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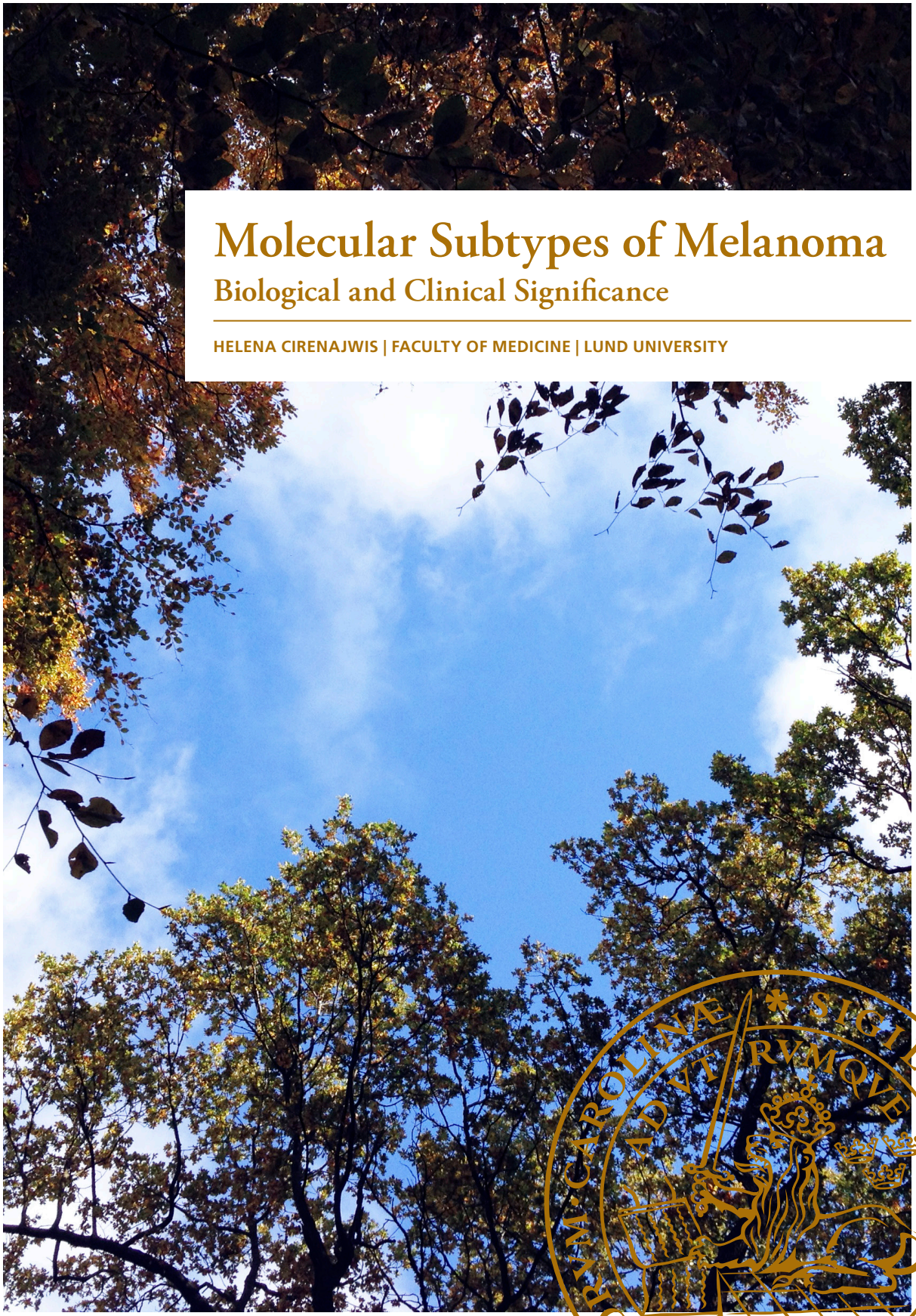

Molecular Subtypes of Melanoma

Biological and Clinical Significance

HELENA CIRENAJWIS | FACULTY OF MEDICINE | LUND UNIVERSITY

Molecular Subtypes of Melanoma

Biological and Clinical Significance

Helena Cirenajwis

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Molecular Subtypes of Melanoma - Biological and Clinical Significance

Abstract

Cutaneous malignant melanoma (CMM) is the most lethal form of skin cancer and its incidence has increased faster than that of any other cancer, rendering it a major public health problem worldwide. High-throughput screenings have opened the door to a new scientific world, which enables molecular-based characterization of large cancer cohort collections. The aim of the research presented in this thesis was to explore the molecular landscapes of melanoma tumors on a genomic and transcriptomic level and subsequently correlate certain molecular features with patient survival, treatment response and tumor evolutionary patterns.

In Paper I, it was concluded that metastatic melanoma could be divided into transcriptomic subtypes (gene expression (GEX) phenotypes) possessing diverse biological and clinical features. Patients harboring melanomas infiltrated by immune cells, i.e. the high-immune subtype, showed a superior survival, whereas highly proliferative melanomas, i.e. the proliferative subtype, was correlated to a poor survival outcome and resistance to targeted therapies. Moreover, it was also shown that, irrespectively of the GEX phenotypes, melanomas could be divided into genomic subtypes based on genetic aberrations in the mitogen-activated protein kinase (MAPK) signaling pathway. In Paper II, it was found that mutations in the tumor suppressor gene neurofibromin 1 *(NF1)*, was linked to inferior survival.

Today, it is well accepted that most tumors possess some level of intratumor heterogeneity (ITH), i.e. subclonality, influencing disease progression. In Papers III and IV, the evolutionary aspects of melanoma were considered by analyzing ITH, as well as disease progression on a molecular basis. When analyzing multiple metastatic lesions from individual patients, we found that most tumors were genetically different, with a common stem of genetic aberrations and the addition of new “private” ones, thus pointing to continued evolution during progression. Moreover, the GEX proliferative phenotype appeared to be correlated to a later disease course. From multiregional biopsies from single tumors, it was found that mutations in the MAPK signaling pathway appeared to be early events in tumorigenesis. Heterogeneous somatic mutations were found in the range of 3-38%, thus highlighting different levels of subclonality in melanoma. A high degree of mutational heterogeneity was associated with a more aggressive disease progression.

In conclusion, melanoma is a complex molecular disease that can be characterized by genomic and transcriptomic signatures with clinical implications. However, a single biopsy might not reflect the true tumor complexity, and subclonality may be one reason behind resistance development.

Key words

Melanoma, molecular subtypes, gene expression, next generation sequencing

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To my lovely boyfriend, dear family and sweet cats

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Content

List of Papers

Papers covered by the thesis

I. **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*, 6(14):12297-309, 2015.

II. **Cirenajwis H**, Lauss M, Ekedahl E, Törngren T, Kvist A, Borg Å, Saal LH, Olsson H, Staaf J, Ingvar C, Carneiro A, Harbst K, Hayward NK, Jönsson G. NF1 mutated melanoma tumors harbor distinct biological characteristics and associate with poor survival. *Submitted.*

III. Harbst K, Lauss M, **Cirenajwis H**, Winter C, Howlin J, Törngren T, Kvist A, Nodin B, Olsson E, Häkkinen J, Jirström K, Staaf J, Lundgren L, Olsson H, Ingvar C, Gruvberger-Saal SK, Saal LH, Jönsson G. Molecular and genetic diversity in the metastatic process of melanoma. *J Pathol*, 233(1):39-50, 2014.

IV. Harbst K, Lauss M, **Cirenajwis H**, Isaksson K, Rosengren F, Törngren T, Kvist A, Johansson MC, Vallon-Christersson J, Baldetorp B, Borg Å, Olsson H, Ingvar C, Carneiro A, Jönsson G. Multiregion whole-exome sequencing uncovers the genetic evolution and mutational heterogeneity of early-stage metastatic melanoma. *Cancer Res*, 76(16):4765-74, 2016.

Papers not included in the thesis

- 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*, 169(5):1049-55, 2013.

- Howlin J, **Cirenajwis H**, Lettiero B, Staaf J, Lauss M, Saal L, Borg Å, Gruvberger-Saal S, Jönsson G. Loss of CITED1, an MITF regulator, drives a phenotype switch in vitro and can predict clinical outcome in primary melanoma tumours. *PeerJ*, 3:e788, 2015.

- Lauss M, Haq R, **Cirenajwis H**, Phung B, Harbst K, Rosengren F, Holm K, Aine M, Jirström K, Borg Å, Busch C, Geisler J, Lönning PE, Ringnér M, Howlin J, Fisher DE, Jönsson G. Genome-wide DNA methylation analysis in melanoma reveals the importance of CpG methylation in MITF regulation. *J Invest Dermatol*, 135(7):1820-8, 2015.

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Abbreviations

AJCC	American Joint Committee on Cancer
ALM	Acral Lentiginous Melanoma
BC	Before Christ
BRAFi	BRAF Inhibitor
CNA	Copy Number Aberration
CMM	Cutaneous Malignant Melanoma
CSD	Chronically Sun-Damaged
dNTP	Deoxyribonucleotide Triphosphates
DSS	Disease-Specific Survival
DTIC	Dacarbazine
FDA	Food and Drug Administration
GEX	Gene Expression
HE	Hematoxylin/Eosin
IHC	Immunohistochemistry
ITH	Intratumor Heterogeneity
LDH	Lactate Dehydrogenase
LM	Lentigo Maligna
LMM	Lentigo Maligna Melanoma
LoF	Loss of Function
MAPK	Mitogen-Activated Protein Kinase
MAPKi	MAPK Inhibitor
MEKi	MEK Inhibitor
MIS	Melanoma In Situ

NGS	Next Generation Sequencing
ORR	Objective Response Rate
OS	Overall Survival
PCA	Principal Component Analysis
RFS	Relapse-Free Survival
RGP	Radial Growth Phase
ROS	Reactive Oxygen Species
SLN	Sentinel Lymph Node
SNV	Single Nucleotide Variant
SSM	Superficial Spreading Melanoma
TCGA	The Cancer Genome Atlas Network
TNM	Tumor/Node/Metastasis
U.S.	United States
UVR	Ultraviolet Radiation
VAF	Variant Allele Frequency
VGP	Vertical Growth Phase
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WT	Wild Type

Abstract

Cutaneous malignant melanoma (CMM) is the most lethal form of skin cancer and its incidence has increased faster than that of any other cancer, rendering it a major public health problem worldwide. High-throughput screenings have opened the door to a new scientific world, which enables molecular-based characterization of large cancer cohort collections. The aim of the research presented in this thesis was to explore the molecular landscapes of melanoma tumors on a genomic and transcriptomic level and subsequently correlate certain molecular features with patient survival, treatment response and tumor evolutionary patterns.

In Paper I, it was concluded that metastatic melanoma could be divided into transcriptomic subtypes (gene expression (GEX) phenotypes) possessing diverse biological and clinical features. Patients harboring melanomas infiltrated by immune cells, i.e. the high-immune subtype, showed a superior survival, whereas highly proliferative melanomas, i.e. the proliferative subtype, was correlated to a poor survival outcome and resistance to targeted therapies. Moreover, it was also shown that, irrespectively of the GEX phenotypes, melanomas could be divided into genomic subtypes based on genetic aberrations in the mitogen-activated protein kinase (MAPK) signaling pathway. In Paper II, it was found that mutations in the tumor suppressor gene neurofibromin 1 (*NF1*), was linked to inferior survival.

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In conclusion, melanoma is a complex molecular disease that can be characterized by genomic and transcriptomic signatures with clinical implications. However, a single biopsy might not reflect the true tumor complexity, and subclonality may be one reason behind resistance development.

