, Sarin KY, Cai E, Kim J, Rock C, Kimbrell HZ, Flake DD 2nd, Warf MB, Nelson J, Davis T, Miller C, Rushton K, Hartman AR, Wenstrup RJ, Clarke LE. Diagnostic Distinction of Malignant Melanoma and Benign Nevi by a Gene Expression Signature and Correlation to Clinical Outcomes. Cancer Epidemiol Biomarkers Prev. 2017 Jul;26(7):1107-1113. doi: 10.1158/1055-9965.EPI-16-0958. Epub 2017 Apr 4. PMID: 28377414.

15. Lezcano C, Jungbluth AA, Busam KJ. PRAME Immunohistochemistry as an Ancillary Test for the Assessment of Melanocytic Lesions. Surg Pathol Clin. 2021 Jun;14(2):165-175. doi: 10.1016/j.path.2021.01.001. Epub 2021 Apr 28. PMID: 34023098; PMCID: PMC8152939.

16. Agaimy A, Specht K, Stoehr R, et al. Metastatic malignant melanoma with complete loss of differentiation markers (undifferentiated/dedifferentiated melanoma): analysis of 14 patients emphasizing phenotypic plasticity and the value of molecular testing as surrogate diagnostic marker. Am. J. Surg. Pathol. 2016;40:181–191. doi: 10.1097/PAS.0000000000000527.

17. Kalpakoff M, Hund S, Musser J, Roach C, Apostolaki A, Vilardo M, Peltz L, Watts B, LaPlaca C, Tabuena-Frolli S, DiMaio MA, Welcher R, Kulangara K. Intrapatient Tumor Heterogeneity in IHC Interpretation Using PD-L1 IHC 22C3 pharmDx. Appl Immunohistochem Mol Morphol. 2021 Oct 1;29(9):667-673. doi: 10.1097/PAI.0000000000000941. PMID: 33973887; PMCID: PMC8505133.

18. Lezcano C, Jungbluth AA, Nehal KS, Hollmann TJ, Busam KJ. PRAME Expression in Melanocytic Tumors. Am J Surg Pathol. 2018 Nov;42(11):1456-1465. doi: 10.1097/PAS.0000000000001134. PMID: 30045064; PMCID: PMC6631376.

19. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977 Mar;33(1):159-74. PMID: 843571.

20. Stanta G, Bonin S. Overview on Clinical Relevance of Intra-Tumor Heterogeneity. Front Med (Lausanne). 2018 Apr 6;5:85. doi: 10.3389/fmed.2018.00085. PMID: 29682505; PMCID: PMC5897590.

21. Geukens T, De Schepper M, Richard F, Maetens M, Van Baelen K, Mahdami A, Nguyen HL, Isnaldi E, Leduc S, Pabba A, Zels G, Mertens F, Vander Borght S, Smeets A, Nevelsteen I, Punie K, Neven P, Wildiers H, Van Den Bogaert W, Floris G, Desmedt C. Intra-patient and inter-metastasis heterogeneity of HER2-low status in metastatic breast cancer. Eur J Cancer. 2023 Jul;188:152-160. doi: 10.1016/j.ejca.2023.04.026. Epub 2023 May 6. PMID: 37247580.

22. Sorroche BP, Teixeira RJ, Pereira CAD, Santana IVV, Vujanovic L, Vazquez VL, Arantes LMRB. PD-L1 Tumor Expression as a Predictive Biomarker of Immune Checkpoint Inhibitors' Response and Survival in Advanced Melanoma Patients in Brazil. Diagnostics (Basel). 2023 Mar 9;13(6):1041. doi: 10.3390/diagnostics13061041. PMID: 36980349; PMCID: PMC10047789.

23. Madore J, Vilain RE, Menzies AM, Kakavand H, Wilmott JS, Hyman J, Yearley JH, Kefford RF, Thompson JF, Long GV, Hersey P, Scolyer RA. PD-L1 expression in melanoma shows marked heterogeneity within and between patients: implications for anti-PD-1/PD-L1 clinical trials. Pigment Cell Melanoma Res. 2015 May;28(3):245-53. doi: 10.1111/pcmr.12340. Epub 2014 Dec 22. PMID: 25477049.

24. Menzies AM, Lum T, Wilmott JS, Hyman J, Kefford RF, Thompson JF, O'Toole S, Long GV, Scolyer RA. Intrapatient homogeneity of BRAFV600E expression in melanoma. Am J Surg Pathol. 2014 Mar;38(3):377-82. doi: 10.1097/PAS.0000000000000136. PMID: 24335665.

25. Kurtenbach S, Sanchez MI, Kuznetsoff J, Rodriguez DA, Weich N, Dollar JJ, Cruz A, Kurtenbach S, Field MG, Durante MA, Decatur C, Sorouri M, Lai F, Yenisehirli G, Fang B, Shiekhattar R, Pelaez D, Correa ZM, Verdun RE, Harbour JW. PRAME induces genomic instability in uveal melanoma. Oncogene. 2024 Feb;43(8):555-565. doi: 10.1038/s41388-023-02887-0. Epub 2023 Nov 29. PMID: 38030788; PMCID: PMC10873199.

26. Li J, Yin J, Zhong J, Yang Z, Tang A, Li S. Clinicopathological and Prognostic Significance of PRAME Overexpression in Human Cancer: A Meta-Analysis. Biomed Res Int. 2020 Dec 10;2020:8828579. doi: 10.1155/2020/8828579. PMID: 33381588; PMCID: PMC7748905.

27. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. IMpassion130 Trial Investigators Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. N Engl J Med. 2018 Nov;379((22)):2108–21. doi: 10.1056/NEJMoa1809615.

28. Pagni F., Guerini-Rocco E., Schultheis A.M., Grazia G., Rijavec E., Ghidini M., Lopez G., Venetis K., Croci G.A., Malapelle U., et al. Targeting Immune-Related Biological Processes in Solid Tumors: We do Need Biomarkers. Int. J. Mol. Sci. 2019;20:e5452. doi: 10.3390/ijms20215452.

29. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, Hassel JC, Rutkowski P, McNeil C, Kalinka-Warzocha E, Savage KJ, Hernberg MM, Lebbé C, Charles J, Mihalcioiu C, Chiarion-Sileni V, Mauch C, Cognetti F, Arance A, Schmidt H, Schadendorf D, Gogas H, Lundgren-Eriksson L, Horak C, Sharkey B, Waxman IM, Atkinson V, Ascierto PA. Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med. 2015 Jan 22;372(4):320-30. doi: 10.1056/NEJMoa1412082. Epub 2014 Nov 16. PMID: 25399552.

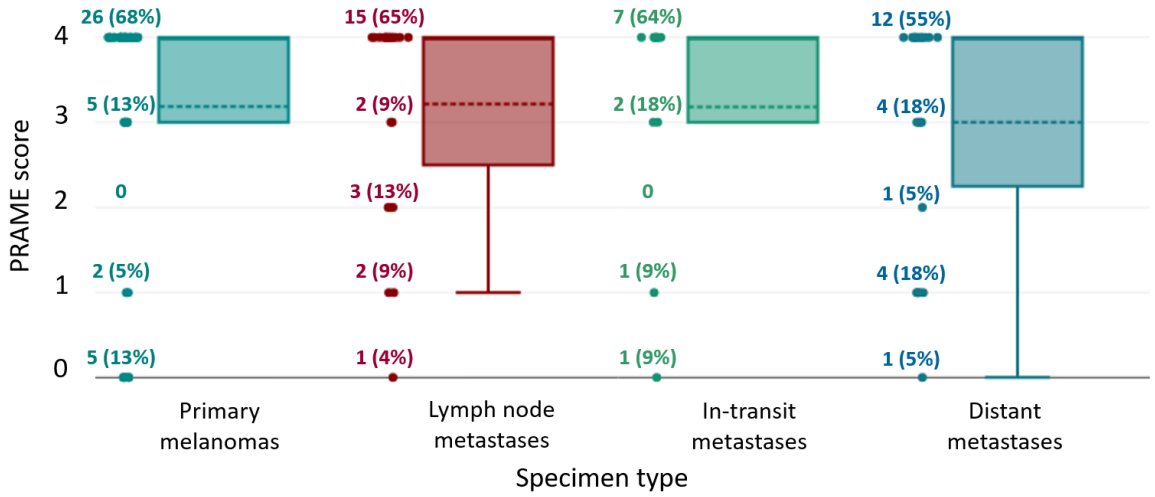

FIGURE 1. Distribution of PRAME immunohistochemistry scores for each group of primary, lymph node, in-transit and distant metastatic melanoma samples (percentage of cases in each group).

