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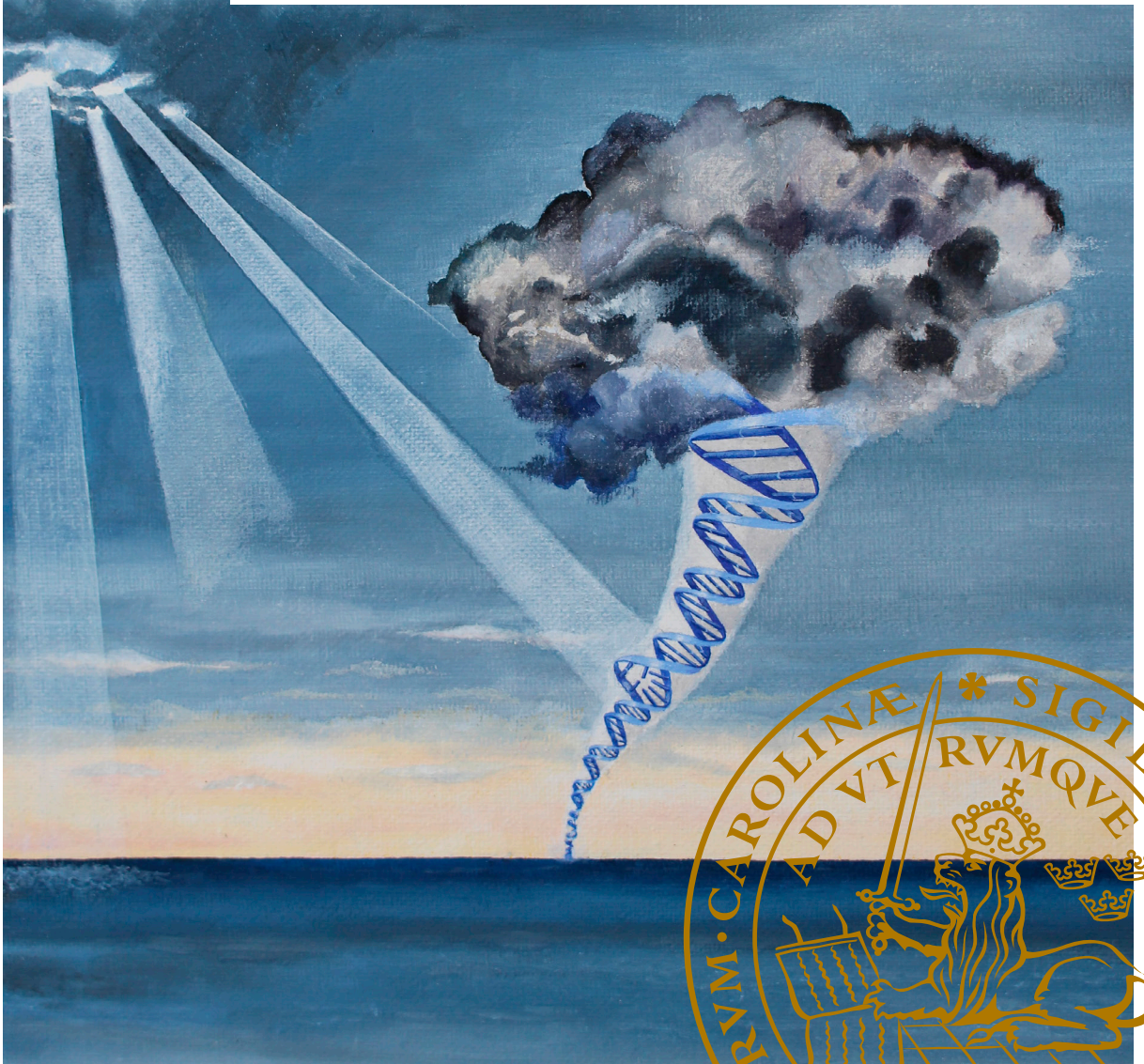
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Clinical aspects of molecular profiles in metastatic malignant melanoma

Abstract:

Malignant melanoma is a heterogeneous, malignant neoplastic disease, most often originating in the skin. Melanoma is characterized by a high mutational load and has a vastly variable prognosis, depending on disease stage. Genetic aberrations in the mitogen-activating protein kinase (MAPK) pathway are important in melanoma, of which mutations in *BRAF* and *NRAS* are the most common. Additionally, recurrent mutations in the promoter of *TERT*, the catalytic subunit of telomerase, have been associated with a poor prognosis in primary melanoma. The introduction of the first T-cell activating antibody, ipilimumab, and the first selective inhibitor of mutant BRAF, vemurafenib, marked the beginning of a new paradigm in the treatment of metastatic melanoma. The rapidly increasing number of treatment options warrants improved prognostic and predictive capability. The aim of this thesis was to examine clinical aspects, in particular prognostic and predictive values, of mutational and transcriptional profiles in metastatic melanoma.

Frozen tumor samples from the Lund Melanoma Study Group molecular melanoma cohort were subjected to mutation analysis of *BRAF*, *NRAS* (paper I), and the *TERT* promoter (paper III), as well as global gene expression analysis and deep targeted sequencing (paper II). Patients with *BRAF*-mutant tumors not treated with BRAF inhibitor showed an inferior overall survival from stage IV disease compared with patients treated with BRAF inhibitor (hazard ratio (HR) 2.35, confidence interval (CI) 1.10-5.01). There was a trend towards better prognosis for patients with wildtype tumors compared with *BRAFV600E*-mutants (HR 0.64, CI 0.39-1.04). *TERT* promoter mutations were not associated with prognosis in non-acral cutaneous metastatic melanoma. Two hundred fourteen melanoma samples, mostly metastases, were classified into four gene expression phenotypes, reflecting distinct biological features: 'proliferative', 'pigmentation', 'high-immune response', and 'normal-like'. Mutational patterns were similar across the phenotypes. Among patients with regional metastatic disease, the proliferative and the pigmentation phenotypes were associated with an increased risk of distant metastasis (HR 2.8, CI 1.43-5.57, and HR 1.9, CI 1.05-3.28) compared with the high-immune response phenotype. In two external datasets, the proliferative phenotype was found to be enriched in tumors progressing on MAPK inhibition.

In paper IV, the one-year clinical use of a next generation sequencing-based 26-genes mutation panel in advanced melanoma was characterized in relation to given treatment. The fraction of *BRAF* hotspot-mutant alleles was highly heterogeneous, and patients with tumors harboring a fraction in the highest and lowest deciles progressed early on MAPK inhibition.

In conclusion, metastatic melanoma displays various mutational and transcriptional profiles, relevant for prognosis and treatment prediction.

Key words
Melanoma, prognosis, BRAF, TERT, gene expression, next generation sequencing

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Clinical aspects of molecular profiles in metastatic malignant melanoma

Henrik Ekedahl

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To my family

Content

List of papers

I.   **Ekedahl H**, Cirenajwis, H, Harbst K, Carneiro A, Nielsen K, Olsson H, Lundgren L, Ingvar C, Jönsson G.

The clinical significance of *BRAF* and *NRAS* mutations in a clinic-based metastatic melanoma cohort.

Br J Dermatol. 2013 Nov;169(5): 1049-55.

II.   Cirenajwis H, **Ekedahl H**, Lauss M, Harbst K, Carneiro A, Enoksson J, Rosengren F, Werner-Hartman L, Törngren T, Kvist A, Fredlund E, Bendahl PO, Jirström K, Lundgren L, Howlin J, Borg Å, Gruvberger-Saal SK, Saal LH, Nielsen K, Ringnér M, Tsao H, Olsson H, Ingvar C, Staaf J, Jönsson G.

Molecular stratification of metastatic melanoma using gene expression profiling: Prediction of survival outcome and benefit from molecular targeted therapy.

Oncotarget. 2015 May 20;6(14): 12297-309.

III. **Ekedahl H**, Lauss M, Olsson H, Griewank KG, Schadendorf D, Ingvar C, Jönsson G.

High *TERT* promoter mutation frequency in non-acral cutaneous metastatic melanoma.

Pigment Cell Melanoma Res. 2016 Sep;29(5):598-600.

IV.   **Ekedahl H***, Nyström H*, Edsjö A, Lindquist KE, Levéen P, Staaf J, Ingvar C, Jönsson G, Carneiro A.

Next generation sequencing-based gene panel analysis in advanced melanoma: one-year clinical experience.

Manuscript. *Contributed equally.

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Related papers not in the thesis

- Cirenajwis H, Lauss M, **Ekedahl H**, Törngren T, Kvist A, Saal LH, Olsson H, Staaf J, Carneiro A, Ingvar C, Harbst K, Hayward NK, Jönsson G.

  NF1-mutated melanoma tumors harbor distinct clinical and biological characteristics.

  Mol Oncol. 2017 Mar 7. doi: 10.1002/1878-0261.12050. [Epub ahead of print]

- Harbst K, Staaf J, Lauss M, Karlsson A, Måsbäck A, Johansson I, Bendahl PO, Vallon-Christersson J, Törngren T, **Ekedahl H**, Geisler J, Höglund M, Ringnér M, Lundgren L, Jirström K, Olsson H, Ingvar C, Borg Å, Tsao H, Jönsson G.

  Molecular profiling reveals low- and high-grade forms of primary melanoma.

  Clin Cancer Res. 2012 Aug 1;18(15):4026-36.

Abbreviations

AJCC	American Joint Committee on Cancer
ALM	Acral lentiginous melanoma
BRAFi	BRAF inhibitor
CI	Confidence interval
CNS	Central nervous system
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CTLA-4i	Cytotoxic T-lymphocyte-associated antigen 4 inhibitor
DMFS	Distant metastasis-free survival
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DSS	Disease-specific survival
DTIC	Dacarbazine
EMA	European Medicine Agency
HR	Hazard ratio
LDH	Lactate dehydrogenase
LMSG	Lund Melanoma Study Group
MAPK	Mitogen-activated protein kinase
MEKi	MEK inhibitor
mRNA	Messenger RNA
NGS	Next generation sequencing
NM	Nodular melanoma
OS	Overall survival
PD-1	Programmed cell death 1

PD-1i	Programmed cell death 1 inhibitor
PFS	Progression-free survival
RFS	Recurrence-free survival
RNA	Ribonucleic acid
SNB	Sentinel node biopsy
SSM	Superficial spreading melanoma
TCGA	The Cancer Genome Atlas Network
UV	Ultraviolet
UVR	Ultraviolet radiation
WT	Wildtype

Abstract

Malignant melanoma is a heterogeneous, malignant neoplastic disease, most often originating in the skin. Melanoma is characterized by a high mutational load and has a vastly variable prognosis, depending on disease stage. Genetic aberrations in the mitogen-activating protein kinase (MAPK) pathway are important in melanoma, of which mutations in *BRAF* and *NRAS* are the most common. Additionally, recurrent mutations in the promoter of *TERT*, the catalytic subunit of telomerase, have been associated with a poor prognosis in primary melanoma. The introduction of the first T-cell activating antibody, ipilimumab, and the first selective inhibitor of mutant BRAF*,* vemurafenib, marked the beginning of a new paradigm in the treatment of metastatic melanoma. The rapidly increasing number of treatment options warrants improved prognostic and predictive capability. The aim of this thesis was to examine clinical aspects, in particular prognostic and predictive values, of mutational and transcriptional profiles in metastatic melanoma.

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In paper IV, the one-year clinical use of a next generation sequencing-based 26-genes mutation panel in advanced melanoma was characterized in relation to given treatment. The fraction of *BRAF* hotspot-mutant alleles was highly heterogeneous, and patients with tumors harboring a fraction in the highest and lowest deciles progressed early on MAPK inhibition.

In conclusion, metastatic melanoma displays various mutational and transcriptional profiles, relevant for prognosis and treatment prediction.

Aims of the thesis

The overall aim of this thesis was to explore the clinical aspects of molecular profiles in metastatic malignant melanoma. The specific aims of the included papers were:

- To investigate the clinical significance of *BRAF* and *NRAS* mutations in metastatic melanoma (paper I).

- To examine the prognostic and predictive value of gene expression phenotypes and their biological characteristics in metastatic melanoma (paper II).

- To explore the prognostic impact of *TERT* promoter mutations in non-acral cutaneous metastatic melanoma and the mutational pattern in multiple metastases (paper III).

- To present the one-year use of a next generation sequencing-based gene mutation panel in a clinical setting of advanced melanoma (paper IV).

Background

Introduction to melanoma

Etiology and risk factors

Malignant melanoma is a malignant neoplasm originating from melanocytes, which are pigment-producing cells, derived from the neural crest (*1*). Melanocytes are most often found in the epidermis of the skin where they produce the pigment melanin, which is transferred to surrounding keratinocytes to protect the DNA from damage caused by exposure to ultraviolet radiation (UVR) (*2*). Melanocytes are also present in various tissues in the body, such as the choroidal layer of the eye, gastrointestinal and genitourinary mucosal membranes and the meninges.

The most important risk factor for developing melanoma is exposure to UVR. In particular, intermittent sun exposure, which can be represented by a history of sunburns, is clearly associated with an increased risk of melanoma (*3, 4*). Exposure to artificial sources of UVR, such as sunbeds, has also been linked to an elevated melanoma risk (*5, 6*). Host factors associated with a raised risk of melanoma include phenotypic features (a high number of nevi, presence of atypical nevi, red hair, freckles, fair skin, light eye color, and an inability to tan), family history, and genetic susceptibility (*3*). Host phenotypic features can reflect both environmental effects, like a high nevi count as a result of a high cumulative exposure to UVR, and the expression of a susceptible genotype (*7*).

A key regulator of pigmentation is the melanocortin-1 receptor (MC1R). Upon UVR exposure, keratinocytes increase the production of melanocortin peptides, which by binding to MC1R induce the production of melanin through upregulation of the microphthalmia-associated transcription factor (MITF) (*8*). There are two forms of melanin: eumelanin, which is brown/black and is present in large amount in dark-skinned people, and pheomelanin, which is reddish/yellow and is abundant in people with red hair and fair skin. The DNA protective capacity of eumelanin is stronger compared with that of pheomelanin. A high level of MC1R activity results in a higher eumelanin/pheomelanin ratio. *MC1R* is a highly polymorphic gene conferring different levels of signaling activity among the receptor variants. Some variants, often resulting in the red hair color phenotype, are associated with

an increased risk of melanoma (*8*). Interestingly, *MC1R* variants appear to contribute to an increased melanoma risk even independently of UVR exposure, possibly due to increased oxidative DNA damage caused by the pheomelanin pigment pathway (*9*).