Aims of the Thesis

The general aims of this thesis were to analyze the molecular landscape and divide the melanoma disease into molecular entities by means of genomic and transcriptomic approaches to ultimately enhance our understanding of melanoma biology and its clinical significance. The specific aims of the papers were:

- To investigate biological characteristics and clinical features of GEX phenotypes in a metastatic melanoma cohort (Paper I)
- To study TCGA genomic subtypes and their clinical implications by integrating our data with a large collection of mutation data from three external studies (Paper II)
- To explore the spatial ITH and clonal evolution in progressing melanoma (Paper III and IV)

Background

The History of Cancer

Cancer is not a newly developed disease in the mankind. In fact, both human beings and animals have had cancer since the dawn of history. The oldest existing manuscript of cancer (the word "cancer" was not yet used) is the Edwin Smith Papyrus, which is part of the ancient Egyptian textbook on trauma surgery, discovered in Egypt, and dates back to about 3000 before Christ (BC). It describes eight cases of breast neoplasms that were removed by cauterization using a tool called fire drill. In addition, the writing points out that "There is no treatment" for this disease. Even earlier evidence of cancer has been found among fossilized bone tumors in human mummies in ancient Egypt.

The Greek physician Hippocrates (460-370 BC), the "Father of Medicine", is credited for the origin of the word cancer, by using the terms *carcinos* and *carcinoma* (in Greek, referring to crab) to describe different forms of tumors (non-ulcer or ulcer forms, respectively). When the Roman physician Celsus (50-28 BC) translated the Greek term into the Latin word for crab, the word *cancer* was established. In addition, the word *oncos* (Greek for swelling) was introduced by Galen (130-200 after Christ), another Greek physician.

The earliest theory about the cause of cancer came from the ancient Egyptians blaming cancers on the gods. Another theory was proposed by Hippocrates. the "Humoral theory", involving four *humors* (body fluids): blood, phlegm, yellow, and black bile. Cancer was thought to arise as a consequence of the humor imbalance and an excess of black bile in the body.

Introduction of autopsies in 1628 improved the understanding of the blood circulation system. Giovanni Morgagni of Padua was the first one using autopsies to correlate patients' illness to pathological findings after death. Still today, this way of examination has a great implication in science. Furthermore, the famous Scottish surgeon John Hunter (1728-1793) suggested that tumors that had not invaded nearby tissue could be surgically removed. A century later, the anesthesia was developed, which further allowed classic cancer operations to develop. The development of the modern microscope in the 19th century, allowed Rudolf

Virchow to correlate microscopic pathology to illness, which could further aid the development of science. [1]

Clinical Features of the Melanoma Disease

Clinical Subtypes of Melanoma

All melanocytic neoplasms (benign nevi, dysplastic nevi and malignant melanomas) originate from melanocytes, which are neural crest-derived cells located mainly in the skin and eye, but also to a lesser degree, in several other tissues throughout the body [1]. Melanocytes in different sites can give rise to phenotypically diverse types of melanomas [2]. In Caucasians, the most common form of melanoma is found on sun-exposed skin. Thus, the original neoplastic lesion might arise in chronically sun-damaged (CSD) skin such as head, neck and dorsal surfaces of distal extremities of elderly people, or from intermittently sun-exposed areas such as trunk and proximal extremities of younger patients (non-CSD). However, melanoma can also arise on sun-protected sites, such as mucosal areas, palms and soles, the latter being additionally shielded by a thicker cornified layer or nails. Thus, several factors, such as site of origin, patient age, level of sun exposure and also genetic alterations distinguish these melanomas from each other. Consequently, melanoma is a heterogeneous disease at many different levels. At the clinical level there are four main classical histopathological subtypes of primary lesions that are distinguished according to their appearance and behavior (Fig. 1).

Three of those lesions are considered to start off as flat patches, i.e. having a radial (superficial) growth phase (RGP):

> Superficial spreading melanoma (SSM) is the most common type of melanoma in Caucasians accounting for about 70% of all melanomas and is most often seen in young people. This type of melanoma can be found anywhere on the skin, but is most likely found on the trunk in men, the legs in women, and the upper back in both. Thus, SSM is a non-CSD melanoma.
>
> Lentigo maligna melanoma (LMM) is most often found in elderly, arising on chronically sun-exposed damaged skin in the head and neck region, arms and upper trunk. Lentigo maligna (LM) is a precursor to LMM, which grows slowly in diameter over 5 to 20 years or longer before it actually forms the LMM. LM is

[1] http://www.cancer.org/cancer/cancerbasics/thehistoryofcancer/index (161101)

more often found in males than females and is not considered to be dangerous unless it develops into an invasive melanoma. LMM is a typical CSD melanoma.

Acral lentiginous melanoma (ALM) originates from glabrous (non-hair-bearing) skin (soles and palms) and nails. It constitutes less than 1% of melanomas in fair skinned people, but it is the most common subtype in people with darker skin accounting for 30-70%. This type of melanoma is not sun-induced and is equally common in males and females.

However, even if the above superficial forms of melanoma generally grow slowly, they may at any time progress from a RGP to a vertical growth phase (VGP) melanoma:

Nodular melanoma (NM) is the most aggressive form of melanoma corresponding to 10-15% of all melanomas. It is mainly found in elderly people and when diagnosed it is recognized as a nodule because of its VGP pattern. Nodular melanoma may arise on any site, but it is commonly found on sun-exposed areas of the head and neck. Thus, this type of melanoma can arise on CSD, non-CSD and actually on glabrous skin as well, and is more common in males than females.

In addition to the four main histopathological subtypes, there are other, more rare types of melanomas, such as: Non-UV-induced mucosal melanomas occurring on mucosal surfaces in the respiratory, gastrointestinal or genitourinary tract; Ocular melanoma, which can be divided into either uveal melanoma (involves the iris, ciliary body, or choroid) or conjunctival melanomas; Spitzoid melanoma, which is

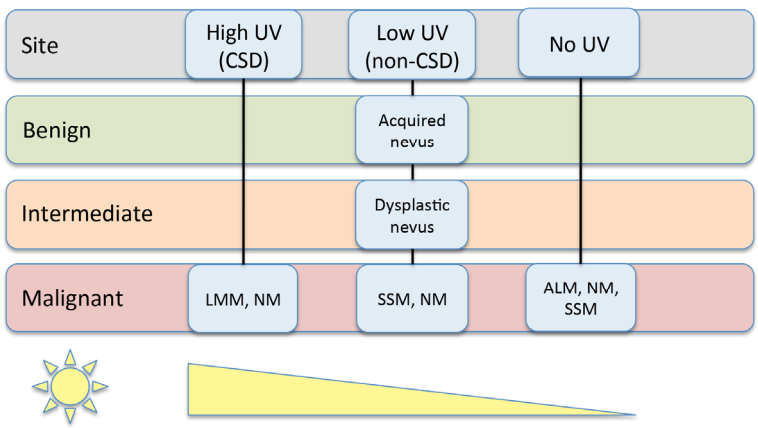

Figure 1. Classical histopathological subtypes and their reationship to UV radiation. Where applicable, benign or intermediate progression stages are noted. SSM, superficial spreading melanoma; LMM, lentigo maligna melanomas; ALM, acral lentiginous melanoma; NM, Nodular melanoma; UV, ultraviolet; CSD, chronically sun-damaged.

histologically similar to a benign skin lesion and usually arises on non-CSD skin; and the desmoplastic melanoma, which is recognized by fibrous tissue surrounding the malignant cells and most commonly arises on CSD skin.

Melanoma Etiology

From epidemiological studies, it has been widely accepted that the principal environmental cause of CMM is the sunlight, with the highest risk associated with intense intermittent ultraviolet radiation (UVR) exposure and severe sunburns in childhood [3, 4]. Moreover, the implication of UVR in melanoma risk has also been demonstrated by the use of artificial tanning devices, such as sunbeds and sunlamps [5]. One response to the UVR is the melanin pigment synthesis by the melanocytes, i.e. the specialized cells located in skin, eye, mucosal epithelia and meninges responsible for pigmentation and photoprotection [6]. Melanocytes can produce either the photoprotective eumelanin (brown/black pigment) or the pheomelanin (red pigment), allowing for a great variety in human skin pigmentation due to ratios of these two melanin types. Pigmentation synthesis is activated through the binding of the α-melanocyte stimulating hormone (α-MSH) secreted by keratinocytes as a response to UVR to melanocortin 1 receptor (MC1R) on melanocytes. MC1R is the main component determining the pigmentation outcome and if not possessing any loss of function (LoF) polymorphisms there will be an activation of cyclic adenosine monophosphate (cAMP) and cAMP response element-binding protein (CREB)-mediated transcriptional activation of microphthalmia-associated transcription factor (MITF), which induces transcription of pigment synthesis genes and eumelanin production. Thus, eumelanin acts as a protector to UVR by directly absorbing both UV photons and reactive oxygen species (ROS). However, certain sequence variants of *MC1R* do not persist the capability to activate the pigmentation pathway, which instead leads to the production of the non-UV protecting melanin, the pheomelanin. Risk of developing melanoma is linked to the most severe LoF *MC1R* alleles producing red hair/fair skin phenotype characterized by the inability to tan and with a tendency to burn due to impaired UVR protection [7, 8]. Even though pigmentation constitutes a UV-protecting mechanism, extensive sun exposure may abrogate this function. The high overall mutation load seen in melanoma is attributable to the mutagenic effect of UVR, inducing C>T transition [9, 10]. There are additional sources of C>T mutations, such as age and chemotherapeutics [11]; however, UVB radiation induces such base substitutions in particular at YpC sites, e.g. cytosines preceded by a pyrimidine, and such mutations dominate melanoma genomes. The mutational landscape of melanoma tumors also reflects UVB-independent processes, such as the UVA signature, the G>T transversion, resulting from absorption of UVA light by pheomelanin leading

to increased levels of ROS and ROS-induced DNA alterations [12]. Moreover, the pheomelanin synthesis can *per se* lead to melanoma carcinogenesis in the red hair/fair skin population through a UVR-independent mechanism involving increased levels of ROS [13]. This is further supported by the fact that the two most frequent genetic targets in melanoma, *BRAF* and *NRAS*, possess driver mutations not attributable to UVR implication. The typical *BRAF V600E* mutation is more frequently found in melanomas on intermittently sun-exposed areas [14]. In all, this clearly indicates that other mutagenic mechanisms are likely associated with the melanoma development and an increased understanding of UVR-independent mechanisms could potentially improve melanoma prevention.

Genetic mutations in the two genes cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase 4 (*CDK4*) confer high risk of melanoma [15]. The *CDKN2A* transcript encodes the tumor suppressor, p16 protein, which inhibits the activity of the cyclinD1 - CDK4/CDK6 complex. In addition, a part of the *CDKN2A* gene is encoding another transcript, the human $p14^{ARF}$ (ARF = alternative reading frame), which is also involved in the regulation of the cell cycle and apoptosis via the retinoblastoma protein (RB) and tumor protein p53 (TP53) pathways [16, 17]. *CDKN2A* mutations have not only been identified in multiple-case CMM families, but also in sporadic multiple primary melanoma [18, 19]. Interestingly, the penetrance of *CDKN2A* germline mutations varies with the geographic origin, with the highest risk found in regions with extensive sun exposure [20]. The *CDKN2A* and *CDK4* genes are involved in the same mechanism of the cell cycle. The *CDK4* gene is an oncogene mutated in the p16-binding domain, which leads to increased cell cycle activity due to loss of the regulatory effects from p16. However, germline mutations in *CDK4* are uncommon and have only been described in a few families worldwide [21].

