Dissertationes Universitatis Helsingiensis
75/2023

Department of Plastic Surgery
Helsinki University Hospital
University of Helsinki
Finland

PROGNOSTIC FACTORS AND SENTINEL LYMPH NODE BIOPSY IN REGIONALLY ADVANCED MELANOMA

Mikko Vuoristo

DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Medicine of the University of Helsinki, in Niilo Hallman Lecture Hall, Park Hospital, on the 3rd of November 2023 at 12 o'clock.

Helsinki 2023

Helsinki University Hospital, Department of Plastic Surgery
University of Helsinki, Faculty of Medicine, Doctoral Programme of Clinical Research

Supervised by

Docent Tiina Jahkola, MD, PhD
Department of Plastic Surgery
Helsinki University Hospital
University of Helsinki
Helsinki, Finland

Reviewed by

Docent Ilkka Koskivuo, MD, PhD
Department of Plastic and General Surgery
Turku University Hospital
University of Turku
Turku, Finland

and

Docent Johanna Palve, MD, PhD
Department of Plastic Surgery
Tampere University Hospital
University of Tampere
Tampere, Finland

Opponent

Professor Marc Moncrieff, MD, FRCS
Department of Plastic & Reconstructive Surgery
Norfolk & Norwich University Hospital
University of East Anglia
Norwich, United Kingdom

The Faculty of Medicine uses the Ouriginal system (plagiarism recognition) to examine all doctoral dissertations.

Publisher: University of Helsinki
Series: Dissertationes Universitatis Helsingiensis 75/2023
ISBN 978-951-51-9486-2 (print)
ISBN 978-951-51-9487-9 (online)
ISSN 2954-2898 (print)
ISSN 2954-2952 (online)
PunaMusta, Joensuu 2023

To melanoma patients and their loved ones

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Vuoristo M, Vihinen P, Vlaykova T, Nylund C, Heino J, Pyrhönen S. Increased gene expression levels of collagen receptor integrins are associated with decreased survival parameters in patients with advanced melanoma. Melanoma Res. 2007 Aug;17(4):215-23.[1]

II Vuoristo M, Muhonen T, Koljonen V, Juteau S, Hernberg M, Ilmonen S, Jahkola T. Long-term prognostic value of sentinel lymph node tumor burden in survival of melanoma patients. Acta Oncol. 2021 Jun;60(6):803-807.

III Vuoristo M, Muhonen T, Koljonen V, Juteau S, Hernberg M, Ilmonen S, Jahkola T. Pelvic sentinel lymph nodes have minimal impact on survival in melanoma patients. BJS Open. 2021 Nov 9;5(6)

IV Vuoristo M, Juteau S, Koljonen V, Hernberg M, Mätzke S, Ilmonen S, Jahkola T. Hot Dots – Which Nodes Should Be Removed in Sentinel Lymph Node Biopsy for Melanoma? Acta Oncol. 2023 Sep;62(9):1021-1027.

The publications are referred to in the text by their roman numerals.

[1] Study I has been previously included in thesis "Collagen binding integrins and cancer testis antigens in prostate cancer and melanoma" by Camilla Nylund, University of Turku, 2016.

ABBREVIATIONS

AJCC	American Joint Committee on Cancer
Bcl-2	B-cell lymphoma 2
CLND	completion lymph node dissection
CT	computed tomography
DeCOG	Dermatologic Cooperative Oncology Group
DFS	disease-free survival
ELND	elective lymph node dissection
ICI	immune checkpoint inhibitor
IHC	immunohistochemistry
ILP	isolated limb perfusion
mRNA	messenger ribonucleic acid
MSLT	Multicentre Selective Lymphadenectomy Trial
MSS	melanoma-specific survival
NSN	non-sentinel lymph node
OS	overall survival
PFS	progression-free survival
PMT	primary melanoma tumour
PSLN	pelvic sentinel lymph node
RT-PCR	reverse transcriptase polymerase chain reaction
SLN	sentinel lymph node
SNB	sentinel (lymph) node biopsy
SPECT	single photon emission computed tomography
^{99m}Tc	technetium-99m
TLND	therapeutic lymph node dissection
TNM	Tumour Node Metastasis
T-VEC	talimogene laherperepvec
WLE	wide local excision

ABSTRACT

Background
Melanoma is a skin malignancy with a reputation for metastatic potential. The incidence of skin melanoma has been increasing in the western world in recent decades. Although most early-stage melanomas can be curatively treated surgically, advanced melanoma is associated with poor survival. Sentinel lymph node biopsy (SNB) was developed to identify patients with microscopic lymph node metastases. The status of the sentinel lymph node (SLN) is the most important prognostic factor in melanoma patients with no clinically detected metastasis. Together with the thickness of the primary melanoma tumour (PMT), SLN status is the most valuable prognostic tool for clinical decision making. New prognostic factors are needed. Integrins are cell adhesion molecules associated with cancer progression. As completion lymph node dissection (CLND) has been abandoned for most melanoma patients, and systemic therapy of melanoma is rapidly evolving, the role of sentinel lymph node biopsy is highlighted. Although SNB has become a standard procedure, several controversies remain.

In this thesis, collagen binding integrins $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha11\beta1$ and SLN tumour burden as prognostic markers for advanced melanoma were studied. The role of pelvic sentinel lymph nodes (PSLN) was evaluated. The radioactivity counts and different criteria for selecting SLNs intraoperatively were compared to determine which lymph nodes should be removed in SNB.

Patients and Methods
The patient population of each study in this thesis consisted of melanoma patients treated in Helsinki University Hospital. The first study consisted of 26 patients with metastatic melanoma who were treated and followed up between December 1988 and January 1996. Tumour samples were taken when first recurrence after primary surgery was suspected, before chemoimmunotherapy initiation. Real-time quantitative PCR was used to study the mRNA expression levels of collagen receptor integrin chains. In studies II (N=173) and IV (N=175), the study population consisted of melanoma patients with no clinically evident metastatic disease who underwent SNB between 2001 and 2008 and presented with one or more positive (*i.e.* metastatic) SLNs. The study group in Study III consisted of 285 cutaneous melanoma patients whose PMT was in the lower limb or trunk and who had no clinically detected metastases at the time of the diagnosis and underwent inguinal or iliac SNB (or both) between 2009 and 2013. Hospital medical records were reviewed, and follow-up data were collected retrospectively.

Results

In Study I, survival of melanoma patients after initiation of chemoimmunotherapy was decreased in all patients whose tumours expressed high mRNA levels of α1β1, α2β1, and α11β1 integrins. High α2 expression levels were associated with unfavourable overall survival. Patients with high mRNA levels of all studied integrins had a significantly shorter survival from the appearance of the first metastasis than patients with low levels of integrins.

In Study II, the most important prognosticators for survival were age, PMT thickness, ulceration, diameter of SLN metastasis, microanatomic location of the SLN metastasis, number of positive SLNs, and presence of positive non-sentinel lymph nodes (NSNs) in CLND. The diameter of the SLN metastasis was a strong independent prognosticator for survival. The strongest predictive factors of positive NSNs were diameter of the SLN metastasis, number of positive SLNs, and location of SLN metastasis. Of patients, 36% with SLN metastasis diameter >4 mm and 7% with SLN metastasis ≤4 mm had positive NSNs. Patients with three or more positive SLNs had poorer survival and increased risk for positive NSNs.

In Study III, all patients had superficial femoral/inguinal SLNs removed and 199 (69.8%) presented with pelvic SLNs. Of patients, 63 (22.1%) had metastases in their SLNs and 7 (2.5%) in PSLNs. A single patient had metastases solely in PSLNs. Removing PSLNs or the number of PSLNs retrieved had no impact on progression-free survival or overall survival. The only predictor of positive PSLNs was radioactivity count greater or equal to the hottest superficial SLNs.

In Study IV, the hottest (*i.e.* most radioactive) node was negative in 38 (22%) patients. By removing the hottest node and all nodes with radioactivity >10% of the hottest node, 97% of patients would have been staged correctly. In five patients, metastasis was found solely in a SLN with radioactivity <10% of the hottest node. Patients with a negative hottest node were associated with a lower SLN tumour burden.

Conclusions

Collagen receptor integrins are associated with the progression and prognosis of melanoma. α2 integrin in particular may be a potential prognostic marker and drug target.

Tumour burden of SLN, especially diameter of the SLN metastasis provides valuable prognostic information. Patients with NSN metastases have significantly poorer prognosis. Patients with SLN metastasis >4 mm and more than two positive SLNs are at greatest risk of harbouring NSN metastases.

Pelvic SLNs have minimal clinical impact on staging and outcomes of melanoma patients especially in cases with negative superficial inguinal SLNs.

To detect SLN metastases with acceptable accuracy, a threshold based on the relative radioactivity count of SLNs should be preferred to removing only one to three of the hottest nodes in SNB.

TIIVISTELMÄ (FINNISH ABSTRACT)

Tutkimuksen tausta

Melanooma on ihosyövistä huonomaineisin, koska se voi lähettää etäpesäkkeitä. Melanooman ilmaantuvuus on ollut kasvussa länsimaissa viimeisten vuosikymmenien ajan. Vaikka useimmat alkuvaiheen melanoomat ovat parannettavissa kirurgisesti, levinneellä taudilla on huono ennuste. Vartijasolmukebiopsia kehitettiin tunnistamaan melanoomapotilaat, joilla on mikroskooppisen pieniä etäpesäkkeitä imusolmukkeissa. Etäpesäkkeen löytyminen vartijasolmukkeesta on tärkein yksittäinen ennustetekijä melanoomapotilailla, joilla ei ole kliinisesti havaittavia etäpesäkkeitä. Yhdessä melanooman primaarikasvaimen paksuuden kanssa vartijasolmukebiopsian tulos on tärkein työkalu kliinisessä päätöksenteossa näillä potilailla. Uusia ennustetekijöitä tarvitaan. Integriinit ovat solukalvon läpäiseviä tarttumisreseptoreja, jotka on yhdistetty syövän leviämiseen. Täydentävästä imusolmukkeiden poistoleikkauksesta on pääosin luovuttu ja melanooman lääkehoidot ovat kehittyneet. Vartijasolmukebiopsian merkitys on korostunut. Vartijasolmukebiopsia on vakiintunut osa melanoomapotilaiden hoitoa, mutta toimenpiteeseen liittyy edelleen useita kiistanalaisuuksia.

Tässä väitöskirjatutkimuksessa arvioitiin kollageenireseptori-integriinejä ja vartijasolmukkeen etäpesäkkeen kokoa melanooman ennustetekijöinä. Tutkimuksessa arvioitiin lantion parailiakaalisten vartijasolmukkeiden merkitystä. Lisäksi selvitettiin, mitkä imusolmukkeet tulisi poistaa vartijasolmukebiopsiassa vertaamalla näiden radioaktiivisuuksia ja etäpesäkkeen löytymistä vartijasolmukkeessa.

Aineisto ja menetelmät

Väitöskirjan osatutkimusten potilaat olivat Helsingin yliopistollisessa sairaalassa hoidettuja melanoomapotilaita. Ensimmäinen osatutkimus koostui 26:sta levinnyttä melanoomaa sairastavasta potilaasta, joita hoidettiin ja seurattiin v. 1988-96. Kasvainnäytteet otettiin ensimmäisen uusiutuman yhteydessä ennen kemoimmunoterapian aloitusta. Integriinien ilmentymistä mitattiin kvantitatiivisella käänteistranskriptaasipolymeraasiketjureaktio-tekniikalla. Osatutkimuksissa II (173 potilasta) ja IV (175 potilasta) tutkittiin melanoomapotilaita, joilla ei ollut kliinisesti havaittavia metastaaseja. Heille tehtiin vartijasolmukebiopsia v. 2001-08 ja todettiin yksi tai useampia positiivisia vartijasolmukkeita eli vartijasolmukkeita, joista löytyi etäpesäke. Osatutkimuksen III aineisto koostui 285 melanoomapotilaasta, joille tehtiin vartijasolmukebiopsia v. 2009-13, missä poistettiin imusolmukkeita nivusten ja lantion alueelta. Seurantatiedot haettiin takautuvasti potilasarkistosta.

Tulokset

Osatutkimuksessa I potilaiden ennuste kemoimmunoterapian aloituksen jälkeen oli huonompi niillä potilailla, joiden kasvainnäytteissä todettiin korkeat kollageeni-integriinien ilmentymistasot. Potilailla, joilla α2β1-integriinin ilmentymistasot olivat korkeat, kokonaiselossaoloajan mediaani oli lyhyempi kuin potilailla, joilla ilmentymistasot olivat matalat.

Osatutkimuksessa II tärkeimmät elinaikaa ennustavat muuttujat olivat ikä, primaarikasvaimen paksuus, primaarikasvaimen haavautuminen, vartijasolmukkeen etäpesäkkeen läpimitta, etäpesäkkeen mikroanatominen sijainti vartijasolmukkeessa, positiivisten vartijasolmukkeiden määrä ja etäpesäkkeiden löytyminen imusolmukkeiden täydentävässä poistoleikkauksessa. Vartijasolmukkeen etäpesäkkeen läpimitta oli vahva, itsenäinen ennustetekijä. Tärkeimmät täydentävässä imusolmukkeiden poistoleikkauksessa etäpesäkkeiden löytymistä ennustavat muuttujat olivat vartijasolmukkeen etäpesäkkeen läpimitta, positiivisten vartijasolmukkeiden määrä ja etäpesäkkeen sijainti vartijasolmukkeessa.

Kolmannessa osatutkimuksessa kaikilta 285 potilaalta poistettiin vartijasolmukkeita pinnalliselta nivusalueelta ja 199 potilaalla (69,8 %) lisäksi parailiakaalialueelta. 63 potilaalla (22,1 %) todettiin etäpesäkkeitä vartijasolmukkeessa ja 7 potilaalla (2,5 %) oli etäpesäke parailiakaalisessa imusolmukkeessa. Yhdellä potilaalla todettiin etäpesäke ainoastaan parailiakaalisessa imusolmukkeessa. Parailiakaalisten solmukkeiden poistaminen tai niiden määrä ei vaikuttanut ennusteeseen. Parailiakaalisen vartijasolmukkeen radioaktiivisuuden ollessa vähintään aktiivisimpien pinnallisten vartijasolmukkeiden tasolla, etäpesäkkeen löytyminen parailiakaalisesta imusolmukkeesta oli todennäköisempää.

Neljännessä osatutkimuksessa 38 potilaalla (22 %) (radio)aktiivisin vartijasolmuke oli negatiivinen. Poistamalla aktiivisin vartijasolmuke ja kaikki imusolmukkeet, joiden radioaktiivisuus oli yli 10 % aktiivisimmista vartijasolmukkeesta, 97 % potilaan levinneisyysaste olisi määritelty oikein. 5 potilaalla ainoa etäpesäke löytyi imusolmukkeesta, jonka aktiivisuus oli alle 10 % verrattuna aktiivisimpaan imusolmukkeeseen.

Johtopäätökset

Kollageeni-integriinit, erityisesti α2β1-integriini on merkittävä ennustetekijä melanoomapotilailla, joilla on todettu etäpesäkkeitä. Vartijasolmukkeen etäpesäkkeen läpimitta on tärkeä, itsenäinen ennustetekijä. Potilailla, joiden vartijasolmukkeen etäpesäkkeen koko on yli 4 mm, ja joilla on enemmän kuin 2 positiivista vartijasolmuketta, todetaan useimmin etäpesäkkeitä täydentävässä imusolmukkeiden poistoleikkauksessa. Parailiakaalisilla vartijasolmukkeilla on vain vähäistä vaikutusta melanoomapotilaiden levinneisyysasteeseen ja ennusteeseen. Vartijasolmukkeita poistettaessa on suositeltavampaa käyttää suhteellista radioaktiivisuuden raja-arvoa kuin poistaa vain 1-3 aktiivisinta solmuketta.

1 INTRODUCTION

Melanoma is the deadliest form of skin cancer, and its incidence has been increasing in western world in recent decades. Surgery is the most important treatment, as 90% of melanomas can be cured if caught in the early stages of the disease. Advanced melanoma is an unpredictable and often aggressive disease. Even stage I melanomas may later progress and metastasize. There is an ongoing need for new prognostic tools to determine which melanomas are likely to metastasize and would therefore benefit from closer follow up and adjuvant therapy.

In most cases, melanoma first metastasizes from the primary tumour site to the adjacent lymph nodes. To accurately determine melanoma staging, the concept and technique sentinel lymph node biopsy (SNB) was introduced in 1992 (1). Since then, SNB has gained wide popularity and has become a standard procedure in early-stage melanomas with no clinically evident metastasis. For these patients, the SLN status is the most important prognostic marker.

For more than two decades, patients with a positive SLN underwent completion lymph node dissection (CLND). Approximately 20% of patients undergoing CLND present with additional metastatic lymph nodes. The procedure is associated with considerable risk of complications, including seroma and lymphedema. Justified concerns were raised whether the benefits of the procedure would outweigh the adverse events. Several studies attempted to identify factors regarding the primary tumour and SLN that would predict the result of CLND and thus identify patients that could be safely spared from further surgery. Two large randomized multicentre trials (MSLT-2 and DeCOG) failed to show any survival benefit for CLND (2, 3). This led to a paradigm shift and CLND is now not performed for most patients. Instead of trying to identify patients who can safely avoid CLND, the focus is now on identifying patients who may benefit from further surgery, if any.

Despite active research on molecular markers in melanoma, Breslow thickness of the primary melanoma tumour (PMT) and SLN status still represent the most important parameters regarding prognosis and clinical decision making. New tools are needed. These markers may be associated with PMT, SLN, metastasis, or blood serum. Integrins are transmembrane receptors known to facilitate cell-cell and cell-extracellular matrix adhesion. Four integrins, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, and $\alpha 11\beta 1$, comprise the subfamily of collagen binding integrins. Integrins have a role in cell migration, tissue invasion, and angiogenesis. Integrins promote primary tumour growth, are involved in several steps in melanoma metastasis, and may be molecular markers and drug targets. B-cell lymphoma 2 (Bcl-2) is an antiapoptotic protein often deregulated in melanoma. The association of Bcl-2 with collagen binding integrin expression in melanoma has not been established.

Tumour burden of SLN is a valuable prognostic tool for both survival and prediction of further metastases in CLND. The most common way to measure this is the maximum diameter of the largest tumour deposit in SLN. The recent abandoning of CLND and the rapid evolution of systemic therapy for advanced melanoma have highlighted the role of SLN. Although SNB has become a standard procedure, several controversies remain. Optimal number of lymph nodes that should be removed in the procedure is debatable and various criteria for defining SLN intraoperatively exist. The role of pelvic SLNs and whether they ever represent true SLNs is unclear.

2 REVIEW OF THE LITERATURE

2.1 Brief history of melanoma

Descriptions of pigmented malignant lesions that frequently present with distant metastasis have been reported since Hippocrates (460-375 B.C.) (4). However, it was not until 1806, when the French physician Rene Laennec described melanoma as a disease entity (5). In 1844, the English surgeon Samuel Cooper described advanced melanoma as untreatable, and early excision of the disease would be the only chance for cure (6). In 1892, Herbert Snow and later in 1904 William Handley suggested removal of regional lymph nodes in addition to wide excision of the PMT; this approach guided surgical treatment of melanoma for over a half century (7, 8).

Improvements in histopathological examination, especially by Wallace Clark (1966) and Alexander Breslow (1970), revealed that melanoma prognosis of appeared to be a function of both tumour size and level of invasion, with primary tumour thickness being the most significant measure of size (9). This raised justified concerns about the extent of surgical treatment for both excision and management of regional lymph nodes. A series of prospective randomized trials failed to show survival benefit for prophylactic or elective lymph node dissection (ELND) (10, 11, 12).

The term “sentinel” lymph node dates to at least as early as 1923 when British surgeon Leonard R. Braithwaite studied lymph drainage from the omentum (13). Ramon M. Cabanas used the term sentinel node to describe the first lymph node to be involved in penile cancer (14). In 1992 Donald Morton described SNB as a minimally invasive procedure for nodal staging and selecting patients who may benefit from a CLND (1). A prospective randomized trial, Multicenter Selective Lymphadenectomy Trial (MSLT-I) commenced in 1994 to compare outcomes of patients who underwent local excision and SNB with patients who underwent local excision and nodal observation (15). Patients with a metastasis in SLN underwent CLND and patients with a clinical nodal relapse underwent a therapeutic lymph node dissection (TLND). The study showed that SLN status is the most important prognostic factor for early-stage melanoma patients. Patients in the SNB group had fewer recurrences than patients in the observation group. Furthermore, among patients with intermediate-thickness (Breslow classification 1.2–3.5 mm) melanomas and nodal metastases, early SNB-guided surgical treatment was associated with increased melanoma-specific survival (MSS). Consequently, SNB remained a fundamental procedure in treatment of melanoma patients. SNB became a standard treatment in Helsinki University Hospital in 2000 (16).

Compared with SNB, the role of CLND was less clear and evidence of survival benefit was lacking. The adverse events related to CLND were more

apparent. Two large multicentre trials, De-COG and Multicenter Selective Lymphadenectomy Trial II (MSLT-II) compared outcomes of patients with positive SLNs who were randomized to CLND and follow-up subgroups (2, 3). Both trials failed to show survival benefit from CLND. Based on these results, routine CLND for patients with SLN metastases has been widely abandoned. This and the rapid evolution of new targeted therapies and immunotherapy for advanced disease have made the 2010s perhaps the most remarkable decade in melanoma treatment (17, 18).

2.2 Epidemiology

The incidence rates of melanoma in 2020 ranged globally from <0.25 (per 100 000) in Southeast Asia to 36.6 in Australia (19). The incidence rate was 19.5 in Finland. According to the Finnish Cancer Registry there where 1807 new melanoma cases in 2019 (20). Of these patients 939 (52%) were men. Although a slight decrease was observed in 2016, when 1909 new cases were reported, incidence has been steadily rising from 1950s. Ultraviolet radiation due to sun exposure is the most important driver of this increase (21). Traveling to sunny locations and sunbathing have increased the risk of melanoma (22).

The age-standardized mortality rate of melanoma in Finland was 1.7 per 100 000 in 2020, which is consistent with the EU average.However, the mortality rate in Finland has increased less since the 1980's (20). This may be due to early diagnosis and treatment advances.

2.3 Pathogenesis

The skin is the largest organ in the human body and consists of two layers, the epidermis and the dermis. Melanocytes are scattered in the epidermis normally surrounded by keratinocytes and produce pigment granules called melanosomes, which contain melanin. Melanosomes are transferred from melanocytes to keratinocytes upon UV radiation to protect against DNA damage (23).

Transformation of melanocytes into metastatic melanoma is a complex process that involves both endogenous and exogenous triggers. Normal melanocytes divide only less than twice a year, but proliferation becomes more rapid as the melanocytic neoplasm evolves (24). Point-mutations and copy-number alterations also become more frequent. Melanomas are associated with a particularly high mutational load. The mutations originate both directly from UV radiation and indirectly from free radicals caused by the biochemical interaction of UV with melanin (25). Although not all melanomas follow a stepwise pattern from a benign nevus–dysplastic nevus–melanoma in situ–invasive melanoma, the malignant transformation into melanoma requires multiple pathogenic mutations. These affect genes in signalling pathways that

regulate growth and metabolism, cell cycle control, proliferation, cell identity and replicative lifespan. Despite the tumour heterogeneity, certain recurrent mutations have been discovered (26). These common mutations differ slightly depending on whether the skin where melanoma develops is chronically sun damaged or not. The $BRAF^{v600}$ mutation is commonly found on benign nevi and further progression to melanoma requires secondary (*e.g.*, mutation in telomerase reverse-transcriptase [TERT] promoter) or tertiary (e.g., mutations in cyclin-dependent kinase inhibitor 2A [CDKN2A]) mutations. Although mutations in phosphatase-and-tensin homologue (PTEN) and tumour-protein 53 (TP53) are associated with metastatic melanoma progression, a conclusive pattern of recurrent alterations for melanoma to gain metastatic potential has not yet been identified (24, 25, 26, 27, 28).