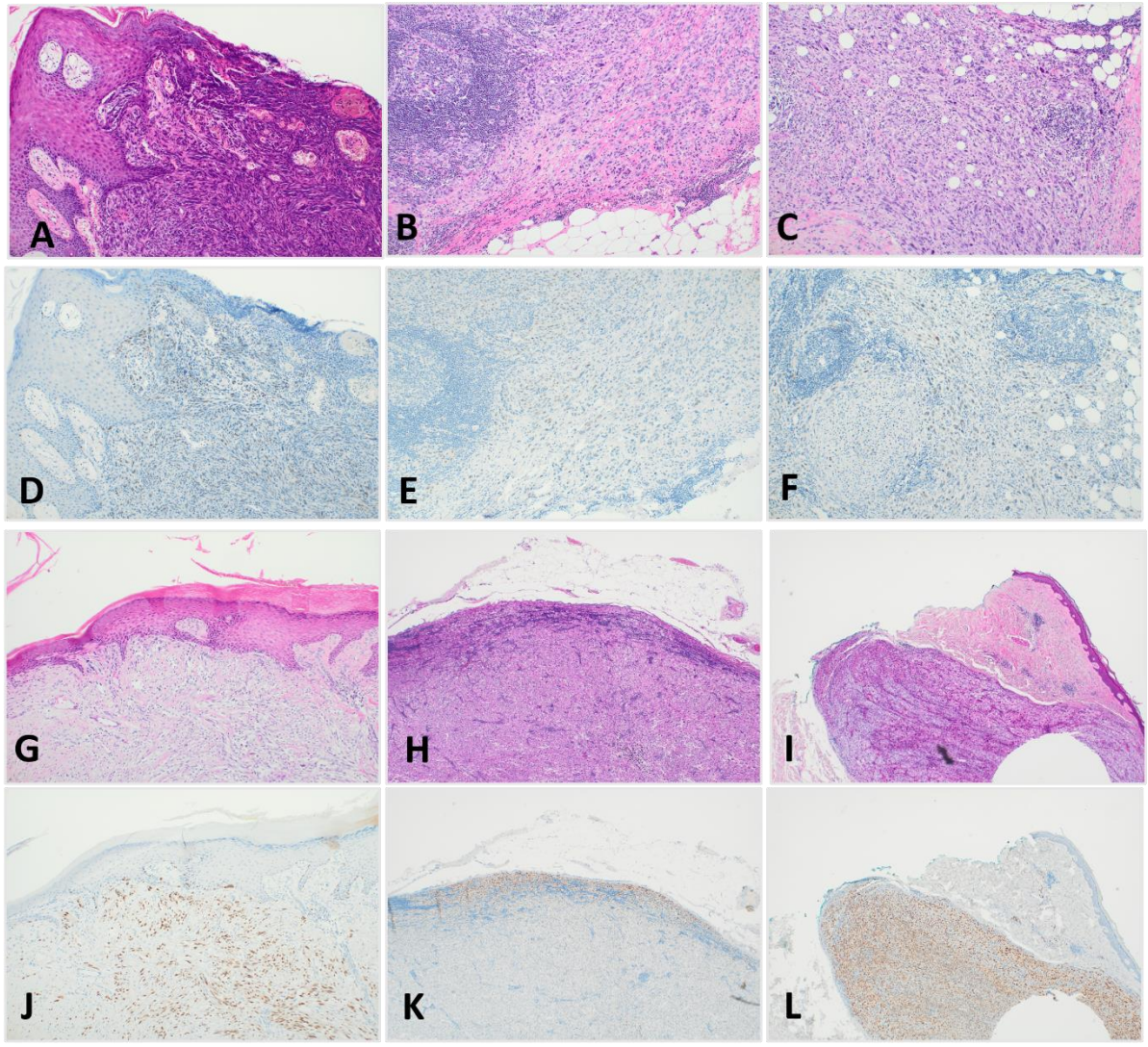

Figure 2. Examples of concordant and discordant PRAME staining.

A-F. Case 9, 62 year old male with (A) primary melanoma of abdominal skin, (B) locoregional lymph node metastasis, and (C) thoracic wall metastasis. PRAME immunohistochemistry of respective specimens (D-F) was concordant, showing weak to moderate intensity staining in less than 25% of tumor cells (score 1).

G-L. Case 2, 61 year old male with (G) subungual melanoma of great toe, (H) groin lymph node metastasis, and (I) subsequent in-transit metastasis of thigh skin. PRAME immunohistochemistry showed score 4 strong staining in the primary (J), which was discordant with score 1 staining in the lymph node metastasis (K). PRAME staining of a subsequent in-transit metastasis (L) showed score 4, which was similar to the primary. (A-C H&E x100, D-F PRAME x100, G-H H&E x40, I H&E x20, J-K PRAME x40, L PRAME x20).

TABLE 1. Clinicopathological characteristics of the studied 38 primary melanoma cases.

Characteristic	No. of cases (n=38)	%
Age (mean = 56, median = 54, range = 16-93)		
<40	3	8%
40-49	14	37%
50-59	5	13%
60-69	10	26%
70-79	3	8%
80+	1	3%
Unknown	2	5%
Gender		
M	20	53%
F	17	45%
Unknown	1	2%
Melanoma subtype		
Superficial spreading	13	34%
Nodular	10	26%
Acral lentiginous	6	16%
Unclassified	5	13%
Desmoplastic	3	8%
Malignant blue naevus	1	3%
Site group		
Lower limb	14	37%
Trunk	12	32%
Head and neck	4	10%
Upper limb	3	8%
Acral or subungual	4	10%
Unknown	1	3%
Mitotic rate (per mm2)		
Mean	9.97	
Std. Deviation	11.64	
Range	0-38	
Ulceration		
Yes	25	66%
No	10	26%
Unknown	3	8%
Microsatellites		
Absent	23	60%
Unknown	12	32%
Present	3	8%

TABLE 2. Types of melanoma specimens tested for PRAME immunohistochemistry

Specimen	Total	%
Primary melanoma	38	40.4%
Lymph node metastasis	23	24.5%
In-transit metastasis	11	11.7%
Distant metastasis	22	23.4%
Total	94	100%
Matched specimens	**Total**	**%**
Primary + lymph node metastasis	8	21.1%
Primary + in-transit metastasis	1	2.6%
Primary + distant metastasis	11	28.9%
Primary + lymph node + in-transit metastasis	7	18.4%
Primary + lymph node + distant metastasis	8	21.1%
Primary + 2 in-transit metastasis	1	2.6%
Primary + in-transit + distant metastasis	1	2.6%
Primary + 2 distant metastases	1	2.6%
Total	38	100%
Distant metastatic sites	**Total**	**%**
Brain	11	37.9%
Skin or subcutaneous tissues (not in-transit)	9	31.0%
Viscera	5	17.2%
Soft tissue/bone	3	10.3%
Non-regional lymph node	1	3.4%
Total	29	100%
Specimen types	**Total**	**%**
Primary melanoma - excision	37	39.4%
Primary melanoma - shave biopsy	1	1.1%
Lymph node metastasis - excision	23	24.5%
In-transit - excision	11	11.7%
Distant metastasis - excision	21	22.3%
Distant metastasis - core biopsy	1	1.1%
Total	94	100%

TABLE 3. PRAME expression for the 38 studied primary melanomas.

PRAME expression score in the primary		
4	26	69%
3	5	13%
2	0	0%
1	2	5%
0	5	13%
PRAME intensity in the primary		
Strong	14	37%
Moderate to strong	7	18%
Moderate	3	8%
Weak to moderate	8	21%
Weak*	1	3%
Absent	5	13%

*The case that had weak PRAME staining also showed a low percentage of staining (5%), corresponding to score 1.