Melanoma Epidemiology

From the early 20th century, when CMM was a relatively uncommon cancer, it has today become a disease with a significant burden to society and public health. CMM is one of the most rapidly growing cancers worldwide with a steadily increasing incidence rate since the mid 1960s. The variation seen in incidence is attributable to latitude and altitude worldwide, with generally higher rates seen in geographic areas closer to the equator and higher in altitude. The highest incidence rates reported worldwide are from Australia and New Zealand with up to 60 affected individuals per 100 000 inhabitants per year [22]. Also implication of sun exposure patterns of the population and their pigmentation type is of importance. Significant phenotypic differences have been observed for all melanoma subtypes with the non-Hispanic white or Hispanic group having the highest incidence rate, while Blacks have the lowest [23]. Even though the increase has leveled off in

both genders especially in younger individuals, incidence rates are still highest among older men and continue to rise sharply in several countries, including the United States (U.S.), Australia and Sweden (reviewed in [24]). According to the "Surveillance, Epidemiology and End Results" (SEER) data, melanoma is currently the sixth and seventh most common cancer among men and women in the U.S. (all races), respectively. On average, 28.5 new cases in men and 16.9 cases in women were diagnosed per 100 000 inhabitants per year based on 2009-2013 cases [25]. In Europe, observed incidence rates range from 6-19 cases per 100 000 inhabitants annually, with the highest incidence rate found in the Northern Europe [26], with Scandinavian countries showing 10-fold increase between 1953 and 1997 [27]. In Sweden, melanoma is the fifth and sixth most common cancer in women and men, respectively; nearly 40 newly diagnosed cases in men and 35 cases in women per 100 000 individuals annually, with an average annual increase of 5% the last five years [28].

Staging and Prognosis of CMM

As all other cancers, melanoma is clinically described by stages. Beyond these stages, a number of compiled prognostic factors with independent role in survival exist. The first staging systems for melanoma were published over three decades ago [29-31], but in order to provide clinicians and patients with accurate prognostic information, a unified staging system was fundamental. Since 1998, the American Joint Committee on Cancer (AJCC) melanoma staging system has served as a foundation for clinical classification, with the latest 7th edition comprising several biological factors with prognostic significance [32].

Localized Melanoma (Stage I and II)

The most important prognostic factor in melanoma is the time of detection of the neoplastic lesion, with early detection associated with improved prognosis. For patients with a localized disease, the prognosis is generally beneficial and the 5-year survival rate is 98.4% [25]. At this stage, there is no dissemination of tumor cells to surrounding tissues (e.g. lymph nodes, distant skin, visceral organs etc.) and the disease is exclusively described by the tumor characteristics (T). Three main independent factors associated with survival are tumor thickness, mitotic rate and the presence of ulceration [32]. Already in 1970, Alexander Breslow described the close association of primary tumor thickness (mm) with survival outcome [33]. Today, the Breslow thickness cut points used in the AJCC staging system to define the T category are 1.0 mm (T1), 2.0 mm (T2) and 4.0 mm (T3) with a significant decrease in survival as primary tumor thickness increases [32]. Sentinel lymph node (SLN) excision is recommended for patients possessing primary tumors of intermediate thickness. However, it was recently reported that tumor thickness can

significantly predict SLN metastasis in thick melanomas and the SLN status was prognostic for overall survival (OS) and disease-specific survival (DSS) in those patients [34]. There is also evidence that histological regression occurring in about 10-35% of primary CMM harbors a prognostic value and is associated with decreased SLN spread (reviewed in [35]). Prognostic implication of mitotic index in the primary tumor dates back to the 1990s [36]. Since then, several studies have confirmed the mitotic rate as an independent predictor of survival of melanoma [37-39], and more recently also as the second most important predictor after tumor thickness, especially among patients with T1 melanomas [32]. Also, increased mitotic rate in the primary tumor could be a predictor of SLN positivity [40]. Thus, melanoma patients with thin melanomas should be considered for SLN biopsy if the primary tumor mitotic rate is $\geq 1/mm^2$. Moreover, histopathological ulceration is defined as the lack of intact dermis overlying the primary tumor. Ulceration has been linked to a more aggressive disease and significant reduction of patient survival [41, 42]. To mark observation of ulceration, primary tumors are given the suffix "a" (non-ulcerating) or "b" (ulcerating) to the tumor nomenclature. Another prognostic factor that has served as a criterion in several melanoma staging systems is the Clark level of invasion [43]. However, in the latest updated AJCC melanoma staging system, the independence of this factor could no longer be confirmed in relation to mitotic rate and ulceration and it is only utilized for predicting prognosis under special circumstances (when mitotic rate cannot be determined) [32].

Stage III Melanoma

For patients with regional metastatic disease (regional lymph node, satellite, and/or in-transit metastasis), other independent factors are of consideration when predicting prognosis and assessing exact staging of this generally heterogeneous group. Herein we find parameters such as the number of affected lymph nodes, the regional node tumor burden (microscopic or macroscopic), and also the ulceration status of the primary tumor [44, 45]. In the AJCC staging system, the N-category is described by the number of nodes involved: 1 node (N1), 2-3 nodes (N2), 4 or more nodes (N3) and this factor is the most important predictor of survival [32, 46]. The second most important prognostic factor is the regional node tumor burden, which is subdividing the N classification further into "*a"* micrometastasis (based on histological analysis of excised lymph node, more commonly SLN biopsy) or *"b"* macrometastasis (clinically or radiographically apparent metastases). For patients with regional spread of the disease, the 5-year survival rate is 62% [25], with poorest survival in patients with >4 affected regional nodes of macrometastasis and concomitant ulceration of the primary tumor [47]. According to the latest version of the staging system, immunohistochemistry (IHC) for at least one melanoma-specific marker, such as human melanoma black 45 (HMB-45) and MLANA, is recommended for identification of nodal

metastases by immunohistochemical assessment [32]. Moreover, patients lacking a nodal metastasis but harboring intralymphatic lesions (in-transit disease or satellite lesions), the suffix *"c"* is used in the AJCC staging system to describe those cases. Actually, the survival rate is higher in patients lacking intralymphatic involvements than those having it [46, 48, 49].

Stage IV Melanoma

In stage IV melanoma, the disease has spread beyond the regional tissues and has reached distant sites and formed metastases (M). The prognosis for patients at this stage is very poor, with 5-year survival rate less than 10% [50]. The most important prognostic factor in stage IV melanoma is the site of distant metastasis, where patients with metastases to distant skin (or distant subcutaneous tissue, and/or lymph nodes) *"a"* have the best one-year survival rate (62%) as compared to patients with lung metastases *"b"* (53%) and non-lung visceral metastases *"c"* (33%) [32]. One additional prognostic factor is the serum marker lactate dehydrogenase (LDH), which assigns patients with an elevated level of LDH to M1c irrespectively of site of distant metastasis, and decreases the survival rates among patients [32].

Additional Prognostic Factors

In above sections, the TNM (tumor/node/metastasis)-based staging system provided by the AJCC foundation was summarized. However, additional prognostic factors exist. In most melanoma studies, increasing patient age has been linked to poor prognosis with a dichotomization of the subjected cohorts at 60-70 years [51, 52]. In melanoma, the median age at diagnosis is 63 years [25]. The prognostic effect of age could perhaps be linked to the primary tumor characteristics such as increased Breslow thickness and presence of ulceration at diagnosis [53]. It has also been discussed whether or not elderly melanoma patients receive the same medical care as the younger subset [54]. Moreover, the female gender is a highly significant predictor of improved prognosis [55-57]. The exact underlying protective effect of the female gender is not clear, but the younger age at disease onset and the difference in tumor location (more often on extremities) have been suggested [53, 55]. One could also speculate if the younger age at diagnosis in females is due to an increase in awareness of the melanoma risk linked to better self-examination than men. It has actually been shown that men living alone are diagnosed at a later stage and thus have a worse survival outcome [58].

Personalized Prognostication

Despite the well functioning AJCC staging system, it is quite constrained in its TNM-based design. Several important predictors, such as gender, age, primary

tumor site, the extent of microscopic tumors and number of distant metastases, which have been linked to prognosis in several studies are not included, even though the data are available in the AJCC database [32]. Today, certain melanoma prediction tools are available to individualize the risk estimate. In 2010, the first electronic predictive tool was published [59]. It was based on the large amount of available data in the AJCC database and it was possible to estimate the 1-, 2- and 5- year survival for individual patients with local melanoma, as well as for those with regional metastases.[2] Recently, Lyth and colleagues presented a Swedish prognostic instrument for primary CMM, which was based on the Swedish Melanoma Register data (>50,000 cases) covering 99% of all CMM diagnosed in Sweden during the last 25 years [60].

Melanoma Genetics

Clinical outcome of patients with similar or even identical clinical and histological features varies considerably [61], especially within the AJCC intermediate risk stages and in patients with advanced disease [62]. This highlights the need of a more personalized disease characterization. Molecular analysis may improve the understanding of the disease biology and provide biomarkers for more correct prognostic assessment.

GEX Phenotypes

DNA microarray technology has facilitated identification and characterization of GEX signatures, i.e. expression patterns of groups of genes, most often linked to specific tumor and patient characteristics. Despite much effort towards identifying independent prognostic molecular signatures in melanoma, this approach lags behind that of other cancers and the initial search for prognostic signatures in melanoma was not performed until 2006 [63]. The reason for this is most likely explained by absence of availability of frozen primary melanoma tissue specimens. However, GEX profiling of 31 melanoma samples was performed already in 2000 with the purpose of classifying the samples, but the identified molecular groups showed no significant association with clinical parameters [64]. Since then, several signatures have been proposed ranging from a single-gene signature of osteopontin in primary melanoma, to different multi-gene signatures in both primary and advanced melanomas [65-68]. In addition, GEX profiling has also been used for examining known points in the tumor progression model, from

[2] http://www.melanomaprognosis.org (161102)

nevus to primary melanoma to advanced disease [69, 70], and recently a 28-gene signature was developed in primary melanomas to predict the metastatic risk in patients of stage I and II [71]. By finding a predictive signature in primary melanoma for metastasis development it could be possible to predict the survival in those patients.

Jönsson and colleagues identified four GEX groups (phenotypes) significantly associated with survival in 57 stage IV metastatic melanomas after performing unsupervised hierarchical clustering of the data [72]. The phenotypes were mainly characterized by differential expression of normal-like genes (e.g. *TRIM29*, *KRT17*, and *KRT10)*, immune-response genes (e.g. *LCK*, *CXCL12*, and *HLA class I* and *II antigen)*, melanocyte-specific genes (e.g. *MITF*, *TYR*, *DCT*, and *MLANA)* and proliferation-related genes (e.g. *Ki67*). Signatures characterizing two of these GEX groups (Pigmentation and Proliferative) correspond closely to the in vitro derived invasive/proliferation signatures that subsequently reflects phenotype switching in melanoma [73]. The proliferative group was also characterized by a lower expression of immune response genes and exhibited the worst prognosis. In addition, Harbst and co-workers applied these signatures to primary melanomas, thus identifying metastatic signatures in primary melanoma associated with poor survival [74]. An increased expression of genes involved in proliferation (DNA replication and repair) has been linked to an inferior prognosis in other studies [68, 75, 76]. Moreover, a superior survival in the immune-response group was found [74], which was in line with previous findings by e.g. Bogunovic and colleagues demonstrating an independent role of the immune system in prognosis irrespectively of the disease stage and mitotic index [68]. Mann and coworkers also found a 46-GEX signature with strong overrepresentation of immune-response genes to be independently correlated to improved outcome [65]. Also, in a comprehensive multicenter study comparing independent reported prognostic gene lists across GEX microarray studies, the authors stated that immune-related molecules were suggested to be strong candidates for valuable biomarkers [77].

If molecular signatures are to be integrated into the present clinical staging system, they must not only support the available pathological classifiers but also complement them. However, for the reported gene signatures during the last years, not all possessed additional prognostic value (reviewed in [78]). It is therefore important to adjust for additional clinical factors when analyzing the prognostic value of molecular markers.

