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# DISCOVERY AND VALIDATION OF PROGNOSTIC MARKERS FOR CUTANEOUS MELANOMA

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# DISCOVERY AND VALIDATION OF PROGNOSTIC MARKERS FOR CUTANEOUS MELANOMA

Thesis for doctoral degree (Ph.D.) to be defended at Radiumhemmet lecture hall at Karolinska University Hospital

**Friday the 4th of May 2018, at 10.00**

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To my father

# ABSTRACT

During the last years there has been an alarming increase in incidence of cutaneous melanoma (CMM) which is the deadliest form of skin cancer. There has been a paradigm shift in treatment of disseminated stage IV disease since the introduction of novel systemic therapies with significant survival benefits. Recently adjuvant therapy has been introduced for stage III CMM. Hence new options to prevent relapses for high-risk categories in stage I-III CMM have appeared. However, the currently used prognostic factors are not enough to identify all high-risk patients. Improved prognostic tools are required to define patient groups with an increased risk of developing metastatic disease who should be offered adjuvant therapies.

The overall aim of these studies was to identify and validate prognostic markers for CMM using microarray, qPCR, DNA sequencing and immunohistochemistry. In papers I-II a tumor set of regional lymph node metastases (n=42) from two stage III CMM patient groups with extremely different disease specific survival after lymph node dissection was used, ≤13months respectively ≥60 months. In papers III-IV a consecutive cohort of ulcerated primary stage I-II CMM tumors (n=71) was used.

In paper I gene expression profiling of tumors from stage III CMM patients identified glycolysis and pigment related gene ontology (GO) categories among the top five GO categories in which overexpression was associated with short survival. GAPDHS was identified as a novel candidate prognostic factor in CMM. Further validation was done on selected genes from these GO categories, three glycolytic genes (GAPDHS, GAPDH and PKM2) and one pigment-related gene (TYRP1), at the protein level. High expression of at least two out of four proteins was found to be of independent adverse prognostic significance.

In paper II a prognostic biomarker panel was found to identify patients with a favorable prognosis in stage III CMM. By combining high expression of CD8+ and FOXP3+ immune cells, low expression of Ki67 and *BRAF* wildtype status a significant independent association with favorable clinical outcome was found, with the best result observed when three out of four factors were present. When adjusting for the previously identified panel in paper I the result remained significant.

In paper III there was a significant association with longer recurrence-free survival (RFS) in ulcerated stage I-II CMM when at least two out of four factors were present in a panel of *BRAF* wildtype/low proportion of *BRAF* mutated alleles, minor ulceration, low proliferation and high presence of TILs, in the multivariate analysis adjusted for Breslow thickness.

In paper IV the presence of TILs was found to be a stronger predictor for RFS in combination with the expression of CD8, FOXP3 and GAPDHS. When at least three of these four factors were present/expressed a significant association with longer RFS was found in multivariate analyses. GAPDHS has been discovered to be a potential prognostic factor in both paper I and IV but may have different functions in stage I-III CMM, a so called moonlighting protein.

This thesis highlights the strength of using a panel of biomarkers instead of using a single biomarker to identify patients with high risk for relapse in stage I-III CMM.

# LIST OF SCIENTIFIC PAPERS

I. **Johan Falkenius**, Joakim Lundeberg, Hemming Johansson, Rainer Tuominen, Marianne Frostvik-Stolt, Johan Hansson, Suzanne Egyhazi Brage.
High expression of glycolytic and pigment proteins is associated with worse clinical outcome in stage III melanoma.
*Melanoma Research, 2013 Dec; 23(6):452-60*

II. **Johan Falkenius**, Hemming Johansson, Rainer Tuominen, Marianne Frostvik Stolt, Johan Hansson, Suzanne Egyhazi Brage.
Presence of immune cells, low tumor proliferation and wild type *BRAF* mutation status is associated with a favourable clinical outcome in stage III cutaneous melanoma.
*BMC Cancer, 2017 Aug 29;17(1):584*