TABLE 4. PRAME scores for matched melanoma specimens

Primary versus lymph node

		Primary PRAME score							
		0	**1**	**2**	**3**	**4**	**Total**	**Kappa**	**p-value**
Lymph node PRAME score	**0**	1	0	0	0	0	1	0.45 (95% CI = 0.04-0.85)	**0.02**
	1	0	1	0	0	1	2		
	2	0	0	0	1	2	3		
	3	0	0	0	1	1	2		
	4	1	0	0	1	13	15		
	Total	2	1	0	3	17	23		

Primary versus in-transit

		Primary PRAME score							
		0	**1**	**2**	**3**	**4**	**Total**	**Kappa**	**p-value**
In-transit PRAME score	**0**	1	o	o	o	o	1	0.32 (95% CI = -0.32-0.97)	0.227
	1	0	0	0	0	1	1		
	2	0	0	0	0	0	0		
	3	0	0	0	1	0	1		
	4	1	1	0	0	5	7		
	Total	2	1	0	1	6	10		

Primary versus distant metastasis

		Primary PRAME score							
		0	**1**	**2**	**3**	**4**	**Total**	**Kappa**	**p-value**
Distant metastasis PRAME score	**0**	0	0	0	0	1	1	0.19 (95% CI = -0.23-0.62)	0.3
	1	1	1	0	1	1	4		
	2	0	0	0	0	1	1		
	3	1	0	0	0	2	3		
	4	1	0	0	2	9	12		
	Total	3	1	0	3	14	21		

Lymph node versus in-transit

		Lymph node PRAME score							
		0	**1**	**2**	**3**	**4**	**Total**	**Kappa**	**p-value**
In-transit PRAME score	**0**	1	0	0	0	0	1	0.67 (95% CI = 0.06-1.27)	0.064
	1	0	0	0	0	0	0		
	2	0	0	0	0	0	0		
	3	0	0	0	0	0	0		
	4	0	1	0	0	5	6		
	Total	1	1	0	0	5	7		

Lymph node versus distant metastasis

		Lymph node PRAME score							
		0	**1**	**2**	**3**	**4**	**Total**	**Kappa**	**p-value**
Distant metastasis PRAME score	**0**	0	0	0	0	0	0	0.68 (95% CI = 0.28-1.09)	**0.03**
	1	0	1	1	0	0	2		
	2	0	0	0	0	1	1		
	3	0	0	0	0	0	0		
	4	0	0	0	0	5	5		
	Total	0	1	1	0	6	8		

TABLE 5. Breakdown of intrapatient intertumoral concordance for PRAME immunohistochemistry for matched melanoma specimens

Primary versus lymph node	**n**	**%**
Concordant	16	70%
Lower PRAME expression in LNM	5	22%
Lower by score of 1+	*2*	
Lower by score of 2+	*2*	
Lower by score of 3+	*1*	
Lower by score of 4+	*0*	
Higher PRAME expression in LNM	2	9%
Higher by score of 1+	*1*	
Higher by score of 2+	*0*	
Higher by score of 3+	*0*	
Higher by score of 4+	*1*	
Primary versus in-transit		
Concordant	7	70%
Patient with lower PRAME expression in one in-transit by score 1, lower in other in-transit by score 3	1	10%
Higher PRAME expression in in-transit	2	20%
Higher by score of 1+	*0*	
Higher by score of 2+	*0*	
Higher by score of 3+	*1*	
Higher by score of 4+	*1*	
Primary versus distant metastasis		
Concordant	10	48%
Lower PRAME expression in metastasis	6	29%
Lower by score of 1+	*2*	
Lower by score of 2+	*2*	
Lower by score of 3+	*1*	
Lower by score of 4+	*1*	
Higher PRAME expression in metastasis	4	19%
Higher by score of 1+	*3*	
Higher by score of 2+	*0*	
Higher by score of 3+	*1*	
Higher by score of 4+	*0*	
One patient with concordant PRAME staining in one met, lower in the second by 1 (partial discordance)	1	5%
Lymph node versus in-transit	**n**	**%**
Concordant	6	86%
Higher PRAME expression in in-transit	1	14%
Higher by score of 1+	*0*	
Higher by score of 2+	*0*	
Higher by score of 3+	*1*	
Higher by score of 4+	*0*	
Lymph node versus distant metastasis	**n**	**%**
Concordant	6	75%
Lower PRAME expression in metastasis	2	25%

Lower by score of 1+	*1*
Lower by score of 2+	*1*
Lower by score of 3+	*0*
Lower by score of 4+	*0*

TABLE 6. PRAME expression as binary positive (score 1-4+) versus negative (score 0) for matched melanoma specimens

Primary versus lymph node metastases (binary PRAME positive versus negative)

		Primary PRAME score				
		Positive	**Negative**	**Total**	**Kappa**	**p-value**
Lymph node PRAME score	**Positive**	21	1	22	0.65	0.062
	Negative	0	1	1		
	Total	21	2	23		

Primary versus in-transit metastases (binary PRAME positive versus negative)

		Primary PRAME score				
		Positive	**Negative**	**Total**	**Kappa**	**p-value**
In-transit metastasis PRAME score	**Positive**	8	1	9	0.62	0.092
	Negative	0	1	1		
	Total	8	2	10		

Primary versus distant metastasis (binary PRAME positive versus negative)

		Primary PRAME score				
		Positive	**Negative**	**Total**	**Kappa**	**p-value**
Distant metastasis PRAME score	**Positive**	16	2	18	0.58	1
	Negative	1	0	1		
	Total	17	2	19		

Lymph node metastases versus in-transit metastases (binary PRAME positive versus negative)

		Lymph node metastasis PRAME score				
		Positive	**Negative**	**Total**	**Kappa**	**p-value**
In-transit metastasis PRAME score	**Positive**	6	0	6	1	**<0.001**
	Negative	0	1	1		
	Total	6	1	7		

Lymph node metastases versus distant metastases (binary PRAME positive versus negative)

		Lymph node metastasis PRAME score				
		Positive	**Negative**	**Total**	**Kappa**	**p-value**
Distant metastasis PRAME score	**Positive**	8	0	8	*	*
	Negative	0	0	0		
	Total	8	0	8		

*Assumptions for the test are not fulfilled.

Table 7. Comparison of clinicopathological characteristics of melanoma patients for concordant and discordant PM and LNM samples

Characteristic	PRAME score concordant (n=16)	%	PRAME score discordant (n=7)	%	p-value
Age (years)					
Mean / median	58 / 59		59 / 61		0.898
Sex					
Male	11	69%	2	29%	
Female	4	25%	5	71%	0.105
Unknown	1	6%	0	0%	
Melanoma subtype					
Superficial spreading	7	44%	2	29%	
Unknown	0	0%	1	14%	
Nodular	3	19%	2	29%	*
Malignant blue naevus	0	0%	0	0%	
Acral lentiginous	3	18%	2	29%	
Desmoplastic	3	19%	0	0%	
Site group					
Lower limb	6	38%	4	57%	
Trunk	5	31%	1	14%	
Head and neck	2	13%	0	0%	*
Upper limb	0	0%	1	14%	
Acral or subungual	3	19%	1	14%	
Unknown	0	0%	0	0%	
Mitotic rate (per mm2)					
Mean	11		5.43		
Std. Deviation	11.25		5.09		0.227
Range	0-38		0-14		
Ulceration					
Yes	11	69%	7	100%	
No	4	25%	0	0%	0.247
Unknown	1	6%	0	0%	
Microsatellites					
Absent	11	69%	4	57%	
Unknown	2	13%	3	43%	0.176
Present	3	19%	0	0%	

*Assumptions for the test are not fulfilled.

Table 8. Features of the 5 cases with negative (score 0) PRAME staining of the primary melanoma

	PRAME score in primary	PRAME score in lymph node	PRAME score in metastasis	Concordance	Comments
Case 3	0	4 (strong)	4 (strong)	Discordant	No sebaceous glands seen to confirm internal control in PM
Case 4	0	0	0	Concordant	Internal control worked in PM
Case 29	0	70	n/a	Discordant	No sebaceous glands seen to confirm internal control in PM
Case 36	0	n/a	80	Discordant	Internal control worked in PM
Case 38	0	n/a	1	Discordant	Internal control worked in PM

Acknowledgements
The authors gratefully acknowledge the expertise and assistance of the immunohistochemistry team and pathologists in the Department of Anatomical Pathology at Peter MacCallum Cancer Centre. The authors also gratefully acknowledge support from colleagues at Melanoma Institute Australia and the Department of Tissue Pathology and Diagnostic Oncology at the Royal Prince Alfred Hospital.