Genomic Subtypes

During recent years, next generation sequencing (NGS) has been introduced to the field of cancer medicine to identify patient- and tumor-specific genetic alterations aiding in prognosis, confirmation of diagnosis and guidance of therapeutic strategy. Vidwans and colleagues highlighted genetic subtypes in melanoma attributable to specific genetic alterations in key molecular pathways linked to certain treatment strategies [79]. In melanoma, the most commonly hijacked pathway is the MAPK pathway, which is often constitutively activated [80] (Fig. 2).

Already before the era of NGS, hotspot mutations in the V600 codon of *BRAF* (in 35-50% of melanomas) and Q61, G12 and G13 codons of *NRAS* (10-25% of melanomas) had been discovered [80, 81]. Despite the long history of the two melanoma key players, the prognostic significance of mutated *BRAF* and *NRAS* is still unclear [82-85]. One reason for the existing contradictions could be the application of MAPK inhibitors (MAPKi) in melanoma. However, in a retrospective study of advanced melanoma where patients had not received prior MAPKi, one could conclude that *BRAF* and *NRAS* mutation status did not impact on survival in metastatic melanoma [86].

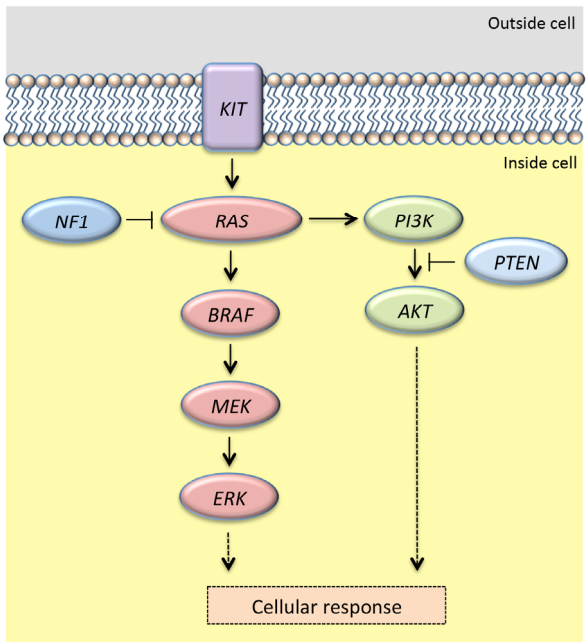

Figure 2. The MAPK signalling pathway and its keyplayers (pink circles). The PI3K-AKT pathway is also depicted (green circles), along with the two pathway inhibitors NF1 and PTEN *(blue circles)*.

Recently, the Cancer Genome Atlas Network (TCGA) established a framework for genomic classification of melanoma based on a large scale NGS analysis [87]. TCGA identified four subtypes based on presence of mutations in key players of the MAPK pathway, i.e. *BRAF*, *RAS*, *NF1* or none of these, the so-called triple-wild-type group. *NF1* is a tumor suppressor gene encoding a RAS-GTPase protein, which is responsible for maintaining the RAS protein in its inactive state by hydrolyzing RAS-GTP to RAS-GDP [88]. Following *BRAF* and *NRAS, NF1* is actually the third most frequently mutated gene in melanoma and is sometimes co-mutated with other RASopathy genes (e.g. *RASA1, RASA2, PTPN11* and *SOS1)* [89].

Several earlier studies have highlighted mutated *NF1* as an important melanoma-linked event. In 2012, it was shown that tumors being significantly enriched for *NF1* mutations or having alterations in *KIT* lacked recurrent mutations in either *BRAF* or *NRAS* [90]. Moreover, Krauthammer and colleagues discerned a class of sun-exposed melanomas with wild-type (WT) *BRAF* and *NRAS* with high mutational load, few copy number aberrations and inactivation of tumor suppressors, such as *NF1, TP53, ARID2* and *PTPRK* [91]. The TCGA genomic subtypes were attributable to specific molecular and clinical features. *NF1* subtype tumors typically occurred in older patients and had a higher mutational burden, the *BRAF* genomic subtype was associated with younger age at diagnosis, whereas the triple WT group was characterized by infrequent UV-signature (30% of samples vs. 90% in the other groups) and comprised a higher fraction of samples with copy number aberrations (CNA) of the 4q12 region containing e.g. *KIT* [87].

However, several sequencing studies have reported additional melanoma-related genes with implication in melanomagenesis and disease progression. In the first whole exome sequencing (WES) based report related to melanoma, recurrent mutations of *GRIN2A* (an N-methyl-D-aspartate receptor) were found in 25% of the twelve examined metastases [92]. Two key players of the MAPK signaling pathway, i.e. *MAP2K1 (MEK1)* and *MAP2K2 (MEK2),* have shown a mutation frequency of about 6% and 2% in melanoma, respectively, and an association with acquired drug resistance observed after BRAF inhibitor (BRAFi) treatment [93-95]. Moreover, the redundant PI3K-AKT signaling pathway can be activated through mutations in *NRAS* (20%) and by mutations in *PIK3CA* and *AKT* at low frequencies in melanoma [96, 97]. The most frequent alteration in this signaling pathway in melanoma is the loss of the AKT-regulator PTEN, occurring in up to 30-50% of melanomas [98, 99]. Interestingly, *BRAF*-mutant tumors are commonly linked to *PTEN* alterations, whereas *NRAS* mutations result in deregulation of both the MAPK and PI3K pathways [100]. The two suppressor genes *CDKN2A* and *TP53* have been found deleted or mutated in up to 50% and 20% of melanomas, respectively [101-103], whereas *CDK4* mutants only accounted for 3% [104]. The key regulator of melanocyte development, *MITF*, has been found amplified in 10-

20% of melanomas [105]. The same study also correlated alterations in *MITF* and mutated *BRAF* with a mutual exclusivity pattern of *NRAS* mutations. In the search for driver mutations, six novel melanomas genes were identified (*PPP6C, RAC1, SNX31, TACC1, STK19* and *ARID2)* with three of them (*RAC1*, *PPP6C* and *STK19*) possessing recurrent mutations mediated by plausible UVB damage [90]. Supporting these findings, another study identified the same recurrent mutation in *RAC1* and highlighted it as the third most common activating mutation in melanoma after those of *BRAF* and *NRAS* [91]. In addition, a subset of melanomas, namely the uveal melanomas, comprises frequent mutations in guanine nucleotide binding proteins, such as *GNAQ* and *GNA11* leading to activation of the MAPK pathway. This has been observed in a mutual exclusive pattern accounting for approximately 85% of uveal melanomas [106]. Thus, genetically altered signaling pathways integrate to form highly complex melanoma tumors.

Through global sequencing projects of thousands of cancers, catalogs of somatic mutations have been generated [107]. A small minority of all observed mutations are "drivers", which confer growth advantage to selected clones, whereas the remaining mutations are "passengers" not subjected to selection during tumor development. The mutations within these catalogs reflect all mutational processes operating throughout the evolution of the neoplasm all the way from the initial fertilized egg [108]. The mutational processes may be a consequence of defective DNA replication and repair machineries, or exposure to endogenous/exogenous mutagens. However, different mutational processes are usually related to certain combinations of mutation types, i.e. signatures. In the melanoma disease, characteristic UV-induced transitions (C:G>T:A and CC:GG>TT:AA) are well known [109]. In order to extract mutational signatures from catalogs of somatic mutations in breast cancer, an algorithm was recently developed and applied to the examined breast cancer cohort [110, 111]. Shortly thereafter, the mutational signatures were investigated in a large spectrum of human cancers, where a UV-signature was observed in malignant melanoma and squamous caracinoma of the head and neck [11].

The Genetic Evolution of Melanoma

Cancer is a disease that arises through accumulation of genetic alterations that promote cell proliferation. However, the exact order of mutations has not been thoroughly investigated until recently. Primary melanomas are thought to arise from distinct precursor lesions such as benign nevi, intermediate lesions (dysplastic nevi) and melanoma in situ (MIS). However, melanoma tumors may have distinct evolutionary trajectories and rarely pass through all of the different phases [112]. In the study by Shain et al, the authors reported that precursor

lesions were initiated by different activating mutations in the MAPK pathway, with benign lesions harboring *BRAF V600E* mutation as the only apparent pathogenic alteration, and intermediate lesions being enriched for *BRAF V600K* or *K601E* and *NRAS* mutations and carrying additional oncogenic alterations [113]. Both intermediate lesions and MIS were enriched for *TERT* promoter mutations, thus suggesting alterations in *BRAF, NRAS* and *TERT* to be early events in neoplastic progression. Biallelic inactivation of *CDKN2A* and genome wide CNA was more pronounced in invasive melanoma, whereas mutations in *PTEN* and *TP53* were associated with advanced primary melanoma [113].

The era of sequencing has not only led to the discovery of driver mutations in tumors, but has also revealed ITH, i.e. multiple clones with varying genetic mutations, as an essential aspect of the tumor landscape [114]. Depending on the level of tumorigenicity of mutations, subclones may eventually outcompete one another or develop in parallel in a "branched" evolution. Subclones can be spatially separated or intermingle in a tumor, however, the subclonal patterns vary throughout the disease course [115, 116]. Interestingly, Wang et al could reveal a level of intercellular genetic variation in breast cancer lesions where no two single cells were genetically identical [117]. In addition, intrapatient heterogeneity can be examined to predict evolutionary paths of disease progression. When analyzing the prevalence of *BRAF*/*NRAS* mutations in paired samples of primary melanomas and secondary metastases from the same patient, most patients showed consistent mutation patterns, whereas a small fraction of the tumor lesions had discrepancies in *BRAF/NRAS* mutation pattern: a mutated primary tumor and a subsequent wild-type metastasis, a wild-type primary tumor and a subsequent mutated metastasis, or perhaps the most intriguing observation where a *BRAF* mutated primary tumor gave rise to a *NRAS* mutated metastasis or the opposite [118]. The authors of the same paper discussed whether those observations were due to coexistence of molecular heterogeneous subclones in the primary melanomas.

In addition to heterogeneity on a genetic level (e.g. mutations, indels, CNA etc), ITH is frequently observed on a transcriptomic level (GEX). This was clearly observed in a large-scale study examining single-cell GEX profiles in glioblastoma, where diversity was found within cells from the same tumor [119]. Interestingly, important therapeutic targets, such as the receptor tyrosine kinases EGFR, FGFR1 and PDGFRA and their ligands, showed a mosaic pattern, thus implying a role in resistance development. Also in melanoma, single-cell GEX signatures have been identified along with subpopulations of cells possessing specific invasion and drug resistance markers, such as *POUF51* (*OCT4*) [120]. The phenotypic diversity may originate from epigenetic alterations, e.g. DNA methylation and/or plasticity (transient changes in GEX) due to drug treatment [121-123]. Thus, ITH can be divided into categories based on intrinsic (genetic and non-genetic factors), but also on extrinsic factors, such as tumor's blood

supply, which may play a role in the complex architecture of ITH [124] (Fig. 3). Thus, cancer is not a homogeneous disease and shall not be treated as such. Even though targeted therapies are now in use (see below section "Treatment Modalities in Melanoma") they are mainly targeting single or sometimes multiple aberrant molecular signatures, which are most of the time based on a single biopsy [125, 126]. The obstacle related to ITH is that only sensitive cells will be removed by therapy, whereas the remaining tumor population will possess some level of resistance, both intrinsic and adaptive [95]. One could argue that novel technologies examining circulating tumor cells and circulating tumor DNA perhaps could depict the full spectrum of ITH in the tumor more accurately.