III. **Johan Falkenius**, Johanna Keskitalo, Lena Kanter, Marianne Frostvik Stolt, Hemming Johansson, Veronica Höiom, Johan Hansson, Suzanne Egyhazi Brage.
Prognostic impact of the proportion of *BRAF* mutated alleles, Ki67 expression, presence of TILs and extent of ulceration on recurrence-free survival in ulcerated primary cutaneous melanoma.
*Submitted for publication*

IV. **Johan Falkenius**, Marianne Frostvik Stolt, Lena Kanter, Hemming Johansson, Veronica Höiom, Johan Hansson, Suzanne Egyhazi Brage.
Presence of TILs with expression of CD8 and FOXP3 as well as high expression of GAPDHS in tumour cells is associated with favourable clinical outcome in ulcerated primary cutaneous melanoma.
*Manuscript*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AJCC | American Joint Committee on Cancer |
| AKT | Protein kinase B, Serine/Threonine kinase |
| ALM | Acral lentiginous melanoma |
| BRAF | Braf, B-Raf proto-oncogene, serine/threonine kinase |
| CDK | Cyclin-dependent kinase |
| CDKN2A | Cyclin-dependent kinase inhibitor 2A |
| cDNA | Complementary deoxyribonucleic acid |
| CD8 | Cluster of differentiation 8 |
| CI | Confidence interval |
| CMM | Cutaneous malignant melanoma |
| CNS | Central nervous system |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| ERK | Extracellular-signal regulated kinase |
| FOXP3 | Forkhead box P3 |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GAPDHS | Glyceraldehyde-3-phosphate dehydrogenase spermatogenic |
| G0 | Gap 0, resting phase |
| G2/M | Gap 2, pre-mitotic phase/ Mitotic phase |
| HIF1α | Hypoxia-inducible factor 1-alpha |
| HR | Hazard ratio |
| IHC | Immunohistochemistry |
| LDH | Lactate dehydrogenase |
| LMM | Lentigo maligna melanoma |
| MAPK | Mitogen-activated protein kinase |
| MEK | Mitogen-activated protein kinase kinase |
| MITF | Melanogenesis associated transcription factor |
| mRNA | Messenger ribonucleic acid |
| NM | Nodular melanoma |
| NRAS | Neuroblastoma RAS viral (v-ras) oncogene homolog |
| OCA2 | Oculocutaneous albinism II |

| | |
|---|---|
| OR | Odds ratio |
| PCR | Polymerase chain reaction |
| PD-1 | Programmed cell death-receptor 1 |
| PD-L1 | Programmed cell death-ligand 1 |
| PFS | Progression-free survival |
| PI3K | Phosphatidylinositol 3-kinase |
| PKM2 | Pyruvate kinase M2 |
| PTEN | Phosphatase and tensin homolog |
| RFS | Recurrence-free survival |
| SNB | Sentinel node biopsy |
| SSM | Superficial spreading melanoma |
| TIL | Tumor infiltrating lymphocyte |
| TNM | Tumor, node, metastasis |
| Treg | Regulatory T cell |
| TYR | Tyrosinase |
| TYRP1 | Tyrosinase-related protein 1 |

# 1 INTRODUCTION

The incidence of cutaneous melanoma (CMM) has steadily increased over the last years in Caucasian populations [1]. There are well known risk factors for CMM with intermittent UV irradiation in combination with pigment-related characteristics, like fair-skinned individuals and excessive number of normal nevi, and documented familial history of CMM [2].

The most important established prognostic markers based on histopathology, according to current TNM classification by the American Joint Committee on Cancer Staging System (AJCC) 2017, include tumor thickness according to Breslow, presence of tumor ulceration and regional lymph node metastasis [3]. Sentinel node biopsy (SNB) of regional lymph nodes has received attention to gain further prognostic information [4]. Today SNB is routinely used as a diagnostic staging tool. So far there is no evident documentation of overall survival benefits with SNB [5,6].