While genetic and epigenetic changes are important, the tumour microenvironment also plays a role in all stages of melanoma development. The melanoma microenvironment differs significantly from that of the normal skin and is altered further as the tumour advances from radial growth phase to vertical growth phase and then to metastatic stage (29). The melanoma microenvironment is very heterogenous, and rapid tumour growth produces hypoxic regions that are associated with resistance to chemo- and radiation therapies (30).

One of the key characteristics of tumour growth is immune evasion. This may involve immune checkpoints, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1). Immune checkpoints are cell-surface proteins that control initiation, duration, and magnitude of immune responses (31).

An advanced understanding of cancer genetics has spurred the development of novel therapeutics (31). Most notably, immune checkpoint inhibitors (ICI) targeting CTLA-4 and PD-1 have revolutionized the management of advanced melanoma (18, 32).

2.4 Lymphatic system

The lymphatic system consists of lymph vessels or lymphatics, lymph nodes, and lymphatic organ (*e.g.* spleen). Excess fluid from intercellular spaces of the skin and other tissues is collected by the lymph capillaries. The fluid, now called lymph, then passes on to small lymph vessels, which join to form larger lymph vessels. The lymph vessels carry lymph to lymph nodes, which are small glands consisting of lymphatic tissue. Histologically lymph nodes have a capsule, cortex, and medulla. Lymph enters the node via afferent channels on its convex surface to subcapsular sinuses. It then passes on to trabecular sinuses and medullary sinuses, which become continuous with efferent lymph channels that leave the node from the hilum.

In general, lymph passes through one or more lymph nodes before it enters large lymph vessels (called lymph trunks) and eventually returns to the

bloodstream. Lymph nodes play an important role in immune defence of the body as they produce antibodies and lymphocytes. Most lymph nodes responsible for the first-line drainage of the skin are in the neck, armpits, and groin. However, lymph may also traverse lymph nodes outside these common basins. Interval nodes are lymph nodes located between the primary tumour site and recognized node fields (33). The lymphatic drainage of the lower limb is illustrated in Figure 1.

As discussed in detail in later chapters, the lymph node or nodes receiving direct lymphatic drainage from the primary skin tumour site is called a SLN. After lymph has passed through the first node, it may continue to the next node in the same lymph node basin or further down the stream. These nodes are referred to as second, third, etc. -echelon or -tier lymph nodes.

Lymphogenous dissemination of cancer cells is typical of melanoma. Accumulating cancer cells causes enlargement of affected lymph node(s), which then present as a palpable tumour. This may be the first manifestation of the disease or a sign of progression if melanoma has been diagnosed previously. The cancer cells may also aggregate in lymph vessels (resulting in *in transit* tumours) and block lymph vessels (thereby altering lymphatic drainage).

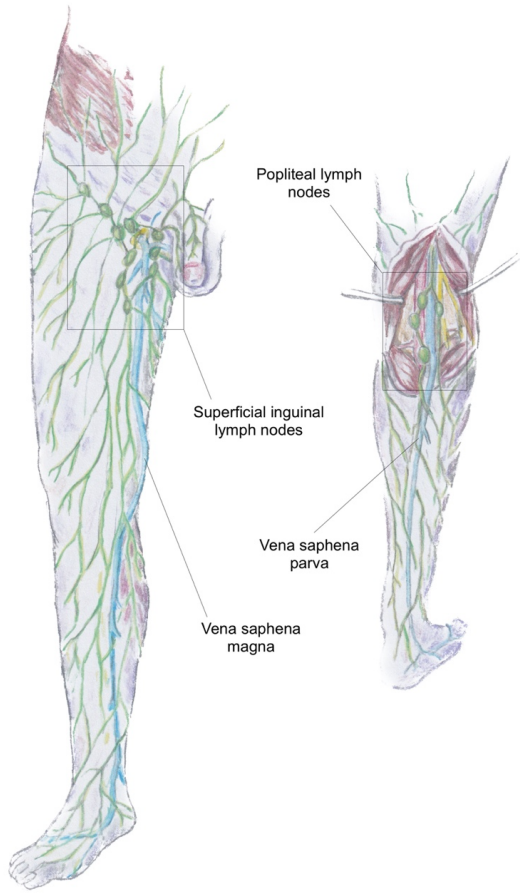

Figure 1. Lymphatic drainage of the lower limb. The superficial inguinal lymph node basin is the most common site of first drainage. From below knee level there may also be drainage to popliteal lymph nodes. © *T. Vuoristo*

2.5 Molecular markers

The diagnosis and treatment of melanoma are based on histopathological parameters of the primary tumour and SLN. Due to advances in molecular technologies in recent decades, there is a growing interest in new molecular markers from the primary tumour, metastatic tumour (including the SLN metastases), or blood serum to help prognosis and guide melanoma treatment. Several markers previously known to participate in tumour progression and metastasis have been analysed using RT-PCR and microarrays to discover genes or gene panels to aid clinical decision making (34, 35, 36). In addition, genome-wide evaluation of melanoma has identified several novel candidate progression markers (26). The gene-expression profiles have been proposed

to *e.g.* help identify melanoma patients with no clinically detectable metastases who may or may not benefit from SNB (37). Despite considerable efforts, the use of genetic alterations in prognosis and treatment of melanoma remains limited (38). However, uncovering genetic alterations, such as BRAF mutation, have already revolutionized melanoma treatment (17, 39). Several commercial gene expression assays have been developed and their clinical value is under investigation (40).

2.6 Integrins

Integrins are heterodimeric proteins that consist of different combinations of 18 α and 8 β subunits, which make up 24 heterodimers encountered in mammals (41). Integrins play a role in the interaction between cells and extracellular matrix (42). Subunits α1, α2, α10, and α11 combine with β1 to form collagen receptor integrins. These integrins differ structurally from other extracellular matrix-binding integrins because they have an additional inserted (αI) domain through which they bind collagen. There are differences in collagen binding, expression patterns, and signalling functions of collagen receptor integrins. Integrin α1 typically binds to basement membrane type IV collagen, whereas α2 prefers fibril-forming collagens (43). Integrin α1 is mainly expressed on mesenchymal cell types and its signalling refers to cell proliferation, whereas α2 is expressed mainly on epithelial cells, platelets, and endothelial cells and is associated with matrix remodelling (44, 45). Integrins α10 and α11 likely have a role in metabolism of bone and cartilage (46, 47).

Integrins have been associated with melanoma pathogenesis for some time and several studies have shown their altered expression in melanoma compared to benign nevi (48, 49). Integrins promote primary tumour growth and are involved in several steps in melanoma metastasis. Integrin α2 is a tumour progression antigen, and its expression is related to the aggressive growth phase of melanoma (50). Integrins promote degradation of basement membrane by increasing expression of matrix metalloproteinases (MMP) (51). Integrins produce growth factors, such as fibroblast growth factor and vascular endothelial growth factor which stimulate tumour angiogenesis (52). Recent studies show that integrins participate in priming the premetastatic niche for metastasis (53, 54).

Given their role in melanoma progression, integrins have motivated studies on their usefulness in risk stratification and as possible therapeutic targets. In a study by Meves et al., expression of genes with roles in epithelial-to-mesenchymal transition including integrin β3 was associated with metastasis in SLN (55). The authors suggested that combining a set of gene-expression variables with clinicopathologic variables improves identification of patients who may forgo SNB due to low risk of metastasis. β3 integrin H score did not significantly improve prediction of SLN metastasis over Breslow depth and patient age (56). Other studies have focused on integrin expression in

circulating tumour cells and exosomes that have detached from the primary tumour and reached peripheral blood, which may be a prognostic tool in the future (57).

Both integrins and molecules that interact with them are promising therapeutic targets. Several drugs targeting integrins have already been developed (58, 59, 60). Although promising in preclinical studies, these drugs are not as effective as monotherapy or when combined with standard chemotherapy. It is hypothesized that novel combination therapies are needed to target integrins at multiple levels.

2.7 Staging and lymph node status

The tumour (T), node (N), and metastasis (M) classification and staging play a critical role in clinical decision making and treatment of melanoma patients. The 8th edition of the American Joint Committee on Cancer (AJCC) staging system was implemented in 2018 (61). The T category is defined by Breslow thickness and ulceration status. The N category was refined in the 6th edition of AJCC Staging Manual published in 2002, which acknowledged the nodal micrometastases, which at the time and ever since represented the result of SNB (62). In the 8th edition, micrometastases are redefined as “clinically occult” opposed to “clinically apparent”, previously termed macrometastases. As most national guidelines no longer recommend CLND after a positive SNB, the N category is based on SNB for most stage II-III melanoma patients (Table 1).

Table 1. T (tumour) and N (node) categories and pathological stage groups for stages I-III according to AJCC 8th edition melanoma staging system.

N Category	Number of tumour-involved regional lymph nodes	Presence of in-transit, satellite and/or micro-satellite metastases	T Category								
			T0	T1a	T1b	T2a	T2b	T3a	T3b	T4a	T4b
			No evidence of primary tumour	<0.8 mm without ulceration	<0.8 mm with ulceration or 0.8-1.0 mm with or without ulceration	>1.0-2.0 mm without ulceration	>1.0-2.0 mm with ulceration	>2.0-4.0 mm without ulceration	>2.0-4.0 mm with ulceration	>4.0 mm Without ulceration	>4.0 mm with ulceration
N0	No regional metastases detected	No	N/A	IA	IA	IB	IIA	IIA	IIB	IIB	IIC
N1a	1 clinically occult (i.e. detected by SNB)	No	N/A	IIIA	IIIA	IIIA	IIIB	IIIB	IIIC	IIIC	IIIC
N1b	1 clinically detected	No	IIIB	IIIB	IIIB	IIIB	IIIB	IIIB	IIIC	IIIC	IIIC
N1c	No regional lymph node disease	Yes	IIIB	IIIB	IIIB	IIIB	IIIB	IIIB	IIIC	IIIC	IIIC
N2a	2 or 3 clinically occult (i.e. detected by SNB)	No	N/A	IIIA	IIIA	IIIA	IIIB	IIIB	IIIC	IIIC	IIIC
N2b	2 or 3, at least 1 of which was clinically detected	No	IIIC	IIIB	IIIB	IIIB	IIIB	IIIB	IIIC	IIIC	IIIC
N2c	1 clinically occult or clinically detected	Yes	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC
N3a	≥4 clinically occult (i.e. detected by SNB)	No	N/A	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIID
N3b	≥4, at least 1 of which was clinically detected, or the presence of any number of matted nodes	No	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIID
N3C	≥2 clinically occult or clinically detected and/or presence of any number of matted nodes	Yes	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIIC	IIID

SNB = sentinel lymph node biopsy.

2.8 Diagnosis and treatment of melanoma

2.8.1 Signs and symptoms

Melanoma classically presents as a dark, asymmetric skin lesion with tendency to grow and change shape. Sometimes the lesion may itch or bleed, but in most cases the suspicion is based on its appearance, and the malignant lesion stands out as an “ugly duckling” among the benign nevi. The ABCDE of melanoma is a checklist of typical characteristics associated with malignant melanocytic lesions, namely Asymmetrical shape, Border irregularities, Colour variation, Diameter typically >6 mm, and Evolution of characteristics. Although these criteria assist in clinical assessment, not all melanomas follow them. Melanomas may appear as amelanotic, nodular lesions, and especially subungual melanomas are often diagnosed late because they are easily misdiagnosed as fungal infections or other more common diseases. Melanoma may first present in advanced stage as a subcutaneous tumour or enlarged

lymph node. In these cases, the primary tumour is often found in the skin nearby. Sometimes the PMT remains unknown. It may have regressed, been removed previously without histopathological assessment, or was misdiagnosed.

2.8.2 Prevention and screening

Primary prevention is based on avoiding UV radiation, the only known risk factor for melanoma (63). There have been public health campaigns around the world to inform people about the risks of sun exposure. In the 1980's the Australian government launched a nationwide "Slip, Slop, Slap" skin cancer prevention campaign to lower the high incidence rates of melanoma, and other skin cancers. The campaign was successful, with an estimated 50 000 cancers prevented and 1400 lives saved (64). The age-standardized incidence of invasive melanoma has stabilized or declined in people aged under 60 years in Australia (65).

Secondary prevention involves identifying at-risk individuals to detect melanomas at an early stage. Thus far there is no evidence to support population-wide screening of melanoma or other skin cancers. Regular surveillance of high-risk individuals with history of pre-malignant or malignant skin lesions (or both) is recommended in several clinical guidelines, including Helsinki University Hospital guidelines (66).

2.8.3 Diagnosis

In most cases, the PMT is removed by physicians in primary care. Finnish guidelines recommend diagnostic excision of the suspicious pigmented lesion with 1-2 mm margins. Skin tension lines should be respected when planning surgery. In the extremities, the excision should be directed axially. The wound should be closed directly, and complex reconstruction avoided to mandate likely further surgery and reliable SNB. Punch biopsy is generally not recommended as it does not represent the entire lesion, and removal of the residual tumour is mandatory for definite classification. In specific cases including large lentigo maligna lesions located on the face, a punch biopsy is acceptable.

Melanoma is diagnosed by histopathology. The pathology report should include Breslow thickness, presence of ulceration, mitotic rate, and subtype of melanoma (superficial spreading, nodular, acral lentigous, lentigo maligna melanoma or another subtype). The margins of surgical excision should be reported. The Clark classification is still widely used, although it is no longer featured in the AJCC manual (61).

After melanoma has been diagnosed, the patient is examined to detect any other suspicious skin lesions that should be excised. Lymph node basins are

palpated, and if enlarged nodes are discovered, and ultrasound-guided core needle biopsy is taken. Ultrasound examination of lymph node basins is routinely performed on patients with thick, >4 mm melanomas in Helsinki University Hospital. Lymph node basins in neck, axillary, and groin areas are examined. A needle biopsy is performed if any suspicious lymph nodes are palpated or detected by ultra-scan.

2.8.4 Local and locally advanced disease

A vast majority of cutaneous melanomas are diagnosed at an early phase and cured with surgical removal of the primary tumour. Tumour thickness and ulceration are the most important prognostic parameters of the primary tumour (61). When the tumour thickness exceeds 1.0 mm, the risk of nodal involvement also increases and SNB is generally recommended. A high-risk group of thin <1 mm melanomas can be determined by including ulceration and mitoses as adverse parameters. SNB may be considered in T1b melanomas, *i.e.* melanomas of 0.8-1.0 mm in thickness or <1 mm with ulceration.

Approximately two thirds of melanoma patients with a recurrent disease present with a locoregional recurrence (67). As discussed in the history chapter, there have been several attempts to improve outcomes of melanoma patients by surgically removing the adjacent lymph nodes. The locally advanced disease may be apparent at the time of diagnosis if a lymph node is palpable or detected with ultrasound. In patients with clinically occult nodes, the locally advanced disease is observed in SNB. Patients with lymph node metastasis undergo a whole-body computed tomography (CT) scan to discover any distant metastases.

2.8.5 Wide excision

The PMT or excision scar is excised with surgical margins determined by Breslow thickness. The recommended margins are 5 mm for in situ melanomas, 1 cm for melanomas ≤1 mm, 1-2 cm for melanomas ≤1-2 mm, and 2 cm for melanomas >2 mm. When the primary tumour is on the face, margins exceeding 1.5 cm are seldom used even in thick melanomas. Excision is continued to the underlaying fascia (Figure 2). Studies comparing narrow (1-2 cm) vs. wide (3-4 cm) margins have shown slightly fewer local relapses with wide margins, but no MSS benefit (68, 69). Currently, no prospective randomized trials have compared 1 cm vs. 2 cm margins, but there is an on-going multicentre trial which should clarify the matter (70). In melanomas located in fingers or toes, including subungual melanomas partial amputation is frequently necessary to attain acceptable margins (71).

The excision wound can be closed directly in most cases. However, for the face and distal extremities, reconstruction options, including skin grafts and local flaps, may be needed for optimal functional or aesthetic result (or both). The reconstructive methods (or lack of thereof) should not compromise oncological safety; aiming for the recommended safety margins is essential (72). Microsurgical flaps are rarely needed but may play a role *e.g.*, in reconstruction of weight-bearing areas of the foot. Lentigo maligna melanomas present a surgical challenge, as they are typically located in the face, are poorly delineated, and often large in diameter. Several re-excisions may be needed to achieve proper safety margins. Local flaps are often the preferred method for reconstruction on the face but should be delayed until the required surgical margins have been histologically confirmed. In some cases, a two-step procedure may be preferable, and the excision wound is left open and reconstructed in a second procedure when the margins are clear.

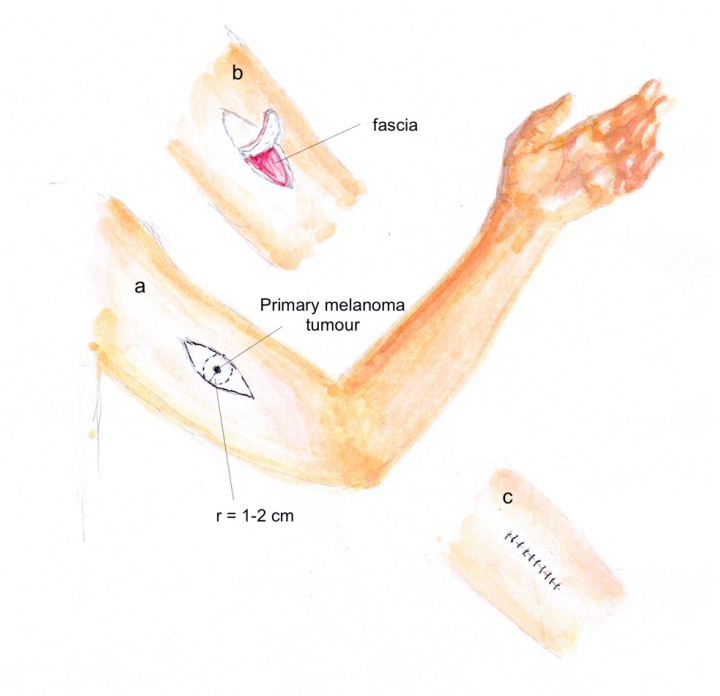

Figure 2. Wide local excision of primary melanoma tumour. a) Axially oriented elliptical incision is planned around the tumour or previous excision scar with 1-2 cm margin as indicated by Breslow thickness. b) Excision is continued to the underlying fascia. c) Direct closure of the wound. © *T. Vuoristo*

2.8.6 Sentinel lymph node biopsy

2.8.6.1 Basis and principle

There are two popular hypotheses explaining the metastatic development of melanoma. The first, the so-called incubator theory, assumes that melanoma spreads through a cascade of orderly progression. According to this theory, the disease will spread from the primary tumour site to the regional lymph nodes before eventually developing distant metastases. The regional lymph nodes serve as governors of disease. Based on this hypothesis, it was postulated that there would be survival benefit from ELND. As discussed before, large, randomized trials failed to demonstrate any survival benefit (11, 12). After the results of these trials, it was postulated that the effect of the procedure was diluted because 80% of the patients where node negative. A more specific way of determining the nodal status was advocated, and eventually SNB was developed (1).

The second hypothesis, the so-called indicator theory, hypothesizes that melanoma undergoes a simultaneous lymphatic and hematogenous spread. According to this theory, lymph node metastases are indicators, not governors, of metastatic disease. In addition to the randomized controlled trials failing to demonstrate survival benefit for ELND, there are more studies to support this theory. For instance, in a study by Meier et al., of 466 melanoma patients, 50.2% had their first metastasis in regional lymph nodes, while 28.1% presented with a distant metastasis as the first metastatic site. The time to distant metastasis was almost identical in both groups (73).

As already discussed in the history chapter of this thesis, the tendency of melanoma to metastasize to lymph nodes has been long known. Imaging techniques have been unable to detect the smallest microscopic metastases and ELND was too mutilating for a vast majority of patients. This was because only the approximately 20% of patients had positive nodes and the lymphatic drainage patterns, especially in head and neck and trunk, varied considerably. Consequently, new ways of detecting possible lymph node metastasis were advocated. The concept of the sentinel node had been established already in the 1960s, but it was Donald Morton and his team that first published the use of SNB in melanomas in 1992 (1). The idea was to inject a tracking dye at the site of primary melanoma or excision scar. The dye then passed to the lymph vessels and on to the lymph nodes, the first of which is now labelled as the sentinel node. Originally Dr Morton used blue dye for intra-operative mapping to visualize lymph vessels and nodes. In up to 82% of cases, at least one sentinel lymph node was detected. The problem with using blue dye as the only tracer was that information of the location of SLNs still relied on the patterns of lymphatic drainage documented by previous studies. This problem was solved by preoperative lymphoscintigraphy, which demonstrated the lymphatic drainage and location of SLNs and thus, provided a roadmap for the surgeon (74). Technetium-99^{m}-labeled radiocolloids soon became the tracer

of choice for the procedure. A few years later the technique was considerably refined when a handheld gamma probe was introduced (75). This made it possible to locate the SLNs intraoperatively both *in vivo* and *ex vivo*. With this combined technique, the number of patients that had at least one lymph node detected went up to 95%. In the next decade SNB gained popularity around the world and became a standard procedure.

Since 2000, SNB has been recommended for patients with intermediate thickness and thick PMTs without evidence of metastasis in Helsinki University Hospital. In 2006, SNB was included in Finnish national clinical practice guideline for melanoma (76). Figure 3 presents the principle of SNB.

For more than two decades, it was recommended that patients with no evidence of metastasis in SLN avoided further surgery, whereas patients with a positive SLN underwent CLND (77). The MSLT-I secured SNB as a staging tool but failed to show undisputed survival benefit for the procedure (78).

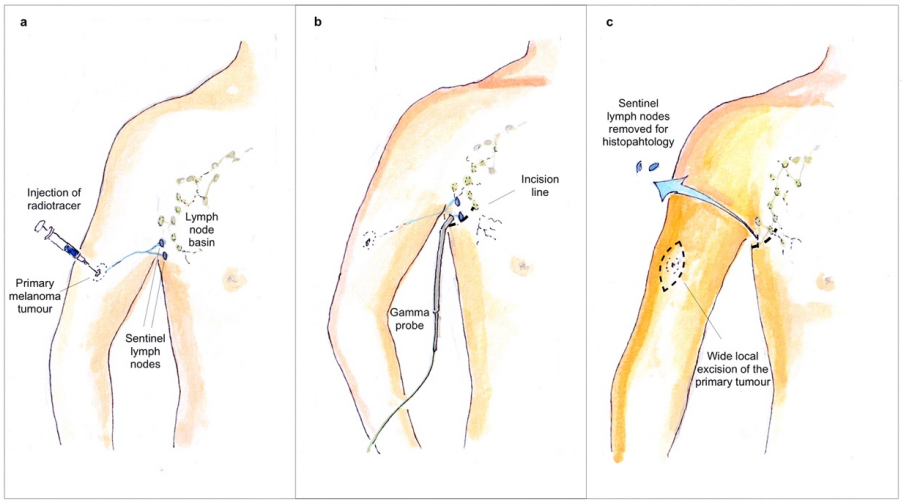

Figure 3. Sentinel lymph node biopsy. a) Radiotracer is injected on both sides of the primary melanoma tumour or excision scar prior to surgery. The radiotracer drains via lymphatic vessels to lymph nodes, shown here in the right axilla. b) The lymph node basin is accessed through axillary incision and a hand-held gamma probe is used to detect the sentinel lymph nodes. c) The sentinel lymph nodes are removed and sent to the pathology work-up. Wide local excision is performed to remove the primary melanoma tumour or previous excision scar. © *T. Vuoristo*

2.8.6.2 Patient selection

SNB is recommended for melanoma patients with primary tumour thickness >1 mm and no clinically detectable metastasis. For thin melanomas, the benefits of the procedure do not outweigh the drawbacks because the likelihood of lymph nodes harbouring metastasis is low. Therefore, T1a

patients do not undergo the procedure. However, there is controversy regarding T1b category patients, as the probability of a positive SLN is 5-10% in this group. There is no consensus on whether to perform SNB routinely on these patients, but most guidelines recommend that it should be discussed with the patient if there are no contraindications to the procedure (79, 80). On the other hand, SNB is recommended for thick melanomas >4 mm in Breslow thickness (81). The risk of an advanced disease in this group is high and theoretically thick tumours may have already metastasized into the lymphatic vessels blocking them (82). This may alter the lymphatic flow and impair the reliability of SNB, but there are no studies on melanoma patients to support this theory.