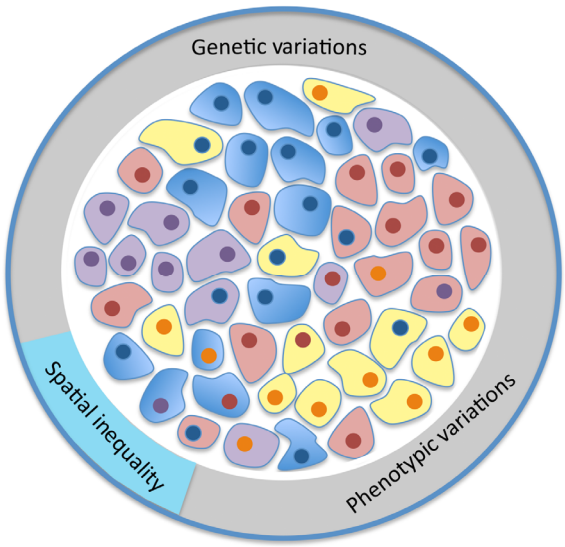

Figure 3. Factors behind intratumor heterogeneity. Both intrinsic (genetic and phenotypic variations) and extrinsic factors (spatial inequality) co-exist and interact with each other to generate intratumor heterogeneity. Intercellular genetic variation is shown as different colors of cell nucleus, whereas phenotypic variation (due to epigeneitc modification and plastic gene expression) is shown as different colors of cytoplasm.

Treatment Modalities in Melanoma

The principal treatment for melanoma is surgical resection. In patients with early-stage melanoma, wide local excision (a minor surgery) with specific safety margins (of 0.5 cm for *in situ* melanomas, 1 cm for tumors with thickness <1 mm, and 2 cm for thicker tumors) is standard care [61]. In addition, SLN biopsy is

performed when tumors are >1 mm in thickness or >0.75 mm with additional risk factors such as mitotic rate and/or ulceration [127]. If the SLN is positive for melanoma cells, the next step has been lymphadenectomy (LND). There are indications at the moment that the MSLT-2 trial will not show any survival benefit for the patients operated with LND compared to the controls having ultrasonography only for the lymph node basin (personal communication with Christian Ingvar, Dept. of Surgery, Lund University). Visceral tumors (small gut obstructions and single brain metastases) that cause symptoms are sometimes removed by surgery, an intervention that can provide long-term survival with good quality of life [128].

Radiotherapy is sometimes considered when the tumors are inoperable because of inadequate resection margins of primary tumors [129] or e.g. in patients with bone or brain metastases [130], or when surgery is not an option for local disease. It has been shown that postoperative radiotherapy after lymph node dissection can improve local tumor control but has no impact on relapse-free survival (RFS) and OS [130, 131].

Systemic chemotherapy is largely ineffective in melanoma. Until 2011, dacarbazine (DTIC) was the only standard agent used in patients with inoperable tumors, but the objective response rate (ORR) was low (<20%) with median response duration of 5-6 months and complete response rate of 5% [132]. The U.S. Food and Drug Administration (FDA) approved this drug already in 1976. More recently, some agents have shown systemic chemotherapeutic activity, such as temozolomide, platin derivates, taxanes and fotemustine [133-135].

Targeted Therapy

In contrast to standard chemotherapy regimens attacking all dividing cells, targeted therapies are directed against a specific molecular alteration. After several attempts to target the hyperactivated MAPK pathway in metastatic melanoma, the U.S. FDA approved vemurafenib (Zelboraf, Roche) in 2011 and dabrafenib in 2013 for the treatment of patients with unresectable or metastatic melanoma with *BRAF V600E* mutations. Initially, a phase 3 clinical trial enrolling 675 melanoma patients showed that vemurafenib treatment yielded improved OS and progression-free survival in melanoma patients with previously untreated *BRAF V600E* mutated melanomas [136]. Sosman and colleagues further supported these findings in a multicenter phase 2 study where patients had received at least one prior systemic therapy before treatment with vemurafenib [137]. Moreover, the approval of dabrafenib was based on a multicenter international randomized phase 3 clinical trial enrolling 250 patients with unresectable stage III or IV melanoma showing similar clinical benefits as vemurafenib when compared to DTIC [138].

In order to inhibit the MAPK pathway, several targets can be considered in the BRAF-MEK-ERK pathway. In addition to BRAF inhibition, the U.S. FDA approved the two MEK inhibitors (MEKi), trametinib in 2013 and cobimetinib in 2015, for treatment of patients with advanced melanomas possessing a *BRAF V600E/K* mutation. In a phase 3 trial (METRIC), trametinib showed potency as a monotherapy for *BRAF V600* mutated advanced melanomas with clear survival benefits as compared to the chemotherapy arm [139]. Instead, cobimetinib was approved in combination with vemurafenib from a recent phase 3 clinical trial [140]. Today, the combination of BRAFi/MEKi is used in *BRAF* mutated melanoma. Long and coworkers demonstrated a beneficial clinical response in patients from a phase 3 clinical trial when obtaining dabrafenib and trametinib in combination as compared to dabrafenib alone [141]. However, the downside of targeted therapy is the resistance development and secondary tumor formation. Resistance mechanisms include reactivation of the MAPK pathway through homodimerization and heterodimerization of wild-type *RAF* isoforms, *BRAF*-mutant amplification or mutations in downstream members of the MAPK pathway [142-144]. Moreover, another resistance mechanism is the activation of the semi-parallel signaling pathway PI3K-AKT-mTOR [145] by activating mutations in *PI3KCA* or loss of *PTEN* [146, 147]. This further emphasizes the importance of targeting this pathway in order to delay/avoid resistance development after MAPKi treatment.

For *BRAF/NRAS* WT melanoma tumors harboring *KIT* mutations in exons 11 and 13, imatinib (Gleevec) can be an alternative treatment [148, 149]. Originally, this drug showed a revolutionary treatment response in adult chronic myelogenous leukemia (CML) already before its approval by the FDA in 2001 [150, 151], and has since then also been approved for treatment of several *KIT*+ unresectable and/or metastatic cancers, such as gastrointestinal stromal tumor (GIST) in 2002 [152]. Moreover, in melanoma patients whose disease progressed after imatinib therapy, disease control was achieved by treatment with the alternative *KIT* inhibitor nilotinib [153].

Immune Therapy

Among the novel approaches to cancer treatment, immunotherapy is achieving much focus. More than 50 years ago, the first pieces of evidence on the role of the immune system in melanoma were reported from studies observing spontaneous regression [154, 155]. Further, T-cell infiltration was found to correlate with clinical benefit [156]. The first immunotherapy drug that was approved by the FDA in 1998 was the T-cell activating cytokine, i.e. the high-dose interleukin-2 (IL-2) [157]. The IL-2 therapy has an ORR of 15-20% in advanced melanoma and a small subset of complete remission occurring in 4-6% of patients [158]. Another

cytokine regimen is the interferon-α (INF-α), given as adjuvant therapy to high-risk melanomas patients, which has demonstrated a similar ORR as the IL-2 therapy and beneficial RFS and OS [159, 160]. However, the use of these two regimens is limited by their high toxicity and low response rates.

One immunotherapy approach is immunization (vaccination) against certain antigenic tumor epitopes, in order to boost the host immune response towards a tumor specific antigen and to eliminate the associated cancer cells. In a randomized phase 2 trial, the clinical activity of melanoma-associated antigen 3 (MAGE-A3) combined with different immunostimulants were investigated in 75 melanoma patients with unresectable MAGE-A3 positive stage III or IV M1a metastatic melanomas [161]. The reason for evaluating different immunostimulants was to improve the otherwise low response rate observed in patients on MAGE-A3 therapy. In melanoma, vaccination with tumor associated antigens induces the immune system but meaningful clinical responses are rare. Another treatment that has shown promising results is adoptive cell transfer, where autologous T cells are harvested from patients' own tumors, expanded and activated in culture (ex vivo), and thereafter re-infused in patients [162].

Although much effort has been devoted to identification of immunotherapy regimens with clinical benefit, the most successful responses have been observed in the recent era of immune checkpoint inhibition. In order to activate a T cell, an interaction between the T cell receptor and an antigenic peptide/MHC complex on the surface of an antigen presenting cell, along with a costimulatory interaction is required [163]. The costimulatory step can have two outcomes, a stimulatory (activating) or inhibitory effect (also called "immune checkpoint"), depending on the specific receptor–ligand pair that interacts [164]. In melanoma, the biggest clinical success has been observed by blocking (targeting) the negative regulators, cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1) expressed on T cells, as well as the PD-1 ligand (PD-L1), which would otherwise diminish the T-cell activation and induce immune tolerance. Ipilimumab, a humanized CTLA-4 blocking monoclonal antibody, was approved by FDA in 2011 after reports of improved OS in advanced melanoma with durable responses [165]. In 2014, two PD-1 inhibitors (monoclonal antibodies), pembrolizumab and nivolumab, were approved by FDA for treatment of patients with unresectable or metastatic melanoma and patients progressing following ipilimumab treatment. Treatment targeting PD-1 or PD-L1 have shown higher response rates and fewer immune-related toxicities as compared to ipilimumab [166].

Melanoma could perhaps turn curable in the future. The biggest challenge now is to increase the fraction of patients that could benefit from checkpoint blockade and therefore investigations of combinatorial therapies are ongoing. Further impressive

results have been demonstrated when combining anti-CTLA-4 and anti-PD-1 treatment [167, 168]. Also, combining immunotherapy with molecular targeted therapy or other therapies could perhaps serve as ultimate treatment regimens.

Tumor Material

For an overview of the tumor material used in Paper I-IV, see Figure 4. The sample cohort used in Paper I-III in this thesis represents a clinic-based retrospective collection of 269 melanoma tumors obtained from 227 patients. Note, some of the patients had contributed with serial tumors to the biobank along the disease course. These patients were considered in Paper III. The majority of tumor specimens were surgically removed at the Department of Surgery in Lund, Skåne University Hospital, during the period of 2000-2012. After surgery, all biopsies were snap frozen in liquid nitrogen and further stored at ultra-low temperature for later experimental use. The major fraction of the tumor collection comprised metastases from patients at different clinical stages of the melanoma disease, whereas a minor fraction comprised biopsies from larger primary melanomas. In addition, blood samples taken before/at surgery time point were available for most of the patients.

In Paper IV, tumors were surgically removed and selected for inclusion in this study based on tumor size. For each tumor (except one), multiple biopsies (n=3-7) were taken and stored as described above. In total, this study comprised six metastases and two primary tumors. Blood samples taken before/at surgery time point were available for seven of the patients, whereas normal skin was used as matched normal control for one patient.

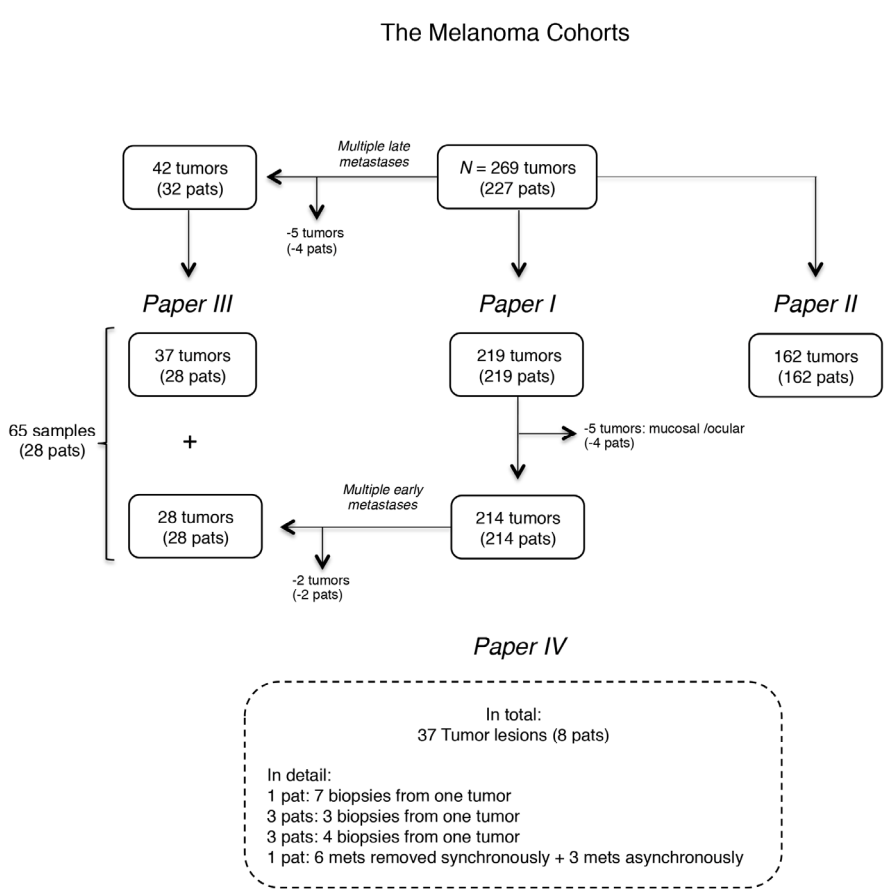

Figure 4. Flow chart of the melanoma samples included in the four different studies.