Approximately 10% of all melanomas occur in patients with a family history of melanoma (*10*). The familial atypical multiple mole and melanoma syndrome (FAMMM) is characterized by a high number of nevi (>50), multiple atypical nevi, specific histological features of the nevi, and at least one first- or second-degree relative with a history of melanoma (*10*). Patients with FAMMM are at high risk of developing melanoma at young age as well as having multiple primary melanomas. Germline mutations of *CDKN2A* are the single most important genetic alterations associated with FAMMM and account for ~40% of familial melanoma (*11*). *CDKN2A* encodes two different proteins: p16, which act as a cell cycle inhibitor through binding to CDK4, and p14^{ARF}, which controls DNA damage repair by interfering with HDM2, a negative regulator of the tumor suppressor p53 (*12*). Germline mutations of *CDK4* also appear in FAMMM, but far less frequently. Patients with FAMMM are also at higher risk of other cancer forms than melanoma, in particular pancreatic cancer. A germline *CDKN2A* mutation increases the risk of pancreatic cancer 38-fold (*10*), which warrants imaging screening for patients at risk, tentatively with MRI (*13*).

Epidemiology

The incidence of cutaneous melanoma has increased rapidly over the past decades in countries of the western world (*14*). Cutaneous melanoma is now the fifth most common cancer in women and the sixth most common in men in Sweden, with almost 4000 new cases per year (*15*). The melanoma incidence in Sweden is comparable to the incidence in the white population in USA, whereas it is almost half of that in Australia and New Zeeland, the countries with the highest incidence in the world (*14*). Melanoma mortality has been rising less rapidly, mainly due to the proportional increase of thin melanomas over the years (*16*).

Diagnosis

Diagnosis of malignant melanoma is based on histopathological examination of the excised lesion (*17*). In order to select appropriate lesions for excision and to detect malignant lesions as early as possible, the ABCD acronym (Asymmetry, Border irregularity, Color variegation, Diameter >6 mm) was introduced in 1985 (*18*), with the addition of 'E' for Evolving in 2004 (*19*). The ABCDE's are based on common characteristics of early pigmented skin melanoma and are widely used

by physicians to assess melanocytic neoplasms (Fig. 1A) (*20*). In addition, dermoscopy allows visualization of subsurface anatomic structures by the use of a hand-held lighted magnifier (Fig. 1B). Dermoscopy increases the diagnostic sensitivity but requires an experienced user (*20*). Several new techniques are emerging, including computerized approaches and noninvasive assessment of genetic markers, which hopefully can contribute to lower melanoma mortality.

A   B
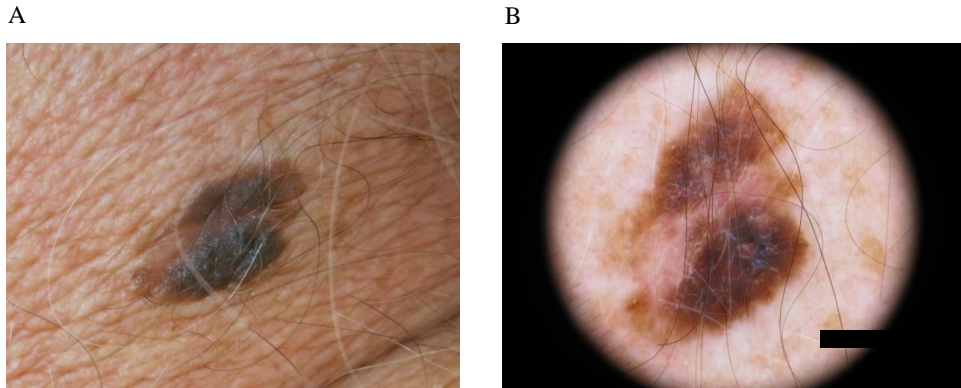
Figure 1. Superficial spreading melanoma (SSM), Breslow thickness 0.5 mm. (A) The melanoma is assymetrical 'A', has irregular borders 'B', displays color variegation, ranging from brown to black, with a hint of blue 'C', has a diameter >6 mm 'D', and has a history of evolution 'E'. (B) The same melanoma is viewed through a dermatoscope; additional colors and structures are being visible. Photos by Dr. Kari Nielsen, Dept. of Dermatology, Helsingborg General Hospital.

Histopathological subtypes

The histogenetic classification of cutaneous melanoma dates back to the 1960's when Wallace Clark and colleagues portrayed melanoma tumors with distinct macro- and microscopic features as well as different biologic behavior (*21*). More subtypes have been added since then, but the most common are yet superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM), and acral lentiginous melanoma (ALM).

SSM accounts for ~70% of all melanomas and is proportionally more common in young patients and in females. SSM tumors are associated with intermittent sun exposure and characterized by a horizontal growth pattern (*22*). In contrast, NM by definition lacks a significant horizontal growth phase, and forms a uniform, elevated nodule. NM makes up approximately 10-15% of all melanomas and is more common in older, male patients (*23*). LMM and ALM have a lentiginous growth pattern, stretching horizontally along the basilar epidermis. LMM constitutes 4-15% of melanoma diagnoses and is mostly found in elderly patients and on skin with chronic sun-induced damage, typically on the cheeks, nose and

ears (*24*). LMM often originates from its in situ form, lentigo maligna. The malignant transformation usually takes several years, and therefore the lesions can be several centimeters wide. The fourth subtype, ALM, is distinguished by its anatomical distribution. ALM appears on non-hair-bearing skin, such as plantar and palmar surfaces and beneath the nails. This subtype accounts for 1-2% of all cutaneous melanomas but occurs at similar rates in all populations, regardless of pigmentation and skin type (*25*). ALM is considered to arise independently of UVR exposure. For ALM, it is common with a considerable patient's delay, and the lesions are often misdiagnosed, leading to a more advanced stage at diagnosis and thus a worse prognosis (*26, 27*). This is particularly true for the amelanotic lesions, which represent ~30% of ALMs.

Several more uncommon subtypes of cutaneous melanoma exist, including spitzoid malignant melanoma (SMM) and desmoplastic melanoma. SMM often presents as a changing, amelanotic nodule on head and extremities. Distinguishing SMM from benign spitz nevi is admittedly difficult (*28*). SMM can occur in pediatric patients, and the association with UVR exposure is dubious. Although SMM seems to confer a better prognosis than conventional skin melanoma, the evidence is scarce (*29*). Desmoplastic melanoma often presents as an amelanotic "scar-like" lesion on chronic sun-damaged skin in elderly patients. Its deceptive appearance can impede a correct diagnosis, and excisions are often non-radical, rendering an increase rate of local recurrences. However, there is no apparent difference in survival compared with conventional melanoma if adjusting for tumor thickness (*30*).

Additionally, non-cutaneous melanoma contributes to the diversity of the disease. Mucosal melanoma shares several similarities with ALM, being non-UVR-induced, growing lentiginously, and displaying similar genomic instability, although having somewhat different genetic profiles (*22*). Uveal melanoma occurs in the iris, ciliary body, and choroid and it is the most common malignant tumor of the eye. Uveal melanoma represents a distinct entity of melanoma with separate genetics and clinical behavior (*31*).

The natural course of melanoma

Although a rest of a pre-existing nevus can be found in ~30% of primary cutaneous melanomas, the majority of cases occur de novo (*32*). Most melanomas occur on the trunk, followed by the lower extremities. The localization varies between men and women, as men more often have trunk melanoma, whereas lower extremities is the most common location in women. After adjusting for body surface and stratifying by age, intermittently sun-exposed sites were the most

common location among young patients, while sites with maximal cumulative sun exposure (typically face and ears) were most common among elderly patients (*22*).

In up to one third of all cutaneous melanoma cases, progressive disease occurs beyond the primary tumor. The most common location for the first recurrence is regional lymph node basins: groin, axilla, or head and neck. In ~20% of cases, the first recurrence comprises satellite or in-transit metastases, while distant metastases occur directly in ~30% (*33*). The median time from primary diagnosis to first recurrence is 17-25 months (*33*). In about 3% of all melanoma cases, metastases occur without a history of primary melanoma or an identifiable primary tumor (*34*). This phenomenon is thought to be due to regression of the primary tumor, as a result of a host immune response, rather than an occult location. This hypothesis is supported by the similar genetic pattern found in metastases of unknown primary and in cutaneous melanoma (*35*), and furthermore by the superior outcome for patients with unknown primary over patients with metastases of a known primary at corresponding stage (*34*).

The most common sites for distant melanoma metastases are skin or subcutaneous tissue, lymph nodes, lung, liver, central nervous system (CNS), and bone, although melanoma can spread to most organs (*33*). CNS involvement is particular harsh, and in most cases multiple metastases occur. Of note, many distant metastases do not cause symptoms and are not detected by imaging, resulting in substantial differences in metastatic rates in autopsy series compared with clinical reports (*33*). For example, gastrointestinal metastases were found in up to 60% of cutaneous melanoma patients at autopsy compared with ~5% being diagnosed before death. In contrast to the metastatic pattern of cutaneous melanoma, the first metastasis of uveal melanoma is found in the liver in >90% (*31*).

Prognostic factors for cutaneous melanoma

The use of a common international staging system is central in cancer management. It serves as a tool for developing guidelines for surveillance and treatment, enables comparisons of patient characteristics in clinical studies, and provides realistic expectations for clinicians and patients. The current cutaneous melanoma staging system, the seventh edition of American Joint Committee on Cancer (AJCC) Cancer Staging Manual, was introduced in 2009 (*36*). It is based on evaluation of the primary tumor (T), regional lymph nodes (N), and distant metastases (M). The eighth edition is planned to be implemented in January 2018 (*37*).

Primary melanoma

In the absence of evident disease beyond the primary tumor, Breslow thickness is the most important prognostic factor. Already in 1970, Alexander Breslow showed that the thickness of the primary tumor was prognostic and suggested that it could be used, together with Clark's level of invasion (21), to select patients for prophylactic lymph node dissections (38). Clark's level was used for staging until the current staging system edition, where it was replaced by mitotic rate for sub-classifying T1 tumors. The third factor used for classification in localized melanoma is the presence of ulceration of the primary tumor. Based on these three factors, invasive melanomas are classified into T1a-4b, with 10-year survival rates ranging from 93% to 39% (Table 1) (36).

Table 1. Summary of primary cutaneous melanoma staging according to the 7th edition of AJCC staging system (36).
Stage 0-IIC have no melanoma disease beyond the primary tumor. Tis=Melanoma in situ.

Stage	T class	Thickness (mm)	Sub-class	Ulceration/(mitosis)
0	Tis			
IA	T1	≤1.00	a	No and mitosis $<1/mm^2$
IB	T1	≤1.00	b	Yes or mitosis $≥1/mm^2$
	T2	1.01-2.00	a	No
IIA	T2	1.01-2.00	b	Yes
	T3	2.01-4.00	a	No
IIB	T3	2.01-4.00	b	Yes
	T4	>4.00	a	No
IIC	T4	>4.00	b	Yes

Satellite, in-transit and regional recurrence

The presence of lymph node metastases is an important prognostic marker in melanoma. The introduction of sentinel node biopsy (SNB) as a routine staging procedure for patients with melanomas >1mm has further improved prognostics (39). In short, a technetium-labeled colloid is injected at the scar of the excised primary tumor, and a pre-operative lymphoscinitigraphy visualizes the drainage to the first lymph node(s), 'the sentinel node'. The lymph node(s) is intra-operatively detected by a hand-held gamma probe, with or without the additional guidance of blue dye. If the sentinel node contains even as little as a single melanoma cell, it confers a stage III diagnosis (39). The value of a subsequent regional complete lymphadenectomy is currently investigated in the MSLT II-trial (40). Although SNB may enhance regional disease control, the procedure has not been shown to improve overall survival (OS) (41). For patients with lymph node metastases, the

number of metastatic nodes is the most important prognostic factor (Table 2). The distinction between microscopic (detected by SNB) or macroscopic (detected clinically or radiologically) disease is also of independent prognostic value (*39*). For patients with nodal micrometastases, primary tumor thickness, increasing number of metastatic nodes, age ≥70 years, presence of ulceration, axial location of the primary tumor, and male gender have been independently associated with a poorer survival (*42*). Contrary, for patients with nodal macrometastases, the thickness of the primary tumor and gender did not independently associate with prognosis. Strikingly, the 5-year survival rates displayed a great variance among subsets of stage III cases, ranging from 87% to 23% (*42*).

Table 2. Summary of satellite, in-transit, and regional metastatic cutaneous melanoma staging according to the 7th edition of AJCC staging system (*36*).
Stage III disease have no evident melanoma disease beyond regional lymph node basins.

Stage	T class	N class	Number of nodes	Sub-class	Micro- or macrometastases
IIIA	T1-4a	N1	1	a	Micro
	T1-4a	N2	2-3	a	Micro
IIIB	T1-4b	N1	1	a	Micro
	T1-4b	N2	2-3	a	Micro
	T1-4a	N1	1	b	Macro
	T1-4a	N2	2-3	b	Macro
	T1-4a	N2	2-3	c	Satellite/in-transit w/o node
IIIC	T1-4b	N1	1	b	Macro
	T1-4b	N2	2-3	b	Macro
	T1-4b	N2	2-3	c	Satellite/in-transit w/o node
	Any	N3	≥4 or nodes with satellite/in-transit mets		Any

Distant metastatic disease

For patients with stage IV disease, the prognosis has traditionally been very poor. Two factors are included in the current AJCC staging system: metastatic site and the level of serum lactate dehydrogenase (LDH) (Table 3). The sites of distant metastases are divided into three prognostic groups with increasingly poor prognosis. Lactate is an important energy source for tumor cells, and LDH is a regulator of the production of lactate. LDH can thus be viewed as a marker of metabolic activity in tumor cells (*43*), and an elevated LDH-level confers a classification as M1c. In addition, increasing number of metastatic sites has also been associated with a shorter survival (*44*). CNS metastases are so far included in

M1c, but are particularly worrisome. Patients with CNS metastases have a poor prognosis and have often been excluded from trials. However, due to improvements in imaging techniques and treatment modalities of CNS metastases, the prognosis have improved (*45*). In fact, with the new treatment options (discussed later), the overall prognosis for patients with stage IV melanoma has improved (*46*).