DISCUSSION AND FUTURE DIRECTIONS

CHAPTER 9: DISCUSSION

The pathologist's role in the assessment of melanoma and melanoma-like lesions in our current molecular era is multi-faceted and dynamic. Pathologists must balance the reporting of rising volumes of skin biopsy specimens, which is driven by increased awareness and clinical monitoring across populations,[37] with the complexity of improving diagnostic accuracy, which is increasingly possible through the discovery and integration of molecular information.[13] Furthermore, the pathologist's role in the selection of treatment for melanoma patients, including those with high risk primary as well as those with metastatic melanoma, will potentially grow through novel predictive biomarker testing.

The works presented in this thesis have focussed on clinicopathological associations within these three domains of melanocytic pathology, namely: the judicious use of resources in high-volume melanoma pathology practice; the integration of molecular information to improve over- and under-diagnosis; and the practical application of emerging immunohistochemical biomarker expression to improve the selection of patients who may potentially benefit from various (mostly drug) therapies. Addressing issues around the careful use of resources and misdiagnosis or incorrect assessment across melanocytic pathology are priority areas of study.

Re-appraising established practices in melanoma pathology

In **Chapter 3 "Evaluation of multiple tissue levels frequently upstages patients with clinically localised thin primary cutaneous melanoma"**, we showed that examination of additional tissue levels in 100 µm increments results in upstaging of a patients' primary tumour stage in 20% (8/40) of cases (15% because of Breslow thickness (BT) alone, 2.5% because of ulceration alone, 2.5% because of BT and ulceration), with most cases affected being thin melanomas (thickness <1 mm). We found

an incremental effect of tissue levelling, such that 80% of cases of melanoma were accurately pathologically staged on the initial level, with roughly another 5% of patients being upstaged with each additional 100 μm interval examined (up to 400 μm). We found only cases initially staged as pT1 (the most common subcategory of invasive melanomas) were eventually upstaged because of increased BT, and the average increase in BT was 0.11 mm.

Our findings suggest that some melanomas are likely to be inadvertently under-staged in daily practice when assessment is based only on a single microscopic level of cuts made at the grossing bench, which is consistent with findings by other investigators[20,21,38-41]. However, our findings advance on this knowledge because, unlike other studies, we performed our tissue levels at consistent intervals and looked at multiple parameters of prognostic estimation. We therefore provide granular information to practically help pathologists choose what depth of tissue is most appropriate to ensure adequate assessment of melanomas, which is likely to improve stratification of patients whose melanomas lie on the cusp of AJCC staging thresholds (ie. pT1a versus pT1b or pT1b versus pT2a).

Our findings are also a counterpoint to investigators who advocate the use of immunohistochemistry (IHC) to improve the assessment of BT,[39,40] because our observations suggest that improved BT evaluation is, at least in part, a function of levelling the tissue rather than the stain used per se. While IHC stains may serve other functions in the assessment of melanoma, particularly around assisting in confirming the diagnosis, the H&E assessment is a more cost-effective stain, which is a priority for health economics.

Finally, we reiterate our discussion in this chapter that the upstaging effect seen in our study may help explain why a small percentage of patients with thin melanomas (≤1.0 mm) have unexpected poor prognoses.[24,25]

In our large cohort study of 640 patients presented in **Chapter 4 "Residual melanoma in wide local excision specimens after 'complete' excision of primary cutaneous in situ and invasive melanomas"**, we found that residual melanoma in WLE specimens after a complete excision biopsy with negative margins is an infrequent occurrence (3.1% of our patients) but, when found, it was associated with lentigo maligna (LM)/lentigo maligna melanoma (LMM) and nodular melanoma (NM) subtypes, higher mitotic rate, larger lesion diameter, and amelanosis. Interestingly, we did not identify any associations with features such as neurotropism, microsatellitosis and lymphovascular invasion, which are all poor prognostic factors and for which tumour beds are typically sampled in entirety according to most international sampling and reporting protocols.[42,43] On the basis of our study's findings, melanomas of LM/LMM and NM subtypes, higher mitotic rate, Breslow thickness >4 mm, larger lesion diameter and amelanosis probably warrant additional sampling of WLE specimens, and this evidence may inform future updates to reporting guidelines.

Evaluating applications of emergent technologies in melanoma pathology

We then turned our attention to how the discovery and integration of molecular knowledge can serve as a powerful ancillary diagnostic tool for morphologically challenging melanocytic lesions.

In **Chapter 5 "Molecular analysis of cutaneous sarcomatoid neoplasms frequently identifies melanoma driver variants"**, we found that nearly 20% of patients with an immunohistologically unclassifiable cutaneous tumour could be re-classified as UM or DM after genomic testing. These mutations were most commonly *NRAS* variants, and less commonly *KIT* and *GNAQ*. Tumours with and without melanoma mutations all had similar demographics, occurring in older males on the head and neck, and an epithelioid cell type was the only clue to the presence of a melanoma driver mutation.

As the first study to consider genomic profiling to stratify this histogenetically diverse group as a whole, we show how upfront molecular testing of sarcomatoid tumours has the potential to improve the detection of UM and DM, including at an earlier stage in a patient's disease course, thereby

opening-up alternative management pathways. By extension, our findings suggest that a small subset of patients with tumours diagnosed as AFX or PDS are likely to have been historically misdiagnosed. This has implications for our understanding of the natural history of these diseases as documented in past medical literature, and may account for the rare occurrence of tumours with rare 'aberrant' behaviour such as metastasising AFX and advanced PDS.[44,45]

The re-appraisal of diagnostic categories because of divergent genomic phenotypes is an area of active discovery across oncology, with molecular re-classification now affecting a wide spectrum of tumour types.[46-48]The recognition of distinct subtypes of melanoma according to aetiological pathways related to, or independent of, chronic sun damage is an example of the molecular refinement of a traditionally morphological classification amongst tumours of the same family.[48] By contrast, the results presented in our study above demonstrate the value of investigators turning their attention to morphologically similar but potentially lineage-diverse tumours, which can help in clarifying our understanding of unexpected disease courses and differing responses to treatment amongst apparently similar tumours. When using the approach of defining tumour lineage based on molecular phenotypes, it is important for studies to define this convention, particularly for tumours that share some genomic similarities (eg. high tumour mutational burden, UV signature, TERT promoter mutations and CDKN2A variants among UM/DM, AFX and PDS). In our study, we argue that because large cohort studies have helped to establish *BRAF, NRAS, KIT, GNAQ* and *GNA11* variants as the characteristic genotype of conventional (differentiated) melanomas,[19] and studies of confidently diagnosed AFX and PDS have shown they typically lack these variants,[27,49,50] there is validity in this approach.

It must be borne in mind that not all melanomas harbour a MAPK variant, so it remains possible that at least some tumours in our study (and in clinical practice) diagnosed as AFX or PDS could theoretically still be UM. The clinical relevance of this to treatment is important, particularly around implications for determining accurate prognosis and appropriate treatment. In our experience at

major skin cancer referral centres, we have encountered occasional advanced PDS tumours that show a response to immunotherapy that is akin to melanoma, a findings that has also been observed by other groups.[51,52]The results presented in Chapter 5 therefore lay the groundwork for future studies to consider whether undifferentiated sarcomatoid skin tumours with immunological and genomic similarities, such as brisk tumour-infiltrating lymphocytes, high tumour mutation burden, UV mutational signatures and TERT promoter mutations, may be more important than the tumour's histogenesis (ie. based on the presence or absence of melanoma-like MAPK variants) when choosing treatment.