Overview of the Main Methods

Microarray - Global GEX

Before the era of global analyzes, scientists were limited to analyze one or a few genes simultaneously. Today it is possible to analyze thousands of genes in a single assay using the microarray technology. This technology was developed during the time when genome information became available from the human sequencing project and elicited much excitement [169, 170]. The basic principle of global GEX microarray is to harvest and fluorescently label the free nucleic acid sample (RNA) from the source of interest, hybridize it to DNA probes (either cloned molecules or oligonucleotides) immobilized on a solid substrate in a matrix pattern, and finally analyze the abundance of a particular sequence (by measuring the fluorescence in a laser scanner) in the target population. Sample RNA is converted to cDNA by using reverse transcriptase and subsequently amplified using PCR [171]. In order to avoid nonlinear amplification between the initial mRNA population and the amplification product (single-stranded cDNA), it is possible to use another approach where cDNA (not amplified) is rendered double-stranded and transcribed into antisense RNA copies (aRNA, also refered to as cRNA since it is complementary with cDNA). Target cDNA or cRNA can be analyzed by either single or dual channel microarray. When two samples are to be compared (e.g. test and reference samples), dual-channel microarrays are co-hybridized with the differently labeled targets (the fluorescent dyes Cy3 and Cy5 are commonly used) in order to obtain a ratio for each probe. However, in a single-channel microarray, only one sample (test sample) is considered and the relative abundance between samples is obtained in subsequent data analysis.

Global GEX analysis was performed in Papers I, III and IV using Illumina Human-HT12v4.0 BeadChip arrays (Illumina) for single-channel detection. BeadChip arrays provide multi-sample format (12 samples/chip) comprising 47231 probes (oligonucleotides) immobilized to beads expressing a specific address tag (29-mer) that enables localization of a bead on the array. The probes cover content from NCBI RefSeq Release 38, and each probe-bead pair is replicated about 30 times on each array. Before scanning of the BeadChips, total RNA from the subjected specimens is converted to cDNA, thereafter biotin-

labeled cRNA (antisense) and finally hybridized to the 50-mer probes on the beads.

NGS

DNA sequencing dates back to the 1970s when the two-dimensional chromatography was used. However, the era of sequencing started when the Sanger chain termination method was developed in 1977 [172] and the sequencing method was automated in 1987, the so called "first-generation" [173]. In 2005, "next-generation" sequencing techniques emerged, allowing massive parallel sequencing of short reads.

There is a variety of sequencing methods to choose among, depending on the experimental question to be answered and the budget. Whole genome sequencing (WGS) is a powerful tool for genomic research. However, WGS is not realistic at the deep coverage needed for clinical cancer diagnostics. Many laboratories have therefore found it advantageous to carry out targeted sequencing of particular regions of interest at high depth of coverage in order to maximize the test's sensitivity and specificity (reviewed in [174]). Examples include WES, where only the protein-coding fraction of the genome is considered, or subsets of cancer-related genes based on different enrichment strategies.

The Illumina NGS workflow includes four basic steps, which are shown in Figure 5. The first step is the library preparation, where DNA or cDNA is randomly fragmented and each sample is given a specific address tag, barcode, in the form of an adapter sequence to both fragment ends. The reason for the use of adapters is to enable parallel sequencing of multiple samples in one flow cell. If targeted sequencing is performed, a capturing system is used for targeting specific regions (Fig. 5a). Subsequently, the targeted fragments are PCR amplified and size separated. Next, the generated library is loaded onto a flow cell comprising surface-bound oligonucleotides complementary to the library adapters, where fragments are captured and clonally amplified (bridge amplification) to create clusters (Fig. 5b), followed by the actual sequencing (Fig. 5c). The principle concept behind the Illumina NGS technology is that a DNA polymerase sequentially adds fluorescently labeled deoxyribonucleotide triphosphates (dNTPs), base by base, into a DNA template, while being identified by the fluorescent excitations. This method is called sequencing by synthesis (SBS) where the nucleotide label serves as a "reversible terminator", which must be enzymatically cleaved off before the next cycle of dNTP incorporation. This is repeated "n" times to create a read length of "n" bases. Finally, the sequenced reads are aligned to a reference genome in order to identify genetic changes.

NGS has been used in all four papers included in this thesis. All studies used the SureSelect Target Enrichment System (Agilent technologies) for targeting specific genes/regions of interest in the samples, which were subsequently subjected to Illumina's Paired-End sequencing system (Illumina). In order to distinguish germline mutations from somatic mutations, each tumor sample was compared against a normal reference sample (normal DNA extracted from tissue or blood). In Papers I-III, a custom enrichment design based on information in the COSMIC database and the literature was used to target 1697 frequently mutated cancer-associated genes before sequencing on a HiSeq2000.

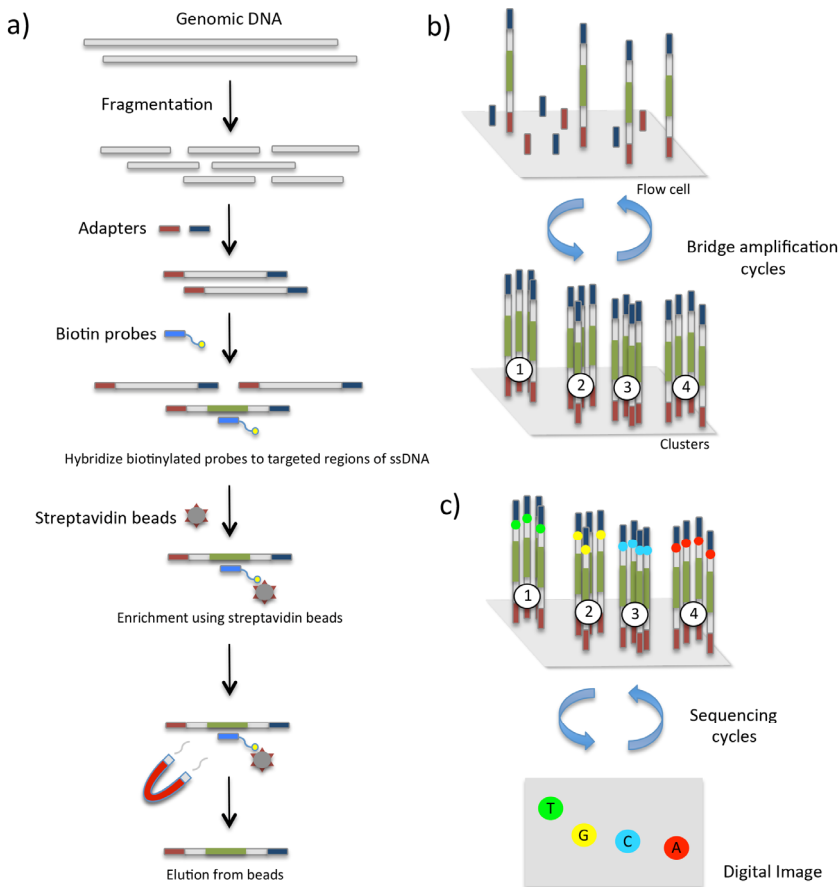

Figure 5. Illumina NGS workflow. a) Genomic DNA is fragmented and the fragments are ligated to adapters on both ends. For targeted sequencing, biotin-labelled probes (complementary to target regions) are hybridized to ssDNA and linked to magnetic beads coated with streptavidin molecules. Targeted regions are captured by using magnetism and finally eluted from the beads. b) The final library (pool of multiple samples) is loaded onto a flow cell and hybridized to its surface. Several cycles of PCR allow clonal amplification of the clusters. The sequencing is performed cycle-wise with the incorporation of one fluorescently-labeled nucelotide at the time. The emission from each cluster is recorded and the cycle is repeated multiple times.

A few samples also underwent WGS in Paper III. In contrast, in Paper IV, all samples underwent WES after capturing DNA complementary to the Agilent Clinical Research Exome oligo panel.

Moreover, today it is also possible to perform RNA sequencing using deep-sequencing technologies in order to determine differential expression, allele-specific expression, as well as detection of splice junctions, isoforms, novel transcripts and gene fusions. In general, a population of RNA transcripts (e.g. poly-A containing mRNA) is converted to a library of cDNA fragments and subsequently sequencing adaptors are added to one or both ends of the cDNA fragments. From each molecule, a short sequence is obtained from one end (single-end sequencing) or both ends (paired-end sequencing) with the typical read length of 30-400 base pairs depending on the selected technology. After sequencing, alignment to the reference genome/transcriptome generates three types of reads: exonic reads, junction reads and poly(A) end-reads, which are used to generate a base-resolution expression profile for each gene. RNAseq was performed in Paper IV as described by Saal and colleagues [175] but instead using TruSeq Stranded mRNA Library Prepration Kit (Illumina) and sequencing on a NextSeq500 (Illumina).

Computational Analysis

When dealing with different types of high throughput techniques, such as global GEX and NGS, much time is spent on subsequent data handling (processing) and final analysis of the massive data output.

Transcriptomics

GEX data in Papers I and III were preprocessed by using the GenomeStudio software to remove outlier beads, calculate average bead signals and detection p-values. Samples were adjusted (normalized) to a common baseline using the algorithm for cubic spline quantile-normalization. Further processing was done using the R statistical software. In Papers I and III, the data were log2 transformed and probes were selected based on their detection p-value. Probes with $p<0.01$ were kept if present in minimum 80% of the samples and thereafter mean centered across the data in order to compare GEX levels across samples. Global GEX was also performed in Paper IV, however, due to the low number of samples, this dataset was integrated with the non-processed quantile-normalized GEX dataset in Paper I. After log-transformation and selection of the most varying probe for each

gene according to the reference set, the median expression level in the reference set was subtracted from the multiregion set.

GEX profiled samples were also classified according to the molecular phenotypes recently presented by our group [72] (Paper I, III-IV). For that purpose we used the centroids from Harbst et al. Samples were assigned to subtypes based on the highest correlation to the four centroids (Pearson's correlation > 0.2, otherwise set as "unclassified") [74]. A centroid may be explained as a list of genes represented by the mean expression of a number of samples. To further describe additional biological patterns in the Paper I data, network clusters (modules) were created based on co-expressing genes (nodes) connected by edges (representing correlations), using a correlation cutoff of 0.6 with a final inclusion of genes with minimum 5 correlating neighbors, as described previously [176]. Five modules with distinct features were identified, further entitled as the "MITF", "cell cycle", "stroma", "immune response" and "interferon module".

The clinical significance of the GEX phenotypes was evaluated in Paper I using: the TCGA RNAseqv2 level 3 data (release 3.1.14.0, 2015-01-28), comprising 20,501 genes from 472 primary and metastatic samples, accessed from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/); and three independent datasets obtained from the Gene Expression Omnibus repository (GSE50509 [177]; GSE61992 [141]; GSE35640 [178]). For details on cohort description and pre-processing of data from the three latter studies, see Supplementary Data in Paper I. Briefly, prior to mean centering and GEX phenotype classification, individual datasets were combined with our large dataset and adjusted in a pairwise manner using the "DWD" method in the R "*InSilicoMerging"* package. This transformation was performed to avoid a skewed mean-centering and biased classification due to the low number of samples in the external studies.