Table 3. Staging of distant metastatic melanoma according to the 7th edition of AJCC staging system (*36*).

Stage	M class	Site	Serum LDH
IV	M1a	Distant skin, subcutaneous, or nodal metastases	Normal
	M1b	Lung metastases	Normal
	M1c	All other visceral metastases	Normal
		All distant metastases	Elevated

Patient characteristics as prognostic factors

Age has repeatedly been independently associated with prognosis in melanoma (*42, 47, 48*). However, a specific cut-off that reflects the impaired prognosis is not known. Furthermore, the underlying mechanism for the association with prognosis is not clear (*47*). It is possible that increased co-morbidity in older patients and age-dependent reduction of the function of the adaptive immune system would contribute to the impaired prognosis (*49*). Age and tumor site, another prognostic factor, are also associated, as older patients more often have head/neck melanoma, which confers a poorer prognosis (*22*). Interestingly, it has recently been shown that age-dependent changes in the microenvironment result in a more invasive phenotype of melanoma tumors (*50*).

Gender has also been independently associated with prognosis in all stages of melanoma (*42, 51*). The favorable prognosis for female patients has been suggested to be caused by differences in tumor sites and health care seeking behavior between men and women, however, this could not fully explain the discrepancy (*51*). Differences in levels of radical oxygen species (ROS) and in immune homeostasis between men and women might offer additional clues to the divergent prognosis (*52, 53*).

Molecular profiles in melanoma

Melanoma is a heterogeneous disease with a complex biology. Several attempts have been made to divide melanoma into biologically and clinically meaningful subtypes (*22*). The histogenetic subtypes have been used for decades in the clinical

management: for description of tumors and to provide prognostic information in primary melanoma. However, they do not qualify as independent prognostic factors to be included in the AJCC staging system and have no impact on the choice of treatment (*54*). Melanoma is generally considered a UVR-driven disease, but clinical and tumor histological features differ depending on pattern of UVR exposure, i.e. for tumors occurring on intermittently, chronically or non-exposed body sites. Studies of tumor genetics over the past decades have revealed that this heterogeneity is largely a reflection of distinct molecular profiles.

Features of genetic events in the MAPK pathway

Activation of the mitogen-activated protein kinase (MAPK) pathway is a central event in melanoma evolution. *NRAS* was the first gene in this pathway to be found mutated in melanoma in a considerable rate (~20%), mainly affecting codon 61, 13 and 12 (*55*). In 2002, *BRAF* mutations were found in several types of malignant tumors and were especially frequent in melanoma (*56*). Mutations occur in approximately 40-50% of melanomas and most often consist of a point mutation (c.1799T>A), causing substitution of a single amino acid residue (V600E). This transversion results in a constitutively active BRAF protein, which is independent of phosphorylation by RAS. As a consequence of this independence, *BRAF* and *NRAS* mutations are mutually exclusive (*56*). Activated BRAF phosphorylates MEK1/2 (encoded by *MAP2K1/2*), which in turn phosphorylate ERK1/2 (Fig. 2). Activated ERK causes transcriptional upregulation of cyclin D1 (encoded by *CCND1*), and other transcription factors stimulating cell proliferation (*57, 58*).

An early association between a mutational event in the MAPK pathway and clinical features were described already in the 1980's, as *NRAS* mutations were described to predominantly occur on chronically sun-exposed body sites (*55*). *BRAF* mutations were later associated with intermittently sun-exposed sites as well as a young patient age and SSM tumors (*59-62*). However, the second most frequent *BRAF* mutation, V600K, which occurs in 10-20% of melanomas, differs from V600E, since it has been found to associate with chronic sun exposure and old age (*62*).

While much research has focused on investigating the features of *BRAF* and *NRAS* mutations, the *BRAF*/*NRAS*-wildtype (WT) melanomas are less thoroughly characterized and constitute a heterogeneous group (*59, 63*). As a result of systematic multi-platform characterization of 333 cutaneous melanomas, The Cancer Genome Atlas Network (TCGA) in 2015 presented a four-group classification of cutaneous melanoma according to the pattern of the most prevalent, significantly mutated genes: mutant *BRAF*, mutant *NRAS*, mutant *NF1*, and triple-WT (*64*). NF1 is a negative regulator of NRAS, and loss-of-function

mutations or deletions of *NF1* have been found in ~14% of melanomas (*65, 66*). *NF1* mutations have been associated with a high mutational burden, old age, and male gender (*65*). Conversely, the triple-WT group was characterized by a low mutational burden and infrequent UVR signature mutations, but with recurrent mutations in *KIT,* a gene most often mutated in acral and mucosal melanoma, as well as in *GNAQ* and *GNA11*, important driver mutations in uveal melanoma. Furthermore, structural rearrangements and copy number changes were enriched in the triple-WT subtype. Affected genes included *KIT* and *PDGFRA*, coding for receptor tyrosine kinases, along with *CCND1* and *CDK4*, coding for important regulators of the cell cycle (*64*). Due to the importance of the MAPK pathway in melanoma development, several studies have investigated the prognostic impact of the *BRAF* and *NRAS* mutations, with inconsistent results (*67-72*). Moreover, proteins in the MAPK pathway have been key targets in the development of molecular targeted therapy (discussed later).
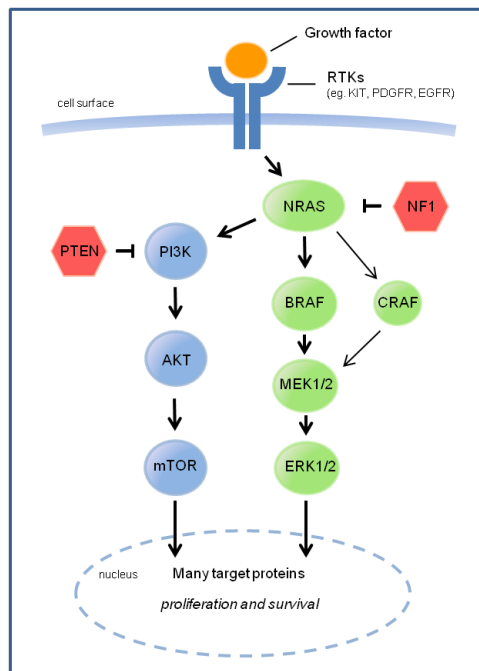
Figure 2. Simplified view over the mitogen-activated protein kinase (MAPK) and the PI 3-kinase (PI3K)/AKT signaling pathways in melanoma. Binding of a ligand to the receptor tyrosine kinase (RTK) leads to activation of the MAPK pathway (green symbols). NRAS can also activate the PI3K/AKT pathway (blue symbols). The activity is inhibited by NF1 and PTEN. Activation of the pathways stimulates cell proliferation and survival through various targets.

Although oncogenic events in the MAPK pathway are considered initial drivers in melanoma development, additional aberrations are required to cause a malignant neoplasm (*73*). This is demonstrated by the presence of *BRAF* mutations in ~80%

of benign nevi (*74, 75*). As *BRAF* mutations trigger melanocytes to proliferate and form nevi, in normal cases innate control mechanisms recognize the abnormal proliferation and prevent further progression by putting the cells in a state of irreversible proliferative arrest, called replicative senescence, or by causing apoptosis. The intense proliferation is recognized by cells through shortening of telomeres or replication-induced genomic instability and DNA replication stress, which in turn activate p16 or p53, key tumor suppressors (*76, 77*). Thus, additional genetic events, compromising the regulatory functions of telomeres and cell cycle progression, are also crucial in the development of an invasive melanoma.

Reactivation of telomerase

Telomeres consist of 2-20 kb of oligonucleotide repeats (TTAGGG) and associated proteins, which protect the end of chromosomes from degradation, recombination, and end-to-end fusion (*78*). In most somatic cells, the telomeres shorten for every cell cycle until a limit when the cells are triggered to enter replicative senescence. On the contrary, in germline cells and in most cancer cells the length of the telomeres is maintained by the protein-RNA complex telomerase. Telomerase is a reverse transcriptase, which carries an RNA template for the telomere sequence (*79*). Although about 90% of human cancers express telomerase, the genetic cause in melanoma was long largely unknown. In 2013, two independent studies described recurrent mutations in the promoter of *TERT*, the gene encoding the catalytic subunit of telomerase (*80, 81*). The mutations carried a UV signature with a C>T transition, or a CC>TT transition, at two dipyrimidine hotspots. The mutations created new binding sites for E-twenty-six transcription factors. *TERT* promoter mutations have been found in ~40% of primary melanomas and in ~70-80% of metastatic melanomas (*64, 80, 82, 83*), with the highest rates in cutaneous melanomas harboring *BRAF*, *NRAS* or *NF1* mutations (*64*). In contrast, *TERT* promoter mutations have been found in <10% of ALMs and triple-WT melanomas (*64, 84*), whereas *TERT* amplification has been described in 15-21% of ALMs and triple-WT tumors (*64, 85*). Other causes of *TERT* expression include *TERT* promoter hypermethylation and structural variants involving the *TERT* gene or its promoter (*86*). The paradoxical effect of methylation on the *TERT* promoter has been explained by the selective binding of the 11-zinc finger factor CTCF, a transcriptional repressor, to the unmethylated *TERT* promoter (*87*). Interestingly, *TERT* promoter mutations and *TERT* amplifications have been associated with a poorer survival in primary cutaneous melanoma and ALM, respectively (*82, 85, 88*). Although several studies indicate functions for TERT independent of telomere elongation, including increasing metastatic potential, the mechanisms are poorly understood, and results are somewhat contradictory (*89-91*).

Additional pathways in melanoma progression

In order to avoid senescence, most cancers inactivate p53, a key player in cell-cycle control, apoptosis, and maintenance of genetic stability. However, inactivating mutations in *TP53* are less frequent in melanoma than in many other cancer forms (*12*). Instead, deletions and inactivating mutations in *CDKN2A* are frequent in melanoma and have a special role in familial melanoma, as discussed earlier. The model describing development of cancer through stimulating proliferation (by mutations in *BRAF*/*NRAS*), avoiding senescence (by inactivating events in *TP53* or *CDKN2A*), and enabling unlimited replication (by expression of *TERT*) thus assumes that these genetic events occur early in tumor progression. Indeed, in an elegant study Shain et al. sequenced 293 cancer-relevant genes in 150 areas of 37 primary melanomas and adjacent distinct precursors, and discovered a typical pattern of genetic events: *BRAFV600E* mutations occurred in benign nevi; V600K, K601E, *NRAS* and *TERT* promoter mutations occurred in intermediate lesions and melanomas in situ; whereas loss of both *CDKN2A* copies was apparent exclusively in invasive melanomas (*73*).

It was early described that NRAS, apart from stimulating proliferation through the MAPK pathway, also activates PI 3-kinase, which by activating AKT stimulates cell growth and survival (*92*). It has also become apparent that *BRAF*-mutant melanoma activates PI 3-kinase by loss of its inhibitor PTEN in ~20% (*64, 93*). Inactivating mutations or deletions in *PTEN* seem to be later events in melanoma progression, as losses mainly have been found in advanced melanomas (*73, 94*). Interestingly, loss of PTEN expression and *PTEN* promoter methylation, but not *PTEN* mutations, have been associated with decreased OS in melanoma (*95, 96*).

Gene expression signatures

The emergence of high-capacity microarray technology in the 1990's enabled researchers to search for patterns of gene expression associated with development and progression of cancers (*97, 98*). Early studies focused on finding signatures, specific for the different stages in melanoma progression, in order to understand the biological features of invasiveness and metastasizing (*99, 100*). Also, driven by the notion that melanoma is predisposed to different forms of immune modulation, Wang et al. in 2002 presented evidence for a variation in immune responsiveness among melanomas (*101*). However, the sparse access to frozen tumors tissue and corresponding long-term follow-up data stalled the development of clinically useful molecular signatures (*102*). In 2006, Winnepenninckx et al. discovered a 254-gene classifier, which was able to predict occurrence of distant metastases within four years, with an accuracy similar to that of the combination of Breslow thickness and ulceration (*103*). The classifier included genes involved

in the cell cycle, DNA replication, and regulation of apoptosis. The same year, Mandruzzatto et al. indentified 70 genes whose expression was associated with survival in metastatic melanoma (*104*). The authors emphasized the importance of the interplay between tumor cells and infiltrating immune cells, as expression of genes related to immune cell activity was associated with a longer survival.

Somewhat challenging the idea of melanoma progressively changing the gene expression during disease progression, Hoek et al. identified two distinct transcriptional signatures in melanoma cell lines, which based on the function of the involved genes were defined as 'proliferative' and 'invasive' (*105*). They presented evidence suggesting that melanoma tumors oscillate between these two states, in response to signals from the microenvironment, in a way that enables repeated spreading and proliferation, and also might provide a resistance mechanism for cytostatic therapy. Interestingly, the signatures were distinguished by their contrasting expression of *MITF*, a master regulator of the melanocyte lineage. A high expression of *MITF* and other melanocytic genes promoted high proliferation and low motility of melanoma cells. Conversely, low *MITF* expression was associated with upregulation of genes involved in modification of the microenvironment. Cells with the latter signature showed low proliferation rates and high motility.

Several studies have subsequently presented gene expression profiles associated with prognosis, but their usefulness is limited in part by the lack of replication, independent prognostic value, and capacity of predicting treatment response (*102*). Our group previously used unsupervised hierarchical clustering of global gene expression data to identify four phenotypes in stage IV melanoma, named 'high-immune response', 'pigmentation', 'proliferative', and 'normal-like', according to the typical genes expressed by each phenotype (*106*). The pigmentation phenotype expressed genes involved in melanin synthesis, such as *MITF* and *TYR*, and high-immune response tumors expressed genes involved in different immunologic processes. In contrast, tumors of the proliferative phenotype showed low expression of both *MITF* and immune response-related genes, but instead expressed cell cycle-associated genes. The MITF-low proliferative phenotype was recently shown to correspond well to the cell line invasive signature proposed by Hoek et al (*107*). The MITF-high pigmentation phenotype concordantly comprised cell lines of the proliferative signature. Importantly, the four groups were significantly associated with OS, with the shortest survival for the proliferative group (*106*). Patients with tumors of the pigmentation phenotype were significantly overrepresented among patients showing objective response or stable disease on treatment with the chemotherapeutic agent dacarbazine. The prognostic value of the four-group classification has furthermore been demonstrated in a cohort of 223 patients with primary melanoma (*108*).