Although CMM patients with favorable prognostic markers according to AJCC classification are treated by surgical excision there is a subset of patients who have a worse clinical outcome. Hence, there is heterogeneity of prognosis regarding relapse and survival which is probably due to yet unknown prognostic factors [7].

The last years there has been a development of novel therapies such as inhibitors of BRAF-mutated protein, MEK-inhibitors, and antibodies against CTLA-4 and PD-1, as well as the PD-1 ligand [8-12]. Today there are more options to treat patients with metastatic CMM disease and a higher probability for long-term survival for a subset of patients. Immunotherapy and a combination of different tumor cell targeted therapies may improve the survival, but so far disseminated stage IV disease is not regarded as curable. It is of great importance to prevent progression from localized disease in stage I and II to regionally advanced CMM in stage III and subsequently a further progression to disseminated stage IV disease. Some of the novel therapies have recently been or are soon expected to be introduced as adjuvant therapies in stage III CMM [13-15].

Improved prognostic tools are required to define patient groups with an increased risk of developing metastatic disease who should be offered adjuvant therapies. Molecular characteristics of the tumor most likely play an important role in clinical outcome but are still insufficiently studied and more molecular studies are therefore needed.

# 2 BACKGROUND

## 2.1 EPIDEMIOLOGY OF CUTANEOUS MELANOMA

There is an alarming increase in incidence of CMM during the last years, both worldwide and as well in Sweden. In countries with a predominance of fair-skinned Caucasian populations the incidence has increased annually with 4-6 % [1,16,17]. In Sweden around 4000 individuals were diagnosed with CMM in 2016 which is the fourth and fifth most common cancer reported in males and females [18].

The steady increase in the incidence of CMM in Sweden may be related to changes in lifestyle behavior regarding sun exposure, with intermittent UV radiation and indoor tanning. Public awareness, including different prevention programs of skin screening, probably explain detection of a higher proportion of thin CMMs (Breslow < 1mm) [19], although there is an increase in incidence of thicker CMM (Breslow > 1mm) as well [20]. The mortality trends have been more stable in Caucasian population, with a trend of slow increase compared to the increase in incidence rates [17,21,22]. Besides earlier detection of CMM there have also been improvements in treatment options of CMM that may to some extent explain the different trends between incidence and mortality.

The incidence differs in populations regarding the ethnicity, geography, age, sex and anatomical locations [17,23].

The highest incidence rates are found in New Zealand followed by Australia, Switzerland, the Netherlands and the Scandinavian countries [24]. There is a gradient of incidence related to the geographical latitude in Australia/New Zealand with a higher incidence closer to the equator, due to higher degree of UV radiation exposure [19]. The inverse gradient is found in the Scandinavian countries, i.e. the highest incidence rates are found in the southern regions [25]. The protection offered by cutaneous melanin explains why there are variations in incidence rates regarding the ethnicity in regions with ethnically mixed populations, with lower incidence among those with darker skin.

CMM is a cancer which affects both genders and a wide range of ages from young to elderly individuals. There is a correlation between higher age and increased incidence rates, with a peak at the seventh and eighth decades of life [26]. Although CMM is a relatively rare disease among younger it is one of the most common cancers among young adults [27].

Overall CMM is more common among male worldwide, but in Europe the female gender is more susceptible to CMM [24]. Among young adults < 40 years of age females are overall in

majority [22]. In Sweden the total number of individuals diagnosed with CMM in 2016 was almost equal between the genders, 2090 males respectively 1953 females [18].

There are also differences between the genders regarding the anatomical distribution of primary CMM.  Among Caucasian males it is more common with CMM on the shoulders and the back, while the lower limbs are more frequently reported among females [28].