In **Chapter 6 "Molecular profiling of noncoding mutations distinguishes naevoid melanomas from mitotically active nevi in pregnancy",** we turned our attention to expanding the evidence base for adjunct molecular features that might help to avoid over-diagnosis and under-diagnosis of ambiguous melanocytic neoplasms as a melanoma or a naevus. By comparing groups of naevoid melanomas (NMs) and mitotically active naevi of pregnancy (MANP), we showed that NMs harboured hotspot variants in *NRAS* (6/8, 75%) versus *BRAF* variants in MANPs (10/12, 83%). We also showed that noncoding mutations, mostly of *TERT* promoter (TERT-p), were significantly more common in NMs than in MANP. These findings are consistent with knowledge that replicative senescence overcome by mutations of *TERT* promoter is a common event in non-naevoid cutaneous melanomas.[53]

Since the publication of our study, we have seen a contribution of findings by other investigators when considering similar differential diagnoses.[32,54,55] In these studies, chromosomal aberrations and TERT-p mutations have been found to be helpful in differentiating between ambiguous benign and malignant melanocytic neoplasms when integrated into careful clinical and histopathological assessment. In an analysis of mitotically active melanocytic proliferations, Mesbah Ardakani *et al* used the presence or absence of cytogenetic evidence of loss of *CDKN2A* gene (chromosome 9p21) to define thirteen cases as NMs and twelve as mitotically active naevi.[32] These cytogenetic findings

were underpinned by reproducible immunomorphological features (deep/marginal mitoses, lack of maturation with depth, pseudo-maturation, loss of p16 immunohistochemistry staining) that could reliably distinguish NMs from mitotically active naevi.

In another group's investigation of eleven locally recurrent melanomas, seventeen recurrent naevi, and non-recurrent melanoma and naevus controls, Walton *et al* showed that hotspot TERT-p mutations were significantly more frequent in recurrent melanomas.

The replication of similar findings to ours strengthens the rationale for ancillary molecular testing to be used in the differential diagnosis of carefully selected types of challenging naevi versus melanoma. However, as previously discussed in Chapter 6, this principle is not necessarily transferable to all ambiguous melanocytic tumours. In a study of unambiguous and ambiguous melanocytic tumours published since ours, Boutko *et al* showed relatively low frequencies of TERT-p mutation in confidently diagnosed benign naevi (1%, 2/34) and dysplastic naevi (6%, 2/35), and a high frequency in confidently diagnosed melanomas (73%, 51/70).[55] By contrast, among their morphologically borderline melanocytic tumours, only 24% of those cases favoured upfront as melanoma had a positive TERT-p mutation status. They suggested that TERT-p mutation is most useful in the differential diagnosis of atypical deep penetrating naevus versus melanoma, has some value in differentiating atypical Spitz tumours and dysplastic naevi from melanomas, but is less informative between atypical and malignant blue naevi.[55]

A conundrum faced by studies investigating molecular differences between borderline melanocytic tumours is the inherent subjectivity of these tumours, such that melanoma cohorts may be contaminated by over-diagnosed melanomas, which in turn dilutes the effect of molecular differences. Study designs can be strengthened by collecting robust clinical follow-up data, which is absent from some of the above studies, but which we included in our study (24 to 60 months).[32,54,55]

In our final chapter in this section on adjunct molecular diagnostics, **Chapter 7 "Extraenteric gastrointestinal neuroectodermal tumour masquerading as mucosal melanoma of the bronchus"**, we considered unusual primary lung tumours where melanoma is a diagnostic consideration. The background literature review in this chapter discussed the rarity of primary melanoma of the lung, while our contemporary case study of a melanoma-like tumour that harboured a *CREB* family fusion, that was ultimately best regarded as extraintestinal gastrointestinal neuroectodermal tumour (GNET), provides an example that may support prior assertions questioning the true existence of primary lung melanoma. As part of this chapter's work, we attempted to expand our contribution beyond a single case study but were ultimately unable to do so. While we found several potential tumours for study in our archives, we were unable to identify a patient where there was appropriate consent that would ethically allow revision of their diagnosis based on a retrospective molecular analysis; neither did we encounter additional cases that could be studied in a prospective manner during the study period. This is not unexpected given that extra-enteric GNET cases are rare across the literature, with only a handful of cases being recognised so far in soft tissues of the trunk, the retroperitoneum, orbit, oral cavity, urinary bladder, falciform ligament/liver and lung.[56-58] The importance of our case in the literature is nonetheless to flag to pathologists that one should always question the diagnosis of a primary melanoma, not only to consider the more common occurrence of metastatic melanoma, but also to consider molecular testing for this unusual sarcoma, which has the potential to be increasingly recognised. Crucially, treatment options vary considerably for these clinical scenarios.

New treatment horizons

Melanoma experienced the first wave of its 'penicillin moment' with the discovery of BRAF-targeted therapies, and a second wave breakthrough with the advent of immunotherapies. There is a third wave potentially on the horizon with trials of immune-based treatments engineered to the PRAME protein currently underway. In the final section of this thesis, **Chapter 8 "Assessment of intrapatient**

intertumoural heterogeneity of PRAME immunohistochemistry expression in melanoma", we addressed the concordance of PRAME expression so that information can be available to clinical investigators early in the study of PRAME-targeted therapies, particularly if PRAME is to be studied as a potential predictive biomarker for these treatments.

In our study, we found moderate intra-patient inter-tumoural concordance of expression for PRAME IHC. PRAME score was concordant between PM and LNM in 70% of patients (16/23, *kappa* = 0.45, *p value* = 0.02); between PM and ITM in 70% (7/10, *kappa* = 0.32, *p value* = 0.227); between PM and DM in 48% (10/21, Kappa = 0.19, *p value* = 0.3); between LNM and ITM in 86% (6/8, *kappa* = 0.67, *p value* = 0.227); and between LNM and DM in 75% (6/8, *kappa* = 0.68, *p value* = 0.03). Only 62.5% (10/16) matched triplet specimens were concordant across all three specimens. We observed bi-directional heterogeneity of PRAME expression.

The importance of this study is underscored by the role that pathologists play in ensuring quality of pathology tests and the valid application of biomarker testing in clinical practice. PDL1 is an example of a widely used predictive biomarker that has previously been used in clinical practice to determine access to anti-PDL1 therapies for several tumour types, but which has recently (in Australia) been deemed no longer essential for future decision-making.[59] This is because of the poor real-world analytical performance of PD-L1 IHC testing, related to a host of factors such as different laboratory protocols and platforms, intra-tumoural heterogeneity, inter- and intra-observer variability, and inducibility of PDL-1 expression resulting in temporal heterogeneity.[59]

The moderate concordance in our study suggests that PRAME IHC may not be immune to these issues, and there is immense value in making these discoveries early in the study of a new treatment. Unfortunately, the recognition of PDL-1 heterogeneity in several tumour types came well after the establishment of anti-PDL1 therapies in practice; in Australia, there is currently a legacy for several tumour types (but not melanoma) where access to treatment is contingent on this poor performing

assay, with consequent ethical implications.[59] Concerningly, there may have been many patients in the anti-PDL-1 era who have not had access to a therapy from which they may have benefited. The value of our study is therefore in identifying potential concordance factors of the PRAME assay upfront, which will alert future investigators to pertinent issues if PRAME IHC is to be used as a criterion for enrolling patients into clinical trials, or studied as a predictive biomarker of treatment response in melanoma patients.

In conclusion, there are enduring difficulties in the pathological assessment of melanocytic lesions, and critical clinicopathological enquiry continues to incrementally advance melanoma oncology through objective findings. In this thesis, we have shown how methodical evaluation of long-standing histological techniques can improve prognostic estimates for patients; we have contributed several studies that illustrate the power of molecular analysis as an adjunctive diagnostic tool in selected morphologically challenging melanoma and melanoma-like lesions; and we have investigated the biological rationale for biomarker testing to ultimately predict response to a promising new melanoma therapy. Collectively these studies emphasise the profound and comprehensive role that pathologists have, and will continue to have, in optimising treatment and improving the outcomes for patients with melanoma.

REFERENCES (FOR CHAPTER 1 INTRODUCTION AND CHAPTER 9 DISCUSSION)

1. Fawdington T. A Case of Melanosis, with General Observations on the Pathology of the Interesting Disease. London: Longman, Orme, Brown, Robinson and Bent; 1826.

2. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. The New England journal of medicine. 2011;364:2507–2516. doi: 10.1056/NEJMoa1103782.

3. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010 Aug 19;363(8):711-23. doi: 10.1056/NEJMoa1003466. Epub 2010 Jun 5. Erratum in: N Engl J Med. 2010 Sep 23;363(13):1290. PMID: 20525992; PMCID: PMC3549297.