Treatment of melanoma

Surgery

Local excision is the standard treatment of primary cutaneous melanoma and can often cure patients with thin tumors. Since abnormal melanocytes often occur in the epidermis surrounding the tumor, a wide local excision is required (*109*). The scar is excised with 1-2 cm margin depending on tumor thickness and site, and SNB is performed for staging in appropriate cases. A metastatic sentinel node or clinically detected regional lymph node metastases confer complete (or selective for head/neck area) lymphadenectomy of the affected regional node basin (*110*), although the DeCOG-SLT study failed to show any survival benefit for complete lymph node dissection over observation, following a positive SNB (*111*). The results of the MSLT-II trial are awaited for definitive answer. Resectable in-transit metastases are excised with narrow but clear margins.

Surgical treatment of distant metastases can be considered in two scenarios: as a strict palliative procedure, for example to relieve symptoms from bleeding or obstructive metastases in the gastrointestinal tract and from ulcerated skin metastases, or with a life prolonging intent. The latter is mostly considered for isolated and slowly growing disease, especially for solitary lesions in the skin, lungs and brain (*112*). Another emerging scenario, where surgery might favor prognosis, is excision of progressive lesions when there is a mixed response on systemic treatment (*113*). Such approach could theoretically eradicate sub-clones of resistant tumor cells.

Targeted therapy

In 2000, the first molecular RAF inhibitor, sorafenib, was tested in clinical trials. Sorafenib is a non-selective RAF inhibitor, initially developed to treat *RAS*-mutant cancer (*114*). Sorafenib is approved to treat renal cell, hepatocellular, and differentiated thyroid cancer, but did not show to be effective in melanoma. In 2011, nine years after the discovery of frequent *BRAF* mutations in melanoma, the first orally available, selective BRAF inhibitor (BRAFi), vemurafenib, was introduced. It binds preferably to BRAF proteins in an active enzyme conformation, caused by V600 mutations (*114*). In the BRIM-3 trial, patients with *BRAFV600* mutations who were treated with vemurafenib had improved response rates (48% vs. 5%) and median progression-free survival (PFS) (5.3 months vs. 1.6 months) compared with patients treated with dacarbazine (*115*). Vemurafenib was subsequently approved in 2012 by the European Medicines Agency (EMA)

for use in unresectable stage III or IV melanoma. In 2013, the second BRAFi, dabrafenib, was approved after it was shown to be associated with a prolonged PFS compared with dacarbazine in *BRAFV600*-mutant melanoma (*116*). The most common adverse effects for BRAFi treatment include fatigue, hyperkeratosis, pyrexia, headache, and arthralgia. Moreover, new cutaneous neoplasms, in particular cutaneous squamous cell carcinoma, are common adverse effects. The formation of new neoplasms is driven by a paradoxical activation of CRAF in *BRAF*-WT cells, promoting growth of pre-malignant *RAS*-mutant cells (*114*).

In 2012, a phase III trial demonstrated improved PFS for patients with *BRAFV600E/K*-mutant metastatic melanoma treated with the MEK inhibitor (MEKi) trametinib compared with chemotherapy-treated patients (*117*). Later, the combinations of dabrafenib and trametinib, and vemurafenib and the MEKi cobimetinib, were associated with a longer median OS compared with single BRAFi (25.6 months and 22.3 months vs. 17.4-18.7 months, respectively (*118-120*). The median PFS for combination treatment was 11-12 months. The most common adverse effects for MEKi treatment include rash, diarrhea, fatigue, and peripheral edema. Impaired left ventricular function or decreased ejection fraction are also common adverse effects, which motivates echocardiogram assessment prior to treatment start. Interestingly, the adverse effects related to the paradoxical activation of the MAPK pathway by BRAF inhibition diminish with the combination of MEKi (*121*). Due to the improved outcome and the manageable side effects, combination therapy has become a standard treatment for *BRAF* hotspot-mutant metastatic melanoma.

Despite the impressive response rates of ~70%, the majority of patients treated with BRAFi and MEKi develops resistance within months. Resistance mechanisms for both single and combination therapy include reactivation of MAPK pathway activity in a majority of cases (*122*). The reactivation can be achieved through various mechanisms, including *BRAF* amplification, secondary mutations in *NRAS* or *MAP2K1*/2, loss of *NF1*, or expression of a *BRAF* splice variant. Resistance can also occur through upregulation of receptor tyrosine kinases (RTKs), such as PDGFRβ (*123*), which activates RAS (*124*). In addition, the tumor microenvironment has been shown to change in response to BRAF inhibition. As a result of paradoxical upregulation of PDGFR in melanoma-associated fibroblasts, these cells produce and modulate matrix, which causes focal adhesion kinase (FAK)-dependent signaling and reactivation of ERK activity in melanoma cells (*125*). Hence, logical strategies of targeting resistance mechanisms to BRAFi/MEKi would include co-targeting PDGFR or ERK.

For patients with *BRAF*-WT tumors, available molecular inhibitors are scarce. Imatinib was developed to target the bcr-abl oncogene in chronic myelogenous leukemia (CML), and due to its inhibitory effect on KIT it is used to treat

gastrointestinal stromal tumors (GIST) (*126*). GIST is characterized by a high rate of *KIT* mutations (~75%). In melanoma, imatinib has been shown to induce responses among the minor fraction of tumors with *KIT* mutations, especially hotspot mutations in exons 11 and 13. Response rates ranged between 16% and 29%, and median PFS were 3-4 months (*127-129*). Responses have also been described for the alternative KIT tyrosine kinase inhibitors sunitinib and nilotinib in melanoma patients with *KIT* alterations (*130, 131*).

Immunotherapy

Cytokines

Interferons are a family of molecules produced by white blood cells as a response to pathogens or foreign antigens. They affect cells in various ways relevant in countering cancer, such as down-regulating cell cycle activity, inducing apoptosis, increasing the expression of tumor antigens, and activating T-lymphocytes (*132*). Recombinant interferon α-2b is approved by EMA since 2000 for adjuvant treatment of melanoma and has been associated with improved OS, although limited, in meta-analyses (*133*). However, the optimal dose, in relation to tumor control and toxicity, is indefinite. Several predictive factors have been proposed, including presence of ulceration and a low disease stage (IIb-IIIN1), but would require validation in large, prospective trials to be implemented (*133*).

Interleukin-2 (IL-2) is a cytokine, which when administered in high doses can generate lymphokine-associated killer (LAK) cells. LAK cells are able to detect and lyse tumor cells (*134*). Recombinant IL-2 has been used in USA for decades, for treatment of metastatic melanoma, and has shown response rates of 5-27% (*135*). Due to the limited effect and the severe toxicity profile, IL-2 treatment is not approved in Sweden.

Checkpoint inhibitors

A breakthrough in treatment of metastatic melanoma came with the introduction of the checkpoint inhibitors. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is an immune checkpoint molecule that negatively regulates T-cell activation (Fig. 3). Ipilimumab, a monoclonal antibody, which blocks CTLA-4 and thus augments anti-tumor T-cell immunity, was the first checkpoint inhibitor that showed improved OS in metastatic melanoma. The results were similar if ipilimumab were administered with dacarbazine, gp100 peptide, or alone, as well as in doses of 3 mg/kg or 10 mg/kg (median OS of 10-11 months) (*136, 137*). Best overall response rates were 10-15%, but in a recent pooled analysis of long-term survival data from phase II and III trials, the survival rate started to plateau at 20% after

three years, extending for 10 years for some patients (*138*). Ipilimumab was approved by EMA for treatment of metastatic melanoma in 2011.
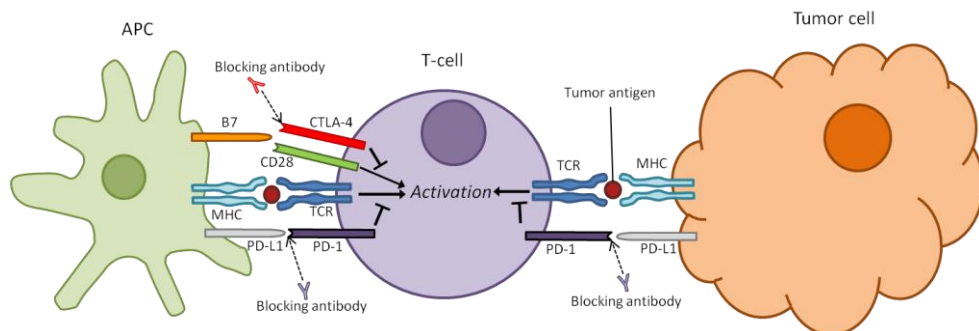

Figure 3. T-cell activation is initiated by binding of a foreign peptide-MHC complex on a tumor cell or a antigen presenting cell (APC) to the T-cell receptor (TCR). Activation by APCs further requires co-stimulatory signals via CD28, which is activated by binding to B7 proteins. CTLA-4 competes with CD28 for binding to B7 proteins and inhibits T-cell activation. PD-1 is activated through binding to PD-L1 and attenuates T-cell receptor signals. By blocking CTLA-4 and PD-1/PD-L1, inhibitory effects on T-cell activation are avoided and T-cells can counter the tumor cells (*139*). MHC= Major histocompatibility complex. CTLA-4=Cytotoxic T-lymphocyte-associated antigen 4. PD-1=Programmed cell death 1. PD-L1=Programmed cell death ligand 1.

Tumor cells can escape the immune system through another checkpoint: the programmed cell death 1 (PD-1). By expressing its ligands, PD-L1 and PD-L2, tumor cells can interact with PD-1 on T-cells, thus inhibiting T-cell activation and proliferation (Fig. 3) (*140*). Two monoclonal antibodies, nivolumab and pembrolizumab, have been shown to increase survival compared with chemotherapy (*141-143*) and compared with ipilimumab (*144, 145*), and were subsequently approved by EMA in 2015 for treatment of metastatic melanoma. To date, long-term survival data is scarce, but a five-year OS rate of 34% was presented from an early phase I trial of nivolumab, where OS rates appeared to plateau after 48 months (*146*). Combining nivolumab and ipilimumab seems to improve the outcome even more, although OS data is not yet published (*145, 147*). However, 55% of the patients who received combination therapy experienced grade 3 or 4 adverse effects, compared with 27% and 16% for ipilimumab and nivolumab alone, respectively (*145*). Specifically, colitis of grade 3 or 4 occurred in 7.7%, 8.7%, and 0.6% of patients treated with combination, ipilimumab, and nivolumab, respectively. In a pooled safety analysis, the most common adverse effects of nivolumab were fatigue, pruritus, diarrhea, and rash, and 3% of patients discontinued treatment due to adverse effects (*148*). Interestingly, treatment-related select adverse effects were independently associated with objective response rate.

Expression of PD-L1 has been associated with an increased response to pembrolizumab and nivolumab, but some patients with PD-L1-negative tumors

still display long-lasting responses, limiting its effect as a useful predictive marker (*145, 149*). Other baseline characteristics associated with a longer OS for patients treated with pembrolizumab include LDH-level <2.5x upper normal limit, no visceral involvement other than lung, relative lymphocyte count ≥17.5%, and relative eosinophil count ≥1.5% (*150*). Furthermore, a high mutational load has been associated with a clinical benefit from inhibition of CTLA-4 and PD-1 (*151, 152*).

Oncolytic viruses

Many viruses are able to infect tumor cells, causing lysis of the tumor cells with subsequent release of pro-inflammatory factors and antigens, resulting in priming of tumor-specific T-cells. Talimogene laherparepvec (T-VEC) is a genetically modified herpes simplex virus 1, which is produced to be injected in cutaneous, subcutaneous, or nodal melanoma metastases to cause local and systemic anti-tumoral responses (*153*). A randomized phase III trial of 436 patients with unresectable stage IIIB-IVM1c melanoma showed an overall response rate of 26% and complete response in 11% for T-VEC-treated patients. Sub-group analysis showed that the clinical benefit was only apparent for patients with stage IIIB-IVM1a disease (*154*). This led to an EMA approval for treatment of unresectable stage IIIB-IVM1a melanoma in December 2015.

Antigen-based active immunotherapy

Melanoma-associated antigens (MAGE) are a group of related proteins that commonly occurs in tumor tissue but not in normal tissue except for testis and placenta (*155*). Research in the past years has focused on developing an immunotherapeutic consisting of MAGE-A3, which is frequently present on the surface of melanoma cells, together with an immunostimulant. The immunotherapeutic is meant to induce an antigen-specific immune response in the host. When recombinant MAGE-A3 and the immunostimulant AS15 were administered intramuscularly in 36 patients with MAGE-A3-positive, unresectable stage III-IVM1a melanoma within a phase II trial, four patients (11%) showed objective response and superior OS compared with the control group (*156*). However, the following phase III trial failed to meet its first co-primary endpoint: extending disease-free survival compared with placebo (*157*). The second co-primary endpoint included using gene expression profiling to identify a subset of MAGE-A3-positive patients, who would benefit from the treatment (*157, 158*). The gene expression signature was not predictive of clinical outcome in a subsequent phase II trial (*159*).

Adoptive cell therapy

The basic principle of adoptive cell therapy is to harvest tumor-specific T-cells from the patient, expanding the T-cell clones in vitro, and transferring them back to the patient after treatment with a lymphocyte-depleting regimen, and followed by high-dose IL-2, thereby increasing the immunologic anti-tumor response. Response rates of ~50% have been demonstrated in phase II trials in metastatic melanoma (*160, 161*). An emerging approach is to genetically engineer tumor-specific, modified T-cell receptors onto the T-cells. In general, adoptive cell therapy is a promising treatment modality, but faces many challenges, one of the most obvious being the requirement of time and resources (*161*).