2.8.6.3 Lymphoscintigraphy

Lymphoscintigraphy is normally performed a day before the operation or on the same day. Radiotracer is injected intradermally on both sides of the primary tumour or excision scar (Figure 4). A dynamic image is taken immediately to discover lymphatic channels from the primary tumour site to the lymph nodes. Static images are taken typically 30 minutes and 2 hours after the injection. The static images show the location and number of lymph nodes that have collected the radiotracer. Technetium-99m has been used as the radionucleotide of choice because of its availability, relatively low cost, and half-life of only 6 hours.

There are several different radiotracers in the market and there are differences between countries in which tracer is used. Technetium-labelled albumin is used mostly in Europe and Australia, whereas technetium-labelled sulphur colloid is most popular in America (83). The choice of the radiotracer impacts lymphoscintigraphy as different tracers have different dynamics. In general, the technetium-labelled albumin produces more nodes visualized in lymphoscintigram (84). This may help to find these nodes but increases the risk of harvesting second echelon nodes.

After the images are taken, the SLNs are marked on the skin of the patient with a pen. This is especially helpful in the neck and groin areas, where it facilitates incision planning and prompt preoperative arrangements. It has less impact in the axilla, where the incision is standard. Lymphoscintigraphy reveals if there is lymphatic drainage to more than one basin, which is often the case with melanomas of the trunk. It also helps detect interval and aberrant SLNs (33). These lymph nodes outside the standard lymph node basins are often located more proximal to the primary tumour site and represent the first nodes (*i.e.*, SLNs). These nodes may harbour metastases and must be excised (85).

Currently, in many hospitals such as Helsinki University Hospital, a single-photon emission computed tomography/computed tomography (SPECT/CT) is performed to more precisely locate the SLNs (86). This assists the surgeon

in determining the exact anatomical location of the lymph nodes, and thus, helps preoperative planning. The shortfall of SPECT/CT scan is that it represents a static image and fails to differentiate the true SLNs from the second echelon nodes (87).

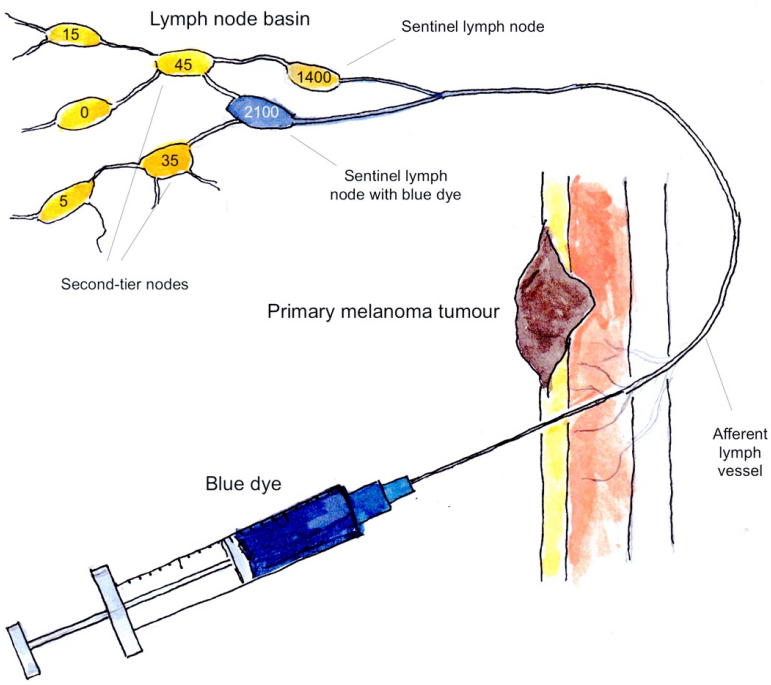

Figure 4. Sentinel lymph nodes and second-tier nodes. Radiotracer is injected on both sides of the primary melanoma tumour. Radiotracer then drains to the lymphatic vessels and to the sentinel lymph nodes. After passing the sentinel node it continues to the second-tier nodes. Similarly, blue dye may be injected just prior to excision. The numbers on the lymph nodes indicate typical radioactivity counts detected during the procedure. © *T. Vuoristo*

2.8.6.4 Surgical procedure

It is recommended that SNB is performed simultaneously with wide local excision of the primary tumour or excision scar (88). The patient is marked preoperatively. The skin tension lines are respected when planning the incisions. The surgeon should also consider possible further surgery, including CLND. The operation is usually performed under general anaesthesia, although local anaesthesia may be sufficient in cases with superficially located SLNs. Sometimes a combination of these is the most practical choice. For example, when the primary tumour is on the back, the patient is first in a

lateral or prone position and WLE is performed under local anesthesia. After this the patient is positioned supine, and SNB is performed in general anaesthesia. In this way, both operation time and the risk of displacement of intubation tube while repositioning the patient may be reduced. At the beginning of the procedure, the radioactivity count from the primary tumour site should be measured, as this confirms the correct placement of radiotracer injection. This is important especially when the patient has several scars from prior surgeries and may not remember which was the site of melanoma. WLE is performed first in most cases; this is recommended particularly in cases where the radioactivity from the primary tumour site might disturb locating SLNs. Blue dye, when used, is injected intradermally at the site of primary tumour or scar just prior to surgery (15). The radioactivity is measured from the site of SLNs before incision to confirm their location and to make necessary refinements concerning the incision. The SLNs are located with a handheld gamma detector and meticulously dissected. The *ex vivo* radioactivity counts are recorded. All blue and radioactive lymph nodes are harvested until no focal residual radioactivity remains (Figure 4). This is further discussed in the definition of SLN chapter. The wound is closed directly. Most patients can be discharged on the same day.

2.8.6.5. Histopathology

Following excision, the formalin-fixed SLNs are sent to the pathologist. Each lymph node is embedded in paraffin and serially cut into 1-mm slices and stained with haematoxylin and eosin. Immunohistochemical staining with S-100, HMB-45 (human melanoma black), and MART-1 (melanoma antigen recognized by T cells, also called Melan-A [melanoma tumour antigen]) is performed. The length and width of the metastatic lesions are measured (Figure 5). In Helsinki University Hospital, also the location of the metastasis (subcapsular or parenchymal) within the lymph node is also reported. Refer to the following chapter on tumour burden.

The use of frozen sections to detect SLN metastasis intraoperatively was investigated especially in the CLND era (89). The rationale to use frozen sections was to discover positive SLNs intraoperatively and continue with CLND in the same session and thereby spare the patient from a second operation. Because of the low frequency of positive SLNs and low sensitivity of frozen sections, the routine use of frozen sections in SNB is not recommended (90). After abandoning CLND as standard procedure, there is minimal use of frozen sections in melanoma management.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was introduced in the mid-1990s to facilitate more precise detection of the smallest metastatic deposits in a SLN (91, 92). Several markers have been used to detect melanoma-specific mRNA, including tyrosinase, MART-1, MAGE (melanoma-associated antigen), GP100 (glycoprotein 100), GalNAc-T

(beta1→4-N-acetylgalactosaminyl-transferase), and Pax3 (paired-box homeotic gene transcription factor 3). Large multicentre trials, including the Sunbelt Melanoma trial and MSLT-II included RT-PCR in the study protocol. Despite the clear potential of this method, its clinical value remains unverified (2, 93).

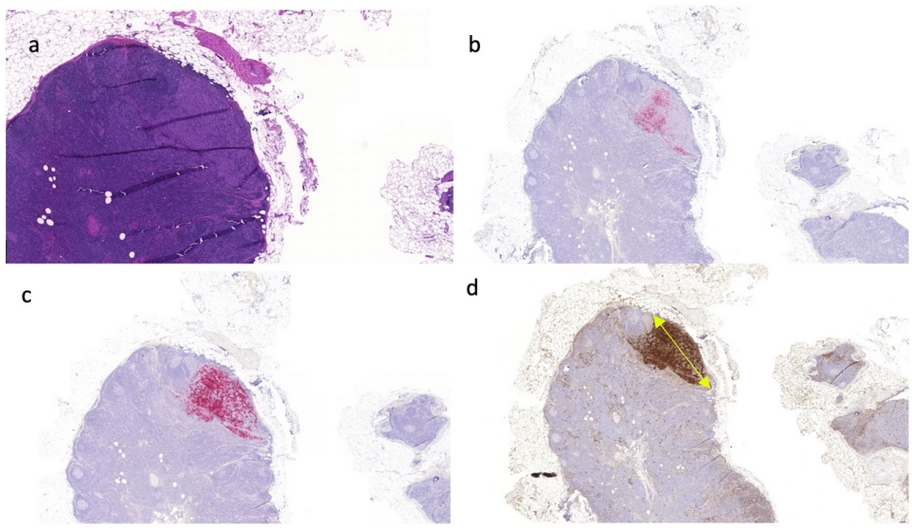

Figure 5. Sentinel lymph node metastasis. The sentinel lymph node is stained with haematoxylin-eosin (a). Immunohistochemistry reveals a metastasis that is positive for HMB-45 (b), MART-1 (c), and S-100 (d). The maximum diameter of the largest metastatic tumour deposit is 2 mm and is indicated by the yellow arrow (d).

2.8.6.6. Definition of SLN

Ever since SNB was described in the treatment of melanoma three decades ago, there has been debate concerning the definition of a SLN. Morton originally described the SLN as the first node draining the primary tumour site (1). Later, when lymphoscintigraphy was adopted as a standard feature of the procedure, Thompson described the SLN as any node or nodes that receive direct lymph drainage from the tumour site (94). In clinical practice, it is common that the lymphoscintigram displays several radioactive nodes. These may not represent true SLNs but rather second-echelon nodes that have received radiotracer after it has passed through the first node (Figure 4). It may be difficult to determine intraoperatively whether a node is truly the first node (*i.e.* SLN) or a second-echelon node. Therefore, several means of determining the actual SLNs have been described. It has been suggested that

only two or three of the hottest nodes should be removed (95). In addition, setting a radioactivity threshold is a common means to delineate the number of nodes that should be removed. The most widely used method of selecting lymph node is the 10% rule. According to this rule, all blue nodes and nodes that have an in vivo radioactivity ≥10% than to the radioactivity of the hottest node should be removed (96). This popular method also has its limits. The number of nodes may still be quite large, and it is often difficult to measure the radioactivity of the nodes in vivo especially when they are located close to each other or are deep (*e.g.* in the pelvis). The most accurate way of determining the SLNs would be a dynamic lymphoscintigram displaying the lymphatic vessels clearly and would thus allow following them to the very first node or nodes representing the true SLN. Indocyanine green has been suggested as a tracer that can be used intraoperatively to follow the lymphatic vessels to the SLN (97, 98). Thus far indocyanine green has not gained popularity in clinical practice (99).

2.8.6.7. Pelvic SLN

In melanoma patients with a PMT in the lower extremities or trunk, the SLNs are frequently located in the groin area (Figure 1). The lymphoscintigraphy in most of these cases displays radioactive nodes superficially in the groin but often also deeper in the pelvic or iliac/obturator area (100, 101, 102) (Figure 6). There have been cases in the literature where the lymphatic drainage from the skin of the trunk and lower extremities flows directly to the deep nodes in the pelvis (103). However, in most cases, these are second-echelon nodes, that receive radiotracer after it has passed the superficial nodes, which in this case represent the true SLNs (102). There are few studies investigating the clinical relevance of these pelvic sentinel lymph nodes (PSLN) (103, 104, 105, 106).

The pelvic lymph nodes can be approached from the same incision as the superficial nodes in the femoral area by continuing the incision in an S-shaped fashion. Another approach is to make a separate incision above the inguinal ligament (Figure 6). Regardless of the choice of entrance, the surgeon must dissect through the inguinal ligament, between the abdominal muscles, or both to reach the pelvic lymph nodes with a proper view. This adds considerably to the surgical trauma and operational time, which raises the question of whether harvesting pelvic lymph nodes is justified.

In the past, when CLND was the standard treatment after a positive SLN, there was debate around the extent of CLND in the groin and pelvis. Some argued that CLND should always include the pelvic lymph nodes, while others would continue dissection to deep lymph nodes only if PSLNs or the most proximal inguinal lymph node in the femoral sheet (called Cloquet's node) were positive (107, 108, 109, 110). However, the predictive value of Cloquet's node in studies is limited (111). More importantly, following the results of MSLT-II and DeGOG trials, CLND has been omitted for most patients, and the

extent of dissection is less relevant. Currently, a laparoscopic approach may be the most feasible method in retrieving clinically metastatic pelvic lymph nodes (112, 113).

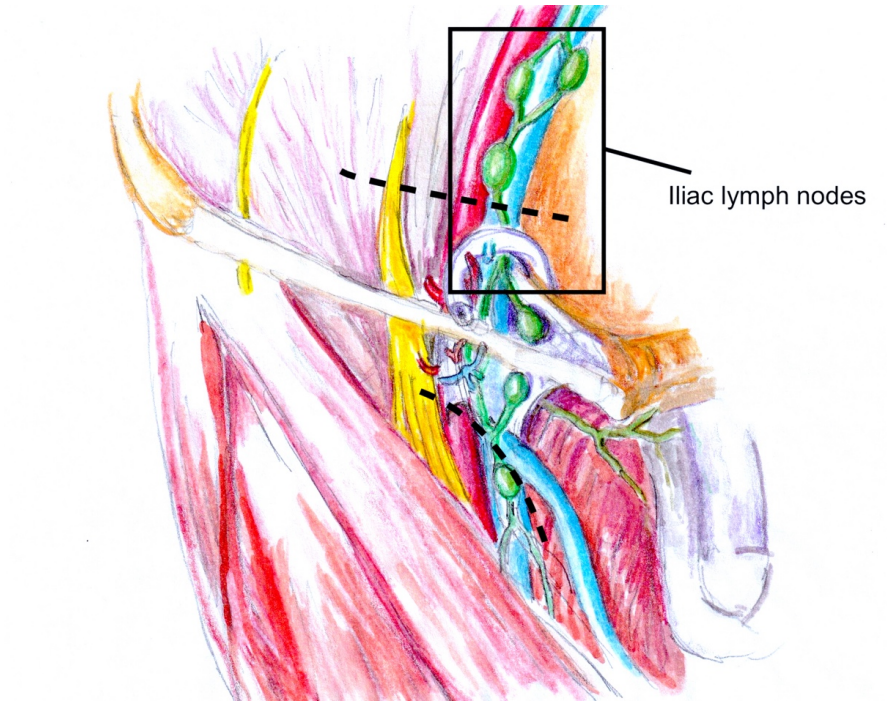

Figure 6. Lymph nodes of the deep inguinal/iliac area. Rectangle indicates pelvic lymph nodes located around iliac vessels proximal to the deep inguinal lymph nodes. The dashed lines indicate surgical incisions commonly used in sentinel lymph node biopsy. © *T. Vuoristo*

2.8.6.8. False-negative SNB

A recurrent lymph node metastasis after a negative SNB is called a false-negative SNB. The false-negative rate should be reported as the number of cases with regional lymph node recurrence after negative SNB divided by the sum of positive SNBs and cases with regional recurrences after negative SNB (114). Studies have reported false negative rates of SNB of 10-30% (78, 115). Lymphoscintigraphy, surgery, and histopathology comprise SNB and each of these steps are susceptible to missing a metastatic node (116). Therefore, each step should be of sufficient standard in a centre that performs SNBs. However, other factors also play a role concerning the sensitivity of the method. Most importantly, tumour biology may affect the result even though the technical

performance at all stages would be of the highest standard. For instance, there may be microscopic in-transit metastases that evolve to lymph node metastasis at a later stage (82). The behaviour of melanoma cells may differ from normal lymphatic flow and metastatic deposits may theoretically develop in lymph nodes further down the stream.

Advancements in techniques in every step of SNB and the experience and education of the personnel involved have reduced the number of false negatives. However, as for every other clinical test, there will always be false-negative results. It is essential to ensure that each stage of the procedure is at the highest level and to discover new techniques to refine and increase the sensitivity of SNB (117). The surgeon should attempt to remove all SLNs but leave NSNs intact. It is important to remember that the purpose of SNB is to be a staging tool with minimal morbidity, and a SNB resembling CLND should be avoided.

Histopathological analysis has been refined over the years and clinical trials require standardized protocols for sample evaluation. RT-PCR has attracted attention as a promising adjunct to the histopathology to increase sensitivity but currently not used universally (116, 118).

2.8.7 CLND

For more than two decades, CLND was a standard treatment for patients who presented with a positive SLN (77). The rationale was to minimize the risk of local recurrence by removing any additional metastatic lymph nodes (*i.e.* positive non-sentinel lymph nodes [NSNs]) in the lymph node basin, and consequently increase survival rate (15). Most guidelines recommended dissecting the remaining lymph nodes of the basin from which the positive SLN was removed. There was controversy regarding the extent of surgery, especially in head and neck area, and groin, as discussed in the pelvic SLN - chapter. The benefits of the procedure were questioned, as only 20% of patients had metastases in NSNs. CLND is associated with significantly more morbidity than SNB alone. Although adverse events such as infections, wound dehiscence, seroma, and lymphoedema are associated with SNB, they are much more frequent after CLND (119, 120, 121). A multicentre trial was therefore warranted and two large, randomized trials were launched. The first one published was the German DeCOG trial, which showed no difference in MSS between the patients who underwent CLND and the follow-up group (3). This study was initially criticized for having a relatively low number of patients. However, the results of the largest trial MSLT-II were similar and the popularity of CLND subsequently rapidly decreased (2). In the beginning, there were doubts about abandoning CLND completely as some subgroups of patients were underrepresented in the trials. The DeCOG trial did not include patients with head and neck melanomas, and thick melanomas were underrepresented in both trials. Therefore, it was postulated that there might

still be a subgroup of patients who would benefit from the procedure. For instance, many positive SLNs, large SLN metastasis, extracapsular extension of SLN metastasis, thick primary tumours, or combinations thereof were suggested to increase the risk of harvesting NSN metastasis, and patients with these features might benefit from CLND for better local control. Thus far, no subgroup of patients has been shown to benefit from CLND, and most present guidelines do not recommend this procedure (79, 122, 123). The probability of better local control does not justify the procedure for any subgroup of patients, as most of them still do not have further metastasis in the CLND. Furthermore, in MSLT-II study, SNB without CLND cleared the affected nodal basin in >80% of patients, providing long-term local control of the disease (124). In cases with a clinically detectable lymph node metastasis with no distant metastasis (*i.e.* a node-only metastasis), dissection of the corresponding basin is still considered standard treatment. Having a recurrence in the lymph node basin rarely means complete loss of local disease control, but patients should be directed to adequate follow-up to detect recurrences early.

2.8.8. Prognostic factors

2.8.8.1 Prediction of SLN metastasis

Several factors are associated with a positive SLN, including increasing Breslow tumour thickness, presence of ulceration, increasing mitotic rate, primary tumour located in trunk or lower extremity, and younger age of the patient (125, 126). The Breslow thickness remains the most important factor, as the decision to perform SNB is mainly based on this. Several risk calculators based on large databases and known predictors of SLN status have been developed to help select patients who may or may not benefit from SNB (127). Clinicians can use these risk-prediction tools in decision making and discussing the procedure with the patient.

2.8.8.2 Prediction of NSN metastasis

Before the results of DeCOG and MSLT-II trials, CLND was standard of care for patients with a positive SLN (77). Considering the low frequency of NSN metastasis and the adverse events of the procedure, it was of great interest to predict the likelihood of additional metastasis in CLND specimens (128). Factors reported to predict NSN positivity include Breslow tumour thickness, ulceration, primary tumour regression, number of positive SLNs, SLN tumour burden, and parenchymal location of the metastasis in SLN (129, 130, 131, 132). As with SNB, several risk-scoring systems were developed to help clinical decision making. The N-SNORE is one the most renowned (133). It is composed of gender (female = 0 points, male = 1), primary tumour regression

(absent = 0, present = 2), proportion of harvested SNs involved in melanoma (absent = 0, present = 2), maximum size of the metastatic deposit in SLN (≤ 0.5 mm = 0, 0.51-2.00 mm = 1, 2.01-10.00 mm = 2, >10.00 mm = 3), and perinodal lymphatic invasion (absent = 0, present = 3). The sum of these parameters, 0, 1-3, 4-5, 6-7, and ≥8, were associated with very low (0%), low (5-10%), intermediate (15-20%), high (40-50%), and very high (70-80%) risk of NSN, respectively. With accurate prediction of metastatic NSNs, it would be possible to select low-risk patients who might be safely spared from the morbidity of CLND. After the DeCOG and MSLT-II trials failed to demonstrate survival benefit from CLND, the procedure was abandoned for most patients. This also led to a paradigm shift. Instead of attempting to select patients for whom the CLND could be omitted, the objective was redirected to identify patients who might benefit from further surgery. In other words, attention was focused on the patients with more tumour burden and highest N-SNOREs and other risk scores.

2.8.8.3 Prognostic Factors for Survival

Increasing age, male gender, and PMT located in trunk or head and neck areas are among the demografic and clinical factors associated with poorer prognosis (134, 135). Breslow tumour thickness and ulceration are the most important prognostic parameters concerning the PMT (134). Patients with increased Breslow thickness and ulcerated primary tumours have poorer prognosis. Breslow thickness and ulceration of the primary tumour constitute the T category in the AJCC Melanoma staging manual (61). Satellites and in-transit metastasis also predict poor survival and are included in the 8th edition of AJCC manual defining the N category. Mitotic rate of the primary tumour is also associated with poorer survival and is among the factors that recommend SNB for patients with T1b melanomas. However, the staging value is debated, and mitotic rate was excluded from the 8th edition of the AJCC staging manual. While tumour regression has also attractred interest as a prognostic factor, its clinical value is unclear. The status of the SLN is the most important prognostic factor in patients with no clinically detected metastasis (78).

The number of metastatic lymph nodes constitutes the N category of the AJCC manual. The metastatic load or tumour burden of the SLN also has prognostic value and will be thoroughly discussed in the next chapter. The presence of metastatic lymph nodes in the CLND specimen (*i.e.*, positive NSNs) represents an independent adverse prognostic factor. Although several other markers have been investigated from the primary tumour, SLNs, and bloodstream, thus far none have achieved popularity in clinical use.