4. Rebecca VW, Sondak VK, Smalley KS. A brief history of melanoma: from mummies to mutations. Melanoma Res. 2012 Apr;22(2):114-22. doi: 10.1097/CMR.0b013e328351fa4d. PMID: 22395415; PMCID: PMC3303163.

5. Urteaga O, Pack GT. On the antiquity of melanoma. Cancer. 1966 May;19(5):607-10. doi: 10.1002/1097-0142(196605)19:5<607::aid-cncr2820190502>3.0.co;2-8. PMID: 5326247.

6. Norris W. Case of Fungoid Disease. Edinb Med Surg J. 1820;16:562–565.

7. Clark WH Jr, Elder DE, Guerry D 4th, Braitman LE, Trock BJ, Schultz D, Synnestvedt M, Halpern AC. Model predicting survival in stage I melanoma based on tumor progression. J Natl Cancer Inst. 1989 Dec 20;81(24):1893-904. doi: 10.1093/jnci/81.24.1893. PMID: 2593166.

8. Breslow A. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. Ann Surg. 1970 Nov;172(5):902-8. doi: 10.1097/00000658-197011000-00017. PMID: 5477666; PMCID: PMC1397358.

9. Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, et al. Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin. 2017; 67:472–491.

10. Cancer Council Australia. Clinical practice guidelines for the diagnosis and management of melanoma. Version 1.1. Published 4 May 2024. https://www.cancer.org.au/clinical-guidelines/skin-cancer/melanoma

11. Weber, Jeffrey. Immunotherapy for melanoma. Current Opinion in Oncology 23(2):p 163-169, March 2011. | DOI: 10.1097/CCO.0b013e3283436e79.

12. Elder DE, Eguchi MM, Barnhill RL, Kerr KF, Knezevich SR, Piepkorn MW, Reisch LM, Elmore JG. Diagnostic error, uncertainty, and overdiagnosis in melanoma. Pathology. 2023 Mar;55(2):206-213. doi: 10.1016/j.pathol.2022.12.345. Epub 2022 Dec 29. PMID: 36642569; PMCID: PMC10373372.

13. Andea AA. Molecular testing in melanoma for the surgical pathologist. Pathology. 2023 Mar;55(2):245-257. doi: 10.1016/j.pathol.2022.12.343. Epub 2022 Dec 28. PMID: 36653236.

14. Brinker TJ, Schmitt M, Krieghoff-Henning EI, Barnhill R, Beltraminelli H, Braun SA, Carr R, Fernandez-Figueras MT, Ferrara G, Fraitag S, Gianotti R, Llamas-Velasco M, Müller CSL, Perasole A, Requena L, Sangueza OP, Santonja C, Starz H, Vale E, Weyers W, Hekler A, Kather JN, Fröhling S, Krahl D, Holland-Letz T, Utikal JS, Saggini A, Kutzner H. Diagnostic performance of artificial intelligence for histologic melanoma recognition compared to 18 international expert pathologists. J Am Acad Dermatol. 2022 Mar;86(3):640-642. doi: 10.1016/j.jaad.2021.02.009. Epub 2021 Feb 11. PMID: 33581189.

15. Moseley H. In the AI science boom, beware: your results are only as good as your data. Nature. 2024 Feb 1. doi: 10.1038/d41586-024-00306-2. Epub ahead of print. PMID: 38302705.

16. Melanoma of the Skin; Australian Institute of Health and Welfare: Canberra, Australia, 2016. https://www.canceraustralia.gov.au/cancer-types/melanoma/statistics

17. Arnold M, Singh D, Laversanne M, Vignat J, Vaccarella S, Meheus F, Cust AE, de Vries E, Whiteman DC, Bray F. Global Burden of Cutaneous Melanoma in 2020 and Projections to 2040. JAMA Dermatol. 2022 May 1;158(5):495-503. doi: 10.1001/jamadermatol.2022.0160. PMID: 35353115; PMCID: PMC8968696.

18. McGovern VJ. The classification of melanoma and its relationship with prognosis. Pathology. 1970 Apr;2(2):85-98. doi: 10.3109/00313027009077330. PMID: 5520514.

19. Hayward, N., Wilmott, J., Waddell, N. et al. Whole-genome landscapes of major melanoma subtypes. Nature 545, 175–180 (2017). https://doi.org/10.1038/nature22071.

20. Bishop J, Tallon B. Number of levels required to assess Breslow thickness in cutaneous invasive melanoma: An observational study. J Cutan Pathol. 2019 Nov;46(11):819-822. doi: 10.1111/cup.13541. Epub 2019 Jul 22. PMID: 31270835.

21. Patrick RJ, Corey S, Glass LF. The use of sequential serial sectioning of thin melanomas in determining maximum Breslow depth. J Am Acad Dermatol. 2007; 57(5): S127-S128.

22. Tan SX, Aung TN, Claeson M, Acs B, Zhou C, Brown S, Lambie D, Baade PD, Pandeya N, Soyer HP, Smithers BM, Whiteman DC, Rimm DL, Khosrotehrani K. Automated scoring of tumor-infiltrating lymphocytes informs risk of death from thin melanoma: A nested case-case study. J Am Acad Dermatol. 2024 Jan;90(1):179-182. doi: 10.1016/j.jaad.2023.09.026. Epub 2023 Sep 19. PMID: 37730017.

23. Claeson M, Tan SX, Lambie D, Brown S, Walsh MD, Baade PD, Pandeya N, Whitehead KJ, Soyer HP, Smithers BM, Whiteman DC, Khosrotehrani K. The association between BRAF-V600E mutations and death from thin (≤1.00 mm) melanomas: A nested case-case study from Queensland, Australia. J Eur Acad Dermatol Venereol. 2023 Sep;37(9):e1168-e1172. doi: 10.1111/jdv.19173. Epub 2023 May 16. PMID: 37147869.

24. Murali R, Haydu LE, Long GV, Quinn MJ, Saw RP, Shannon K, Spillane AJ, Stretch JR, Kefford RF, Thompson JF, Scolyer RA. Clinical and pathologic factors associated with distant metastasis and survival in patients with thin primary cutaneous melanoma. Ann Surg Oncol. 2012 Jun;19(6):1782-9. doi: 10.1245/s10434-012-2265-y. Epub 2012 Feb 14. PMID: 22350600.

25. Hothem Z, Bayci A, Thibodeau BJ, Ketelsen BE, Fortier LE, Uzieblo AF, Cosner D, Totoraitis K, Keidan RD, Wilson GD. Using global gene expression to discriminate thin melanomas with poor outcomes. Mol Cell Oncol. 2016 Nov 8;4(1):e1253527. doi: 10.1080/23723556.2016.1253527. PMID: 28197532; PMCID: PMC5286964.

26. Santamaria-Barria JA, Mammen JMV. Surgical Management of Melanoma: Advances and Updates. Curr Oncol Rep. 2022 Nov;24(11):1425-1432. doi: 10.1007/s11912-022-01289-x. Epub 2022 Jun 3. PMID: 35657482.

27. Ak M, Kahraman A, Arnold FM, Turko P, Levesque MP, Zoche M, Ramelyte E, Dummer R. Clinicopathological and Genomic Profiles of Atypical Fibroxanthoma and Pleomorphic Dermal Sarcoma Identify Overlapping Signatures with a High Mutational Burden. Genes (Basel). 2021 Jun 25;12(7):974. doi: 10.3390/genes12070974. PMID: 34202213; PMCID: PMC8303615.

28. Agaimy A., Specht K., Stoehr R., Lorey T., Märkl B., Niedobitek G., Straub M., Hager T., Reis A.C., Schilling B., et al. Metastatic Malignant Melanoma With Complete Loss of Differentiation Markers (Undifferentiated/Dedifferentiated Melanoma): Analysis of 14 Patients Emphasizing Phenotypic Plasticity and the Value of Molecular Testing as Surrogate Diagnostic Marker. Am. J. Surg. Pathol. 2016;40:181–191. doi: 10.1097/PAS.0000000000000527.

29. Ferreira I, Droop A, Edwards O, et al. Clinicopathological spectrum and genomic landscape of de-/trans-differentiated melanomas. Mod Pathol. 2021 Nov;34(11):2009-2019. doi: 10.1038/s41379-021-00857-z.