Chemotherapy

Before 2011, when ipilimumab and vemurafenib were introduced, chemotherapy was the standard treatment for metastatic melanoma. Dacarbazine (DTIC) is an alkylating agent, which introduces alkyl groups to guanine bases in DNA and thereby causes apoptosis. DTIC has been used for decades for treatment of metastatic melanoma with response rates of 10-20%. However, DTIC has never been shown to increase OS in a phase III trial (*162*). Attempts have been made to improve OS by using combination chemotherapy, but without success. Instead, temozolomide was often preferred as it is an orally available DTIC analog, which has shown similar effects in treatment of metastatic melanoma (*162*). A specific clinical situation in which chemotherapy has a role is the presence of multiple in-transit metastases on extremities, not eligible for surgical treatment. Here, isolated limb perfusion, using the alkylating agent melphalan, can be a safe treatment option, providing complete responses in 47-65% of cases (*163, 164*).

Radiotherapy

Melanoma has traditionally been considered radioresistant (*165*). However, adjuvant radiotherapy can decrease the risk of regional relapse after lymphadenectomy in patients with high-risk stage III melanoma (*166-168*). The effect seems to be greatest for cervical regional nodes, while radiation to inguinal regional nodes is associated with the highest risk of complications, such as lymphedema, delayed wound healing, and fibrosis. While most studies, including a recent randomized trial, have not shown any survival benefit from adjuvant radiotherapy (*166, 168*), a retrospective study including >600 patients showed that radiotherapy was independently associated with a longer disease-specific survival (DSS) (*167*). Of note, in the latter study, the absolute majority of patients were treated with a hypofractionated regime of 30 Gray (Gy) delivered twice weekly at

6 Gy per fraction, while in the prospective trial, 48 Gy in 20 fractions was given (*166*). In another retrospective study, only a total dose of >50 Gy was associated with an improved survival (*169*).

Radiotherapy is also used in palliative treatment of distant metastases. Stereotactic radiosurgery (SRS) is a feasible alternative to surgery for small metastases (<3 cm) and oligometastatic disease in the brain (*170*). Whole brain radiation therapy (WBRT) is considered for patients with multiple brain metastases and as adjuvant treatment following surgery or SRS. Although WBRT can improve CNS disease control, its adjuvant use is highly debated (*171*). Lastly, radiotherapy targeting bone metastases can relief pain and enhance local disease control (*110*).

Materials and methods

Study cohorts

A flowchart of included and excluded patients in the studies is shown in Fig. 4. The studies were approved by the ethics committee at Lund University (Dnr. 191/2007 and 101/2013).

LMSG molecular melanoma cohort (paper I-III)

Lund Melanoma Study Group (LMSG) molecular melanoma cohort consists of patients who received surgical treatment for melanoma between 1993 and 2012 and from whom tumor tissue were sampled and stored in ultra-low temperature for later experimental analysis. The majority of patients were referred for surgical treatment for metastatic melanoma at the Department of Surgery, Skåne University Hospital, Lund, Sweden. The tumor specimens mostly include regional metastases but also distant metastases, local/in-transit metastases, and locally advanced primary tumors. A peri-operative blood sample was collected in most cases. In the majority of cases, a representative tumor sample was cut from the resected tumor specimen at the operating theatre and was immediately frozen and stored in the biobank of the Department of Oncology, while the main sample was sent for standard pathological assessment. A pathologically confirmed melanoma diagnosis was required for inclusion in the analysis. For several patients serial samples were collected. Most patients had not received systemic treatment prior to surgery, but treatments used were typically regional or systemic chemotherapy and interferon. Clinical and histopathological data was retrieved from patient records, pathology reports, and the National Population Registry.

The inclusion of patients in the cohort stretched over almost two decades, which has affects on the patient and tumor data. The time of follow-up has a wide range, where patients included late contribute less to survival analysis. Approximately 60% of the patients had died at the time of last follow-up, which enables consistent survival analysis. Important changes in treatment guidelines during the period include the introduction of sentinel node biopsy (SNB) as a standard procedure for staging between 2004 and 2006 at Skåne University Hospital. However, SNB has

not contributed to an increased survival in melanoma (*41*). The use of ipilimumab in metastatic melanoma was approved in Sweden in July 2011, and thus had limited effect on survival data for this cohort. Nine patients in the cohort were treated with BRAFi in a clinical study setting. These patients were analyzed separately in paper I, together with other patients treated with BRAFi within clinical trials at the Department of Oncology, who were not in the LMSG molecular melanoma cohort (n=13). The report of tumor features has also changed during the inclusion period. For example, mitotic rate was introduced as a prognostic marker in the 7th edition of AJCC staging system in 2009 (*36*) and was reported in a non-standardized way in most cases. Thus, many cases lack information about certain tumor characteristics, which limits statistical analysis of confounding factors. The described chain of tumor sample management ensures a good quality of the tumor samples, but requires macroscopically identifiable tumors, which allow sampling without jeopardizing diagnostics. In that sense the cohort is biased towards bulky and locally advanced disease, and the results should be very carefully considered before being translated to the entire melanoma population.

Next generation sequencing melanoma cohort (paper IV)

From January 2015, mutational analyses of *BRAF* and *KIT* were performed by using a next generation sequencing (NGS)-based mutation panel of 26 cancer-related genes in melanoma patients at Skåne University Hospital, Lund, Sweden. Mutation analysis was performed as a routine step in clinical evaluation of patients who were referred for surgical or systemic treatment of metastatic or locally advanced melanoma and for discussion at the regional multidisciplinary conference. In paper IV, patients subjected to mutation analysis during the period of January 2015 through June 2016 were included (Fig. 4). Hence, the cohort represents a consecutive series of patients, mainly with stage III-IV melanoma, from the Departments of Oncology, Ear- Nose and Throat, and Surgery. Clinical data was retrieved from patient records. Time of follow-up was through September 2016, which limits analysis of survival and treatment effect duration. The standard treatment of stage IV melanoma has changed in multiple steps during the recent years, which is reflected in this cohort. Halfway through the inclusion period, standard treatment for *BRAF*-WT melanoma changed from CTLA-4 inhibitor (CTLA-4i) or chemotherapy to PD-1 inhibitor (PD-1i). After the inclusion period, it has become more common to combine PD-1i and CTLA-4i.
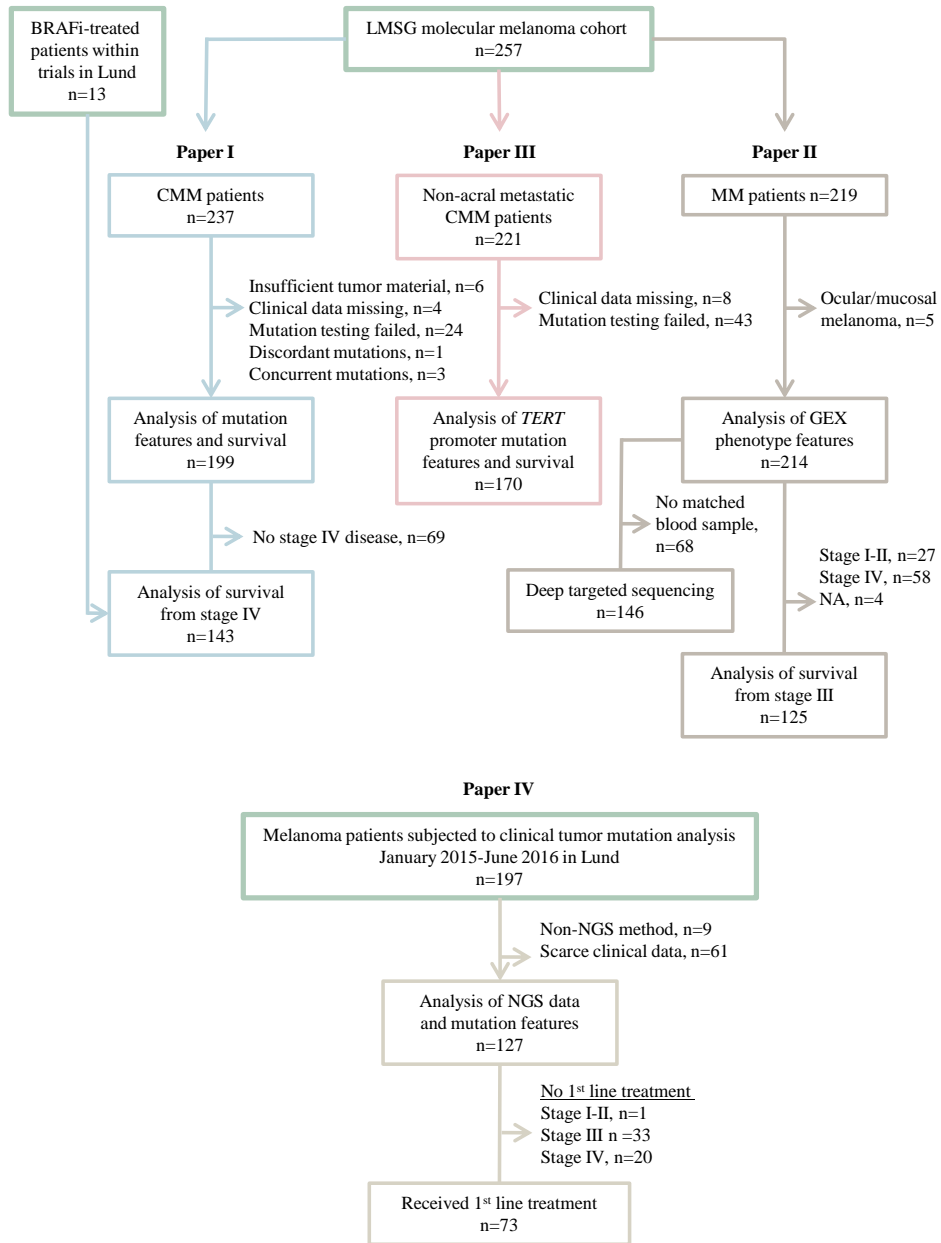
Figure 4. Flowchart of included and excluded patients in the four studies included in the thesis. BRAFi=BRAF inhibitor. CMM=Cutaneous malignant melanoma. MM=Malignant melanoma. LMSG=Lund Melanoma Study Group. GEX=Gene expression. NGS=Next generation sequencing.

Extraction of nucleic acids

For experimental analysis in paper I-III, nucleic acids were extracted from samples of the LMSG molecular melanoma cohort. Frozen tumor samples were homogenized using a TissueLyser (Qiagen). DNA and RNA extracts were isolated using the AllPrep DNA/RNA Mini Kit (Qiagen). Sample concentrations and purity were assessed using the NanoDrop ND-1000 (NanoDrop Products). The quality of the RNA extracts was analyzed on the Agilent Bioanalyzer 2100, and only samples with an RIN value >6 were included. DNA was extracted from blood using the DNeasy Blood and Tissue Kit (Qiagen).

In paper IV, formalin-fixed, paraffin-embedded (FFPE) tumor samples were selected from the archives of the Department of Pathology, Skåne University Hospital, Lund, Sweden. Hematoxylin and eosin stained tissue sections were assessed by a pathologist, and a suitable area for mutation analysis was selected. An estimated tumor cell content of at least 10% was required. Tissue sections (6x5µm) were cut and re-evaluated to ensure a representative material before DNA was extracted using the AllPrep Kit (Qiagen).

Sanger sequencing

In 1977, Sanger et al. described a new method to sequence DNA, which became gold standard for decades (*172*). The method utilizes the inhibitory effect of 2'3'-dideoxynucleoside triphosphate (ddNTP) on elongation of oligonuclotide chains by DNA polymerase I. ddNTPs are dNTP analogues lacking a 3'-hydroxyl group. Incubating the DNA template, a primer, DNA polymerase, all four dNTPs (A, T, G, and C), and all four ddNTPs, which are labeled with four unique fluorescent dyes, creates fragments of different lengths, covering the entire region of interest. All DNA fragments contain a labeled ddNTP at the last position in the oligonucleotide chain (*173*). The fragments are then separated in order of length by capillary electrophoresis, and the base at last position of each fragment can be read using laser-based fluorescence detection (*174*). Sanger sequencing is a robust and cheap technique but has limited sensitivity for mutations occurring at low frequencies (*175*).

Sanger sequencing was used for analysis of hotspot mutational status of *BRAF*/*NRAS* and the *TERT* promoter in paper I and III, respectively. DNA was amplified by polymerase chain reaction (PCR) using primers covering the hotspot areas (*56, 82*). The PCR products were cleaned by using vacuum-based PCR-filter clean-up for *BRAF*/*NRAS* analysis and column clean-up (QIAquick PCR

Purification Kit) for *TERT* promoter analysis. Sequencing analysis was performed in both directions using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Since the *TERT* promoter is GC-rich, 5% DMSO and 3% glycerol was added to the reaction mix to prevent secondary structures from causing inefficient DNA sequencing (*176*). Sequencing traces were analyzed using Sequencher v.4.5 (Gene Codes Corporation).

Next generation sequencing

The Human Genome Project accomplished to sequence the first human genome in 2000 and was driving the development of high-throughput DNA sequencing (*177*). NGS emerged in 2005 and has become essential in cancer research and as a method for analysis of patient-specific tumor genetics in clinical oncology (*178*). A variety of methods and platforms are available, suitable for different purposes. Whole-genome sequencing (WGS) is an important method for cancer genomics. However, the low depth of coverage achieved by WGS is not sufficient to detect oncogenetic events with the statistical accuracy required for clinical diagnostics, since mutations can occur in sub-clones of tumor cells or be diluted by the presence of non-tumor cells (*178*). Instead, only the genes of interest can be targeted for sequencing, which allows sequencing to a required depth of approximately 1000x.