2.8.8.4. Predicting response to systemic therapy

Ever since systemic therapies were introduced in the treatment of advanced melanoma, there has been a constant demand for prediction tools to select the optimal treatment and identify patient subgroups who are likely to benefit most. For decades, the systemic therapies had frustratingly minimal effect on survival. The introduction of targeted therapies and checkpoint inhibitor-based immunotherapy have revolutionized the field. Subsequently, it has become more important to predict response and outcome. BRAF gene status is routinely determined, whereas PD1 remains controversial (136, 137, 138). For $BRAF^{V600}$-negative patients, clinically validated next-generation sequencing (NGS) panels covering key oncogenic drivers are increasingly performed routinely (139, 140). Tumour mutational burden (TMB), defined as the number of somatic mutations per megabase of genes studied, has been used to predict response to immune checkpoint inhibitors (ICIs); cancer cells with higher TMB or mutation rate are more likely to be recognized as foreign by the immune system, thus improving the efficacy of immunotherapy (141). High level of serum lactate dehydrogenase (LDH) activity remains a robust indicator of poor prognosis and poor therapeutic outcome (142). Biomarkers, such as a normal LDH and low disease burden, are predictors of a favourable response to both ICIs and targeted therapy, and as such cannot be used to select optimal treatment (142). New predictive tools are needed due to the rapid evolution of new therapies.

2.8.9.5 SLN tumour burden

SLN tumour burden has prognostic value in both survival and predicting NSN metastasis (129). Several means of measuring the metastatic load in SLN have been described (131, 132, 143). The depth of invasion from the cortex of the lymph node was described by Starz in 2001 and has proved to be a simple and reliable substaging system (144, 145). Some studies have attempted to count the surface area of metastasis and the ratio of the area compared to the area of the lymph node (129, 146). The problem with this approach is that it requires specific equipment and is affected by interobserver variability (143). The most widely used method of reporting SLN tumour burden is the maximum diameter of the largest metastatic tumour deposit in SLN (147)(Figure 5). This straightforward way of measuring tumour load has gained popularity because it is reliable and has less interobserver variability (148). The maximum diameter of the SLN metastasis was further popularized by the Rotterdam classification, which stratifies the maximum diameter into four classes (149). Although there are not many studies comparing the different means of measuring tumour burden, according to a study by Egger et al., the maximum diameter of SLN metastasis prevailed (143). While SLN tumour burden is not currently incorporated in the AJCC staging manual, its importance was

discussed and it is recommended that the pathology reports should include the maximum diameter of the SLN metastases (61). This should be reported at an accuracy of 0.1 mm. Future staging manuals will likely include tumour burden to refine the N category. SLN tumour burden has already been acknowledged in adjuvant systemic therapy trials (39, 150).

2.8.9 Adjuvant therapy

Adjuvant treatment is considered for patients who have no evidence of macroscopic metastasis but are at high risk of having microscopic metastasis and developing recurrent disease. Before the development of novel therapies, interferon-α was the first therapy shown to significantly improve disease-free survival (DFS) and to impact overall survival (151). However, interferon-α is associated with significant toxicity. ICI and targeted therapies are more effective and better tolerated than interferon-α, and therefore interferon-α is no longer recommended for adjuvant treatment of melanoma.

After showing successful results in metastatic melanoma, checkpoint inhibitor-based immunotherapy has been tested as an adjuvant treatment of patients with completely resected local or regionally advanced melanoma (18, 150, 152). Use of BRAF/MEK inhibitors in the adjuvant setting in patients with completely resected locoregional metastatic melanoma has also been successfully tested (39). Subsequently, national clinical guidelines have adopted protocols that include immunotherapies and targeted therapies (79, 122, 123). In Helsinki University Hospital, dabrafenib plus trametinib combination therapy may be offered as an adjuvant treatment for $BRAF^{v6}$-positive patients. Anti-PD1 adjuvant therapy may be offered to stage IIIB-IIID melanoma patients and for completely resected stage IV disease for patients without autoimmune diseases or significant comorbidities. The decision to use adjuvant treatment is made in an interdisciplinary setting and the adverse effects of drug therapy, especially with anti-PD1 therapy, must be carefully weighed against the risk of recurrent disease.

Encouraged by these novel therapies, there is a growing interest towards their use in the neoadjuvant setting and for stage IIB-C melanoma patients (153, 154, 155). This has provoked discussion about a subgroup of patients for whom SNB could be omitted and replaced with adjuvant therapy (156).

2.8.10 Radiotherapy

Melanoma is generally considered a radio-resistant disease. Radiotherapy is not routinely considered as adjuvant therapy. In rare selected cases, radiotherapy may be considered to improve local control or as palliative care (157, 158). In Helsinki University Hospital guidelines, radiotherapy may be

considered for selected patients with a massive tumour or multiple recurrencies, for patients with positive surgical margins who are not suitable for re-excision, or if there are multiple positive lymph nodes or extracapsular extension after therapeutic lymph node dissection.

2.8.11 Isolated limb perfusion and T-VEC

Isolated limb perfusion (ILP) is a good therapeutic option for melanoma patients with multiple recurrences or non-resectable disease limited to the limb and no evidence of distant disease (159, 160). Before the procedure, the blood circulation of the limb must be confirmed by a vascular surgeon. ILP is indicated if there is a considerable and measurable tumour load in the limb and not in the adjuvant setting. Melphalan is the drug of choice for this procedure. More than 50% of patients respond to this therapy (160). In cases with complete response, response may be durable (*i.e.* >2 years).

As an alternative to ILP, the oncolytic Talimogene laherperepvec (T-VEC) and anti-PD1 antibodies may be considered. T-VEC is a genetically modified herpes simplex 1 virus based oncolytic immunotherapy (161). It has been approved by the United States Food and Drug Administration and European Medicines Agency for the treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with recurrent melanoma after initial surgery (162). The drug is injected intratumourally to replicate and stimulate anti-tumour responses both locally and systemically. The adverse events that have been reported are mild and easily reversible. T-VEC has been used both in monotherapy and in combination with ICIs. In a recently published multicentre trial, T-VEC combined with pembrolizumab did not significantly improve DFS or overall survival than with pembrolizumab alone (163). Ongoing trials are futher evaluating the clinical value of T-VEC.

2.8.12 Systemic and recurrent melanoma

Metastatic melanoma has a bad reputation; in the past, distant metastasis indicated very poor survival. It was well recognized that at this stage, the surgical procedures represented palliative therapy. However, the recent renaissance of systemic therapies in melanoma has revolutionized the treatment. This has also changed the surgical aspect in the advanced disease. For patients with single or oligo metastasis, surgery still represents a valuable treatment with a 100% response rate. Even in patients with a diffusely metastasized melanoma, removal of large, bleeding tumours may provide valuable palliative treatment.

ICIs and targeted therapy constitute the basis of systemic therapy for stage IV melanoma patients. For BRAF-mutated melanoma, BRAF and MEK inhibitors have been used as first-line treatment (17). A combination of BRAF

and MEK inhibitors improved progression-free survival (PFS) compared with single-agent BRAF inhibitor and resulted in reduced skin toxicity and development of non-melanoma skin cancer lesions (164). Checkpoint inhibitors against CTLA-4 (ipilimumab) and PD-1 (pembrolizumab and nivolumab) have further improved systemic therapy for advanced melanoma, and their efficacy both as monotherapy and combination therapy has been tested in several trials (138, 165, 166). At present, both monotherapy with PD-1 inhibitors and combination of ipilimumab and nivolumab are considered standard of care. However, the field is rapidly evolving and treatments are planned individually considering patient and tumour characteristics. Drug resistance and immune-related adverse events are the main drawbacks with the current standard treatments (167).

2.8.13 Follow-up

Although follow-up of melanoma patients after surgery depends on staging, there is currently no international consensus on follow-up schedule (122, 168). In Helsinki University Hospital, the follow-up guidelines states that *in situ* melanomas usually do not require routine follow-up. Stage I melanomas are directed for follow-up in primary healthcare. Stage IIA melanoma patients visit the outpatient plastic surgery clinic for the first 2 years and then primary health care for the remaining 5-year follow-up period. Stage IIB-IV patients are followed in the department of oncology. The frequency and need for imaging (*e.g.* CT scan) depends on staging. Furthermore, the follow-up schedule may vary if a patient is recruited in a clinical trial. For instance, patients in MSLT-II trial were followed according to a specific protocol (2). The patients are informed about the lifetime risk of disease and advised to contact their hospital whenever suspicious new lesions, tumours, or symptoms develop between hospital visits.

3 AIMS OF THE STUDY

The aim of this study was to investigate and report prognostic factors and sentinel lymph node biopsy in patients with locoregionally metastasized melanoma.

The specific aims were:

I To investigate the role of collagen receptor integrins as prognostic factors in patients with advanced melanoma.

II To assess the prognostic value of SLN tumour burden in patients with a metastasis in SLN and to evaluate the impact of SLN tumour burden on survival and presence of non-sentinel node metastasis.

III To evaluate the impact of pelvic SLNs on survival and staging of melanoma patients.

IV To analyse different criteria of selecting SLNs by radiotracer uptake and blue dye and their impact on nodal staging and to evaluate the association between SLN tumour burden and radiotracer uptake.

4 PATIENTS AND METHODS

4.1 Patients

Study I

The study population consisted of patients with metastatic melanoma who were treated and followed up in Helsinki University Central Hospital between December 1988 and January 1996. Originally, 102 patients participated in a phase II study with combined chemotherapy consisting of dacarbazine, vincristine, bleomycine and lomustine combined with natural leukocyte interferon-α. Approvals by the local ethics committees were obtained before study initiation. All patients provided written informed consent. Of 102 patients, 30 patients provided frozen tissue samples of metastatic melanoma tumour. Four patients were omitted due to poor quality of extracted tumour RNA, resulting in a study population of 26 patients. The tumour samples were taken when first recurrence after primary surgery was suspected. All samples were collected before starting the chemoimmunotherapy.

Study II

An electronic search using the QPATI database of the Department of Pathology of Helsinki University Hospital for all patients who underwent melanoma re-excision and SNB from January 2001 to December 2008 yielded 191 patients with a metastatic SLN. Altogether 173 patients were included. Eighteen patients were excluded for the following reasons: 8 patients presented with a recurrent or metastasized disease at the time of SNB and 10 patients did not have follow-up data available. Patients had a recently diagnosed melanoma with Breslow thickness ≥1 mm, other high-risk histological features (Such as ulceration), or both. Patients with Breslow thickness ≥4 mm were routinely examined with ultrasound preoperatively to exclude clinical metastases. Patients with no palpable tumours or metastasis detected by ultrasound underwent wide local excision and SNB.

Study III

The study group consisted of 285 cutaneous melanoma patients whose primary melanoma was in the lower limb or trunk with no clinically detected metastases at the time of diagnosis and who underwent inguinal or iliac SNB (or both) at Helsinki University Hospital between January 2009 and

December 2013. The criteria for SNB included PMT Breslow classification ≥ 1 mm, ulceration, or mitotic level ≥1/mm2.

Study IV

A total of 930 consecutive patients underwent SNB during the period 2001-08 at the Department of Plastic Surgery, Helsinki University Hospital, Finland. The criteria for SNB were Breslow thickness of the primary tumour of 1 mm or more or other pathological features suggesting a more aggressive behavior of melanoma (*i.e.* ulceration and mitotic activity). Patients with no clinically evident metastatic disease at the time of surgery, and with one or more positive. (*i.e.* metastatic SLNs) were included. The inclusion criteria resulted in 175 melanoma patients who had at least one positive SLN.

4.2 Tissue sample handling and total RNA isolation (Study I)

Tissue samples of melanoma metastasis were obtained when the first melanoma recurrence was suspected. The samples were cut in half and one part was put in liquid nitrogen and stored at -80°C. The other part was formalin fixed for pathological diagnosis and immunohistochemistry analysis. Of each sample, 50-150 mg was used for RNA isolation. The frozen samples were cut into sections. The first and last sections were placed on a glass slide and stained to confirm the tumour cells in the sample. The sections were then put into TRIzol™ reagent and mechanically homogenized. Total RNA was prepared with TRIzol™ reagent according to the manufacturer's instructions (Life Technologies Inc., Carlsbad, California, USA).

4.3 Real-time quantitative PCR (Study I)

RT-PCR was performed using fluorescent probes (TaqMan™, Applied Biosystems, Waltham, Massachusetts, USA) as previously described. β-actin was chosen for an internal control and represents a housekeeping gene. Forward and reverse primers and probes for α1, α2, and α11 integrins, and β-actin were designed using primer express software (PE Biosystems, Foster City, California, USA). RT-PCR was performed in 96-well plates. In a final volume of 10 µl, one well contained 5 µl of TaqMan™ Universal PCR Master mix (Applied Biosystems, Waltham, Massachusetts, USA), 2 µl of cDNA, 200 nm of probe, 300 nm of forward primer, and 300 nm of reverse primer. Three parallel qRT-PCR samples were prepared from every cDNA. qRT-PCR was run on a ABI PRISM 7700 or 7900HT sequence detection system (Applied Biosystems, Waltham, Massachusetts, USA). Accumulation of PCR products

was measured real time as an increase in fluorescence. The cycle threshold (C_T) value was determined for each sample representing the cycle number in which the detected fluorescence exceeds the threshold value. A constant threshold value of 0.05 relative fluorescence units was used in all measurements. For each sample $C_T^{integrin}$ and $C_T^{\beta\text{-actin}}$ were determined as mean threshold values of the triplicates. C_T value of integrins was normalized with the C_T value for the endogenous reference gene β-actin. The mean relative mRNA expression was calculated for each sample using the following formula: relative expression = $2^{-\Delta C_T}$, where $\Delta C_T = C_T^{integrin} - C_T^{\beta\text{-actin}}$ and presented as percentage by multiplying the relative integrin expression by a factor of 100.

4.4 Immunohistochemistry (Study I)

Immunohistochemistry (IHC) of Bcl-2 protein was performed using a streptavidin-biotin method, as described by Vlaykova et al. (169). Frozen sections of tumour samples from the patients were also stained for expression of α1 and α2 integrin. Samples from 22 patients were available for IHC, which was performed using monoclonal anti-Bcl-2 mouse antibody (1:300; Clone 124, DAKO A/S, Glostrup, Denmark), monoclonal anti-α1 integrin antibody (1:750, CD49a/ SR84; BD Pharmingen, Franklin Lakes, New Jersey, USA), and a mouse monoclonal anti-α2 integrin antibody (1:1000 MAB 1950/P1E6; Chemicon, Temecula, California; USA). Evaluation of Bcl-2 IHC was performed blinded patient clinical history. Infiltrating lymphocytes, sweat glands, and dermal papillae of hair follicles served as positive internal controls for Bcl-2 IHC. Bcl-2 expression was analysed at a 100x magnification on a Leitz Orthoplan microscope (Leitz Microscope, Ernst Leitz, Wetzlar, Germany) and classified as negative, focal, or diffuse. In Bcl-2-positive tumours, the expression pattern was considered “diffuse” when staining was seen throughout the whole tumour, and “focal” when positive cells were observed only in scattered aggregates of the specimens (169).

4.5 Lymphoscintigraphy (Studies II-IV)

Lymphoscintigraphy was performed on the day before surgery. Patients received Technetium-99m-labelled colloidal albumin (Albu-Res/Nanocoll, GE Healthcare, Amersham, UK) 80 MBq in a 0.2 ml injection intradermally into the primary tumour site on both sides of the excision scar and then proceeded to lymphoscintigraphy with static images at 30 min and 2 h after injection.

4.6 Sentinel lymph node biopsy (Studies II-IV)

For most patients in Studies II and IV, blue dye was used to help visualize SLNs intraoperatively. Blue dye (Patent Blue V, 1ml) was injected intradermally into the site of the primary tumour just prior to surgery. The surgeon used a gamma-detecting probe (Navigator, Tyco Health Care and Neo2000, Neoprobe Corp.) intraoperatively and harvested all blue-stained or radioactive nodes (or both) until no focal residual activity remained. To retrieve deep pelvic lymph nodes, a separate incision on the abdominal wall was made in most cases. The radioactivity count of each sentinel node was recorded. The radioactivity counts of the primary tumour site and all harvested nodes were collected prospectively from the beginning for research and quality-control purposes. A specific form was completed for every patient undergoing SNB; the form included date, primary tumour site and radioactivity, *in vivo* and *ex vivo* radioactivity of harvested nodes, presence of blue dye for each node, and residual activity of operated lymph node basins.

4.7 Histopathology of SLN and CLND specimen (Studies II-IV)

Each SLN was sent for histopathological analysis. The nodes were embedded in paraffin and serially cut into 1-mm slices and stained with haematoxylin-eosin. Immunohistochemical staining with melanoma-specific antigens S-100, Melan-A, and HMB-45 was performed. The length and width of the SLN metastases were measured and the location of the metastases within the node (*i.e.* subcapsular or parenchymal) was reported.

The CLND specimen was weighed, and half of each node was subjected to histopathological analysis (haematoxylin-eosin). Immunohistochemistry was not performed routinely. Metastases were recorded according to size in one dimension and according to the number of positive nodes of all nodes in the basin.

4.8 Follow-up (Studies I-IV)

The patients were routinely followed for a minimum of 5 years. Imaging was performed regularly for high-risk patients according to hospital guidelines and in case of clinical suspicion of recurrent disease. In Studies II-IV, patients who were randomized to the MSLT-II trial were followed according to the MSLT-II study protocol (2). Hospital medical records were reviewed in detail and follow-up data were retrospectively collected for each patient.

4.9 Statistical analysis (Studies I-IV)

In Study I, cumulative survival curves for DFS, overall survival, survival from the appearance of first metastasis and survival after initiation of chemoimmunotherapy were drawn by the Kaplan-Meier method. The difference between the curves was analysed by the Mantel-Cox (log rank) test. The contingency tables analysed by the w2 test and Fisher's exact test were used to assess the correlation between integrin α subunit expression and Bcl-2 expression. The expression levels of the studied integrins were compared for each sample by Wilcoxon signed-rank test. Correlations between integrin levels were analysed using Spearman's rank correlation test. To investigate the correlation with survival parameters, the samples were dichotomized according to the integrin expression levels. An optimal cutoff value was set for each of three collagen receptor integrins. The most appropriate cutoff point for dividing the expression levels of α integrin chains into two groups was defined by assigning different values to the cutoff point and determining the corresponding P-values, using the Mantel-Cox (log rank) test to analyse the Kaplan-Meier survival curves. Kaplan-Meier survival curves were created to visualize the difference between the two groups. This procedure of determining the cut-off point, instead of using mean or median values, has been described previously (170).

In Study II, DFS and MSS were calculated from the time of SNB until first recurrence or death from melanoma, respectively, and censored if no such events had occurred by the last follow-up. Different cutoff values for the diameter of the largest metastatic focus were set to demonstrate the impact on survival and to determine a high-risk group of patients for poor survival and NSN positivity. The diameter of the largest metastatic focus was also analysed as a continuous variable. Univariate analyses of survival were performed using the Kaplan-Meier method and the log-rank test. Co-variables showing statistical significance in univariate analysis were evaluated in a multivariate Cox proportional hazards model. Due to multicollinearity of different tumour burden parameters, separate analyses were performed. The hazard ratios, confidence intervals, and P-values regarding age, gender, primary melanoma thickness, and ulceration were obtained when using the median cut-off value of 1 mm for the maximum diameter of SLN metastasis. χ^2 test and univariate logistic regression model were used to test the association of various parameters with NSN positivity. Factors with a significant univariate association were analysed in a multivariate logistic regression model.

In Study III, the clinicopathological co-variables of patients with and without PSLNs and patients with and without positive SLNs were compared using a χ^2 test for categorical variables and Mann-Whitney *U* test for continuous variables. A multivariable logistic regression model was applied to

analyse predictors of PSLNs, positive SLNs and positive PSLNs. PFS and MSS were calculated from the time of SNB until first recurrence or death from melanoma, respectively, and censored if no such events had occurred by the last follow-up. Univariable analyses of survival were performed using the Kaplan-Meier method and the log-rank test. Covariables showing statistical significance in univariable analysis or considered to be of clinical importance were evaluated in a multivariable Cox proportional hazards model. No violation of proportional hazards assumption was found. To elucidate any impact of melanoma staging according to the 8th edition of AJCC staging manual, the TNM classification and stage grouping of patients with the staging based solely on harvested SSLNs (where no PSLNs had been removed) were compared.

In Study IV, statistical analysis was performed by χ^2 test for categorical variables and Mann-Whitney *U* test for continuous variables.

In Study I, the RT-PCR results and clinical data were analysed using the StatView package for Windows, version 4.53 (Abacus Concepts Inc., Berkley, California, USA). In studies II-IV, SPSS version 25 (IBM, Armonk, NY, USA) was used to perform statistical analyses. In all studies, P-values <0.05 were considered statistically significant.

5 RESULTS

5.1 mRNA expression of collagen receptor integrins in patients with advanced melanoma (Study I)

The patient characteristics of Study I are presented in Table 2. The quantity of α1, α2, and α11 in advanced melanoma was determined with RT-PCR, and mRNA expression of α1 and α2 was higher than α11. The expression levels of different integrins are not strictly comparable due to differences in primer/probe sets, and RT-PCR is most useful when expression levels of the same gene are compared between different samples. Despite these limitations, the expression level of α11 integrin was significantly lower than those of α1 and α2 ($P<0.001$).

The immunostained frozen sections of tumour samples showed that α1 integrin was expressed in melanoma cells and vessels while α2 integrin was expressed mostly in tumour cells. We compared α1 and α2 IHC results with PCR results and observed that patients with higher integrin mRNA expression also had higher expression of IHC in their tumour cells. In this study, these integrins were more positive in tumour cells than in stroma.

5.2 Impact of mRNA expression of collagen receptor integrins on survival in patients with advanced melanoma (Study I)

High expression levels of α1, α2, and α11 each predicted poor outcome after initiation of chemoimmunotherapy. Integrin α2 had impact on overall survival. The median overall survival was 53 months in patients with low α2 expression levels and 35 months in patients with high α2 expression ($P=0.033$). Expression of α1 and α11 showed no statistically significant impact on overall survival. However, low levels of both α1 and α11 integrins predicted improved survival after beginning of treatment and first metastasis.

The focal Bcl-2 expression pattern was associated with a poorer prognosis than that of diffuse expression or a lack of Bcl-2 staining ($P < 0.001$)(169). Similarly, low median mRNA expression levels of integrin α2 and α11 chains were associated with diffuse Bcl-2 protein expression. Tumours with high median α1 or α2 integrin mRNA levels were mostly related to tumours negative for Bcl-2, or with only focal Bcl-2 expression patterns.

Table 2. Study I patient characteristics.

Patients	26
Gender	
Female	8
Male	18
Age at time of melanoma diagnosis (years)	
Median (range)	51 (30-74)
Location of primary melanoma	
Head and neck	2
Trunk	12
Upper limb	4
Lower limb	8
Stage	
III	12
IV	14
Karnofsky performance status index[a]	
70	1
90	12
100	12
Number of organs with metastases[a]	
1	13
2	9
≥3	3
Visceral metastases[a]	
Yes	12
No	13
Serum LDH[a]	
<450 U/l (normal)	18
≥450 U/l (high)	7
Disease-free survival (months)	
Median (range)	11.8 (0.4-200.0)
Overall survival (months)	
Median (range)	40.7 (7.5-204.2)
Survival from first metastasis (months)	
Median (range)	26.4 (1.4-131.8)
Survival after initiation of chemoimmunotherapy (months)	
Median (range)	11.2 (0.9-103.7)
Treatment response to DOBC-IFN-α therapy	
Complete response	7
Partial response	8
Stable disease	4
Progressive disease	6

DOBC=dacarbazine, vincristine, bleomycin and lomustine; IFN=interferon; LDH=lactate dehydrogenase
[a]At the beginning of chemoimmunotherapy

5.3 SLN tumour burden as prognostic factor for survival (Study II)

Of the study population of 173 patients in Study II, 150 patients underwent a subsequent CLND and 23 did not for the following reasons: randomized into the follow-up group of the MSLT-II trial (n=10), refusal (n=7), poor general condition (n=5), metastasis in an interval node (n=1).