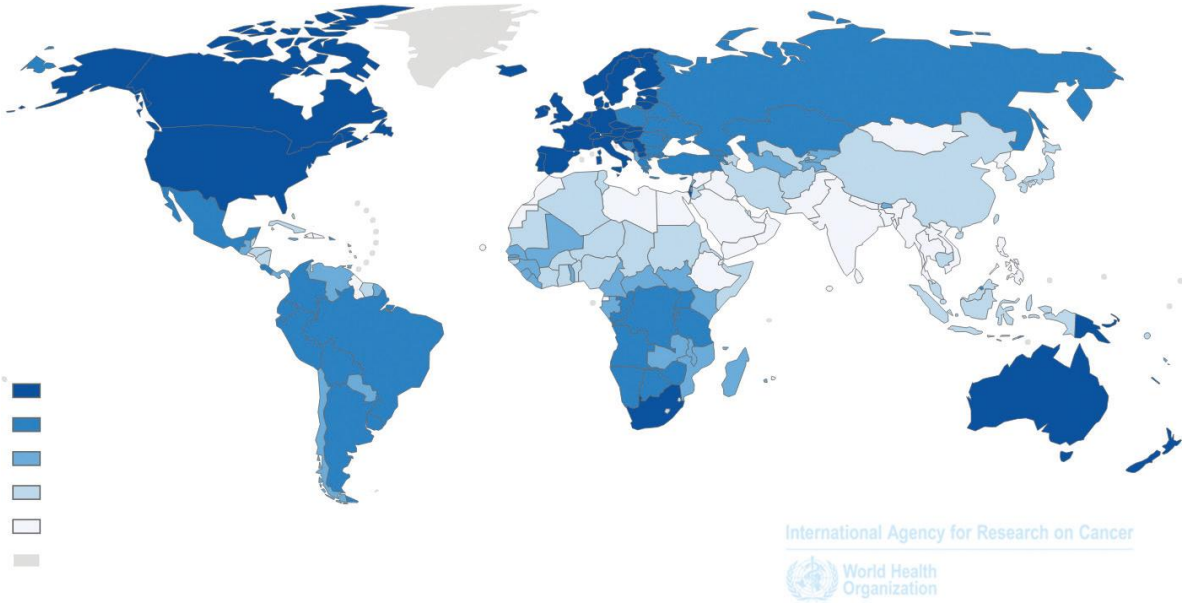


Figure 1. Estimated CMM incidence worldwide 2012 (GLOBOCAN 2012, www.globocan.iarc.fr).

## 2.2 HISTOLOGICAL SUBTYPES OF CUTANEOUS MELANOMA

CMM is generally divided into four subtypes based on different growth patterns and anatomical distribution; superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM) [29-31].

SSM has a lateral growth and often arise in pre-existing nevi. This subtype accounts for about 70 % of all CMM and occurs more often among younger individuals. The anatomical distribution is more often on locations associated with intermittent UV exposure, like the trunk and extremities. SSM has the highest frequency of *BRAF* mutation compared with other subtypes [32].

NM represents the second largest subtype of CMM and is characterized by a vertical growth, which is associated with rapid invasiveness. Therefore the tumor thickness is in general higher in NM. In comparison to SSM there is no correlation to specific anatomical distribution and the median age at diagnosis is higher. *NRAS* mutation is present more commonly in NM and the frequency of *BRAF* mutation is lower compared with SMM [32].

LMM is rarely seen among young individuals. The typical patient with LMM is an elderly individual with tumor location in chronically sun-exposed areas in the head and neck. LMM evolves slowly compared to other subtypes.

ALM evolves predominately from the skin in distal acral parts of the body, like palms, soles and nailbeds. This subtype is most common among non-Caucasian individuals. There is a high proportion of *KIT* mutation in ALM, whereas *BRAF* or *NRAS* mutations are rarely present [33].

The clinical outcome is considered to be worse for ALM compared to the other histological subtypes. However, a recent retrospective study with a large cohort of ALMs has pointed out the same prognostic factors in multivariate analysis as other subtypes of CMM [34].