In paper II, 1697 cancer-related genes were selected for deep targeted sequencing, and in paper IV the use of a clinical NGS mutation panel of 26 cancer-associated genes was described. Both analyses used the Illumina sequencing by synthesis chemistry. The Illumina NGS workflows include four steps. First, library preparation is performed. The DNA is fragmented and specific adapters are ligated to the ends of the DNA fragments. For targeted sequencing, the regions of interest are in this step selected by use of one of two methods: target enrichment or amplicon generation. Target enrichment was used in paper II, namely the SureSelect Target Enrichment System (Agilent technologies). Here, biotinylated complementary probes are hybridized to the fragments of interest and are captured by magnetic pull-down. In paper IV, the Illumina TrueSight Tumor 26 gene panel was used, which utilizes amplicon generation. Probes are hybridized upstream and downstream of the regions of interest, and DNA polymerase extends the sequences to connect the probes. The products are amplified by PCR, and index sequences, which allow several samples to be analyzed simultaneously, and adaptors are attached. Secondly, the library is loaded into an oligonucleotide-coated flow cell, where the adaptors bind to the complementary oligos on the flow cell surface. Each DNA fragment is then amplified through a process called bridge

amplification. Next, fluorescently labeled dNTPs are added to the flow cell. When a complementary dNTP is bound to the first nucleotide of the DNA template, the fluorescent label is detached and the emission is imaged. The four dNTPs emit specific wavelengths and so the sequence is identified by the detected emissions, base-by-base. In the last step, the reads are aligned to a reference sequence to identify genetic discrepancies. In paper II, the tumor samples were compared with sequences from blood sample to distinguish somatic mutations from germline mutations. In paper II, sequencing was performed on an Illumina HiSeq2000, and in paper IV on an Illumina MiSeq.

Data processing in paper II was performed as previously described (*179*). In brief, DNA sequencing reads were cleaned and demultiplexed using Illumina inherent software before alignment to the human reference genome hg19 by using Novoalign (Novocraft Technologies). Local realignment was performed using GATK (*180*) in order to minimize false-positive calls due to misalignment. Duplicate fragments were marked using the Picard tool (*181*). Variant calling was performed using VarScan2 (*182*), and variants were annotated using ANNOVAR (*183*). Copy number estimates for tumor-normal pairs were generated using Contra (*184*). Data processing in paper IV was performed as described earlier (*185*): using the MiSeq Reporter and VariantStudio analysis pipeline (Illumina).

Microarray-based gene expression analysis

While sequencing of tumor DNA provides information about tumor-specific changes in the genome, analysis of levels of mRNA in tumor samples present a snapshot of which genes are actually expressed in the analyzed cells. This level of cellular information can provide insight into important biological features of sub-groups of tumors. The improvements in gene expression profiling methods during the last decades have made it possible to study thousands of genes in a single experiment using DNA microarrays.

In paper II, gene expression analysis was performed using Illumina-HT12v4.0 BeadChip arrays for single-channel detection. In brief, mRNA from the tumor samples was converted to complimentary DNA (cDNA) by reverse transcriptase, and in turn was made double-stranded by DNA polymerase. Through in vitro transcription linear amplification, amplified amounts of cRNA were generated, keeping the relative levels of RNA intact (*186*). Biotinylated cRNA was hybridized to the 50-mer probes on the beads, washed, and fluorescent-labeled with streptavidin-Cy3 before scanning with iScan. The BeadChip array targets >47000 probes, covering the NCBI RefSeq Release 38.

Preprocessing of the gene expression data was performed using the GenomeStudio software (Illumina) to remove outlier beads, calculate average bead signals and detection p-values. Samples were normalized to a common baseline using the algorithm for cubic spline quantile-normalization (*187*). Further data processing was performed in R statistical environment. The data was log2 transformed, and probes with a detection p-value <0.01 in at least 80% of the samples were selected and mean centered, to enable comparisons between samples. The samples were classified according to the four gene expression phenotypes previously described by our group, using the centroids from Harbst et al. (*106, 108*). The centroid with the highest Pearson correlation coefficient (r) was selected for each sample, if r>0.2, otherwise set as 'unclassified'. Principal component analysis (PCA) was applied on the gene expression data to validate that the variations in gene expression levels mainly were due to biological and not technical factors. For this purpose the swamp package in R was used (*188*).

For validation of the prognostic significance of the gene expression phenotypes, TCGA RNA sequencing data of 20,501 genes from 472 samples, mostly metastases, was obtained from the TCGA data portal (*189*). Furthermore, three independent gene expression datasets were used to investigate the predictive role of the gene expression phenotypes (*158, 190, 191*). The datasets were accessed from the Gene Expression Omnibus repository (*192*). In brief, all individual datasets were merged with the 'Lund' dataset and adjusted in a pairwise manner using the Distance Weighted Discrimination method in the R '*InSilicoMerging*' package before mean centering and phenotype classification. This method was used to compensate for systemic bias when comparing the datasets (*193*).

Immunohistochemistry

To study if the major characteristics of the gene expression profiles investigated in paper II also were reflected on protein expression, a subset of the tumor samples were subjected to immunohistochemistry. Tumor specimens were formalin-fixed and paraffin-embedded, and cut in 4µm tissue sections. Hematoxylin and eosin staining was performed to visualize tissue structural patterns. Staining was performed using antibodies against CD3 (polyclonal, DAKO), MITF (clone C5, Thermo Fisher), and Ki67 (clone MIB-1, DAKO), combined with the DAKO EnVision horseradish peroxidase rabbit/mouse kit system and the DakoCytomation Autostainer (DAKO).

Statistical methods

Statistical analyses were performed in the R environment or using IBM SPSS Statistics v. 20 or 24. All *P*-values were two-tailed, and *P*<0.05 was considered significant. For associations between categorical values, Pearson chi-square test or Fisher's exact test were used. Mann-Whitney U-test or Kruskal-Wallis test were used to compare continuous variables between two or more groups, respectively. Pearson correlation was used to describe the association between continuous variables if the variables were normally distributed and the correlation was linear, otherwise Spearman's rank correlation was used. Survival curves were generated by the Kaplan-Meier method, and *P*-values were calculated using the log-rank test. Cox regression analysis was used for univariate or multivariate survival analysis with a 95% confidence interval (CI). Since *BRAF*, *NRAS,* and *TERT* promoter mutations are early events in melanoma development and are preserved through progression (*73, 194*), survival in metastatic disease was calculated from diagnosis of first recurrence and stage IV disease in paper I and III. In paper II, survival was calculated from the date of sampling as the gene expression profiles are dynamic during tumor progression (*179*).

Results and discussion

In the modern era of cancer treatment, when precision medicine is the goal, it has become ever more important to find clinically relevant prognostic markers. Ideal prognostic markers can identify patients at high risk of progression, who would benefit from more intensive surveillance and treatment, as well as patients at low risk, who safely could be spared the toxicity of treatment. Of the same reasons, it is important to find predictive biomarkers which could assist in the choice of treatment. To be able to identify such biomarkers, it is necessary to conduct studies in metastatic settings. This thesis focuses on investigating the clinical aspects of molecular profiles in metastatic melanoma based on mutational status and gene expression patterns.

Mutational profiles in metastatic melanoma

After the discovery of *BRAF* mutations in ~50 % of melanomas in 2002 (*56*), the idea of genetic subtypes has been intensively explored (*59, 61, 69, 194, 195*), and much work has focused on finding selective blockers for constitutively activated mutated tumors (*196*). However, the clinical significance of *BRAF* and *NRAS* mutations is still not clear.

Frequencies of *BRAF* and *NRAS* mutations in metastatic melanoma

Sanger sequencing was used to screen for *BRAF* and *NRAS* hotspot mutations in paper I. *BRAF* mutations were found in tumors from 82 out of 191 patients (43%) with metastatic melanoma. The most common mutation was V600E (88%), followed by V600K (10%). In paper IV, the mutational pattern in 127 patients with mainly stage III-IV melanoma was described using an NGS panel. The *BRAF* mutation frequency was similar (46%), however 22% of the *BRAF* mutations were V600K. The difference in V600K mutation rate could be explained partly by the composition of the cohorts, as the cohort in paper I originated from the Department of Surgery and thus included few tumors originating from head/neck melanomas, which previously have been associated with V600K mutations (*62*).

The cohort in paper IV comprised all melanoma patients eligible for *BRAF* mutation testing during the study period at Skåne University Hospital and thus included patients with head/neck melanomas treated at the Ear- Nose and Throat Department. Mutations in *NRAS* were found in 30% in paper I and in 31% in paper IV. *BRAF* and *NRAS* mutations appeared mutually exclusive, and although a few tumors harbored mutations in both genes, the majority of these comprised one non-hotspot mutation, corroborating previous studies (*64, 66*).

BRAF and *NRAS* mutations are preserved in multiple metastases

Since the initial concept of targeted therapy presumes that all tumor cells carry the target mutation, it is of interest to examine multiple samples from the same patient. In both paper I and IV, multiple samples were analyzed for *BRAF* and *NRAS* mutations in a total number of 73 tumors from 33 patients. *BRAF* mutational status matched in all cases but one, where the last of three lymph node metastases had lost its mutation. The discordant result could be explained by a low tumor cell content, resulting in a too low *BRAF*-mutant allele fraction to be detected by Sanger sequencing, which is supported by a low copy number profile in that sample in subsequent analysis (*179*). Although *BRAF* mutations occur already in benign nevi, intra-tumoral heterogeneity in melanoma has been described (*197*). Furthermore, discordant *BRAF* mutational status in paired samples of primary tumor and metastasis has also been shown (*197, 198*). However, concerns have been raised that the heterogeneity could be due to limited sensitivity for mutation detection by certain sequencing methods used in some studies, or due to the presence of occult second primary tumors (*199*).

NRAS mutational status matched in all samples but one, where a lymph node metastasis and a distant skin metastasis contained a Q61R and a Q61K mutation, respectively. This type of *NRAS* heterogeneity has been described in previous studies, where different *NRAS* mutations have been found within the same primary tumor, giving rise to metastases with different mutations, and thus suggests sub-clones within the primary tumor (*55, 200*). However, none of the metastases had lost its *NRAS* mutated allele.

Clinical significance of *BRAF* and *NRAS* mutations in metastatic melanoma

BRAF and *NRAF* mutations have previously been associated with certain host and tumor characteristics, which might reflect different etiologies (*59, 61, 69, 194, 195*). Corroborating these results, in paper I, patients with *BRAF*-mutant tumors were significantly younger than patients with *NRAS*-mutant or WT tumors. There

were also non-significant trends towards that *BRAF*-mutant metastases originated from SSMs (*P*=0.07) and from primary melanomas on the trunk (*P*=0.12), whereas *NRAS* mutated metastases most often were from NMs and extremities. There was no association between *BRAF* or *NRAS* status and type of first recurrence, number of involved lymph nodes, or metastatic sites. In paper IV, *BRAF* mutations were significantly associated with lower age, low Breslow thickness, and trunk location compared with *BRAF*-WT tumors. Yet, patients with V600K mutations were older than patients with V600E mutations (*P*=0.02). *NRAS* mutations were not significantly associated with any tumor or host characteristic.

The prognostic value of *BRAF* and *NRAS* mutations are uncertain since several studies with different designs have been performed and shown discordant results. Most studies show no difference in survival from primary melanoma (*67, 69, 194, 201*), but in a recent large retrospective study, *BRAF* and *NRAS* mutations were independently associated with poor melanoma-specific survival in a subgroup of high-stage primary melanoma (*201*). Some studies have shown inferior survival for *BRAF* and *NRAS* mutations in stage III-IV melanoma (*70, 71*). *BRAF* mutations have also been associated with a worse outcome in stage III melanoma in a small study (*72*). Conversely, *BRAF* status had no impact on survival before distant metastatic disease occurred, but was associated with a worse OS thereafter, in a study of 197 patients (*67*). In a study of 519 patients, *NRAS* mutations were associated with the shortest OS in stage IV melanoma (*68*).

Corroborating most previous studies, there was no significant difference in recurrence-free survival (RFS), distant metastasis-free survival (DMFS) or OS in relation to *BRAF/NRAS* status in Paper I. However, OS from stage IV disease was significantly different among four groups: *BRAFV600E*-mutant, *NRAS*-mutant, WT, and BRAFi-treated patients, with *BRAFV600E*-mutants having the worst prognosis. In a univariate Cox regression model, patients with *BRAF*-mutant tumors not treated with BRAFi were significantly associated with an inferior outcome compared with BRAFi-treated patients (hazard ratio (HR) 2.35, confidence interval (CI) 1.10-5.01). Adjusting for age in a multivariate Cox regression model displayed a similar result. There was a trend for better prognosis for patients with WT and *NRAS*-mutant tumors compared with V600E-mutants (HR 0.64, CI 0.39–1.04 and HR 0.76, CI 0.48–1.21, respectively).

The routine use of BRAFi will hinder future studies of the roles of *BRAF* and *NRAS* mutations in the natural course of melanoma. Altogether, the studies to date indicate that *BRAF* and *NRAS* mutations do not affect OS, but *BRAF* mutations, and to a lesser extent *NRAS* mutations, may perhaps confer a poorer prognosis in advanced stage melanoma. The reason for this delayed prognostic impact is unknown, but it seems reasonable that additional factors occurring in metastatic disease may contribute to create a more aggressive phenotype. Interestingly, *BRAF*

and *NRAS* mutations were associated with CNS involvement at diagnosis of stage IV disease in a study of 519 patients with stage IV melanoma (*68*). Results from studies in a mouse model harboring a *BRAFV600E* mutation and loss of *CDKN2A* revealed that activation of AKT1 initiated metastasizing to the brain, further enhanced by loss of the upstream negative regulator PTEN (*202*). High expression of phosphorylated AKT and low PTEN expression was also demonstrated in human brain metastases, as opposed to lung and liver metastases (*203*). In line with these results, loss of PTEN has been associated with shorter OS, but not shorter DMFS, as well as shorter time to brain metastasis in stage IIIB-C melanoma (*95*). In a subgroup analysis, this association appeared in *BRAF*-mutant tumors, but not in tumors WT for *BRAF* and *NRAS*. *BRAF* mutations often co-occur with *PTEN* mutations, while mutated NRAS is able to activate PI3K directly (*64, 92*). Besides, additional proteins in the PI3K/AKT pathway are expressed at different levels among the mutation subtypes (*64*). Given the disparity in mechanisms for activating the PI3K/AKT pathway among the mutation subtypes, this might be a cause for the prognostic variation in metastatic melanoma.