The median follow-up was 8.3 years (100 months, range 4-189 months). The overall 5-year DFS and MSS were 55.3% (SE 0.04) and 67.4% (SE 0.04), respectively. Altogether 80 (46%) patients had recurrent disease during follow-up. Seventy-nine patients (46%) were alive at the last follow-up, 66 (38%) had died due to melanoma, and 28 (16%) had died from another cause.

The median primary melanoma thickness was 2.5 mm. The median number of harvested SLNs was 4. The average number of positive SLNs was 1.5 with a median of 1 (range 1-6). Mean diameter of the largest metastatic focus in SLN was 2.1 mm, with a median of 1.0 mm (range 0.05-15 mm). The location of SLN metastasis was parenchymal in 107 patients (62%) and subcapsular in 66 patients (38%).

The most important adverse prognosticators for survival were older age, increasing primary melanoma thickness, presence of ulceration, increasing maximum diameter of SLN metastasis, parenchymal location of the SLN metastasis, increasing number of positive SLNs, and presence of positive NSNs in univariate analysis. In multivariate analysis, maximum diameter of SLN metastasis, number of positive SLNs (>2), location of metastasis within SLN, and presence of positive NSNs remained independent prognosticators for survival. Maximum diameter of SLN metastasis was also analysed as a continuous variable and was a powerful prognosticator for survival in both univariate and multivariate analyses (P<0.001).

The location of the primary tumour was not a significant prognosticator for survival. Neither the number of removed nodes nor the ratio of positive SLNs to harvested SLNs had significant prognostic value.

The diameter of the SLN metastasis was an independent prognostic factor both as a continuous variable (P<0.001) and with every cutoff value (0.2 mm, 0.3 mm, 1 mm, 2 mm, 3 mm, and 4 mm) tested. Patients with smaller metastases had better outcomes in all subgroups according to diameter. Patients with a metastasis diameter <0.2 mm had 77.0% (SE 0.08) 5-year DFS (P=0.035) and 86.2% (SE 0.06) 5-year MSS (P=0.042). Conversely, patients with metastasis of >4 mm had 33.6% (SE 0.10) 5-year DFS (P=0.003) and 38.5% (SE 0.10) 5-year MSS (P<0.001) (Figure 7). Cut-offs at 3 mm and 4 mm significantly delineated the subgroup of patients with shortest DFS, MSS, and OS. This is evident in Figure 7, which shows Kaplan-Meier-estimated MSS and DFS according to maximum diameter of SLN metastasis with cutoffs at 1 mm (Figure 7a-b) and with cutoffs at 0.5 mm and 4 mm (Figure 7 c-d).

Parenchymal location of metastasis within SLN was an adverse prognosticator for survival. Patients with SLN metastases located only in the

subcapsular area had more favourable outcome, with 64.7% (SE 0.06) 5-year DFS (P=0.068) and 84.1% (SE 0.05) 5-year MSS (P=0.007). For patients with parenchymal metastases, the 5-year DFS and MSS were 49.4% (SE 0.05) and 56.9% (SE 0.05), respectively.

Patients with three or more positive nodes had poorer outcomes. The 5-year DFS and MSS for patients with three or more positive SLNs were 20.9% (SE 0.10) and 43.2% (SE 0.12), respectively. For patients with less than three positive SLNs DFS and MSS were 59.3% (SE 0.04) and 70.3% (SE 0.04), respectively (P<0.001).

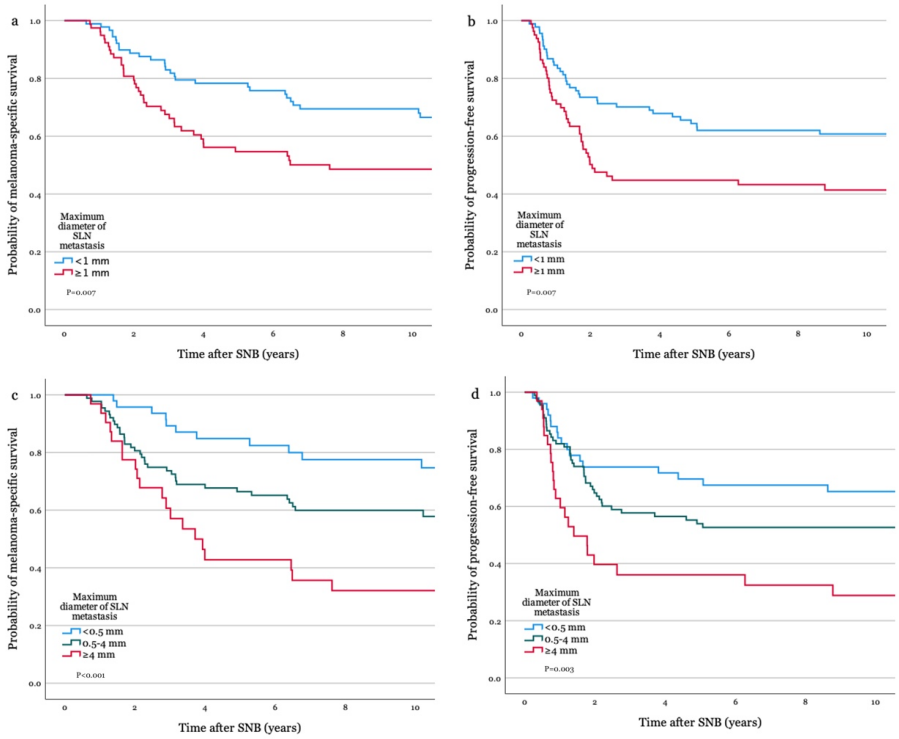

Figure 7. Kaplan-Meier plots of progression-free survival and melanoma-specific survival according to sentinel lymph node (SLN) tumour burden. In (a) and (b), the cutoff is set at 1 mm, which represents the median value of the maximum diameter of SLN metastasis. In (c) and (b), cutoffs at 0.5 mm and 4 mm clearly delineate low-risk and high-risk subgroups. SNB = sentinel lymph node biopsy.

5.4 NSN metastasis - predictive factors and prognostic value (Study II)

Of 150 patients who had a subsequent CLND, 17 (11%) had additional metastatic lymph nodes in the CLND specimen. The number of positive NSNs was 1 in 14 cases (82%), and 2 in one case (6%), and 3 in two cases (12%).

The strongest predictive parameters of positive NSNs in univariate analysis were the diameter of the largest tumour foci in SLN, number of positive SLNs, and location of metastasis in SLN. Of patients with diameter of SLN metastasis >4 mm, 36% and of patients with SLN metastasis ≤ 4 mm 7% had one or more positive NSNs ($p<0.001$). Of patients with three or more positive SLNs, 29% and of patients with less than three positive SLNs 9% presented with further positive nodes in CLND ($p=0.013$). Of patients with parenchymal SLN metastasis, 15% had NSN metastases compared with 5% of patients with subcapsular SLN metastasis ($p=0.040$).

In multivariate analysis, the maximum diameter of SLN metastasis – both as a continuous variable and with cut-offs >2 mm – and the number of positive SLNs (>2) were the most important prognosticators for NSN metastases.

Presence of additional metastatic lymph nodes in the CLND specimen predicted an unfavorable outcome. Patients with positive NSNs had 18.8% (SE 0.10) 5-year DFS and 26.9% (SE 0.12) 5-year MSS and patients without positive NSNs 58.0% (SE 0.04) and 70.5% (SE 0.04), respectively ($p<0.001$).

5.5 Presence and predictive factors of pelvic sentinel lymph nodes and SLN metastasis (Study III)

The median age of the study population of 285 patients was 58 years and two-thirds were female. The PMT was in the lower extremity (*i.e.* in or below the thigh) in 78% of cases.

The median follow-up was 6.1 years. Of 285 patients, 62 (21.8%) had recurrent disease. The type of recurrence was local in 28 (45%), regional in 13 (21%), and systemic in 21 (34%) patients. All 285 patients had at least superficial femoral/inguinal SLNs. The median number of removed sentinel nodes was five (range 1-16). In addition, 199 patients (70%) also had PSLNs. The median number of PSLNs was three (range 1-7). The most radioactive node was superficial in 183 cases (92%).

Of all 285 patients, 63 (22%) had metastases in one or more SLNs. Seven patients (2.5% of all patients and 11% of those with positive SLN) had positive PSLNs. A single patient had metastases solely in PSLNs, while superficial SLNs remained negative.

Of 63 patients with positive SLNs, 39 (62%) had a subsequent CLND. The reasons for not undergoing CLND were as follows: randomized to MSLT-II

follow-up group (n=19), patient refusal (n=4), and contraindicated due to poor general health (n=1). In four cases (10%) metastatic lymph nodes were detected in the CLND specimen. The CLND was continued to the iliac/obturator area in 4 patients (10%) with positive pelvic SLNs. In this group, the patients had no further positive nodes in the CLND specimen.

Table 3 presents the baseline characteristics for 285 patients in Study III stratified by presence of PSLNs, for 199 patients with PSLNs stratified by presence of positive SLNs. Table 4 presents the characteristics of 63 patients with positive SLNs stratified by presence of pelvic SLNs and positive pelvic SLNs.

The location of PMT was predictive of pelvic SLNs, as they were more frequently harvested when the PMT was located below knee level. The number of SLNs and PSLNs was also higher when PMT was located below knee level. Mean PMT thickness was higher in the group with pelvic SLNs. Other parameters, including ulceration, were not predictive of pelvic SLNs.

PMT thickness and ulceration were predictive of positive SLN. The number of harvested nodes (P=0.58), the presence of PSLNs (P=0.54), or the number of harvested pelvic nodes (P=0.33) had no impact on SLN status.

Radioactivity count of the SLNs was a predictor of positive PSLNs. When the radioactivity count of the PSLNs was equal or greater than the hottest superficial SLN it was more likely to harbour metastasis. In multivariate analysis, radioactivity count was the only significant predictor of positive PSLNs. The likelihood of positive PSLNs was greater when the PMT was in the foot (P=0.05). Interestingly, however, all 12 patients with PMT in their toe presented with PSLNs, but none of these patients had positive PSLNs. Age, PMT thickness, ulceration, and number of SLNs were not predictive of positive PSLNs.

Table 3. Clinical and histopathological characteristics of 285 patients by presence of PSLNs and 199 patients with PSLNs by presence of positive SLNs

		All patients				**Patients with PSLNs**		
		All	No PSLNs	PSLNs	P-value	No SLN+	SLN+	P-value
Number of patients		285	86	199		157	42	
Age (years)	Mean	57	55	57	0.61	57	58	0.75
	Median (Range)	58 (20-90)	58 (20-86)	58 (20-90)		58 (21-90)	60 (20-88)	
Gender	Male	93 (32.6)	32 (37.2)	61 (30.7)	0.28	52 (33.1)	9 (21.4)	0.14
	Female	192 (67.4)	54 (62.8)	138 (69.3)		105 (66.9)	33 (78.6)	
Location of primary tumour	Trunk, groin, or buttock	60 (21.1)	28 (32.6)	32 (16.1)	<0.001	27 (17.2)	5 (11.9)	0.22
	Thigh	89 (31.2)	33 (38.4)	56 (28.1)		44 (28.0)	12 (28.6)	
	Leg or ankle	86 (30.2)	15 (17.4)	71 (35.7)		59 (37.6)	12 (28.6)	
	Foot	50 (17.5)	10 (11.6)	40 (20.1)		27 (17.2)	13 (31.0)	
Breslow thickness (mm)	Mean	2.2	1.9	2.4	0.05	1.9	4.0	<0.001
	Median (Range)	1.5 (0.4-11)	1.3 (0.4-8)	1.5 (0.5-11)		1.3 (0.5-11)	3.5 (0.6-11)	
Ulceration	Yes	67 (23.5)	20 (23.3)	47 (23.6)	0.89	25 (15.9)	22 (52.4)	<0.001
	No	208 (73.0)	64 (74.4)	144 (72.4)		124 (79.0)	20 (47.6)	
	Unknown	10 (3.5)	2 (2.3)	8 (4.0)		8 (5.1)	-	
Number of SLNs	Mean	5.1	2.7	6.2	<0.001	6.1	6.7	0.30
	Median (Range)	5 (1-16)	2 (1-8)	6 (2-16)		6 (2-15)	6 (2-16)	
Number of pelvic SLNs	Mean	1.9	-	2.7		2.6	3.1	0.07
	Median (Range)	2 (0-7)	-	3 (1-7)		2 (1-7)	3 (1-7)	
Most radioactive SLN	Superficial	269 (94.4)	-	183 (92.0)		144 (91.7)	39 (92.9)	0.81
	Pelvic	16 (5.6)	-	16 (8.0)		13 (8.3)	3 (7.1)	
Patients with positive SLNs		63 (22.1)	21 (24.4)	42 (21.1)	0.54	-	42 (100.0)	
Follow-up time in years, mean		6	5.7	6.5		5.7	6.5	
Recurrent disease		62 (21.8)	18 (20.9)	44 (22.1)	0.31	19 (12.1)	25 (59.5)	<0.001
Groin recurrence		16 (5.6)	2 (2.3)	14 (7.0)	0.11	4 (2.5)	10 (23.8)	<0.001
Vital status, Alive		225 (78.9)	72 (83.7)	153 (76.9)	0.19	131 (83.4)	22 (52.4)	<0.001

Values in parentheses are percentages unless indicated otherwise. SLN = Sentinel lymph node, PSLN = pelvic sentinel lymph node, SLN+ = positive sentinel lymph node

Table 4. Clinical and histopathological characteristics of 63 patients with positive SLNs by presence of PSLNs and presence of positive PSLNs

		All	No PSLNs	PSLNs	P-value	No PSLN+	PSLN+	P-value
Number of patients		63	21	42		56	7	
Age (years)	Mean	57	56	58	0.77	57	56	0.56
	Median (Range)	60 (20-88)	58 (20-81)	60 (20-88)		60 (20-84)	52 (37-88)	
Gender	Male	16 (25.4)	7 (33.3)	9 (21.4)	0.31	15 (26.8)	1 (14.3)	0.47
	Female	47 (74.6)	14 (66.7)	33 (78.6)		41 (73.2)	6 (85.7)	
Location of primary tumour	Trunk, groin or buttock	12 (19.0)	7 (33.3)	5 (11.9)	0.11	11 (19.6)	1 (14.3)	0.18
	Thigh	18 (28.6)	6 (28.6)	12 (28.6)		17 (30.4)	1 (14.3)	
	Leg or ankle	18 (28.6)	6 (28.6)	12 (28.6)		17 (30.4)	1 (14.3)	
	Foot	15 (23.8)	2 (9.5)	13 (31.0)		11 (19.6)	4 (57.1)	
Breslow thickness (mm)	Mean	3.6	2.9	4.0	0.05	3.6	3.9	0.47
	Median (Range)	3.0 (0.6-11)	2.5 (1.1-8)	3.5 (0.6-11)		3.0 (0.6-11)	4.5 (1.4-6.3)	
Ulceration	Yes	30 (47.6)	8 (38.1)	22 (52.4)	0.29	26 (46.4)	4 (57.1)	0.59
	No	33 (52.4)	13 (61.9)	20 (47.6)		30 (53.6)	3 (42.9)	
Number of SLNs	Mean	5.3	2.7	6.7	<0.001	5.1	7.1	0.04
	Median (Range)	5 (1-16)	2 (1-7)	6 (2-16)		4 (1-16)	8 (4-9)	
Number of PSLNs	Mean	2.1	-	3.1		1.9	3.9	0.02
	Median (Range)	2 (0-7)	-	3 (1-7)		1.5 (0-7)	3 (2-7)	
Number of SLN+	Mean	1.7	1.7	1.7	0.95	1.4	3.7	<0.001
	Median (Range)	1 (1-7)	1 (1-4)	1 (1-7)		1 (1-4)	3 (2-7)	
Most radioactive SLN	Superficial	60 (95.2)	21 (100)	39 (92.9)		34 (97.1)	5 (71.4)	0.02
	Pelvic	3 (4.8)	-	3 (7.1)		1 (2.9)	2 (28.6)	
Completion lymph node dissection	Positive	4	2	2	0.38	3	1	0.44
	Negative	35	10	25		31	4	
Follow-up time in years, mean		5.2	5.4	5.1	0.99	5.3	4.6	0.51
Recurrent disease		33 (52.4)	8 (38.1)	25 (59.5)	0.13	27 (48.2)	6 (85.7)	0.17
Groin recurrence		11 (17.5)	1 (4.8)	10 (23.8)	0.06	7 (12.5)	4 (57.1)	0.003
Vital status, Alive		39 (61.9)	17 (80.6)	22 (52.4)	0.03	36 (64.3)	3 (42.9)	0.27

Values in parentheses are percentages unless indicated otherwise. SLN = Sentinel lymph node, PSLN = pelvic sentinel lymph node, SLN+ = positive sentinel lymph node, PSLN+ = positive pelvic sentinel lymph node

5.6 Impact of PSLNs on outcome and staging (Study III)

In both univariate and multivariate analyses, age, PMT thickness, ulceration, PMT location in foot, and positive SLNs were the strongest prognosticators for survival. The presence of PSLNs had no impact on PFS or MSS. The number of harvested pelvic nodes also did not affect PFS or MSS (P=0.44 and P=0.25, respectively). In 63 patients with positive SLNs, patients with positive PSLNs showed a trend towards shorter PFS; there was no difference in MSS.

Fourteen patients had a groin recurrence during follow-up; 4 of these patients simultaneously presented with a systemic disease. Four patients with no SLN metastases had a groin recurrence, suggesting a false-negative rate of 6.0%. Two patients who underwent a superficial CLND developed metastases in pelvic nodes later in follow-up. PSLNs of six and three nodes, respectively, were negative in the previous SNB for both patients.

When we compared the TNM classification and stage grouping of the patients to the staging based solely on harvested superficial SLNs, we discovered that for 283/285 patients the staging remained the same. The N category would have changed in 6/7 patients who had positive PSLNs. The patient who had metastases only in PSLNs would have been upstaged from IIA to IIIB group. In addition, one patient would have been upstaged from IIIC to IIID group.

Of the overall study group, 74 patients (26%) and 52 patients (21%) who did not undergo CLND presented with lymphoedema during follow-up. In multivariate analysis, female sex, location of PMT (*i.e.*, foot), and total number of SLNs harvested were prognosticators for lymphoedema. No difference in lymphoedema was present between patients who had PSLNs removed and those who had not. A seroma after SNB was present in 132 patients (46%). No predictive covariates were discovered and no difference between groups emerged regarding the presence of PSLNs.

5.7 Radiotracer uptake and status of SLNs (Study IV)

Study IV included 175 melanoma patients; among these, at least one SLN was positive and a total of 783 SLNs were sampled from 221 lymph node basins. A total of 133 patients had operation on just one basin. Of the 221 basins, 188 (85%) harboured one or more metastatic nodes. Of the 175 patients, 163 (93%) had a single positive basin, 11 (6%) had two positive basins, and a single patient had three positive basins. The median number of SLNs per patient was four (range 1-15). At least two nodes were removed in 160 (91%) cases. Altogether 270 (35%) nodes were positive (mean positive nodes per patient 1.5). Of the 175 patients, 67 (38%) had more than one positive SLN.

The hottest node (node with the highest radiotracer uptake measured by radioactivity count) was positive in 137 (78%) patients. When the hottest node was negative, the second hottest node was positive in 26 (14%) cases, the third hottest node in 4 (2.3%) cases, and other less active node in 8 (4.6%) cases, (Figure 8). Patients with less tumour load (*i.e.* lower Breslow thickness [P=0.011], smaller diameter of SLN metastasis [P<0.001], fewer positive SLNs [P<0.001] or combinations thereof) were less likely to present with a metastasis in the hottest node.

For the 38 patients with a negative hottest node, the median ratio of the radioactivity of the most radioactive positive node versus the radioactivity of the hottest node was 0.46 (range 0.02-0.96).

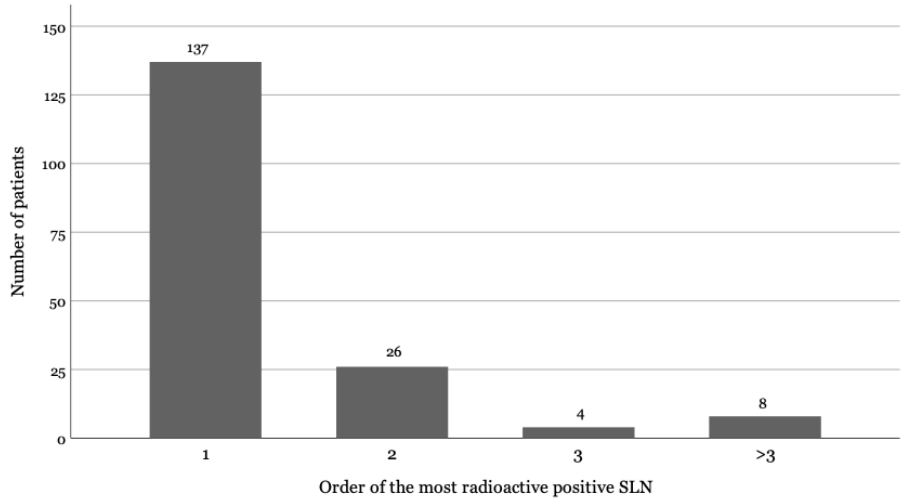

Figure 8. Order of the most radioactive or hottest positive SLN in 175 patients of study IV according to radiotracer uptake. Number 1 represents 137 patients with a positive hottest SLN. Numbers 2 and 3 represent patients with a negative hottest SLN who presented with a metastasis in the second or third hottest SLN, respectively. In eight cases (indicated by >3), the three of the most radioactive SLNs were negative and a less radioactive node harboured metastasis. SLN = sentinel lymph node.

5.8 Blue dye (Study IV)

Of all 783 lymph nodes, 220 (28%) were blue stained; the corresponding value of the 270 positive nodes was 125 (46%). In 57 (33%) patients, no blue dye in SLNs was detected. Of 118 patients with any blue dye in SLNs, 104 had blue-stained metastatic SLNs, all of which were also radioactive. In 87 cases, the hottest node was blue and positive. In 15 patients, the hottest node was

negative, but the hottest positive node was blue. In three of these 15 patients, the radioactivity of the positive node was <10% of the hottest node. Two patients had a positive hottest node with no blue dye but another less radioactive positive blue node. In 14 patients blue dye was found only in non-metastatic lymph nodes, and 10 of these patients presented with a blue but negative hottest node.

5.9 Various criteria for SLN (Study IV)

Table 5 presents different criteria set for a SLN to test their impact on miss rate of positive nodes and nodal and overall staging of patients according to the 8th edition of AJCC staging manual (61). By removing the hottest node only, 114 (42%) of all 270 positive nodes would have been missed and 34 (19%) of 175 patients would have been under-staged. By following the "10% rule", the number of missed positive nodes would have been 14 (5.2%) and the number of under-staged patients would have been 2. Following the "10% rule" would have decreased the total number of harvested lymph nodes from an average of 4.5 to 3.4 per patient. However, 18 patients had positive SLNs with radioactivity <10% of the hottest node and 5 patients presented with a metastasis solely in such a node. Of these 5 patients, 3 had blue dye in the positive SLN.

Table 5. SNB according to various retrospectively applied SLN criteria based on which nodes are removed from a lymph node basin.

Definition of SLN	Number of SLNs	Positive SLNs	Positive nodes missed (%)	Number of patients with a change in N category (*	Number of under-staged patients (*
All hot and blue nodes	783	270	-	-	-
All hot nodes	782	270	-	-	-
>10% and blue	588	256	14 (5.2)	8	2
>10%	560	250	20 (7.4)	12	6
>50%	317	196	74 (27)	51	17
Three hottest	512	239	31 (11)	19	8
Two hottest	390	213	57 (21)	33	14
Hottest only	220	156	114 (42)	78	34

* According to the 8th edition of AJCC staging manual. SNB = sentinel lymph node biopsy, SLN = sentinel lymph node.