## 2.3 STAGING OF CUTANEOUS MELANOMA

The current TNM classification by The American Joint Committee on Cancer Staging System (AJCC) includes the most important established prognostic markers for local, regional and distant disease [35]. The last updated 8$^{th}$ edition was recently implemented in January 2018 and is based on a large database platform of > 50 000 CMM patients in stage I-IV from different centers worldwide [3]. The staging system involves results from histopathological examination regarding localized (stage I-II) and regional nodal (stage III) disease, and includes imaging technology and serum samples to assess distant disease (stage IV).

### 2.3.1 **Tumor thickness**

Localized CMM, stage I-II, is primarily defined by tumor thickness according to Breslow. Tumor thickness is categorized in four groups, T1-T4, with an increasing thickness for each higher T-level. The thickness is measured in mm and is rounded to the nearest 0.1 mm. The thickness is divided into the subsequent intervals; ≤ 1.0 mm (T1), >1.0-2.0 mm (T2), >2.0-4.0

(T3) and > 4.0 mm (T4). There is a subcategory of each T-level, T1a-T4a versus T1b-T4b depending on the absence or presence of tumor ulceration. T1 CMMs have in general a favorable prognosis and are regarded as low risk tumors. However, there is a threshold at Breslow thickness of 0.8 mm, which distinguishes patients in favorable T1-level with exceptionally good prognosis with thickness < 0.8mm and without presence of ulceration (T1a) from patients with tumor thickness 0.8-1.0 or <0.8 with ulceration (T1b). Presence of ulceration defines the advanced subcategories of T2b-T4b.

There is an evident difference in prognosis between localized disease T1aN0 and T4bN0 from 98% to 75% in 10-year CMM-specific survival, as illustrated in Figure 2.