Lastly, principle component analysis (PCA) was used to confirm that the observed variation in the GEX data were mainly due to biological factors and not systematic experimental artifacts (e.g. technical batch effects). In PCA, vectors describing the variation in the data (principal components, PCs) were tested for association with the biological and technical variables to elucidate their impact on the variation in the data [179]. We performed PCA in Paper I by using the swamp package in R [180].

In paper IV, RNA sequencing data were demultiplexed using an in-house software. Reads aligning to ribosomal sequences were discarded [181], and the remaining reads were aligned to the human genome reference GRCh37/hg19 using TopHat2 [182]. Cufflinks v2.1.1 was used to determine the RNA expression levels and fragments per kilobase of exon per million mapped reads [183]. The Bioconductor *Rsamtools* v.1.12.4 was used to check for duplicates, extracting read counts at single nucleotide variants (SNVs) base positions (mpileup) and for

routine quality assessment.[3] Based on the findings that mutant RNA variant allele frequency (VAF) correlated well with the DNA-VAF obtained from DNA sequencing, a linear regression model was used to calculate the expected RNA-VAF for a given DNA-VAF. Consequently, allele-specific expression was defined as when the RNA-VAF deviated from the expected value of the genomic background. Correction for multiple testing was performed using Benjamini-Hochberg test.

DNA Sequencing

DNA sequencing data were cleaned and demultiplexed using Illumina supplied software. Reads were aligned to the human reference genome hg19 using Novoalign (Novocraft Technologies) (targeted sequencing and WES, Paper I-IV) or the Burrows-wheeler algorithm (WGS, Paper III) [184]. Local realignment was performed to minimize false-positive calls due to misalignment and recalibration of base quality scores to account for machine cycle and dinucleotide context, using the GATK [185]. The Picard tool was used to mark duplicate fragments (http://picard.sourceforge.net). Variant calling of SNVs/indels for targeted sequencing and WES data were performed using VarScan2 (Papers I-III) [186] or MuTect (Paper IV) [187], respectively, and variants were annotated using Annovar [188]. Copy number estimates were obtained from tumor-normal pairs using Contra (targeted sequencing and WES, Papers I-IV) [189] or Control-FREEC (WGS, Paper III) [190] and segmented using GLAD (targeted sequencing and WES in Papers III-IV) [191]. BreakDancer was used to assess structural rearrangements from WGS data (Paper IV) [192].

In Paper II, somatic mutation data from the three external studies were downloaded from the supplementary section of each of the publications [89, 90], whereas mutation data provided by TCGA were collected from the data portal (frozen March 14, 2016) [87]. In order to annotate the identified somatic mutations for their effect on the protein level, Oncotator was used.[4] To circumvent the problem of a high mutational background rate in melanoma, we used the MutSigCv algorithm to screen for significantly mutated genes across the cohort in Paper II [9]. The mutational landscape was visualized using *"Oncoprinter"* [193, 194] in Paper I. Furthermore, mutational signatures in the compiled cohort (Paper II) were evaluated using the "*deconstructSigs"* R package [111]. To further assess the implication of the observed mutations on protein function, MutationAssessor was used (http://mutationassessor.org/r3/).

[3] http://www.bioconductor.org/packages/release/bioc/html/Rsamtools.html (161101)

[4] https://confluence.broadinstitute.org/display/CGATools/Oncotator (161101)

In Paper IV, the fraction of tumor cells containing a specific SNV was determined using the ABSOLUTE algorithm [195]. Moreover, phylogenetic trees were generated in Paper IV to visualize the evolutionary genetics of the different clones (based on SNVs) comprised in the tumors. This was done using the parsimony method provided by the *"phangorn"* R package with subsequent tree evaluation [196], rooting by germline outgroup using the "*ape"* R package [197] and revision of the edge lengths based on the observed mutation number in the data using the *"geiger"* R package [198].

Nucleic Acid Extraction

Frozen tissues were homogenized using a TissueLyser (Qiagen) and DNA and RNA extracts were isolated using the AllPrep kit (Qiagen). Assessment of concentration and sample purity was performed using the NanoDrop ND-1000 (NanoDrop Products). To confirm RNA quality, all extracts were analyzed on an Agilent Bioanalyzer 2100 (Agilent) and only samples with a RIN value larger than 6 was included in the global GEX. DNA from blood samples was extracted using the DNeasy Blood and Tissue kit (Qiagen).

IHC

The principle of IHC is based on an antigen-antibody binding reaction, involving a primary antigen-specific antibody, a second enzyme-linked antibody (usually peroxidase) and a substrate (e.g. diaminobenzidine) that is converted by an enzyme to create a colorimetric stain of the tissues. Nuclei are counterstained with hematoxylin and observed as blue. Often, to obtain a full overview of the structural patterns (i.e. nuclear, membrane and cytosol) in the tissues, sections from the same specimen are often stained with the combination of hematoxylin and Eosin (HE).

In Paper I, a subset of the examined melanoma tumors was subjected to IHC. Staining was performed using antibodies against MITF (clone: C5, Thermo Fisher), cluster of differentiation 3 (CD3; polyclonal, DAKO) and Ki67 (clone: MIB-1, DAKO). In Paper III, three different metastatic tumors from patient 1 were evaluated for the MITF expression (same antibody as above). Also, Paper I and Paper IV included HE staining of selected tumors.

Results and Discussion

Prognostic Implication of GEX Profiling in Melanoma

Paper I can be considered as a validation study of recently published data from our group [72, 74] with the specific aim to investigate the four molecular subtypes and their clinical and biological significance in a large cohort of melanoma samples. For that purpose, tumor material from 214 melanoma patients, most of the metastatic type, were collected and analyzed. All of the four molecular subtypes (high-immune, normal-like, pigmentation and proliferative) were identified in the cohort without any observed difference in distribution across clinical factors such as gender, age and tumor type (with the exception for the normal-like group comprising most primary tumors). In the metastatic compartment of the cohort, metastases of the proliferative type had passed a longer time since the diagnosis/removal of the associated primary tumor. However, this observation was not due to a later clinical phase of the disease in the patients currently possessing the proliferative metastases because most metastases analyzed in this study represented the first relapse. Since the proliferative group also correlated with a poor distant-metastasis-free survival and overall outcome, one could assume that a certain number of genetic aberrations were needed before the formation of this aggressive proliferative phenotype characterized by high expression of proliferative genes and decreased expression of melanocyte-specific genes such as *MITF*. In Paper III it was demonstrated that patients harboring a proliferative metastasis were unlikely to develop additional metastases of different molecular subtypes, i.e. it appears difficult for a tumor to switch back to any of the other GEX phenotypes after transforming into a proliferative state.

The survival rate for the pigmentation group was also poor, although not as poor as for the proliferative group (Paper I). The pigmentation group could be further subdivided into low and high cell cycle activity (based on network cluster analysis) with the latter group having the worst survival. This phenomenon was also observed on a protein level, where pigmentation-classified tumors expressed both Ki67 (proliferation marker) and MITF (melanocyte differentiating marker) to various degrees. In Paper III, focusing on patient 1 comprising three subsequent metastases (one pigmentation- and two proliferative-classified), metastasis 1 showed a high prevalence of MITF positive cells, whereas the second metastasis

comprised an intermediate level of MITF positive cells and finally the proliferative classified tumor with almost no such cells. Thus, this suggests a close relationship between the pigmentation and the proliferative phenotypes, with selection for non-pigmented tumor cells during progression of the melanoma disease.

In line with other studies demonstrating an association of the host immune system with beneficial survival and/or response to treatment [72], it was not surprising that the high-immune GEX group showed superior survival in Paper I. The infiltration of immune cells in these tumors was demonstrated on tumor sections stained for CD3 (T cell marker) using immunohistochemistry.

As already pointed out, the three main GEX phenotypes in metastatic melanoma could be well explained also on a protein level. However, in order to implement the GEX phenotypes into the clinic in the future, one could argue that perhaps a protein panel representing the GEX phenotypes would be easier to implement since most molecular prognostication today is based on protein levels using immunohistochemistry. However, the GEX phenotypes are described by a number of differentially expressed genes that also explains the biology, information that would otherwise get lost in a strict protein-based analysis.

Recurrent Mutations in Melanoma Molecular Subtypes and Activation of Signaling Pathways

From previous studies, little was reported on the genetic aberrations associated with the GEX phenotypes. To thoroughly explore the mutational landscape in the four different molecular subgroups, targeted deep sequencing of 1697 cancer-associated genes was performed in 146 patients (having a matched blood sample) out of the 214 melanoma patients included in Paper I. The most frequently mutated genes, *BRAF* and *NRAS*, were equally mutated across the GEX phenotypes suggesting that these alterations are likely early events in the melanomagenesis. This was further supported in Paper III, where *BRAF* and *NRAS* mutations were preserved across subsequent multiple metastases within single patients. Paper IV also highlighted the conservative means of these mutants after probing all intra-tumor regions investigated. In addition, mutated *TP53* and *PTEN* showed the same prevalence across the GEX phenotypes in Paper I, whereas *CDKN2A* alterations were more commonly found in the proliferative group with a high proportion of homozygous deletions, consistent with our previous study [72]. Not surprisingly, mutations in *CTNNB1* were more frequently found in tumors of the pigmentation type, supporting a role of Wnt/beta-catenin in activating MITF [199].

In Paper II, a comprehensive analysis of the mutational landscape was performed, with special focus to assess the clinical implication of the *BRAF*, *RAS*, *NF1* and the triple WT genomic groups in melanoma. Sequencing data from the Lund study (Paper I with the addition of 16 new samples) were integrated with three external studies (described in the "DNA sequencing" section in "An Overview of the Main Methods") comprising 870 unique melanoma samples in total. *NF1* mutated melanoma tumors appeared to be a molecular group with distinct biological and clinical features. This genomic subtype showed the highest mutational load across all genomic groups and it also possessed a strong correlation to the UV mutagenic process, whereas the *BRAF* genomic group had the weakest UV correlation. In line with previous reports, tumors arising on CSD skin usually comprise aberrations in *NF1,* whereas non-CSD usually harbor the *BRAF V600E* alteration [112]. After correcting for the high background mutational rate in the *NF1* genomic group, a number of putative driver genes (e.g. *PTPN11*, *RASA2, RASSF2)* were identified. The genes *PTPN11* and *RASA2* are known RASopathy genes possessing similar functions as *NF1* [89], whereas the *RAS* effector *RASSF2* might be a novel tumor-suppressor gene [200, 201]. In the *BRAF* genomic group, *PTEN* mutations were significantly enriched, whereas in the triple WT group, *KIT* and *GNA11* mutations were found enriched. The data in Paper II were also analyzed for activity of key molecular pathways, described by Vogelstein and colleagues [202]. The RAS pathway (*BRAF, CIC, HRAS, KRAS, NF1, NRAS, PTPN11*) was the most frequently mutated pathway observed across the compiled data (89%). Thus, melanoma is highly dependent on the MAPK signaling pathway and usually one "hit" in *BRAF, RAS* or *NF1* is enough to perturb the downstream signaling. A mutual exclusive pattern was observed between these genes in Papers I-IV.

The analysis in Paper II showed that *NF1* mutants had an increased risk of death from melanoma (5-year DSS; HR, 1.9; 95% CI, 1.21-3.10; $P = 0.046$) and poor OS (5-year OS; HR, 2.0; 95% CI, 1.28-2.98; $P = 0.01$). Moreover, patients with *NF1* mutated tumors were generally older, whereas *BRAF* mutants were associated with a younger age at diagnosis. This was also in line with the previous report from TCGA [87]. The compiled data comprised more males than females, however, the within-group male/female proportion was more pronounced in the *NF1* subtype. Previous studies have linked poor survival to older age and male gender in melanoma [203]. Therefore, we adjusted for these variables in a multivariable Cox regression model and found that *NF1* mutational status still added independent prognostic value.