5.10 SLN radioactivity and tumour burden (Study IV)

Patients with a negative hottest node presented with lower SLN tumour burden measured by the maximum diameter of the largest tumour deposit of the SLN. The correlation was tested with the maximum diameter as a continuous variable (P<0.001) and using a cutoff at 1 mm, which represents the median diameter (P=0.004).

Of 67 patients with >1 positive SLNs, 45 (67.2%) had the largest tumour deposit in the hottest positive node and 22 (38.8%) in a less active positive node. From these 67 patients, altogether 163 positive SLNs were recorded. The largest metastasis was found in the hottest positive node in 45 (27.6%), and a less active SLN in 22 (13.5%) of all 163 positive SLNs in patients with >1 positive SLN (P<0.001). Correspondingly, the higher relative radioactivity of positive SLN vs hottest positive SLN correlated with the presence of the largest metastasis (P<0.001).

6 DISCUSSION

Most melanomas are diagnosed in early stages and treated with curative surgery. Advanced melanoma is a potentially aggressive disease with a poor prognosis despite modern treatments. Melanoma may be unpredictable; even stage I melanomas may progress while stage III melanomas may remain in remission.

Breslow thickness, ulceration, and mitosis are still the most important prognostic parameters for primary melanoma tumour (134). SNB presents the most important prognostic marker in early-stage melanomas with no clinically detectable metastases (78). New prognostic tools are needed and could be obtained from the primary tumour, sentinel lymph node and blood serum. Despite years of active research, no prognostic tests have gained wide popularity or surpassed Breslow thickness and SLN status as prognostic tools.

6.1 Collagen receptor integrins as prognostic factors in Advanced Melanoma

In Study I, expression levels of two integrins previously associated with metastatic potential in melanoma (α1 and α2) and a less established integrin α11 were detected. We found that high expression levels of all three integrins correlated with poor prognosis. This was most prominent with α2 integrin, which was associated with poor overall survival. Although our study was conducted in the era of chemotherapy and prior to novel systemic treatments, our results suggest a role for α2 integrin as a possible drug target or prognostic tool. Integrin α11 was also considered an interesting new marker. To our knowledge, there are not many other studies on α11 and melanoma. In a study by Yu et al. on drug resistance to targeted therapy for melanoma, drug tolerance was mediated by α3β1 and α11β1 signalling, and knockdown of integrins α3, α11, and β1 significantly inhibited the proliferation of drug-resistant sublines (171). Integrin α11 has also been linked to lung cancer and breast cancer (172, 173). High expression of integrin α11 is associated with advanced stage and lower recurrence-free survival in patients with non-small cell lung cancer (174). Furthermore, upregulated integrin α11 expression was found in the desmoplastic tumour stroma of cutaneous squamous cell carcinomas, and the highest α11 expression was detected in high-grade tumours (175). In our study, we discovered that diffused expression of Bcl-2 correlated with lower levels of α2 and α11 integrin chains and thus with better overall prognosis. Although the antiapoptotic members of the Bcl-2 family represent a promising therapeutic target in melanoma, the role of Bcl-2 expression alone as a prognostic marker remains unclear (176, 177, 178).

Despite the promising findings suggesting a role as a prognostic tool for melanoma, α1 and α2 integrins are not yet present in current commercial gene expression profile tests. Thus far, β3 integrin is the only integrin included (55). The commercial tests are focused on primary tumours and are intended to stratify stage I-II patients into low-risk and high-risk subgroups. SNB may theoretically be omitted for both groups, because low-risk patients are unlikely to harbour SLN metastasis, and high-risk groups may benefit from adjuvant therapies (179). Fewer gene-expression panels are available to predict progression of advanced melanoma (180). This may be due in part to less tumour tissue available for analysis or the tumour heterogeneity of metastatic melanoma.

Further research is warranted to assess the validity and clinical applicability of present prognostic gene-expression profiles. New tests are needed to inform recommendations regarding SNB, intensity of follow-up or imaging surveillance, and postoperative adjuvant therapy. Collagen receptor integrins may increase the accuracy of these tests.

6.2 SLN tumour burden in melanoma

In our study, the diameter of the largest metastatic deposit in SLN, the number of positive nodes, and the presence of NSNs each provided valuable prognostic information.

Abandoning CLND as a routine procedure for melanoma patients with positive SLNs has brought new challenges to treatment and follow-up. Although the prognostic value of NSN metastases was evident in our study and in others (181, 182), the morbidity of the CLND procedure is simply too high for a staging tool with little if any therapeutic value. As discussed above, new tools are needed to select patients for future trials and adjuvant treatments. The role of SLN has been highlighted and any information derived from the metastatic nodes may be useful in the future. SLN tumour burden is an essential addition to the toolbox.

Several methods of measuring the SLN tumour burden have been proposed (129, 143). The diameter of the largest SLN tumour deposit is used most often because of its simplicity and rather low inter-observer variability (143, 147).

We also investigated the prognostic value of the microanatomic location (subcapsular or parenchymal) of the SLN metastasis. We found a more favourable outcome for patients with subcapsular metastases. Although this did not achieve the statistical significance of the maximum diameter of SLN metastasis, the microanatomic location adds to the prognostic information acquired from SNB. A study by van der Ploeg et al. reported that patients with SLN metastasis <0.1 mm located in the subcapsular area had the most favourable prognosis and recommended the combination of size and site of SLN metastasis to be standardized (183).

The MSLT-II and DeCOG-SLT studies were unable to identify a subgroup of patients who would benefit from CLND according to the characteristics of the primary tumour or SLN, including tumour burden (2, 3). The relatively low number of patients with large SLN tumour deposits in these trials may partly explain this. However, it is uncertain whether early CLND will bring a meaningful survival benefit to any group of patients. The rationale for considering the procedure is reducing nodal recurrences in a high-risk group of patients. However, these patients are also at a high risk of harbouring distant metastases.

In Study II, the diameter of SLN metastasis was correlated with both survival and positive NSNs. The relatively low number of CLND-positive patients did not allow detailed analysis of different subgroups according to the diameter of sentinel node metastasis. However, patients with metastasis >4 mm stood out as having non-sentinel node metastases in 36% of cases, whereas those with metastasis ≤4 mm had additional metastatic nodes in <7% of cases. Metastasis >4 mm in diameter may be palpable or at least detectable with ultrasound examination. Whether CLND or a more selective procedure should be favoured for patients with a single clinically detected lymph node metastasis is debatable and warrants further randomized clinical trials. Currently, the decision to perform CLND in rare cases is individual, and potential risks and benefits are carefully discussed with each patient. Various clinicopathological factors and eligibility for and availability of adjuvant therapies are considered. Study II suggests that diameter of SLN metastasis >4 mm and number of positive SLNs >2 are among the high-risk factors. These criteria can be used to select patients for adjuvant treatments and for more intense follow-up protocols.

The microscopic tumour burden of the SLN is not included in the 8th edition of the AJCC staging manual. However, its importance and the growing evidence were discussed, and the panel recommended at a minimum that the single maximum dimension of the largest discrete, metastatic melanoma deposit in SLNs be recorded in pathology reports (61). In a substudy by the MSLT Study Group, MSLT-II patients randomized to nodal observation were investigated for nodal recurrence in the lymph node basin(s) of the previously removed positive SLN. SLN tumour burden and age, primary tumour thickness, ulceration status, and basin location were all independently associated with non-SLN status (124). Furthermore, SLN tumour burden has already been incorporated in inclusion criteria in clinical trials (39, 150). Future AJCC staging manuals will undoubtedly feature SLN tumour burden to further clarify the N category. The key question around implementing SLN tumour burden into the classification is determining optimal cutoffs (182, 184, 185, 186). In several studies, including Study II, the cutoff value of 1 mm for the diameter of the largest metastatic deposit in SLN represents the median and has been used to differentiate between patient subgroups for survival (184, 187) (Figure 7). Previous studies have demonstrated that patients with minimal tumour burden. (*i.e.*, SLN metastasis of <0.1 mm or <0.2 mm) have

favourable prognosis and may safely avoid CLND (149, 184). As CLND is now omitted for most patients, the focus should be on selecting patients for follow-up and adjuvant therapies. Risk stratification based on SLN tumour burden seeks to identify. a low-risk (for whom adjuvant therapy may be avoidede) and high-risk (for whom adjuvant therapy and intense follow-up are indicated) subgroup of patients. It seems that more than one cutoff for the maximum diameter of SLN metastasis is necessary for specific treatment algorithms. For low-risk patients, a cutoff at 0.1-0.3 mm may be adequate, whereas cutoffs at 3 and 4 mm may be considered for the very high-risk subgroup, as suggested by Study II (Figure 7). In a study by Palve et al., a cutoff at 4 mm similarly delineated a high-risk subgroup (188). Combining other parameters (*e.g.*, primary tumour ulceration status and microanatomic location of SLN metastasis) to this classification may increase its accuracy (189).

The number of SLNs harvested per patient was rather high in our study (129, 190). Although this may play a role in avoiding false-negative sentinel node biopsies, the impact of the number of harvested SLNs on survival was less than anticipated. The number of positive SLNs had an impact on both survival and positive NSNs. In particular, patients ≥3 positive SLNs had significantly poorer prognosis and increased risk of additional metastases on CLND. Sabel et al. and Frankel et al. also reported an increased risk of NSN metastases for patients with ≥3 positive SLNs (130, 191). Kim et al. found increased NSN involvement for patients with ≥2 positive nodes (186). A few studies have reported no correlation (129, 190). In these studies, the number of patients with ≥3 positive nodes in SNB was low. In our study, we retrieved >2 SLNs in 138 of 173 cases (78%). The number of patients with >1 positive SLN was 63 (36%). This allowed us to compare the subgroups of patients according to the number of positive SLNs; patients with more positive nodes, both 1> vs. <1 and >2 vs. <2, had poorer prognosis and increased risk for additional involvement in CLND.

Our results suggest a significant role of SLN tumour burden as a prognostic factor for melanoma patients with positive lymph nodes. Meticulous performance of SNB provides prognostic information that is useful in planning the treatment and follow-up of melanoma patients. More intense follow-up aims to find distant metastatic melanoma early enough to be cured with immuno-oncological treatments.

6.3 Pelvic sentinel nodes in melanoma

The role of PSLNs has been controversial since the early days of SNB. However, only few studies on PSLN exist (103, 104, 105, 106). In Study III, 69.8 % of patients had PSLNs removed in SNB with a median of three nodes, a frequency far higher than that published previously. Only 11.1 % of patients with positive SLNs had positive PSLNs. Only one patient had positive pelvic nodes without any positive superficial SLNs in SNB. Most importantly, no

difference in MSS related to the presence of PSLNs in the overall study group or positive PSLNs among patients with positive SLNs was found. The harvesting procedure of pelvic SLNs extends operation time and causes additional surgical trauma and scarring.

Before the results of MSLT-II and DeCOG-SLT, positive PSLNs were an indicator for CLND of iliac/obturator lymph nodes in addition to superficial lymph nodes (102, 108, 109). As CLND has been omitted for most patients with a positive SLN, the extent of CLND becomes less important. New adjuvant therapies may well compensate for whatever benefit CLND would theoretically provide for stage III patients (192, 193). Follow-up with ultrasonography or computed tomography is recommended instead of CLND. A therapeutic lymph node dissection may be advocated if nodal metastases are detected. Robot-assisted videoscopic surgery offers a less invasive approach and has gained popularity in pelvic lymph node dissection (112, 113).

CLND status is a prognostic factor and has been useful for staging and patient selection for clinical trials of adjuvant treatments (18, 138). Removing all potential SLNs, including PSLNs would theoretically compensate for the missing information from CLND. Study III does not support this hypothesis, as the number of harvested nodes was not predictive of either positive SLNs or survival. Furthermore, only 2 of 199 patients with PSLNs were upstaged based on their PSLN status.

The median number of harvested SLNs in Study III was 5 and the median number of harvested PSLNs was 2 per patient in the overall study group. SLNs were removed until no focal radioactivity remained, rather than strictly following the widely used "10 % rule" (*i.e.,* harvesting SLNs with radioactivity ≥10 % of that of the most radioactive node) (96). The high number of harvested lymph nodes suggests that more second-tier nodes were removed than in other studies. However, for the interests of this study, it was essential to remove all potential sentinel nodes to identify any clinical impact. Despite the large number of harvested pelvic lymph nodes, they appear minimally important in the treatment and prognosis of melanoma patients.

It is advisable to remove the pelvic lymph node in cases where it is the only or most radioactive SLN or equal to the most radioactive superficial SLN. This may be difficult to determine intraoperatively, as the SLNs may be located deep in the pelvis and *in vivo* radioactivity cannot be detected accurately without dissecting through the abdominal wall. The role of lymphoscintigraphy must be highlighted when selecting true SLNs from the second-tier nodes. SPECT/CT helps compare the radiotracer uptake of individual lymph nodes and dynamic imaging of sentinel nodes reveals potential direct pathways to pelvic lymph nodes, which indicates their removal (87).

Although only few studies exist on the importance of PSLNs, they mostly agree on the very limited impact of PSLNs on staging and treatment of melanoma (104, 105, 106). A slightly shorter PFS was found in patients with positive PSLNs in Study III. Karakousis and colleagues observed a marginal

association of the presence of deep pelvic nodes with PFS in SLN-negative patients, suggesting that it may be a marker of more aggressive tumour biology (104). In Study III, the presence of PSLNs was associated with higher Breslow thickness, supporting their theory. Most recently, in a study by Laakkonen et al., drainage to PSLNs was not associated with shorter survival or increased nodal recurrence rate, and the authors concluded that PSLNs should not be harvested unless they are first-tier nodes (194). Creating precise criteria for harvesting pelvic lymph nodes warrants randomized controlled trials or at least a large multicentre retrospective study, as suggested by Swords et al. (106).

Apart from the rare cases where PSLN is the only or most radioactive SLN, or preoperative imaging reveals a direct lymphatic pathway to PSLN, it seems reasonable to avoid routine retrieval of PSLNs. Based on studies on PSLNs the current guideline by the Finnish Melanoma Group does not recommend routine retrieval of PSLNs when superficial SLNs are present (123).

6.4 Which lymph nodes should be harvested in SNB?

The definition of SLN is the node or nodes that receive direct lymph drainage from the primary tumour (94). All true SLNs should be removed in SNB but second-echelon nodes and other lymph nodes in the basin should be left intact to limit the morbidity and surgical trauma of the procedure. As discussed earlier, in real-world practice it may be challenging to differentiate between true SLNs and second-tier nodes. Several methods have been suggested to delineate the number of harvested nodes intraoperatively (195, 196, 197, 198). The most popular method is to use a radioactivity count threshold, comparing radioactivity of lymph nodes to the radioactivity of the hottest node (96). In Study IV, 175 patients with a positive SLN were investigated retrospectively to see which nodes harboured metastasis and how many nodes where needed to find them. We employed different criteria of harvesting SLNs to optimize the extent of the procedure.

The broad range of relative radioactivity levels of SLNs observed in Study IV explains why a cutoff at 50% of the hottest node or an absolute amount of sentinel nodes represent suboptimal ways to limit the number of harvested nodes. A relative radioactivity cutoff (such as 10% of the hottest node) is advisable. We found that by following the 10% rule, 97% of positive sentinel lymph nodes would have been detected.

SNB became a standard procedure in Helsinki University Hospital in 2000 (16). Since then, we sought to determine the optimal number of nodes and aimed to target very low residual activity and careful recording of radioactivity of every SLN removed. In previous studies on the optimal number of SLNs, the average number per patient ranged from two to three (195, 196, 197, 198, 199). In Study IV, the mean number of SLNs was 4.5 per patient. This was due in part to the protocol described above, but the tracer also plays a role. We use

technetium-99m-labelled colloidal albumin, which yields higher numbers of nodes compared to sulphur colloid (84). In Study IV, by removing the hottest node and all nodes with radioactivity >10% of the hottest node yielded an average of 3.4 SLNs per patient, which still represents a rather high number of lymph nodes. However, there is currently no better way to limit the number of harvested nodes intra-operatively. Today, SPECT/CT is in routine use in Helsinki University Hospital and helps locate the SLNs but has less value in limiting their number (*i.e.*, selecting the true SLNs from second-tier nodes) (87, 200). Dynamic imaging and discussion between the nuclear medicine physician and the surgeon are important. Although new imaging techniques, such as indocyanine green, should further help clarify this issue, lymphoscintigraphy currently remains the gold standard (98, 99).

Blue dye was used routinely in the early years of the procedure but has lost popularity due to allergic reactions (201, 202). Although it is considered helpful in visualizing SLNs and lymph vessels, the use of blue dye does not significantly increase the accuracy of SNB (196, 197). In Study IV, in three out of five cases where the radioactivity count was <10% of that of the hottest node, the positive node was stained blue, suggesting a positive impact on the sensitivity of the procedure. However, only 46% of all positive SLNs were stained blue. Considering the possible risks of blue dye, it is no longer in routine use in Helsinki University Hospital.

6.5 Limitations

A major limitation to the studies in this thesis was their retrospective nature. The different stages of the SNB procedure, lymphoscintigraphy, surgery, and histopathology, may have been more susceptible to subjective interpretation. The data of complications in Study III, such as lymphoedema and seroma, were not collected in a prospective, standardized manner. However, they were routinely reported whenever present, and there was no difference between patients with pelvic nodes harvested and those without. Other complications, such as chronic pain, were not investigated and may play a role when considering the drawbacks of harvesting PSLNs. In Study II and III, there were differences between MSLT-II patients and other patients due to the randomization and follow-up protocol (2). However, the same guidelines for SNB applied to all patients and most patients underwent CLND according to the previous paradigm. A similar prospective analysis is therefore no longer possible as CLND has been rightfully abandoned.

6.6 Future prospects

Sentinel node biopsy has been an essential staging procedure for melanoma patients for over 2 decades and remains essential. The evolving landscape of melanoma treatment, especially the major advancements in immuno-oncological treatments, have emphasized the need for prognostic factors to guide individual planning of treatment and follow-up.

SLN status is still the most important prognostic factor for melanoma patients with no clinically detectable metastases. The lack of predictive tools for melanoma is somewhat striking considering the vast research on the subject. However, it is inevitable that new prognostic markers will be developed and the need for the information gained from SNB may eventually become less relevant. Until then, SNB remains the cornerstone of melanoma staging. Further studies to improve the accuracy of the procedure are warranted. These include refinements and innovations concerning every step of the procedure (lymphatic mapping, surgery, and histopathology). The first two of these are likely to benefit from new imaging techniques, and new molecular markers will enhance the pathological analysis.

Several controversies remain including the management of single macroscopic lymph node metastases and the role of SNB in very thin and very thick melanomas. A multidisciplinary approach and individual treatment planning for melanoma patients in different stages are paramount. Every effort should also be made in the future to support primary prevention of melanoma and to improve early detection of primary melanoma lesions to improve survival.

7 CONCLUSIONS

I Collagen receptor integrins are associated with the progression and prognosis of metastatic melanoma. In particular, α2 integrin has a role as a potential prognostic marker and drug target.

II Tumour burden of SLN provides valuable prognostic information. Patients with SLN metastasis >4 mm or >2 positive SLNs are at greatest risk of harbouring NSN metastases. This prognostic information is useful in selecting patients for more intense follow-up and immuno-oncological treatment.

III Pelvic sentinel lymph nodes have minimal impact on the outcome of melanoma patients. Removal of PSLNs should be considered in rare cases when they are the only or most radioactive nodes or preoperative lymphoscintigraphy indicates a direct pathway to PSLN.

IV A threshold based on relative radioactivity count of SLNs should be preferred to removing only one to three hottest nodes. By removing the hottest node and all nodes with radioactivity >10% of the hottest node, more than 97% of patients with SLN metastases are discovered. Blue dye may add to the accuracy of SNB but is not essential.

8 ACKNOWLEDGEMENTS

First, I want to thank my supervisor Tiina Jahkola for acknowledging my previous melanoma research and launching this thesis project. I am thankful for her endless support and guidance. It has been a privilege to share thoughts along the way and to travel around the world to present our scientific work.

I am sincerely grateful to Professor Virve Koljonen for participating in the study and mentoring me at crucial stages of the project. Her dynamic approach to research and publishing is truly remarkable and I have learned a lot from her.

I want to thank Professor Erkki Tukiainen for his kind support for my thesis. He has been a tremendous role model in both surgery and science. I feel privileged having completed my plastic surgery training under his supervision.

I thank Docent Ilkka Koskivuo and Docent Johanna Palve for reviewing this thesis. Their comments and suggestions were pivotal and really cut the diamond.

I am grateful to my steering committee members Professor Antti Lauerma and Professor Johan Lundin for their advice and the discussions we had along the project.

I wish to thank Professor Marc Moncrieff for agreeing to act as an opponent.

I am grateful to the head of our department Docent Patrik Lassus, his predecessor Professor Hannu Kuokkanen, and Dr Tuija Ylä-Kotola for promoting research and allowing me to have scientific leave. I owe my gratitude to Docent Nina Lindfors for her great impact on research activity in our division and for making it possible to concentrate on research projects regularly. I thank Jaana Jäppinen for her kind help in the early stages of this thesis.

I am thankful to the Finnish Melanoma Group, Biomedicum Foundation, Vappu Uuspää Foundation, Kurt and Doris Palander Foundation, Paulo Foundation, and the Cancer Society of Finland for financially supporting this thesis project.

I owe my deepest gratitude to my co-writers and members of our research group. Docent Susanna Juteau's vast expertise in skin tumours and histopathology is stellar and our dialogue in all stages of the project was indispensable. Docent Micaela Hernberg helped my thesis project in numerous ways. I have huge respect for her dedication to melanoma research and treatment and for her input to the national and international network of melanoma professionals. I thank Dr Sorjo Mätzke for bringing the essential expertise in nuclear medicine and imaging. I am extremely grateful to Dr Suvi Ilmonen for sharing thoughts and experience related to both research and clinical work. I thank her for our co-operation in skin tumour MDT and, of course, for introducing me to Timo. Docent Timo Muhonen's way of explaining

all aspects of clinical research and statistics is unparalleled. The discussions we had in person, on the telephone, and via e-mail taught me way more than any research course or meeting I have ever attended. I thank him for making me a better scientist and giving me several tools for my future research.

I wish to thank my honourable Turku colleagues, especially Professor Seppo Pyrhönen and Docent Pia Vihinen for guiding me in the very early stages of my research career. I am grateful to the other co-writers in Study I, Dr Camilla Nylund, Professor Jyrki Heino, and Dr Tatyana Vlaykova. I owe special gratitude to Dr Ilari Sauroja for introducing me to melanoma research a long time ago. The lab, medical school, Turku, somewhere in Finland, abroad, army, work, off-work, Kirsi and Pekka Taimen, and Mikko Ovaska have always made me feel at home, thanks!

I want to thank all my co-workers in Helsinki University Hospital from East to West and my friends who have encouraged me. Anna Höckerstedt and Andrew Lindford have been not only work colleagues but also very good friends during training and ever since I arrived in Töölö Hospital. I want to thank Andy for helping me on several occasions related to this thesis and sharing the football pitch with me regularly.

The music of Foo Fighters has given me a lot of strength time and time again.

I owe my sincerest gratitude to all the members of the skin tumour MDT, the cornerstone of proper, up-to-date melanoma treatment. They make this thesis work feel worthwhile. It is a privilege to be a part of our team of dedicated professionals working together every week for the good of the patients.