30. Theodosiou G, Johansson I, Hamnerius N, et al. Naevoid malignant melanoma: a diagnosis of a naevus that you later regret. Acta Derm Venereol. 2017;97:745–746.

31. Chan MP, Chan MM, Tahan SR. Melanocytic nevi in pregnancy: histologic features and Ki-67 proliferation index. J Cutan Pathol. 2010;37:843–851.

32. Mesbah Ardakani N, Singh S, Thomas C, Van Vliet C, Harvey NT, Calonje JE, Wood BA. Mitotically Active Nevus and Nevoid Melanoma: A Clinicopathological and Molecular Study. Am J Dermatopathol. 2021 Mar 1;43(3):182-190. doi: 10.1097/DAD.0000000000001721. PMID: 32649342.

33. Yousef S, Joy C, Velaiutham S, Maclean FM, Harraway J, Gill AJ, Vargas AC. Dedifferentiated melanoma with MDM2 gene amplification mimicking dedifferentiated liposarcoma. Pathology. 2022 Apr;54(3):371-374. doi: 10.1016/j.pathol.2021.05.096. Epub 2021 Aug 20. PMID: 34420795.

34. Xu, Y.; Zou, R.; Wang, J.; Wang, Z.W.; Zhu, X. The role of the cancer testis antigen PRAME in tumorigenesis and immunotherapy in human cancer. Cell Prolif. 2020, 53, e12770.

35. Kirkey DC, Loeb AM, Castro S, McKay CN, Perkins L, Pardo L, Leonti AR, Tang TT, Loken MR, Brodersen LE, Loeb KR, Scheinberg DA, Le Q, Meshinchi S. Therapeutic targeting of PRAME with mTCRCAR T cells in acute myeloid leukemia. Blood Adv. 2023 Apr 11;7(7):1178-1189. doi: 10.1182/bloodadvances.2022008304. PMID: 35984639; PMCID: PMC10111362.

36. Hamid O, Williams A, Lopez JS, Olson D, Sato T, Shaw HM, Friedman CF, Thistlethwaite F, Middleton MR, Lebbe C, Ma VT, Izar B, Kar Han Lau P, Bechter OE, Kirk P, Yuan Y, Marshall S, Davar D. Phase 1 safety and efficacy of IMC-F106C, a PRAME × CD3 ImmTAC bispecific, in post-checkpoint cutaneous melanoma (CM).. JCO 42, 9507-9507(2024).DOI:10.1200/JCO.2024.42.16_suppl.9507

37. Leachman SA, Latour E, Detweiler-Bedell B, Detweiler-Bedell JB, Zell A, Wenzel E, Stoos E, Nelson JH, Wiedrick J, Berry EG, Lange J, Etzioni R, Lapidus JA. Melanoma literacy among the general

population of three western US states. Pigment Cell Melanoma Res. 2023 Nov;36(6):481-500. doi: 10.1111/pcmr.13106. Epub 2023 Aug 13. PMID: 37574711.

38. Dyson SW, Bass J, Pomeranz J, Jaworsky C, Sigel J, Somach S. Impact of thorough block sampling in the histologic evaluation of melanomas. Arch Dermatol. 2005 Jun;141(6):734-6. doi: 10.1001/archderm.141.6.734. PMID: 15967919.

39. Pop AM, Monea M, Olah P, Moraru R, Cotoi OS. The Importance of Immunohistochemistry in the Evaluation of Tumor Depth of Primary Cutaneous Melanoma. Diagnostics (Basel). 2023 Mar 7;13(6):1020. doi: 10.3390/diagnostics13061020. PMID: 36980327; PMCID: PMC10046945.

40. Kamyab-Hesary, K., Ghanadan, A., Balighi, K. et al. Immunohistochemical Staining in the Assessment of Melanoma Tumor Thickness. Pathol. Oncol. Res. 26, 885–891 (2020). https://doi.org/10.1007/s12253-019-00635-y.

41. Solomon AR, Ellis CN, Headington JT. An evaluation of vertical growth in thin superficial spreading melanomas by sequential serial microscopic sections. Cancer. 1983 Dec 15;52(12):2338-41. doi: 10.1002/1097-0142(19831215)52:12<2338::aid-cncr2820521229>3.0.co;2-t. PMID: 6357428.

42. Scolyer RA, Balamurgan T, Busam K, Elder D, Evans A, Gershenwald J, Frishberg DP, McMenamin M, Prieto VG, Shiau C, Swetter S, van den Oord J (2019). Invasive Melanoma Histopathology Reporting Guide. 2nd edition. International Collaboration on Cancer Reporting; Sydney, Australia. ISBN: 978-1-925687-32-3.

43. Royal College of Pathologists of Australasia. Invasive Melanoma Structured Reporting Protocol (3rd Edition, 2023). Available at: https://www.rcpa.edu.au/Library/Practising-Pathology/Structured-Pathology-Reporting-of-Cancer/Cancer-Protocols/Skin-Adnexal/Protocol-Melanoma.aspx

44. Wang WL, Torres-Cabala C, Curry JL, Ivan D, McLemore M, Tetzlaff M, Zembowicz A, Prieto VG, Lazar AJ. Metastatic atypical fibroxanthoma: a series of 11 cases including with minimal and no subcutaneous involvement. Am J Dermatopathol. 2015 Jun;37(6):455-61. doi: 10.1097/DAD.0000000000000237. PMID: 25590287.

45. Tolkachjov SN, Kelley BF, Alahdab F, Erwin PJ, Brewer JD. Atypical fibroxanthoma: Systematic review and meta-analysis of treatment with Mohs micrographic surgery or excision. J Am Acad Dermatol. 2018 Nov;79(5):929-934.e6. doi: 10.1016/j.jaad.2018.06.048. Epub 2018 Jul 5. PMID: 29981390.

46. Casuscelli J, Vano YA, Fridman WH, Hsieh JJ. Molecular Classification of Renal Cell Carcinoma and Its Implication in Future Clinical Practice. Kidney Cancer. 2017 Jul 26;1(1):3-13. doi: 10.3233/KCA-170008. PMID: 30334000; PMCID: PMC6179110.

47. Bertero L, Mangherini L, Ricci AA, Cassoni P, Sahm F. Molecular neuropathology: an essential and evolving toolbox for the diagnosis and clinical management of central nervous system tumors. Virchows Arch. 2024 Feb;484(2):181-194. doi: 10.1007/s00428-023-03632-4. Epub 2023 Sep 2. PMID: 37658995; PMCID: PMC10948579.

48. Elder DE, Bastian BC, Cree IA, Massi D, Scolyer RA. The 2018 World Health Organization Classification of Cutaneous, Mucosal, and Uveal Melanoma: Detailed Analysis of 9 Distinct Subtypes Defined by Their Evolutionary Pathway. Arch Pathol Lab Med. 2020 Apr;144(4):500-522. doi: 10.5858/arpa.2019-0561-RA. Epub 2020 Feb 14. PMID: 32057276.

49. Griewank KG, Wiesner T, Murali R, et al. Atypical fibroxanthoma and pleomorphic dermal sarcoma harbor frequent NOTCH1/2 and FAT1 mutations and similar DNA copy number alteration profiles. Mod Pathol. 2018 Mar;31(3):418-428. doi: 10.1038/modpathol.2017.146.

50. Sakamoto A, Oda Y, Itakura E, Oshiro Y, Tamiya S, Honda Y, Ishihara A, Iwamoto Y, Tsuneyoshi M. H-, K-, and N-ras gene mutation in atypical fibroxanthoma and malignant fibrous histiocytoma. Hum Pathol. 2001 Nov;32(11):1225-31. doi: 10.1053/hupa.2001.28953. PMID: 11727262.

51. Klein S, Persa OD, Mauch C, Noh KW, Pappesch R, Wagener-Ryczek S, Buettner R, Quaas A, Helbig D. First report on two cases of pleomorphic dermal sarcoma successfully treated with immune checkpoint inhibitors. Oncoimmunology. 2019 Sep 20;8(12):e1665977. doi: 10.1080/2162402X.2019.1665977. PMID: 31741767; PMCID: PMC6844321.