The major role for *BRAF* mutations in the clinic is nonetheless not as a prognostic marker, but as a predictive marker for BRAFi and MEKi. This is illustrated by the OS curves for stage IV disease from paper I and IV, with the patients divided into groups according to *BRAF* status (Fig. 5A-B). There was a non-significant trend towards poorer prognosis for the *BRAF*-mutant group not treated with BRAFi compared with WT tumors in paper I (Fig. 5A). As described above, the difference in OS was significant among patients with *BRAF*-mutated tumors depending on if the patient received BRAFi treatment. In fact, in study IV, the OS from stage IV disease was significantly longer for patients with *BRAF*-mutant tumors, the majority treated with BRAFi (75%), compared with *BRAF*-WT (Fig. 5B). However, relatively few patients in this cohort received PD-1i as the trend is to increasingly use PD-1i upfront, and the follow-up was limited. Thus, no conclusions can be drawn regarding the outcome, according to *BRAF* mutational status, for patients with access to the approved treatments to date.

Moreover, a substantial part of the patients with stage IV disease in the study died of melanoma without receiving systemic treatment (18 of 89, 20%). Of these patients, only two (11%) carried a *BRAF* mutation. Although these findings were not covered by the aim of the study and hence were not included in the manuscript, they contribute to give a more comprehensive picture of the disease. These findings probably reflect the possibility of treating patients with more advanced disease with targeted therapy and emphasize the need for new targeted therapies for patients with *BRAF*-WT tumors as a complement to immunotherapy.
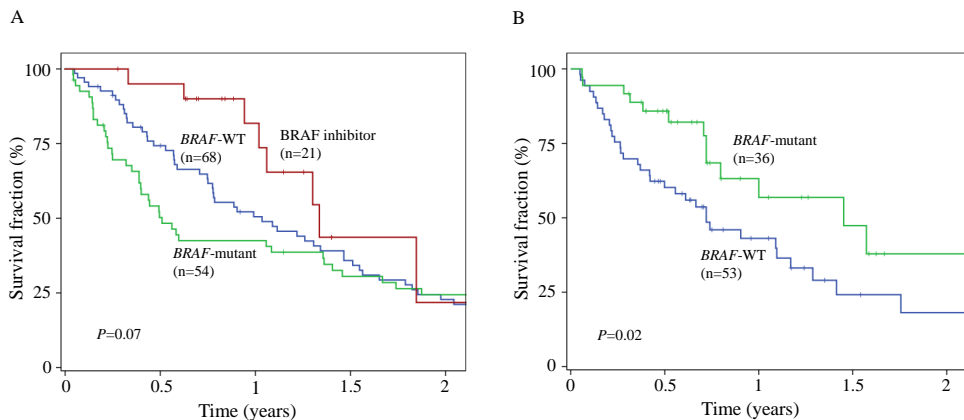
Figure 5. Overall survival from stage IV melanoma in relation to *BRAF* mutational status in the cohorts of paper I (A) and paper IV (B). In paper IV, most patients with *BRAF*-mutant tumors were treated with a BRAF inhibitor.

Frequent *TERT* promoter mutations without evident prognostic value in non-acral cutaneous metastatic melanoma

In paper III, tumor samples from 170 patients with non-acral cutaneous metastatic melanoma were screened for *TERT* promoter hotspot mutations, and the prognostic impact was investigated. Mutations were found in 81% of the cases. Hotspot mutations were mutually exclusive. The -124C>T and -146C>T mutations were most common and equally frequent (44%), followed by -138/139CC>TT (7%), -124/125CC>TT (4%), and -124C>A (0.7%). *TERT* promoter mutational status matched in all 27 patients with multiple tumor samples, supporting previous studies of benign, premalignant, and malignant lesions, indicating that *TERT* promoter mutations occur early in tumor development: in intermediate stages (*73, 204*). As expected, *TERT* promoter mutations were significantly more frequent in tumors harboring a *BRAF* or *NRAS* mutation. In fact, the mutated *TERT* promoter has recently been shown to be a key target for phoshorylated ERK in *BRAF*- and *NRAS*-mutant melanoma, as ERK signaling maintains the *TERT* promoter in an active chromatin state, which is necessary for transcriptional activation of mutant *TERT* (*205*).

There was no association between *TERT* promoter mutations and characteristics of the corresponding primary tumor, patient age, or gender. Furthermore, there was no association with type of first metastasis, sentinel node status, or number of affected lymph nodes in regional disease. *TERT* promoter mutational status did not correlate with RFS, or with OS from first metastasis or from first distant metastasis. Survival was also analyzed combining *TERT* promoter and *BRAF*/*NRAS* mutational status, resulting in four groups (*TERT*+/*BRAF*+,

TERT+/*NRAS*+, *TERT*+/*BRAF*-/*NRAS*-, and *TERT*-), but still without difference in survival. Since less than 20% of the metastatic lesions were WT, as compared with more than 60% in primary melanoma (*82, 83*), it is plausible that the high mutation rate reflects a greater metastatic potential among *TERT* promoter mutated primary tumors. It is also possible that the *TERT* promoter-WT tumors which nonetheless have metastasized represent a selection of the most aggressive tumors, which in the absence of *TERT* promoter mutations have gained metastatic potential through a different mechanism.

Gene expression phenotypes in metastatic melanoma

Melanoma displays heterogeneity not only at a genomic level but also at a transcriptional level. Several attempts have been made to utilize the transcriptional heterogeneity to find clinically useful gene expression subtypes (*59, 64, 105*). Our group has previously demonstrated that melanoma can be divided into four gene expression phenotypes, reflecting distinct biological features (*106, 108*). The phenotypes provide prognostic information in primary and stage IV melanoma (*106, 206*). In paper II, the prognostic value of this classification in stage III melanoma was further established, and its role as a predictive marker for targeted therapy was investigated.

Characteristics of the gene expression phenotypes

Tumors from 214 patients were classified into the gene expression signatures and all phenotypes were represented: high-immune response (30%), normal-like (6%), pigmentation (44%), and proliferative (15%) (Fig. 6A). Of the 16 primary tumors included, eight (50%) were classified as normal-like, whereas only four out of 188 (2%) of the metastatic tumors belonged to this phenotype, and thus these were excluded from survival analysis. There was no significant difference in age or gender according to gene expression phenotype, and furthermore no difference in Breslow thickness or presence of ulceration of the corresponding primary tumors. Interestingly, the time from primary melanoma to diagnosis of the analyzed tumor differed significantly between the phenotypes, with the longest period in the proliferative group. However, type of metastasis (local/in-transit/regional/distant) did not vary between the phenotypes.

In order to examine if the gene expression phenotypes are reflected in protein expression, 59 tumors were analyzed for expression of MITF, Ki67, and CD3 by immunohistochemical analysis. As expected, tumors of the high-immune response phenotype showed a strong infiltration of CD3-positive T-lymphocytes. Tumors of

50

the pigmentation phenotype frequently expressed MITF, a key regulator of pigmentation, while it was absent in proliferative tumors. Ki67, which is a general marker of proliferation, was expressed to a large extent by melanoma cells of both the pigmentation and proliferative phenotypes.

Gene expression phenotypes provide independent prognostic information in stage III melanoma

Among patients with regional and in-transit metastases, 5-year DMFS varied between the gene expression phenotypes, with a poorer outcome for patients with tumors of the proliferative (HR 2.8, CI 1.43-5.57) or the pigmentation (HR 1.9, CI 1.05-3.28) phenotypes compared with the high-immune response phenotype. Adjusting for age and gender in a multivariate Cox regression model yielded a similar result (HR 2.7, CI 1.37-5.36 and HR 1.8, CI 1.00-3.17, respectively). There was also a difference in 5-year DSS, with an inferior outcome in the proliferative (HR 3.5, CI 1.56-7.80) and the pigmentation (HR 1.7, CI 0.83-3.28) phenotypes compared with the high-immune response phenotype (corresponding survival curves are shown in Fig. 6B). In a multivariate analysis including age, gender, and type of metastasis, the difference in DSS was only significant for the proliferative phenotype compared with the high-immune phenotype (HR 2.8, CI 1.19-6.65). The superior DSS for the high-immune response phenotype was confirmed in an external dataset (TCGA) comprising 309 regional and distant metastases.
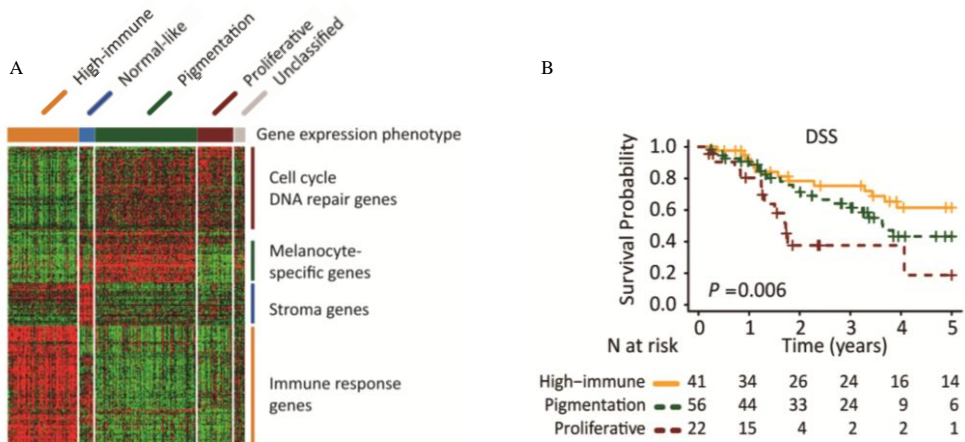

Figure 6. (A) Heat map demonstrating the difference in expression of 299 genes, reflecting distinct biological features, according to the four phenotypes in 214 melanoma tumors. (B) Disease-specific survival (DSS) in stage III melanoma in relation to gene expression phenotypes.

Mutational patterns in metastatic melanoma and the relation to gene expression phenotypes

The mutational landscape of metastatic melanoma was analyzed in both Paper II (deep targeted sequencing of 1697 cancer-associated genes in 146 patients) and Paper IV (clinical NGS panel of 26 genes in 127 patients). The mutation rates were generally similar between the cohorts. As described earlier, *BRAF* and *NRAS* mutations occurred in a mutual exclusive manner. In paper II, tumors WT for *BRAF* and *NRAS* harbored mutations in *NF1* and *KIT* significantly more often than *BRAF*/*NRAS*-mutants. Similarly, in Paper IV, 31% of the *BRAF*/*NRAS*-WT tumors carried a mutation in *GNAQ*, *KIT* or *MAP2K1* compared with none of the *BRAF/NRAS*-mutants. Furthermore, both cohorts comprised one *KRAS*-mutant tumor each WT for *BRAF*/*NRAS*. Thus, oncogenic driver mutations in the MAPK pathway were present in the absolute majority of cases, corroborating previous comprehensive mutational landscape studies (*64, 66*), indicating the importance of the MAPK pathway in melanoma. In both cohorts, *TP53* was the third most commonly mutated gene, and in Paper IV *TP53* mutations correlated with head/neck location of the primary tumor. These findings seems rational knowing that *TP53* mutations are associated with UV signature mutations (*207*), but at the same time surprising, since alterations of *TP53* long was considered infrequent in melanoma (*196*). p53 is activated by p14^{ARF}, a transcript of the *CDKN2A* gene, which is commonly affected by deletions or loss-of-function mutations (*66*). In Paper II, *CDKN2A* alterations were present in 45% of the tumors, and other genes in the CDKN2A-RB1 pathway were altered mainly in the *CDKN2A*-WT tumors (*RB1* 5%, *CDK4* 4%, and *CCND1* 9%). Hence, the high rate of events in the CDKN2A-RB1 pathway is considered to diminish selection pressure for *TP53* mutations in melanoma (*66*).

The landscape of driver mutations in melanoma is at this stage rather well explored and new highly recurrent driver mutations are unlikely to be found, at least in coding sequences (*64, 66*). The interplay between oncogenic alterations and gene expression, on the other hand, is far less scrutinized. In Paper II, the distribution of genetic alterations among the four gene expression phenotypes was analyzed. The mutational burden ranged between 5 and 768, but did not vary significantly between the phenotypes. *BRAF* and *NRAS* mutations were not significantly associated with any of the phenotypes. This finding is in line with results from Paper III, showing no significant difference in *TERT* promoter mutations among the phenotypes, but an association between *BRAF* and *NRAS* mutations and *TERT* promoter mutations. Conversely, *CDKN2A* alterations most often occurred in tumors of the proliferative phenotype (*P*=0.05), corroborating previous findings from our group (*106*), and supporting studies demonstrating

poorer prognosis for melanomas with loss of *CDKN2A* (*208, 209*). The pigmentation phenotype was as expected significantly associated with amplifications in *MITF*. In addition, the pigmentation phenotype was significantly associated with mutations in *CTNNB1* and amplifications in *CCND1,* supporting the roles for Wnt/β-catenin as activators of MITF (*196, 210*) and cyclin D1 as a target of MITF signaling, promoting cell proliferation (*211*).

Treatment prediction

With the rapid advances in novel treatment modalities of melanoma, it is necessary to find useful predictive markers for respective treatments. *BRAF* hotspot mutations are established in clinical practice as a required predictive marker for BRAFi treatment and for combination with MEKi. However, it is now clear that despite initial responses, acquired resistance occurs in the majority of patients treated with BRAFi only, or in combination with MEKi (*122, 212*). In addition, a minority of tumors show intrinsic resistance, i.e. they do not respond at all. A question currently being investigated in clinical trials is in which sequential order treatments should be given. Early studies indicated an increased immune activity in tumors after BRAFi treatment (*213, 214*), motivating upfront targeted therapy treatment, which also has a more rapid effect compared with immunotherapy. However, several clinical studies now point towards that pre-treatment with BRAFi actually confers poorer response to immunotherapy (*215, 216*). The same negative effect does not seem to appear in the opposite order (*215, 217*). A seemingly reasonable strategy, while awaiting the results from prospective trials, is to treat patients with a large tumor burden, who need quick tumor volume reduction, with targeted therapy upfront, and to switch to immunotherapy after induction or at progression. For patients with low tumor burden, immunotherapy is given upfront. In Paper IV, the patients treated with BRAFi upfront were indeed significantly associated with elevated LDH and presence of CNS metastasis compared with patients receiving immunotherapy in first line. In all, a better understanding of the underlying mechanisms of divergent responses hopefully will assist in selecting treatment for each patient.