My parents, I do not know how to thank you enough for all your love and support. You have been extremely encouraging in this thesis project and in all aspects of life. Thanks, mom, also for the excellent pictures. I am grateful to my sister Auli, my brother-in-law Ville, and my god-daughter Leia for encouraging me and for helping me relax on vacations.

Finally, my wife Ilona and our children Erik and Ellen, you are what matters the most and every day I am grateful for sharing my life with you. Your love, joy, and happiness are my inspiration and what keeps me going. My heroes.

Mikko Vuoristo

Helsinki, September 2023

9 REFERENCES

1. Morton DL, Wen DR, Wong JH, Economou JS, Cagle LA, Storm FK, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Archives of surgery (Chicago, Ill: 1960). 1992;127(4):392-9.
2. Faries MB, Thompson JF, Cochran AJ, Andtbacka RH, Mozzillo N, Zager JS, et al. Completion Dissection or Observation for Sentinel-Node Metastasis in Melanoma. The New England journal of medicine. 2017;376(23):2211-22.
3. Leiter U, Stadler R, Mauch C, Hohenberger W, Brockmeyer N, Berking C, et al. Complete lymph node dissection versus no dissection in patients with sentinel lymph node biopsy positive melanoma (DeCOG-SLT): a multicentre, randomised, phase 3 trial. The LancetOncology. 2016;17(6):757-67.
4. Urteaga O, Pack GT. On the antiquity of melanoma. Cancer. 1966;19(5):607-10.
5. Roguin A. Rene Theophile Hyacinthe Laennec (1781-1826): the man behind the stethoscope. Clin Med Res. 2006;4(3):230-5.
6. Cooper S. The First Lines of the Theory and Practice of Surgery. London: Longman; 1840.
7. Snow H. Melanotic cancerous disease. Lancet. 1892;2:872-4.
8. Handley WS. The pathology of melanotic growths in relation to their operative treatment. Lancet. 1907;1.
9. Breslow A, Macht SD. Optimal size of resection margin for thin cutaneous melanoma. Surg Gynecol Obstet. 1977;145(5):691-2.
10. Balch CM, Soong S, Ross MI, Urist MM, Karakousis CP, Temple WJ, et al. Long-term results of a multi-institutional randomized trial comparing prognostic factors and surgical results for intermediate thickness melanomas (1.0 to 4.0 mm). Intergroup Melanoma Surgical Trial. Ann Surg Oncol. 2000;7(2):87-97.
11. Cascinelli N, Morabito A, Santinami M, MacKie RM, Belli F. Immediate or delayed dissection of regional nodes in patients with melanoma of the trunk: a randomised trial. WHO Melanoma Programme. Lancet. 1998;351(9105):793-6.
12. Veronesi U, Adamus J, Bandiera DC, Brennhovd IO, Caceres E, Cascinelli N, et al. Inefficacy of immediate node dissection in stage 1 melanoma of the limbs. N Engl J Med. 1977;297(12):627-30.
13. Braithwaite LR. The flow of lymph from the ileocaecal angle, and its possible bearing on the cause of duodenal and gastric ulcer. British Journal of Surgery. 1923;11:7-26.
14. Cabanas RM. An approach for the treatment of penile carcinoma. Cancer. 1977;39(2):456-66.
15. Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Elashoff R, Essner R, et al. Sentinel-node biopsy or nodal observation in melanoma. The New England journal of medicine. 2006;355(13):1307-17.

16. Jahkola T, Virolainen S, Leppanen E, Suominen S, Hernberg M, Hahka-Kemppinen M, et al. [Sentinel lymph node biopsy in patients with cutaneous melanoma]. Duodecim. 2002;118(22):2289-94.
17. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. N Engl J Med. 2015;372(1):30-9.
18. Eggermont AM, Chiarion-Sileni V, Grob JJ, Dummer R, Wolchok JD, Schmidt H, et al. Prolonged Survival in Stage III Melanoma with Ipilimumab Adjuvant Therapy. The New England journal of medicine. 2016;375(19):1845-55.
19. GLOBOCAN 2020: Estimated age-standardized incidence rates (World) in 2020, melanoma of skin [Internet]. 2020 [cited February 9th 2023]. Available from: https://gco.iarc.fr.
20. Cancer statistics [Internet]. 2022 [cited February 9th 2023]. Available from: https://cancerregistry.fi/statistics/cancer-statistics/.
21. Welch HG, Mazer BL, Adamson AS. The Rapid Rise in Cutaneous Melanoma Diagnoses. N Engl J Med. 2021;384(1):72-9.
22. Chang C, Murzaku EC, Penn L, Abbasi NR, Davis PD, Berwick M, et al. More skin, more sun, more tan, more melanoma. Am J Public Health. 2014;104(11):e92-9.
23. Kondo T, Hearing VJ. Update on the regulation of mammalian melanocyte function and skin pigmentation. Expert Rev Dermatol. 2011;6(1):97-108.
24. Shain AH, Yeh I, Kovalyshyn I, Sriharan A, Talevich E, Gagnon A, et al. The Genetic Evolution of Melanoma from Precursor Lesions. N Engl J Med. 2015;373(20):1926-36.
25. Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, et al. A landscape of driver mutations in melanoma. Cell. 2012;150(2):251-63.
26. Cancer Genome Atlas N. Genomic Classification of Cutaneous Melanoma. Cell. 2015;161(7):1681-96.
27. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, et al. TERT promoter mutations in familial and sporadic melanoma. Science. 2013;339(6122):959-61.
28. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA. Highly recurrent TERT promoter mutations in human melanoma. Science. 2013;339(6122):957-9.
29. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res. 1996;56(19):4509-15.
30. Wachsberger P, Burd R, Dicker AP. Tumor response to ionizing radiation combined with antiangiogenesis or vascular targeting agents: exploring mechanisms of interaction. Clin Cancer Res. 2003;9(6):1957-71.
31. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci U S A. 2010;107(9):4275-80.
32. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med. 2011;364(26):2517-26.

33. Uren RF, Howman-Giles R, Thompson JF, McCarthy WH, Quinn MJ, Roberts JM, et al. Interval nodes: the forgotten sentinel nodes in patients with melanoma. Arch Surg. 2000;135(10):1168-72.
34. Hsueh EC, DeBloom JR, Lee JH, Sussman JJ, Covington KR, Caruso HG, et al. Long-Term Outcomes in a Multicenter, Prospective Cohort Evaluating the Prognostic 31-Gene Expression Profile for Cutaneous Melanoma. JCO Precis Oncol. 2021;5.
35. Greenhaw BN, Covington KR, Kurley SJ, Yeniay Y, Cao NA, Plasseraud KM, et al. Molecular risk prediction in cutaneous melanoma: A meta-analysis of the 31-gene expression profile prognostic test in 1,479 patients. J Am Acad Dermatol. 2020;83(3):745-53.
36. Soikkeli J, Lukk M, Nummela P, Virolainen S, Jahkola T, Katainen R, et al. Systematic search for the best gene expression markers for melanoma micrometastasis detection. J Pathol. 2007;213(2):180-9.
37. Bellomo D, Arias-Mejias SM, Ramana C, Heim JB, Quattrocchi E, Sominidi-Damodaran S, et al. Model Combining Tumor Molecular and Clinicopathologic Risk Factors Predicts Sentinel Lymph Node Metastasis in Primary Cutaneous Melanoma. JCO Precis Oncol. 2020;4:319-34.
38. Grossman D, Okwundu N, Bartlett EK, Marchetti MA, Othus M, Coit DG, et al. Prognostic Gene Expression Profiling in Cutaneous Melanoma: Identifying the Knowledge Gaps and Assessing the Clinical Benefit. JAMA Dermatol. 2020;156(9):1004-11.
39. Long GV, Hauschild A, Santinami M, Atkinson V, Mandala M, Chiarion-Sileni V, et al. Adjuvant Dabrafenib plus Trametinib in Stage III BRAF-Mutated Melanoma. The New England journal of medicine. 2017;377(19):1813-23.
40. Amaral TMS, Hoffmann MC, Sinnberg T, Niessner H, Sulberg H, Eigentler TK, et al. Clinical validation of a prognostic 11-gene expression profiling score in prospectively collected FFPE tissue of patients with AJCC v8 stage II cutaneous melanoma. Eur J Cancer. 2020;125:38-45.
41. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673-87.
42. Campbell ID, Humphries MJ. Integrin structure, activation, and interactions. Cold Spring Harb Perspect Biol. 2011;3(3).
43. Kern A, Eble J, Golbik R, Kuhn K. Interaction of type IV collagen with the isolated integrins alpha 1 beta 1 and alpha 2 beta 1. Eur J Biochem. 1993;215(1):151-9.
44. Voigt S, Gossrau R, Baum O, Loster K, Hofmann W, Reutter W. Distribution and quantification of alpha 1-integrin subunit in rat organs. Histochem J. 1995;27(2):123-32.
45. Zutter MM, Santoro SA. Widespread histologic distribution of the alpha 2 beta 1 integrin cell-surface collagen receptor. Am J Pathol. 1990;137(1):113-20.
46. Camper L, Holmvall K, Wangnerud C, Aszodi A, Lundgren-Akerlund E. Distribution of the collagen-binding integrin alpha10beta1 during mouse development. Cell Tissue Res. 2001;306(1):107-16.
47. Tiger CF, Fougerousse F, Grundstrom G, Velling T, Gullberg D. alpha11beta1 integrin is a receptor for interstitial collagens involved in cell migration and collagen reorganization on mesenchymal nonmuscle cells. Dev Biol. 2001;237(1):116-29.

48. Yoshinaga IG, Vink J, Dekker SK, Mihm MC, Jr., Byers HR. Role of alpha 3 beta 1 and alpha 2 beta 1 integrins in melanoma cell migration. Melanoma Res. 1993;3(6):435-41.
49. Danen EH, van Muijen GN, van de Wiel-van Kemenade E, Jansen KF, Ruiter DJ, Figdor CG. Regulation of integrin-mediated adhesion to laminin and collagen in human melanocytes and in non-metastatic and highly metastatic human melanoma cells. Int J Cancer. 1993;54(2):315-21.
50. Klein CE, Steinmayer T, Kaufmann D, Weber L, Brocker EB. Identification of a melanoma progression antigen as integrin VLA-2. J Invest Dermatol. 1991;96(2):281-4.
51. Riikonen T, Westermarck J, Koivisto L, Broberg A, Kahari VM, Heino J. Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression. J Biol Chem. 1995;270(22):13548-52.
52. Senger DR, Claffey KP, Benes JE, Perruzzi CA, Sergiou AP, Detmar M. Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. Proc Natl Acad Sci U S A. 1997;94(25):13612-7.
53. Huang R, Rofstad EK. Integrins as therapeutic targets in the organ-specific metastasis of human malignant melanoma. J Exp Clin Cancer Res. 2018;37(1):92.
54. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. 2005;438(7069):820-7.
55. Meves A, Nikolova E, Heim JB, Squirewell EJ, Cappel MA, Pittelkow MR, et al. Tumor Cell Adhesion As a Risk Factor for Sentinel Lymph Node Metastasis in Primary Cutaneous Melanoma. J Clin Oncol. 2015;33(23):2509-15.
56. Quattrocchi E, Sominidi-Damodaran S, Murphree DH, Meves A. beta3 integrin immunohistochemistry as a method to predict sentinel lymph node status in patients with primary cutaneous melanoma. Int J Dermatol. 2020;59(10):1241-8.
57. Tucci M, Mannavola F, Passarelli A, Stucci LS, Cives M, Silvestris F. Exosomes in melanoma: a role in tumor progression, metastasis and impaired immune system activity. Oncotarget. 2018;9(29):20826-37.
58. Casal JI, Bartolome RA. RGD cadherins and alpha2beta1 integrin in cancer metastasis: A dangerous liaison. Biochim Biophys Acta Rev Cancer. 2018;1869(2):321-32.
59. Saviola AJ, Burns PD, Mukherjee AK, Mackessy SP. The disintegrin tzabcanin inhibits adhesion and migration in melanoma and lung cancer cells. Int J Biol Macromol. 2016;88:457-64.
60. Pickarski M, Gleason A, Bednar B, Duong LT. Orally active alphavbeta3 integrin inhibitor MK-0429 reduces melanoma metastasis. Oncol Rep. 2015;33(6):2737-45.
61. Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, Long GV, Ross MI, et al. Melanoma staging: Evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA: a cancer journal for clinicians. 2017;67(6):472-92.
62. Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG, et al. Final version of the American Joint Committee on Cancer staging system

for cutaneous melanoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2001;19(16):3635-48.
63. Evans RD, Kopf AW, Lew RA, Rigel DS, Bart RS, Friedman RJ, et al. Risk Factors for the Development of Malignant Melanoma—I: Review of Case-Control Studies. The Journal of Dermatologic Surgery and Oncology. 1988;14(4):393-408.
64. Shih STF, Carter R, Heward S, Sinclair C. Skin cancer has a large impact on our public hospitals but prevention programs continue to demonstrate strong economic credentials. Aust N Z J Public Health. 2017;41(4):371-6.
65. Aitken JF, Youlden DR, Baade PD, Soyer HP, Green AC, Smithers BM. Generational shift in melanoma incidence and mortality in Queensland, Australia, 1995-2014. Int J Cancer. 2018;142(8):1528-35.
66. Garbe C, Amaral T, Peris K, Hauschild A, Arenberger P, Basset-Seguin N, et al. European consensus-based interdisciplinary guideline for melanoma. Part 1: Diagnostics: Update 2022. Eur J Cancer. 2022;170:236-55.
67. Reintgen DS, Cox C, Slingluff CL, Jr., Seigler HF. Recurrent malignant melanoma: the identification of prognostic factors to predict survival. Ann Plast Surg. 1992;28(1):45-9.
68. Veronesi U, Cascinelli N. Narrow excision (1-cm margin). A safe procedure for thin cutaneous melanoma. Arch Surg. 1991;126(4):438-41.
69. Balch CM, Soong SJ, Smith T, Ross MI, Urist MM, Karakousis CP, et al. Long-term results of a prospective surgical trial comparing 2 cm vs. 4 cm excision margins for 740 patients with 1-4 mm melanomas. Ann Surg Oncol. 2001;8(2):101-8.
70. Moncrieff MD, Gyorki D, Saw R, Spillane AJ, Thompson JF, Peach H, et al. 1 Versus 2-cm Excision Margins for pT2-pT4 Primary Cutaneous Melanoma (MelMarT): A Feasibility Study. Ann Surg Oncol. 2018;25(9):2541-9.
71. Cohen T, Busam KJ, Patel A, Brady MS. Subungual melanoma: management considerations. Am J Surg. 2008;195(2):244-8.
72. Sullivan SR, Liu DZ, Mathes DW, Isik FF. Head and neck malignant melanoma: local recurrence rate following wide local excision and immediate reconstruction. Ann Plast Surg. 2012;68(1):33-6.
73. Meier F, Will S, Ellwanger U, Schlagenhauff B, Schittek B, Rassner G, et al. Metastatic pathways and time courses in the orderly progression of cutaneous melanoma. Br J Dermatol. 2002;147(1):62-70.
74. Uren RF, Howman-Giles R, Thompson JF, Shaw HM, Quinn MJ, O'Brien CJ, et al. Lymphoscintigraphy to identify sentinel lymph nodes in patients with melanoma. Melanoma Res. 1994;4(6):395-9.
75. Krag DN, Meijer SJ, Weaver DL, Loggie BW, Harlow SP, Tanabe KK, et al. Minimal-access surgery for staging of malignant melanoma. Arch Surg. 1995;130(6):654-8; discussion 9-60.
76. Suominen E, Jahkola T, Jeskanen L, Knuuttila E, Mustonen P, Pyrhonen S, et al. [Not Available]. Duodecim. 2006;122(17):2157-8.
77. Cochran AJ, Balda BR, Starz H, Bachter D, Krag DN, Cruse CW, et al. The Augsburg Consensus. Techniques of lymphatic mapping, sentinel lymphadenectomy, and completion lymphadenectomy in cutaneous malignancies. Cancer. 2000;89(2):236-41.

78. Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Nieweg OE, Roses DF, et al. Final trial report of sentinel-node biopsy versus nodal observation in melanoma. The New England journal of medicine. 2014;370(7):599-609.
79. Garbe C, Amaral T, Peris K, Hauschild A, Arenberger P, Basset-Seguin N, et al. European consensus-based interdisciplinary guideline for melanoma. Part 2: Treatment - Update 2022. Eur J Cancer. 2022;170:256-84.
80. Wong SL, Faries MB, Kennedy EB, Agarwala SS, Akhurst TJ, Ariyan C, et al. Sentinel Lymph Node Biopsy and Management of Regional Lymph Nodes in Melanoma: American Society of Clinical Oncology and Society of Surgical Oncology Clinical Practice Guideline Update. Annals of surgical oncology. 2018;25(2):356-77.
81. Han D, Han G, Duque MT, Morrison S, Leong SP, Kashani-Sabet M, et al. Sentinel Lymph Node Biopsy Is Prognostic in Thickest Melanoma Cases and Should Be Performed for Thick Melanomas. Ann Surg Oncol. 2021;28(2):1007-16.
82. Leijte JA, van der Ploeg IM, Valdes Olmos RA, Nieweg OE, Horenblas S. Visualization of tumor blockage and rerouting of lymphatic drainage in penile cancer patients by use of SPECT/CT. J Nucl Med. 2009;50(3):364-7.
83. Bedrosian I, Scheff AM, Mick R, Callans LS, Bucky LP, Spitz FR, et al. 99mTc-human serum albumin: an effective radiotracer for identifying sentinel lymph nodes in melanoma. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 1999;40(7):1143-8.
84. Pijpers R, Borgstein PJ, Meijer S, Krag DN, Hoekstra OS, Greuter HN, et al. Transport and retention of colloidal tracers in regional lymphoscintigraphy in melanoma: influence on lymphatic mapping and sentinel node biopsy. Melanoma research. 1998;8(5):413-8.
85. Verwer N, Scolyer RA, Uren RF, Winstanley J, Brown PT, de Wilt JH, et al. Treatment and prognostic significance of positive interval sentinel nodes in patients with primary cutaneous melanoma. Ann Surg Oncol. 2011;18(12):3292-9.
86. Even-Sapir E, Lerman H, Lievshitz G, Khafif A, Fliss DM, Schwartz A, et al. Lymphoscintigraphy for sentinel node mapping using a hybrid SPECT/CT system. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2003;44(9):1413-20.
87. Uren RF. SPECT/CT Lymphoscintigraphy to locate the sentinel lymph node in patients with melanoma. Annals of surgical oncology. 2009;16(6):1459-60.
88. Gannon CJ, Rousseau DL, Jr., Ross MI, Johnson MM, Lee JE, Mansfield PF, et al. Accuracy of lymphatic mapping and sentinel lymph node biopsy after previous wide local excision in patients with primary melanoma. Cancer. 2006;107(11):2647-52.
89. Fahy AS, Grotz TE, Keeney GL, Glasgow AE, Habermann EB, Erickson L, et al. Frozen section analysis of SLNs in trunk and extremity melanoma has a high false negative rate but can spare some patients a second operation. J Surg Oncol. 2016;114(7):879-83.
90. Stojadinovic A, Allen PJ, Clary BM, Busam KJ, Coit DG. Value of frozen-section analysis of sentinel lymph nodes for primary cutaneous malignant melanoma. Ann Surg. 2002;235(1):92-8.

91. Van der Velde-Zimmermann D, Roijers JF, Bouwens-Rombouts A, De Weger RA, De Graaf PW, Tilanus MG, et al. Molecular test for the detection of tumor cells in blood and sentinel nodes of melanoma patients. Am J Pathol. 1996;149(3):759-64.
92. Takeuchi H, Morton DL, Kuo C, Turner RR, Elashoff D, Elashoff R, et al. Prognostic significance of molecular upstaging of paraffin-embedded sentinel lymph nodes in melanoma patients. J Clin Oncol. 2004;22(13):2671-80.
93. McMasters KM, Egger ME, Edwards MJ, Ross MI, Reintgen DS, Noyes RD, et al. Final Results of the Sunbelt Melanoma Trial: A Multi-Institutional Prospective Randomized Phase III Study Evaluating the Role of Adjuvant High-Dose Interferon Alfa-2b and Completion Lymph Node Dissection for Patients Staged by Sentinel Lymph Node Biopsy. J Clin Oncol. 2016;34(10):1079-86.
94. Thompson JF, Uren RF. What is a 'sentinel' lymph node? European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2000;26(2):103-4.
95. Carlson GW, Murray DR, Thourani V, Hestley A, Cohen C. The definition of the sentinel lymph node in melanoma based on radioactive counts. Annals of surgical oncology. 2002;9(9):929-33.
96. McMasters KM, Reintgen DS, Ross MI, Wong SL, Gershenwald JE, Krag DN, et al. Sentinel lymph node biopsy for melanoma: how many radioactive nodes should be removed? Annals of surgical oncology. 2001;8(3):192-7.
97. Patel N, Allen M, Arianpour K, Keidan R. The utility of ICG fluorescence for sentinel lymph node identification in head and neck melanoma. Am J Otolaryngol. 2021;42(5):103147.
98. Knackstedt R, Gastman BR. Indocyanine Green Fluorescence Imaging with Lymphoscintigraphy Improves the Accuracy of Sentinel Lymph Node Biopsy in Melanoma. Plast Reconstr Surg. 2021;148(1):83e-93e.
99. Stoffels I, Dissemond J, Poppel T, Schadendorf D, Klode J. Intraoperative Fluorescence Imaging for Sentinel Lymph Node Detection: Prospective Clinical Trial to Compare the Usefulness of Indocyanine Green vs Technetium Tc 99m for Identification of Sentinel Lymph Nodes. JAMA Surg. 2015;150(7):617-23.
100. Uren RF, Howman-Giles R, Thompson JF. Patterns of lymphatic drainage from the skin in patients with melanoma. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2003;44(4):570-82.
101. Pan WR, Wang DG, Levy SM, Chen Y. Superficial lymphatic drainage of the lower extremity: anatomical study and clinical implications. Plastic and Reconstructive Surgery. 2013;132(3):696-707.
102. van der Ploeg IM, Kroon BB, Valdes Olmos RA, Nieweg OE. Evaluation of lymphatic drainage patterns to the groin and implications for the extent of groin dissection in melanoma patients. Annals of surgical oncology. 2009;16(11):2994-9.
103. Kaoutzanis C, Barabas A, Allan R, Hussain M, Powell B. When should pelvic sentinel lymph nodes be harvested in patients with malignant