Clonal Evolution in Melanoma

Today, it is well accepted that most tumors possess some levels of ITH, i.e. subclonality, influencing disease progression. The evolutionary aspects of melanoma were investigated by analyzing ITH in multiregional biopsies from metastatic lesion in eight patients (Paper IV). In addition, intrapatient heterogeneity can be examined to predict evolutionary paths of disease progression and this was further studied in Paper III using metachronous lesions from 28 patients. Both Paper III and Paper IV supported recent findings suggesting that MAPK signaling pathway aberrations are early events in the tumorigenesis (reviewed in [112]). In Paper IV, heterogeneous somatic mutations within tumors were found in the range of 3-38% across all analyzed tumors, thus implying different levels of subclonality in melanoma. Of all somatic mutations in the data, 129 were mapped to previous reported mutations in driver genes (Cosmic Cancer Gene Census list), where only 12% were affected by ITH. A high degree of mutational heterogeneity was associated with a more aggressive disease progression. When analyzing multiple lesions within a patient (Paper III), most tumors were genetically different with a common stem of genetic aberrations and the addition of some additional "private" aberrations observed in the later progressing tumors.

However, there was no consistence between ITH based on mutation spectrum and phenotypic diversity (GEX); In paper IV, regions with similar mutational profile belonged to different transcriptomic groups and regions with similar trancriptomic signatures harbored different mutational profiles, and in Paper III, half of the melanoma patients comprising multiple metastases were of different GEX phenotypes even though multiple metastases occasionally comprised exactly the same genetic information. Thus, in all four papers presented in this thesis, the GEX phenotypes did not show consistence across genomic aberrations. This further suggests environmental and/or epigenetic implications of the GEX subtypes. It was recently shown that *MITF* and its target genes are differently methylated across the GEX phenotypes, with hypometylation observed in the pigmentation subtypes and hypermetylation observed in the MITF-low proliferative subtype [204].

Different mutational processes seem to operate during the melanoma evolution. Most of the trunk mutations in Paper IV (80%) had the UVB signature, whereas branch mutations had a significant increase of the UVA signature (T>G). Thus, the UVB signature seemed to be enriched early in melanoma tumor evolution, whereas mutations occurring later in the progression may be caused by other mutational processes. Of relevance, a large proportion of the melanoma driver mutations are attributable to UV-induced mutations with 46% accounting for C>T

and 9% the G>T alteration [90]. These mutations are usually found in tumor suppressor genes like *CDKN2A*, *TP53* and *PTEN,* but sometimes also at hotspots in oncogenes such as *RAC1* [90, 91]. However, the two most frequently altered genetic targets in melanoma, *BRAF* and *NRAS*, possess driver mutations mostly not attributable to UVR. Also, the typical *BRAF V600E* mutation is more frequently found in intermittently as opposed to chronically sun-exposed areas [14]. This clearly indicates that other mutagenic mechanisms are also associated with melanoma development. In Paper III, a non-UV signature (A:T -> G:C) was observed in the latest progressing metastasis (M3) from patient 1. A possible explanation for this specific case could be the alkylating agent DTIC that the patient received prior to the last metastasis.

In summary, our integrated investigations of mutational spatial ITH and clonal evolution provide an important molecular foundation for improved understanding of tumorigenesis and progression in melanoma.

Treatment Predictive Potential of Molecular Subtypes and ITH in Melanoma

Despite initial response to treatment, a major obstacle in melanoma is the development of resistance to MAPKi. In order to circumvent this, novel predictive biomarkers are needed. To assess the predictive potential of the GEX phenotypes, GEX data from three external studies were included in Paper I [141, 177, 178]. Tumors classified as proliferative responded poorly to treatment with either BRAFi (vemurafenib or dabrafenib) or BRAFi/MEKi (dabrafenib and trametinib) with poor objective response and shorter progression-free survival. Moreover, when comparing the GEX phenotypes in pre-treatment tumors with that of post-relapse tumors, we found an enrichment of MITF-low proliferative-classified melanoma tumors in the resistant fraction after treatment. This corroborates recent findings relating the MITF-low state to both intrinsic and acquired resistance to MAPK pathway inhibition [205, 206]. In vivo models have shown that melanoma cells can undergo transcriptional signature switching between a proliferative and invasive state, a phenomenon that is likely regulated by local microenvironmental conditions [73]. However, it should be mentioned that the GEX phenotype referred to as “proliferative” by our group, also contains genes related to invasiveness, i.e. a more dedifferentiated tumor status and intrinsic resistance to therapy. Moreover, proliferative-classified tumors also showed a poor clinical benefit from MAGE-A3 vaccine therapy. Generally, tumors with high influence of the immune system, the “high-immune” GEX phenotype, showed a clinical beneficial response to molecular targeted therapies. Several studies have confirmed an important role of

the tumor microenvironment for an improved immunotherapy response [207], as well as the tumor mutational load correlating with more available neo-antigens capable of activating an immune response [208]. In Paper II, melanomas with somatic mutations in *NF1* were found to have a high mutational load, indicating potential clinical benefit from immunotherapy in these tumors.

Several studies have highlighted a strong link between genetic heterogeneity and therapy resistance as a consequence of ITH suggesting a positive selection of subclones with varying degree of resistance and survival advantages. In Paper IV, one patient with a large lymph node metastasis receiving BRAFi treatment, but relapsed 6 months after treatment initiation. Analysis of the treatment naïve tumor revealed the existence of heterogeneous mutations in the PI3K pathway, such as activating mutations in *PIK3CA* and stop mutation in *PIK3R1*, across the tumor lesions, which could be a potential reason for the observed relapse.

Conclusions and Future Perspectives

Melanoma is a highly complex disease that can be classified by at least two layers of molecular information, i.e. genomic and transcriptomic. However, there is little consistence between these two layers. The GEX phenotypes comprised prognostic information highlighting the important role of the immune system as a general suppressor of tumor progression, whereas a proliferative signature was associated with opposite features. Molecular features reflected by the GEX phenotypes may predict response to immune therapy and molecular targeted therapies. However, future studies comprising more patients are needed to fully explore the predictive potential of the GEX phenotypes.

Melanoma is a tumor disease with a high mutational burden with mainly UVR induced mutations. However, a range in mutational load was observed in the cohort and one subtype, the *NF1* genomic group, appeared to have the highest mutational load. This subgroup could also be linked to a poor patient survival outcome, which was not due to the presence of more males and older patients in this group. Since others have linked a high mutational burden with an activation of the immune system, future studies should focus on molecular analyzes of *NF1* mutants and their response to immunotherapy. In general, further studies are needed to determine the clinical benefit of GEX and mutational profiles in population-based metastatic melanoma cohorts including patients treated with immune checkpoint blockade agents and targeted therapies as first-line treatment.

Molecular analysis of ITH suggested a branched rather than linear tumor evolution and analysis of multiple metastatic lesions from individual patients confirmed that melanomas continue to evolve molecularly during disease progression. ITH may have prognostic potential in metastatic melanoma but extensive studies are needed to draw any further conclusions.

Populärvetenskaplig Sammanfattning

Malignt melanom är den cancerform som ökar mest just nu, detta trots den ökade medvetenheten om hur farligt det är att bränna sig i solen. Det är svårt att förändra gamla vanor, det vet vi alla. Men faktum är att malignt melanom är den sjätte vanligaste cancerformen idag och det är således ingen överraskning att denna sjukdom kommer att göra sig påmind framöver. Trots att tumören opereras bort och ca 80-85% blir kvitt sin cancer för gott, så drabbas vissa av spridd sjukdom ändå. Drygt 500 personer har avlidit av melanom de senaste 3 åren. Prognosbedömning är inte bara viktig för behandlingen och hur patienten skall kontrolleras men även patienten själv vill veta hur utsikterna är även om man inte i det enskilda fallet kan ge ett precist besked. Det finns en väl definierad vägledning för detta (AJCC graderingssystem). Beroende på när sjukdomen upptäcks så kommer också överlevnadstiden att se annorlunda ut. Ju tidigare sjukdomen upptäcks, desto bättre överlevnad. Patienter med redan metastaserat melanom har sämst utsikter, det kan handla om en överlevnadstid på bara några månader ibland.

Syftet med denna avhandling var att undersöka malignt melanom på ett molekylärt plan för att få ökade kunskaper om viktiga biologiska processer i tumörer och hur signaturer av dessa har betydelse för överlevnaden i sjukdomen, men också förutspå hur patienter svarar på cancerterapi. Genom att vi använde oss av en idag välkänd metod, s.k. ”gene microarray” kunde vi titta på de individuella tumörernas DNA och se hur olika gener var aktiverade. Efter en sammanställning av alla resultat kunde vi konstatera att metastaserande malignt melanom kan indelas i fyra olika grupper, också kallade ”subtyper”, som visade sig ha olika överlevnad.

Behandling av metastaserande melanom har länge varit oerhört svårt att nå framgång med. Först år 2011 lanserades några godkända behandlingsalternativ på marknaden. Dessa behandlingar riktar sig mot tumörceller med tumörspecifika förändringar som i huvudsak saknas hos normala celler. Genom att undersöka tumörers gener och dess genetiska förändringar (mutationer) med hjälp av DNA-sekvenseringsteknik, så har ytterligare fyra molekylära grupper, så kallade ”genomiska subtyper”, kunnat identifieras. En av dessa grupper karakteriseras av en mutation i genen *BRAF*, vilket i sin tur bidrar till ett muterat BRAF protein som kan hämmas med hjälp av ovanstående behandlingsalternativ. Utöver en behandlingsprediktiv förmåga så visade sig även dessa genomiska subtyper ha betydelse för överlevnadstiden hos dessa patienter. Framförallt en av grupperna

hade en väldigt kort överlevnad där flertalet av tumörerna inom denna grupp kom från äldre patienter och män. Dessa grupper visade inte ha någon direkt överlapp med ovanstående grupper som var baserat på DNA-aktivitet. Vidare studerades också melanom ur ett evolutionärt perspektiv där ackumulation av genetiska förändringar kunde härledas till olika steg i tumörutvecklingen under bildandet av olika kloner av tumörceller, samt till olika faser av spridningen av sjukdomen.

Sammanfattningsvis konstateras att själva tumörutvecklingen styrs av genetiska förändringar, likt mutationer, men utöver dessa förändringar kan tumörer ur ett molekylärt perspektiv se väldigt olika ut där andra mekanismer tycks styra vad som faktiskt översätts från DNA nivå och blir brukbart för tumören. Denna kunskap bör beaktas vid valet av behandling för patienten för att erhålla bästa möjliga tumördödande effekt, och samtidigt minimera biverkningarna och risken för resistensutveckling.

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About the Thesis

Cutaneous malignant melanoma is the most lethal form of skin cancer and its incidence has increased faster than that of any other cancer, rendering it a major public health problem worldwide. The main cause for this cancer is exposure to UV radiation. As all other cancers, melanoma is clinically described by stages. Clinical outcome of patients with similar or even identical clinical and histological features varies considerably, especially within the intermediate risk stages and in patients with advanced disease. This highlights the need of a more personalized disease characterization. Molecular analysis may improve our understanding of the disease biology and provide biomarkers for more correct prognostic assessment and putative targeted therapies. The general aims of this thesis were to analyze the molecular landscape of melanoma and divide the disease into molecular entities by means of genomic and transcriptomic approaches, with the ultimate goals of enhancing our understanding of melanoma biology and its clinical significance.

About the Author

Helena Cirenajwis is a graduated molecular biologist from Skåne with a keen interest in cancer research, especially in the melanoma disease.
In her spare time she enjoys playing classical piano, cooking, being out in nature and rescuing cats. Besides, she loves spending time with her fantastic family and her lovely boyfriend.

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