52. Cheuque, R.P. Román et al. Pembrolizumab treatment for advanced pleomorphic dermal sarcoma, a three-case series in a spanish tertiary-level hospital. EJC Skin Cancer, Volume 2, 100205. doi: 10.1016/j.ejcskn.2024.100205

53. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA. Highly recurrent TERT promoter mutations in human melanoma. Science. 2013 Feb 22;339(6122):957-9. doi: 10.1126/science.1229259. Epub 2013 Jan 24. PMID: 23348506; PMCID: PMC4423787.

54. Walton KE, Garfield EM, Zhang B, Quan VL, Shi K, Mohan LS, Haugh AM, VandenBoom T, Yazdan P, Isales MC, Panah E, Gerami P. The role of TERT promoter mutations in differentiating recurrent nevi

from recurrent melanomas: A retrospective, case-control study. J Am Acad Dermatol. 2019 Mar;80(3):685-693. doi: 10.1016/j.jaad.2018.09.030. Epub 2018 Oct 1. PMID: 30287318.

55. Boutko A, Asadbeigi S, Roth A, Lampley N, Olivares S, Dittmann D, Dittmann D, Jennings L, Gerami P. TERT Promoter Mutational Analysis as an Ancillary Diagnostic Tool for Diagnostically Challenging Melanocytic Neoplasms. Am J Dermatopathol. 2023 May 1;45(5):289-299. doi: 10.1097/DAD.0000000000002366. Epub 2023 Feb 17. PMID: 36898007.

56. Ulici V, Hornick JL, Davis JL, Mehrotra S, Meis JM, Halling KC, Fletcher CDM, Kao E, Folpe AL. Extraenteric Malignant Gastrointestinal Neuroectodermal Tumor-A Clinicopathologic and Molecular Genetic Study of 11 Cases. Mod Pathol. 2023 Jul;36(7):100160. doi: 10.1016/j.modpat.2023.100160. Epub 2023 Mar 17. PMID: 36934861.

57. Allanson BM, Weber MA, Jackett LA, Chan C, Lau L, Ziegler DS, Warby M, Mayoh C, Cowley MJ, Tucker KM, Long GV, Maher A, Anazodo A, Scolyer RA. Oral malignant gastrointestinal neuroectodermal tumour with junctional component mimicking mucosal melanoma. Pathology. 2018 Oct;50(6):648-653. doi: 10.1016/j.pathol.2018.07.002. Epub 2018 Aug 31. PMID: 30177220.

58. Zheng Q, Chen H, Li Y. Primary Gastrointestinal-Type Clear Cell Sarcoma-like Tumor of the Bronchus: A Hitherto Unreported Bronchial Tumor. J Thorac Oncol. 2019 Sep;14(9):e202-e205. doi: 10.1016/j.jtho.2019.04.030. PMID: 31445737.

59. Medical Services Advisory Committee. MSAC Position Statement on programmed death-ligand 1 (PD-L1) immunohistochemistry testing to determine eligibility for treatment with PD-(L)1 checkpoint inhibitors. Accessed at: https://www.msac.gov.au/search/funnelback?query=pdl1&search_scope=0 (accessed 15 February 2025).

APPENDIX

CONTRIBUTION OF THE CANDIDATE

Contribution of the candidate

Jackett LA, Scolyer RA. A Review of Key Biological and Molecular Events Underpinning Transformation of Melanocytes to Primary and Metastatic Melanoma. Cancers (Basel). 2019 Dec 17;11(12):2041. doi: 10.3390/cancers11122041. PMID: 31861163; PMCID: PMC6966527.

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Writing of the manuscript

Preparation of figures

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

Richard A. Scolyer

Contribution of the candidate

Jackett LA, Gullifer JP, Scolyer RA. Evaluation of Multiple Tissue Levels Frequently Upstages Patients With Clinically Localized Thin Primary Cutaneous Melanoma. J Cutan Pathol. 2024 Oct 2. doi: 10.1111/cup.14726. Epub ahead of print. PMID: 39357975.

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Development of study concept and design

Identification of cases

Correlation of clinical data and tissue samples

Histopathological review

Data analysis and interpretation

Writing of the manuscript

Preparation of tables and figures

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

James P. Gullifer Richard A. Scolyer

Contribution of the candidate

Jackett LA, Satgunaseelan L, Roper E, Lo SN, Thompson JF, Scolyer RA. Residual melanoma in wide local excision specimens after 'complete' excision of primary cutaneous in situ and invasive melanomas. Pathology. 2022 Feb;54(1):71-78. doi: 10.1016/j.pathol.2021.05.094. Epub 2021 Aug 13. PMID: 34392983.

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Development of study concept and design

Identification of cases

Correlation of clinical data and tissue samples

Histopathological review

Data analysis and interpretation

Writing of the manuscript

Preparation of tables and review of figures

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

Laveniya Satgunaseelan	Edward Roper	Serigne Lo
John F. Thompson	Richard A. Scolyer	

Contribution of the candidate

Jackett LA, Mitchell C, Snell C, Hewitt C, Yellenki S, Snow H, Speakman D, Angel C, Khoo C, Pang J, Lo S, Scolyer RA, Fox S, Gyorki D. Molecular analysis of cutaneous sarcomatoid neoplasms frequently identifies melanoma driver variants. Accepted to American Journal of Surgical Pathology, February 2025.

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Development of study concept and design

Identification of cases

Correlation of clinical data and tissue samples

Histopathological review

Review of molecular data

Data analysis and interpretation

Writing of the manuscript

Preparation of tables and figures

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

Catherine Mitchell	Cameron Snell	Chelsee Hewitt	
Shravan Yellenki	Hayden Snow	David Speakman	
Chris Angel	Christine Khoo	Jia-Min Pang	
Serigne Lo	Richard A Scolyer	Stephen Fox	David Gyorki

Contribution of the candidate

Jackett LA, Colebatch AJ, Rawson RV, Ferguson PM, Thompson JF, McCarthy SW, Wilmott JS, Scolyer RA. Molecular Profiling of Noncoding Mutations Distinguishes Nevoid Melanomas From Mitotically Active Nevi in Pregnancy. Am J Surg Pathol. 2020 Mar;44(3):357-367. doi: 10.1097/PAS.0000000000001406. PMID: 31743128.

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Development of study concept and design

Identification of cases

Correlation of clinical data and tissue samples

Histopathological review

Data analysis and interpretation

Writing of the manuscript

Preparation of tables and review of figures

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

Andrew J. Colebatch	Robert V. Rawson	Peter M. Ferguson
John F. Thompson	James S. Wilmott	Richard A. Scolyer

(Stan W. McCarthy *deceased*)

Contribution of the candidate

Jackett LA, Mitchell C, Chu J. Extraenteric gastrointestinal neuroectodermal tumour masquerading as mucosal melanoma of the bronchus. Submitted to: Pathology, February 2025

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Development of study concept

Identification of case

Correlation of clinical and outcome data

Histopathological review

Correlation of FISH and molecular testing

Writing of the manuscript

Preparation of figure

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

Catherine Mitchell Julie Chu

Contribution of the candidate

Jackett LA, Scolyer RA, Bishop JF, Thompson JF (2017) Primary melanoma of the lung, In: Raghaven D, Ahluwalia MS, Blanke CD, Brown J, Kim ES, Reaman GH, Sekered MA (eds) Textbook of Uncommon Cancer 5th Edition, Wiley-Blackwell, west Essex, pp 285–292

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Collaborative writing of the manuscript

Preparation of tables

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

Richard A. Scolyer J. F. Bishop John F. Thompson

Contribution of the candidate

Jackett LA, Conway J, Thompson JF, Scolyer RA. Assessment of intrapatient homogeneity of PRAME immunohistochemistry expression in melanoma. Submitted to: Modern Pathology, February 2025.

The below named authors have confirmed that the following is a fair and accurate description of the contribution made by **Louise A. Jackett**:

Literature review

Development of study concept and design

Correlation of clinical data and tissue samples

Histopathological review

Immunohistochemistry assessment

Data analysis and interpretation

Writing of the manuscript

Preparation of tables and figures

Submission and approval of the manuscript and associated administrative work

Electronically signed/acknowledged:

Jordan Conway John F. Thompson Richard A. Scolyer

Contribution of the candidate

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Louise A. Jackett

February 2025

As **supervisor** for the candidature upon which this thesis is based, I can confirm that the authorship attributions statements above are correct.

Professor Richard A. Scolyer AO BMedSc MBBS MD FRCPA FRCPath

February 2025