melanoma? Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2012;65(1):85-90.
104. Karakousis GC, Pandit-Taskar N, Hsu M, Panageas K, Atherton S, Ariyan C, et al. Prognostic significance of drainage to pelvic nodes at sentinel lymph node mapping in patients with extremity melanoma. Melanoma research. 2013;23(1):40-6.
105. Schuitevoerder D, Leong SPL, Zager JS, White RL, Avisar E, Kosiorek H, et al. Is pelvic sentinel node biopsy necessary for lower extremity and trunk melanomas? American Journal of Surgery. 2017.
106. Swords DS, Andtbacka RHI, Bowles TL, Hyngstrom JR. Routine retrieval of pelvic sentinel lymph nodes for melanoma rarely adds prognostic information or alters management. Melanoma research. 2019;29(1):38-46.
107. Egger ME, Brown RE, Roach BA, Quillo AR, Martin RC, 2nd, Scoggins CR, et al. Addition of an iliac/obturator lymph node dissection does not improve nodal recurrence or survival in melanoma. Journal of the American College of Surgeons. 2014;219(1):101-8.
108. Mozzillo N, Pasquali S, Santinami M, Testori A, Di Marzo M, Crispo A, et al. Factors predictive of pelvic lymph node involvement and outcomes in melanoma patients with metastatic sentinel lymph node of the groin: A multicentre study. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2015;41(7):823-9.
109. Verver D, Madu MF, Oude Ophuis CMC, Faut M, de Wilt JHW, Bonenkamp JJ, et al. Optimal extent of completion lymphadenectomy for patients with melanoma and a positive sentinel node in the groin. The British journal of surgery. 2018;105(1):96-105.
110. Pasquali S, Mocellin S, Bigolin F, Vecchiato A, Montesco MC, Di Maggio A, et al. Pelvic lymph node status prediction in melanoma patients with inguinal lymph node metastasis. Melanoma research. 2014;24(5):462-7.
111. Chu CK, Zager JS, Marzban SS, Gimbel MI, Murray DR, Hestley AC, et al. Routine biopsy of Cloquet's node is of limited value in sentinel node positive melanoma patients. Journal of surgical oncology. 2010;102(4):315-20.
112. Dossett LA, Castner NB, Pow-Sang JM, Abbott AM, Sondak VK, Sarnaik AA, et al. Robotic-Assisted Transperitoneal Pelvic Lymphadenectomy for Metastatic Melanoma: Early Outcomes Compared with Open Pelvic Lymphadenectomy. J Am Coll Surg. 2016;222(4):702-9.
113. Sohn W, Finley DS, Jakowatz J, Ornstein DK. Robot-assisted laparoscopic transperitoneal pelvic lymphadenectomy and metastasectomy for melanoma: initial report of two cases. Journal of robotic surgery. 2010;4(2):129-32.
114. Nieweg OE. False-negative sentinel node biopsy. Ann Surg Oncol. 2009;16(8):2089-91.
115. Testori A, De Salvo GL, Montesco MC, Trifiro G, Mocellin S, Landi G, et al. Clinical considerations on sentinel node biopsy in melanoma from an Italian multicentric study on 1,313 patients (SOLISM-IMI). Ann Surg Oncol. 2009;16(7):2018-27.
116. Karim RZ, Scolyer RA, Li W, Yee VS, McKinnon JG, Li LX, et al. False negative sentinel lymph node biopsies in melanoma may result from

deficiencies in nuclear medicine, surgery, or pathology. Annals of Surgery. 2008;247(6):1003-10.
117. Kretschmer L, Altenvoerde G, Meller J, Zutt M, Funke M, Neumann C, et al. Dynamic lymphoscintigraphy and image fusion of SPECT and pelvic CT-scans allow mapping of aberrant pelvic sentinel lymph nodes in malignant melanoma. European journal of cancer (Oxford, England : 1990). 2003;39(2):175-83.
118. Cook MG, Green MA, Anderson B, Eggermont AM, Ruiter DJ, Spatz A, et al. The development of optimal pathological assessment of sentinel lymph nodes for melanoma. J Pathol. 2003;200(3):314-9.
119. Wrightson WR, Wong SL, Edwards MJ, Chao C, Reintgen DS, Ross MI, et al. Complications associated with sentinel lymph node biopsy for melanoma. Annals of surgical oncology. 2003;10(6):676-80.
120. Morton DL, Cochran AJ, Thompson JF, Elashoff R, Essner R, Glass EC, et al. Sentinel node biopsy for early-stage melanoma: accuracy and morbidity in MSLT-I, an international multicenter trial. Annals of Surgery. 2005;242(3):302-11; discussion 11-3.
121. Kretschmer L, Thoms KM, Peeters S, Haenssle H, Bertsch HP, Emmert S. Postoperative morbidity of lymph node excision for cutaneous melanoma-sentinel lymphonodectomy versus complete regional lymph node dissection. Melanoma research. 2008;18(1):16-21.
122. Michielin O, van Akkooi ACJ, Ascierto PA, Dummer R, Keilholz U, clinicalguidelines@esmo.org EGCEa. Cutaneous melanoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-updagger. Ann Oncol. 2019;30(12):1884-901.
123. Suomen Melanoomaryhmä Ry. Melanoomaryhmän kansalliset hoitosuositukset 2022 [Available from: https://www.onkologiayhdistys.fi/fin/alaryhmat/melanoomaryhma/hoitosuositukset/.
124. Multicenter Selective Lymphadenectomy Trials Study Group, Crystal JS, Thompson JF, Hyngstrom J, Caraco C, Zager JS, et al. Therapeutic Value of Sentinel Lymph Node Biopsy in Patients With Melanoma: A Randomized Clinical Trial. JAMA Surg. 2022;157(9):835-42.
125. Chang JM, Kosiorek HE, Dueck AC, Leong SPL, Vetto JT, White RL, et al. Stratifying SLN incidence in intermediate thickness melanoma patients. Am J Surg. 2018;215(4):699-706.
126. Moncrieff MD, Lo SN, Scolyer RA, Heaton MJ, Nobes JP, Snelling AP, et al. Evaluation of the Indications for Sentinel Node Biopsy in Early-Stage Melanoma with the Advent of Adjuvant Systemic Therapy: An International, Multicenter Study. Ann Surg Oncol. 2022;29(9):5937-45.
127. Lo SN, Ma J, Scolyer RA, Haydu LE, Stretch JR, Saw RPM, et al. Improved Risk Prediction Calculator for Sentinel Node Positivity in Patients With Melanoma: The Melanoma Institute Australia Nomogram. J Clin Oncol. 2020;38(24):2719-27.
128. Cochran AJ, Wen DR, Huang RR, Wang HJ, Elashoff R, Morton DL. Prediction of metastatic melanoma in nonsentinel nodes and clinical outcome based on the primary melanoma and the sentinel node. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2004;17(7):747-55.

129. Gershenwald JE, Andtbacka RH, Prieto VG, Johnson MM, Diwan AH, Lee JE, et al. Microscopic tumor burden in sentinel lymph nodes predicts synchronous nonsentinel lymph node involvement in patients with melanoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26(26):4296-303.
130. Sabel MS, Griffith K, Sondak VK, Lowe L, Schwartz JL, Cimmino VM, et al. Predictors of nonsentinel lymph node positivity in patients with a positive sentinel node for melanoma. Journal of the American College of Surgeons. 2005;201(1):37-47.
131. Dewar DJ, Newell B, Green MA, Topping AP, Powell BW, Cook MG. The microanatomic location of metastatic melanoma in sentinel lymph nodes predicts nonsentinel lymph node involvement. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2004;22(16):3345-9.
132. Cochran AJ, Wen DR, Huang RR, Abrishami P, Smart C, Binder S, et al. Sentinel lymph node melanoma metastases: Assessment of tumor burden for clinical prediction of outcome in the first Multicenter Selective Lymphadenectomy Trial (MSLT-I). Eur J Surg Oncol. 2022;48(6):1280-7.
133. Murali R, Desilva C, Thompson JF, Scolyer RA. Non-Sentinel Node Risk Score (N-SNORE): a scoring system for accurately stratifying risk of non-sentinel node positivity in patients with cutaneous melanoma with positive sentinel lymph nodes. J Clin Oncol. 2010;28(29):4441-9.
134. Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, et al. Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. J Clin Oncol. 2001;19(16):3622-34.
135. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Ding S, Byrd DR, et al. Multivariate analysis of prognostic factors among 2,313 patients with stage III melanoma: comparison of nodal micrometastases versus macrometastases. J Clin Oncol. 2010;28(14):2452-9.
136. Barbour AP, Tang YH, Armour N, Dutton-Regester K, Krause L, Loffler KA, et al. BRAF mutation status is an independent prognostic factor for resected stage IIIB and IIIC melanoma: implications for melanoma staging and adjuvant therapy. Eur J Cancer. 2014;50(15):2668-76.
137. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. Cancer. 2010;116(7):1757-66.
138. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. 2015;16(4):375-84.
139. Reiman A, Kikuchi H, Scocchia D, Smith P, Tsang YW, Snead D, et al. Validation of an NGS mutation detection panel for melanoma. BMC Cancer. 2017;17(1):150.
140. Castillo P, Marginet M, Jares P, García M, Gonzalvo E, Arance A, et al. Implementation of an NGS panel for clinical practice in paraffin-embedded tissue samples from locally advanced and metastatic melanoma patients. Explor Target Antitumor Ther. 2020;1(2):101-8.
141. Huang T, Chen X, Zhang H, Liang Y, Li L, Wei H, et al. Prognostic Role of Tumor Mutational Burden in Cancer Patients Treated With Immune

Checkpoint Inhibitors: A Systematic Review and Meta-Analysis. Front Oncol. 2021;11:706652.
142. Long GV, Grob JJ, Nathan P, Ribas A, Robert C, Schadendorf D, et al. Factors predictive of response, disease progression, and overall survival after dabrafenib and trametinib combination treatment: a pooled analysis of individual patient data from randomised trials. Lancet Oncol. 2016;17(12):1743-54.
143. Egger ME, Bower MR, Czyszczon IA, Farghaly H, Noyes RD, Reintgen DS, et al. Comparison of sentinel lymph node micrometastatic tumor burden measurements in melanoma. Journal of the American College of Surgeons. 2014;218(4):519-28.
144. Starz H, Siedlecki K, Balda BR. Sentinel lymphonodectomy and s-classification: a successful strategy for better prediction and improvement of outcome of melanoma. Annals of surgical oncology. 2004;11(3 Suppl):162S-8S.
145. Koskivuo I, Talve L, Vihinen P, Mäki M, Vahlberg T, Suominen E. Sentinel lymph node biopsy in cutaneous melanoma: a case-control study. Ann Surg Oncol. 2007;14(12):3566-74.
146. Vuylsteke RJ, Borgstein PJ, van Leeuwen PA, Gietema HA, Molenkamp BG, Statius Muller MG, et al. Sentinel lymph node tumor load: an independent predictor of additional lymph node involvement and survival in melanoma. Ann Surg Oncol. 2005;12(6):440-8.
147. Ranieri JM, Wagner JD, Azuaje R, Davidson D, Wenck S, Fyffe J, et al. Prognostic importance of lymph node tumor burden in melanoma patients staged by sentinel node biopsy. Annals of surgical oncology. 2002;9(10):975-81.
148. Murali R, Cochran AJ, Cook MG, Hillman JD, Karim RZ, Moncrieff M, et al. Interobserver reproducibility of histologic parameters of melanoma deposits in sentinel lymph nodes: implications for management of patients with melanoma. Cancer. 2009;115(21):5026-37.
149. van Akkooi AC, de Wilt JH, Verhoef C, Schmitz PI, van Geel AN, Eggermont AM, et al. Clinical relevance of melanoma micrometastases (<0.1 mm) in sentinel nodes: are these nodes to be considered negative? Annals of oncology : official journal of the European Society for Medical Oncology. 2006;17(10):1578-85.
150. Eggermont AMM, Blank CU, Mandala M, Long GV, Atkinson V, Dalle S, et al. Adjuvant Pembrolizumab versus Placebo in Resected Stage III Melanoma. The New England journal of medicine. 2018;378(19):1789-801.
151. Ives NJ, Suciu S, Eggermont AMM, Kirkwood J, Lorigan P, Markovic SN, et al. Adjuvant interferon-α for the treatment of high-risk melanoma: An individual patient data meta-analysis. Eur J Cancer. 2017;82:171-83.
152. Weber J, Mandala M, Del Vecchio M, Gogas HJ, Arance AM, Cowey CL, et al. Adjuvant Nivolumab versus Ipilimumab in Resected Stage III or IV Melanoma. The New England journal of medicine. 2017;377(19):1824-35.
153. Menzies AM, Amaria RN, Rozeman EA, Huang AC, Tetzlaff MT, van de Wiel BA, et al. Pathological response and survival with neoadjuvant therapy in melanoma: a pooled analysis from the International Neoadjuvant Melanoma Consortium (INMC). Nature Medicine. 2021;27(2):301-9.

154. Amaria RN, Postow M, Burton EM, Tezlaff MT, Ross MI, Torres-Cabala C, et al. Neoadjuvant relatlimab and nivolumab in resectable melanoma. Nature. 2022;611(7934):155-60.
155. Luke JJ, Rutkowski P, Queirolo P, Del Vecchio M, Mackiewicz J, Chiarion-Sileni V, et al. Pembrolizumab versus placebo as adjuvant therapy in completely resected stage IIB or IIC melanoma (KEYNOTE-716): a randomised, double-blind, phase 3 trial. Lancet. 2022;399(10336):1718-29.
156. Hindie E. Adjuvant therapy in stage IIB and IIC melanoma: is sentinel biopsy needed? Lancet. 2022;400(10352):559.
157. Henderson MA, Burmeister BH, Ainslie J, Fisher R, Di Iulio J, Smithers BM, et al. Adjuvant lymph-node field radiotherapy versus observation only in patients with melanoma at high risk of further lymph-node field relapse after lymphadenectomy (ANZMTG 01.02/TROG 02.01): 6-year follow-up of a phase 3, randomised controlled trial. Lancet Oncol. 2015;16(9):1049-60.
158. Olivier KR, Schild SE, Morris CG, Brown PD, Markovic SN. A higher radiotherapy dose is associated with more durable palliation and longer survival in patients with metastatic melanoma. Cancer. 2007;110(8):1791-5.
159. Grünhagen DJ, Brunstein F, Graveland WJ, van Geel AN, de Wilt JHW, Eggermont AMM. One Hundred Consecutive Isolated Limb Perfusions With TNF-α and Melphalan in Melanoma Patients With Multiple In-Transit Metastases. Annals of Surgery. 2004;240(6):939-48.
160. Moreno-Ramirez D, de la Cruz-Merino L, Ferrandiz L, Villegas-Portero R, Nieto-Garcia A. Isolated limb perfusion for malignant melanoma: systematic review on effectiveness and safety. Oncologist. 2010;15(4):416-27.
161. Liu BL, Robinson M, Han ZQ, Branston RH, English C, Reay P, et al. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther. 2003;10(4):292-303.
162. Andtbacka RHI, Collichio F, Harrington KJ, Middleton MR, Downey G, Öhrling K, et al. Final analyses of OPTiM: a randomized phase III trial of talimogene laherparepvec versus granulocyte-macrophage colony-stimulating factor in unresectable stage III–IV melanoma. Journal for ImmunoTherapy of Cancer. 2019;7(1):145.
163. Chesney JA, Ribas A, Long GV, Kirkwood JM, Dummer R, Puzanov I, et al. Randomized, Double-Blind, Placebo-Controlled, Global Phase III Trial of Talimogene Laherparepvec Combined With Pembrolizumab for Advanced Melanoma. J Clin Oncol. 2023;41(3):528-40.
164. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: a multicentre, double-blind, phase 3 randomised controlled trial. Lancet. 2015;386(9992):444-51.
165. Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, et al. Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. J Clin Oncol. 2015;33(17):1889-94.
166. Hodi FS, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Cowey CL, et al. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. Lancet Oncol. 2018;19(11):1480-92.

167. Haanen J, Obeid M, Spain L, Carbonnel F, Wang Y, Robert C, et al. Management of toxicities from immunotherapy: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. Ann Oncol. 2022;33(12):1217-38.
168. Moncrieff MD, Bastiaannet E, Underwood B, Francken AB, Garioch J, Damude S, et al. Follow-up Schedule for Patients With Sentinel Node-negative Cutaneous Melanoma (The MELFO Study): An International Phase III Randomized Clinical Trial. Ann Surg. 2022;276(4):e208-e16.
169. Vlaykova T, Talve L, Hahka-Kemppinen M, Hernberg M, Muhonen T, Collan Y, et al. Immunohistochemically detectable bcl-2 expression in metastatic melanoma: association with survival and treatment response. Oncology. 2002;62(3):259-68.
170. Kronqvist P, Kuopio T, Collan Y. Morphometric grading of invasive ductal breast cancer. I. Thresholds for nuclear grade. Br J Cancer. 1998;78(6):800-5.
171. Yu C, Zhang M, Song J, Zheng X, Xu G, Bao Y, et al. Integrin-Src-YAP1 signaling mediates the melanoma acquired resistance to MAPK and PI3K/mTOR dual targeted therapy. Mol Biomed. 2020;1(1):12.
172. Navab R, Strumpf D, To C, Pasko E, Kim KS, Park CJ, et al. Integrin α11β1 regulates cancer stromal stiffness and promotes tumorigenicity and metastasis in non-small cell lung cancer. Oncogene. 2016;35(15):1899-908.
173. Primac I, Maquoi E, Blacher S, Heljasvaara R, Van Deun J, Smeland HY, et al. Stromal integrin α11 regulates PDGFR-β signaling and promotes breast cancer progression. J Clin Invest. 2019;129(11):4609-28.
174. Ando T, Kage H, Matsumoto Y, Zokumasu K, Yotsumoto T, Maemura K, et al. Integrin α11 in non-small cell lung cancer is associated with tumor progression and postoperative recurrence. Cancer Sci. 2020;111(1):200-8.
175. Martínez-Nieto GA, Teppo HR, Petrelius N, Izzi V, Devarajan R, Petäistö T, et al. Upregulated integrin α11 in the stroma of cutaneous squamous cell carcinoma promotes skin carcinogenesis. Front Oncol. 2022;12:981009.
176. Ilmonen S, Hernberg M, Pyrhonen S, Tarkkanen J, Asko-Seljavaara S. Ki-67, Bcl-2 and p53 expression in primary and metastatic melanoma. Melanoma Res. 2005;15(5):375-81.
177. Espindola MB, Corleta OC. Bcl-2 expression is not associated with survival in metastatic cutaneous melanoma: a historical cohort study. World J Surg Oncol. 2008;6:65.
178. Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Zhang XD, Thompson JF, et al. Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. Mod Pathol. 2007;20(4):416-26.
179. Amaral T, Sinnberg T, Chatziioannou E, Niessner H, Leiter U, Keim U, et al. Identification of stage I/II melanoma patients at high risk for recurrence using a model combining clinicopathologic factors with gene expression profiling (CP-GEP). Eur J Cancer. 2022.
180. Ulloa-Montoya F, Louahed J, Dizier B, Gruselle O, Spiessens B, Lehmann FF, et al. Predictive gene signature in MAGE-A3 antigen-specific cancer immunotherapy. J Clin Oncol. 2013;31(19):2388-95.

181. Ariyan C, Brady MS, Gonen M, Busam K, Coit D. Positive nonsentinel node status predicts mortality in patients with cutaneous melanoma. Annals of surgical oncology. 2009;16(1):186-90.
182. Baehner FL, Li R, Jenkins T, Hwang J, Kashani-Sabet M, Allen RE, et al. The impact of primary melanoma thickness and microscopic tumor burden in sentinel lymph nodes on melanoma patient survival. Annals of surgical oncology. 2012;19(3):1034-42.
183. van der Ploeg AP, van Akkooi AC, Rutkowski P, Nowecki ZI, Michej W, Mitra A, et al. Prognosis in patients with sentinel node-positive melanoma is accurately defined by the combined Rotterdam tumor load and Dewar topography criteria. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(16):2206-14.
184. van Akkooi AC, Nowecki ZI, Voit C, Schafer-Hesterberg G, Michej W, de Wilt JH, et al. Sentinel node tumor burden according to the Rotterdam criteria is the most important prognostic factor for survival in melanoma patients: a multicenter study in 388 patients with positive sentinel nodes. Annals of Surgery. 2008;248(6):949-55.
185. Satzger I, Leiter U, Grager N, Keim U, Garbe C, Gutzmer R. Melanoma-specific survival in patients with positive sentinel lymph nodes: Relevance of sentinel tumor burden. European journal of cancer (Oxford, England : 1990). 2019;123:83-91.
186. Kim C, Economou S, Amatruda TT, Martin JC, Dudek AZ. Prognostic significance of microscopic tumor burden in sentinel lymph node in patients with cutaneous melanoma. Anticancer Research. 2015;35(1):301-9.
187. Borgognoni L, Bellucci F, Urso C, Manneschi G, Gerlini G, Brandani P, et al. Enhancing the prognostic role of melanoma sentinel lymph nodes through microscopic tumour burden characterization: clinical usefulness in patients who do not undergo complete lymph node dissection. Melanoma research. 2019;29(2):163-71.
188. Palve J, Ylitalo L, Luukkaala T, Jernman J, Korhonen N. Sentinel node tumor burden in prediction of prognosis in melanoma patients. Clinical & experimental metastasis. 2020;37(2):365-76.
189. Verver D, van Klaveren D, van Akkooi ACJ, Rutkowski P, Powell BWEM, Robert C, et al. Risk stratification of sentinel node-positive melanoma patients defines surgical management and adjuvant therapy treatment considerations. European journal of cancer (Oxford, England : 1990). 2018;96:25-33.
190. Satzger I, Volker B, Meier A, Kapp A, Gutzmer R. Criteria in sentinel lymph nodes of melanoma patients that predict involvement of nonsentinel lymph nodes. Annals of surgical oncology. 2008;15(6):1723-32.
191. Frankel TL, Griffith KA, Lowe L, Wong SL, Bichakjian CK, Chang AE, et al. Do micromorphometric features of metastatic deposits within sentinel nodes predict nonsentinel lymph node involvement in melanoma? Annals of surgical oncology. 2008;15(9):2403-11.
192. Dummer R, Hauschild A, Santinami M, Atkinson V, Mandala M, Kirkwood JM, et al. Five-Year Analysis of Adjuvant Dabrafenib plus Trametinib in Stage III Melanoma. The New England journal of medicine. 2020;383(12):1139-48.

193. Eggermont AMM, Blank CU, Mandala M, Long GV, Atkinson VG, Dalle S, et al. Longer Follow-Up Confirms Recurrence-Free Survival Benefit of Adjuvant Pembrolizumab in High-Risk Stage III Melanoma: Updated Results From the EORTC 1325-MG/KEYNOTE-054 Trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2020:JCO2002110.
194. Laakkonen J, Kaarela O, Tervala T, Nuutinen H. A role for pelvic sentinel lymph nodes in lower extremity melanoma? Scand J Surg. 2023:14574969221149968.
195. Porter GA, Ross MI, Berman RS, Sumner WE, 3rd, Lee JE, Mansfield PF, et al. How many lymph nodes are enough during sentinel lymphadenectomy for primary melanoma? Surgery. 2000;128(2):306-11.
196. Liu LC, Parrett BM, Jenkins T, Lee W, Morita E, Treseler P, et al. Selective sentinel lymph node dissection for melanoma: importance of harvesting nodes with lower radioactive counts without the need for blue dye. Annals of surgical oncology. 2011;18(10):2919-24.
197. Ranson JM, Pantelides NM, Pandit DG, Laitung J. Sentinel lymph node biopsy in melanoma: Which hot nodes should be harvested and is blue dye really necessary? Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2018;71(9):1269-73.
198. Abou-Nukta F, Ariyan S. Sentinel lymph node biopsies in melanoma: how many nodes do we really need? Annals of Plastic Surgery. 2008;60(4):416-9.
199. Murphy AD, Britten A, Powell B. Hot or not? The 10% rule in sentinel lymph node biopsy for malignant melanoma revisited. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2013.
200. Moncrieff M, Pywell S, Snelling A, Gray M, Newman D, Beadsmoore C, et al. Effectiveness of SPECT/CT Imaging for Sentinel Node Biopsy Staging of Primary Cutaneous Melanoma and Patient Outcomes. Annals of Surgical Oncology. 2022;29(2):767-75.
201. Hunting AS, Nopp A, Johansson SG, Andersen F, Wilhelmsen V, Guttormsen AB. Anaphylaxis to Patent Blue V. I. Clinical aspects. Allergy. 2010;65(1):117-23.
202. Leong SP, Donegan E, Heffernon W, Dean S, Katz JA. Adverse reactions to isosulfan blue during selective sentinel lymph node dissection in melanoma. Annals of surgical oncology. 2000;7(5):361-6.