Treatment prediction for targeted therapy

Apart from the required presence of *BRAF* hotspot mutations there are limited factors that can predict response to BRAFi +/- MEKi. In general, factors associated with shorter PFS to date are prognostic factors associated with a more aggressive disease and advanced disease stage, such as elevated LDH and

increased number of disease sites (*218*). A high fraction of *BRAF* hotspot-mutant alleles (BRAF-M%) has been associated with prolonged PFS in one study (*219*). This finding could not be replicated in two following studies (*220, 221*). In Paper IV, the BRAF-M% varied between 5% and 92%. After adjustment for tumor cell content, the relation between BRAF-M% and PFS on MAPK inhibition was examined. A high BRAF-M% was not associated with PFS. On the other hand, tumors within the highest and lowest deciles of BRAF-M% displayed short PFS. Amplification of *BRAF* is present in 5-15% of *BRAF*-mutant melanoma (*64, 222*) and is an important resistance mechanism for BRAFi +/- MEKi (*223-225*). It is possible that tumors with the highest BRAF-M%, which are caused by increased copy numbers of the *BRAF*-mutant allele (*222*), contain sub-clones of *BRAF* amplified cells that continue to proliferate during MAPK inhibition, giving rise to a rapid acquired resistance.

The role of the gene expression signatures as a predictive marker for targeted therapy was evaluated using two publicly available external gene expression datasets in Paper II (*190, 191*). The sets included pre-treatment and post-relapse samples from patients treated with BRAFi, with or without the addition of MEKi (n=21 and n=10, respectively), and were evaluated for best response according to the RECIST criteria and PFS. After classification of these samples into the gene expression phenotypes, no clear correlation to response appeared due to low number of cases. However, the only two pre-treatment samples carrying the proliferative signature responded poorly and rapidly progressed. Contrary, six out of seven of the samples with the high-immune response signature showed responses better than median. In both datasets, the proportion of phenotypes appeared to have changed after treatment with an increase in samples with the proliferative signature and a decrease in the high-immune response. When combining the datasets, the increase in proportion of samples with the proliferative signature was statistically significant. These results corroborate findings from preclinical studies, showing that absence of MITF expression conferred intrinsic resistance to MAPK inhibition, and that MITF-absent samples were abundant among samples with acquired resistance (*226, 227*). In these studies, the MITF-low samples expressed high levels of the receptor tyrosine kinase AXL and a NF-κB-related signature. AXL expression is associated with cell survival, proliferation, and migration in several cancers, as through AKT-dependent activation of NF κB (*228*). Moreover, a recent study of RNA expression in patients with complete response versus progressive disease in combined trials of BRAFi +/- MEKi treatment indicated that gene signatures of immune response were enriched among complete responders (*229*).

Treatment prediction for immunotherapy

Predictive factors for the two established immune checkpoint inhibitors, CTLA-4i and PD-1i, have been found, but are not consensual, and still none is used in the clinic. Expression of PD-L1, low LDH-levels, and a high mutational load have been associated with improved response to PD-1i (*145, 152, 230*). *NRAS* mutations have been proposed to be predictive of increased response to immunotherapy (*231*), however this could not be replicated in a follow-up study (*152*). Instead, *NF1* mutations were enriched among responders to PD-1i, perhaps more expected given the higher mutational load in this genotype. Furthermore, mutational load measured by two NGS panels of 236 and 315 genes showed excellent correlation to global mutation load from whole-exome sequencing (R=0.995) and were associated with PFS (*152*). In fact, mutations in one single gene, *LRP1B*, correlated with mutational load and were significantly more frequent among responders to PD-1i. In Paper IV, the total number of mutations found by the 26-genes mutation panel ranged 0-5, but was not significantly correlated with PFS on PD-1i treatment. Notable, the gene panel did not include *NF1*. Moreover, *NRAS* mutations were not significantly associated with PFS on PD-1i treatment.

In Paper II, it was evaluated whether the gene expression phenotypes could predict response to MAGE-A3 immunotherapeutic. External data was used from a study where a gene expression signature predictive of response to MAGE-A3 immunotherapeutic was derived (*158*). The study included pre-treatment samples from 56 patients, who were treated with the MAGE-A3 immunotherapeutic, evaluated for RECIST response and PFS, and subsequently were divided into clinical benefit (n=22) or no clinical benefit (n=34). Only two out of 11 samples with the proliferative signature had clinical benefit, while the highest proportion of responders were found among the high-immune response signature (6/10), however, the difference was not significant. Intriguingly, the gene signature derived in the original study by Ulloa-Montoya et al. failed to predict response in a recent prospective phase II trial (*159*), highlighting the complexity of gene expression and the need of validation studies.

Conclusions

- Stage III melanoma can be divided into four gene expression phenotypes with different biological and prognostic impact.

- Mutated *BRAF* is a frail prognostic marker, but has a central role in melanoma management as a predictive factor for treatment with BRAFi and MEKi.

- The fraction of *BRAF* hotspot-mutant alleles showed substantial inter-patient heterogeneity and might play a role in predicting response to targeted therapy.

- *TERT* promoter mutations were highly recurrent, but were not associated with prognosis, in non-acral cutaneous metastatic melanoma.

- *BRAF, NRAS,* and *TERT* promoter mutational status showed high concordance in multiple metastases.

Future perspectives

The great advancements during the last two decades in the understanding of biological features driving melanoma development and progression have clearly led to a remarkable improvement in the ability to treat metastatic melanoma. However, new challenges appear ahead and need to be overcome. As new therapies are added to the arsenal, the need for prognostic and predictive markers will increase even more. The gene expression classification was shown to independently associate with DMFS in stage III melanoma. Nonetheless, several issues need to be solved before gene expression analysis may be a valuable tool in clinical melanoma management. Among others, the role for the gene expression phenotypes as treatment predictive factors needs to be clarified. No systemic therapy with reasonable toxicity is used in the adjuvant setting at present, but results from ongoing studies of PD-1i and BRAFi+/-MEKi might change this fact (*232*). In order to select patients for adjuvant treatment, the risk for recurrence and the chance of preventing it by adjuvant treatment, must motivate taking the risk of treatment-related side effects. In that sense, treating tumors with the proliferative phenotype appears challenging, since previous pre-clinical studies and the results presented here, although very immature, indicate that the proliferative phenotype, which associates with the poorest prognosis, also seems to predict poor response to targeted therapy and immunotherapy. Hopefully, future studies will reveal suitable treatment options for tumors of all phenotypes. An alternative approach to tackle the proliferative tumors might be to pharmacologically induce a phenotype switch to make the melanoma cells susceptible to therapy. A pharmacologically induced switch has been described previously by Sáez-Ayala et al. (*233*). A potential way forward might be to induce a switch, from MITF-low proliferative to MITF-high pigmentation, and evaluate the outcome on the effectiveness of BRAFi treatment.

Resistance mechanisms to BRAFi treatment are rather well explored, and efforts are taken to overcome these by trying additional combinations of drugs. However, little is known about the inter-tumoral heterogeneity of resistance mechanisms, as well as possible features that can predict if and how resistance will occur. To gain this knowledge would require examining multiple post-relapse samples from the same patient, and also comparing these with pre-treatment samples, by means of mutation, amplification, and gene expression analyses.

The role for telomerase, apart from that of maintaining telomere length, needs to be further elucidated. *TERT* promoter mutations have been associated with a poorer prognosis in primary melanoma, but little is known about the features of other events causing regained telomere lengthening capacity and non-telomere-dependent effects of these events. It would certainly be interesting to study telomerase expression in relation to different *TERT* promoter mutations, amplifications, and methylation status, and the effect on RFS in primary melanoma.

Populärvetenskaplig sammanfattning

Malignt melanom är en typ av cancersjukdom som utgår ifrån melanocyter, celler som producerar det pigment som ger huden, håret och ögonen dess färg. Pigmentet skyddar cellernas arvsmassa från att skadas av solens ultravioletta strålning. Det finns två olika typer av pigment: ett brunsvart, som ger ett bra skydd, samt ett rödgult, som utgör ett sämre skydd. Personer med rött eller blont hår, fräknar och ljusa ögon har mer av det rödgula pigmentet och löper därför större risk att utveckla malignt melanom. Malignt melanom uppkommer oftast i huden, men kan i ovanliga fall uppstå i ögonen eller i slemhinnor, t.ex. i tarmen. De senaste decennierna har malignt melanom blivit allt vanligare och drabbar nu närmare 4000 personer i Sverige årligen, vilket gör att det nu är den femte vanligaste cancerformen hos kvinnor och den sjätte vanligaste hos män. Sjukdomen beror på att flera olika skador uppstår i en melanocyts arvsmassa. Skadorna är ofta orsakade av UV-strålning och resulterar i att viktiga reglersystem i cellen sätts ur spel. Detta leder till att cellen kan föröka sig obehindrat och forma en tumör. Denna kan börja växa in i intilliggande vävnader samt även ge upphov till dottertumörer, så kallade metastaser, på andra ställen i kroppen, såsom i lymfkörtlar i armhålorna eller ljumskarna, lungorna, hjärnan eller skelettet. Prognosen skiljer sig avsevärt beroende på hur tjock primärtumören är samt huruvida tumören har spridit sig. Tunna tumörer som skärs bort medför en mycket liten risk för återfall, medan riktigt tjocka tumörer ger upphov till metastaser i mer än hälften av fallen. Det är alltså av största vikt att malignt melanom upptäcks tidigt.

Malignt melanom har traditionellt sett varit en mycket svårbehandlad sjukdom när den väl spridit sig och de cellgifter som användes som standard har inte visats förlänga överlevnaden. Sedan 2011 har det dock introducerats flera nya läkemedel som har bevisad effekt på överlevnaden. Det finns två typer av nya läkemedel som i huvudsak används idag. Den ena är antikroppar som aktiverar kroppens eget immunförsvar, vilket därpå kan bekämpa tumörerna. Den andra kallas ofta målriktad behandling eftersom den mycket specifikt angriper tumörceller som bär på en viss förändring i arvsmassan, i en gen som kallas *BRAF*. Tyvärr svarar inte alla patienter på dessa behandlingar och i många fall uppstår resistens mot behandlingen efter några månader. I takt med att alltfler nya läkemedel introduceras, och med hänsyn till att behandlingarna medför biverkningar av varierande grad, blir det allt viktigare att få kunskap om vilka faktorer som kan

förutsäga hur det kommer att gå för en viss grupp av patienter, samt vilka som svarar på respektive behandling. Målet med denna avhandling var att undersöka hur olika typer av förändringar i tumörcellernas arvsmassa påverkar sjukdomsprognosen och möjligheten att svara på behandling.

I avhandlingens tre första delstudier studerades en grupp av patienter som mellan 1993 och 2012 opererats för spridd melanomsjukdom, oftast till lymfkörtlar, där en liten tumörbit frysts ner för att kunna användas i forskningssyfte. Tumörproverna analyserades för att avgöra vilka förändringar som förekom i tumörernas arvsmassa samt även vilka gener som var aktiva i respektive tumör. Resultaten visade att förändringar, eller mer specifikt mutationer, i *BRAF*-genen förekom i 43 % av tumörerna och oftast hos yngre patienter, vilket är i linje med vad som beskrivit tidigare. Mutationer i *BRAF*-genen tenderade att vara kopplade till en sämre prognos från den tidpunkt då spridning i kroppen diagnostiserats, men skillnaden var inte statistiskt säkerställd. Däremot hade patienter med spridd sjukdom som fick målriktad behandling en förlängd överlevnad.

Alla celler i kroppen bär på samma arvsmassa, men olika typer av celler använder olika delar av arvsmassan, d.v.s. olika gener, för att göra sin specifika uppgift. Med andra ord är olika gener aktiva i t.ex. en melanocyt jämfört med en levercell. Genom att studera mönster av vilka gener som var aktiva i cellerna som utgjorde melanomtumörer har vår forskargrupp tidigare kunnat visa att malignt melanom kan delas in i fyra grupper. Resultaten i avhandlingen visade att melanommetastaser i lymfkörtlar kunde delas in i dessa fyra grupper. Den grupp som uppvisade aktiva gener kopplade till immunförsvar hade den bästa prognosen, medan gruppen med aktiva gener relaterade till cellförökning hade sämst prognos. Dessutom indikerade analyserna att den senare gruppen var kopplad till resistens mot målriktad behandling.

Införandet av målriktade läkemedel som standardbehandling vid avancerat malignt melanom har inneburit att analyser av tumörers arvsmassa nu blivit en del av den kliniska vardagen. Sedan 2015 görs på Skånes universitetssjukhus i Lund rutinmässigt tumöranalyser av metastaserade maligna melanom med hjälp av en analyspanel, som analyserar 26 cancerrelaterade gener, däribland *BRAF*-genen. I den sista delstudien beskrevs resultaten av de första 18 månadernas användande av denna analyspanel. Genförändringar identifierades i 91 % av de 127 tumörer som analyserats och förekomsten av de olika genförändringarna var väl överensstämmande med vad som tidigare beskrivits internationellt. Resultat från en tidigare studie i USA indikerade att mutationer i den så kallade *NRAS*-genen kunde förutspå ett bättre svar på immunterapi. Denna hypotes kunde inte bekräftas här. Däremot observerades att av de patienter som fått målriktad behandling mot *BRAF*-muterade tumörer hade de patienter med tumörer som bar på ovanligt hög eller låg andel av den muterade genen endast kortvarig effekt av behandlingen.

Sammantaget visar resultaten i denna avhandling att malignt melanom har olika genetiska profiler, både gällande vilka gener som är förändrade samt vilka som är aktiva. De olika profilerna har betydelse för prognosen och även chansen att svara på behandling. Vidare studier krävs för att säkrare kunna avgöra vilka patienter som ska få respektive behandling, hur man ska undvika att resistens uppstår samt eventuellt kunna påverka tumörer att bli mer känsliga för behandling.

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Advancements in the understanding of molecular mechanisms responsible for development and progression of malignant melanoma have paved the way for the last years' astonishing breakthroughs in treatment of metastatic malignant melanoma. Modern immunotherapy and targeted therapy provide treatment options proven to prolong survival in patients with metastatic malignant melanoma. This development calls for more accurate prognostic and predictive factors. The aim of this thesis was to investigate clinical aspects of molecular profiles in metastatic melanoma based on mutational status and gene expression patterns.

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