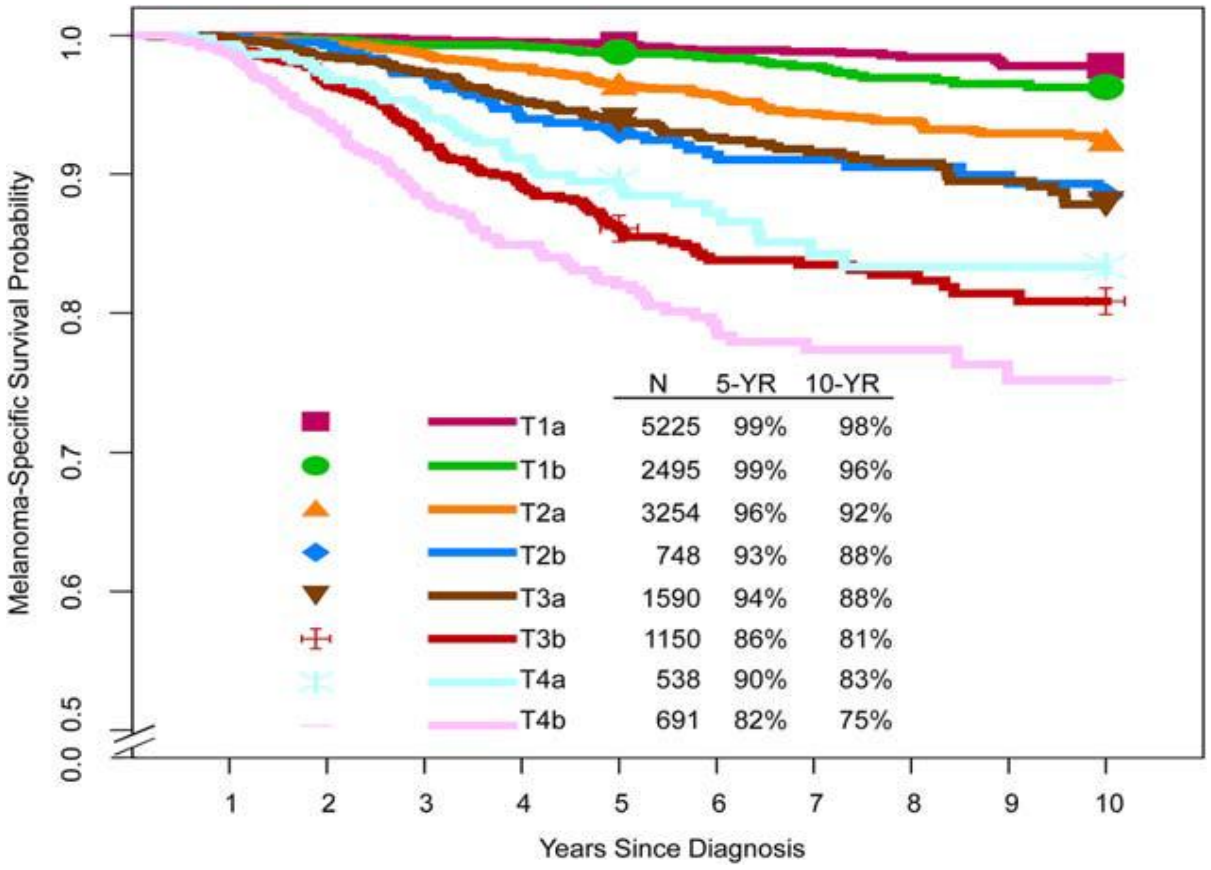


Figure 2. Survival curves according to T subcategory for patients from the 8th Edition AJCC Database. Patients with N0 CMM have been filtered, so that patients with T2 to T4 CMM were included only if they had negative SN, whereas those with T1N0 CMM were included regardless of whether they underwent SNB. Adapted from Gershenwald et al. CA Cancer J Clin 2017.

### 2.3.2 **Ulceration**

The presence of ulceration in primary CMM is a strong adverse independent prognostic factor and upstages patients by both subcategories and stages in the AJCC classification compared to the same tumor thickness without ulceration, as mentioned previously. Although there is today no evidence-based definition of ulceration it is often defined as absence of an intact epidermis above any portion of the primary tumor with an associated host reaction (characterized by a fibrinous and acute inflammatory exudate) based on histopathological examination [36]. Not only the presence but the extent of ulceration seems to be of importance, although this is not included in the AJCC classification [37]. When the ulcerated CMM has been subdivided into excessive or minimal/moderate ulcerations this has provided additional prognostic information, showing that an excessive ulceration is associated with a more adverse clinical outcome [37].
The molecular background of ulceration is still unclear. Ulceration is more often present in CMMs with a higher Breslow thickness and in NM [38]. Recent gene expression studies have shown overexpression of genes associated with tumor-related inflammation, for example interleukin-6, and pathways associated with wound healing, proliferation and angiogenesis [39,40]. Tumor related inflammation and suppression of adaptive immune system supports the clinical results with benefit from adjuvant interferon-alpha reported in patients with ulcerated tumors [41]. Hence ulceration may be a predictive factor for immunotherapy.

### 2.3.3 **Regional metastatic CMM**

Stage III CMM is defined as metastatic disease with regional spreading to lymph node or presence of in-transit or satellite metastasis. The prognosis in stage III is correlated with the number of affected lymph nodes (N1-N3 level) and the extent of regional tumor involvement (subcategories a-c of the N-level). Tumor thickness and presence of ulceration of the primary tumor have prognostic impact in stage III CMM and in the current edition of AJCC there is an important change in stage III with added information of tumor thickness besides ulceration, i.e combination of T-level and N-level. In the recently updated 8$^{th}$ AJCC edition stage III CMM has been extended from three to four subgroups, stage IIIA-D. There is a very variable clinical outcome in stage III, from reported 5-year survival of 93% in stage IIIA to 32% in stage IIID, as illustrated in Figure 3.

The extent of tumor involvement is based on clinically occult lymph node metastasis detected by sentinel node biopsy (SNB) (N1-3a) and clinically detected disease, i.e. macroscopic nodal

disease. Presence of in-transit or satellite metastasis without or in combination with nodal metastases is prognostically unfavorable (N1-3c level). SNB has been routinely used as a staging tool for confirmed tumors with Breslow thickness >1.0 mm and is also considered in T1b tumors with ulceration or other prognostic risk factors.

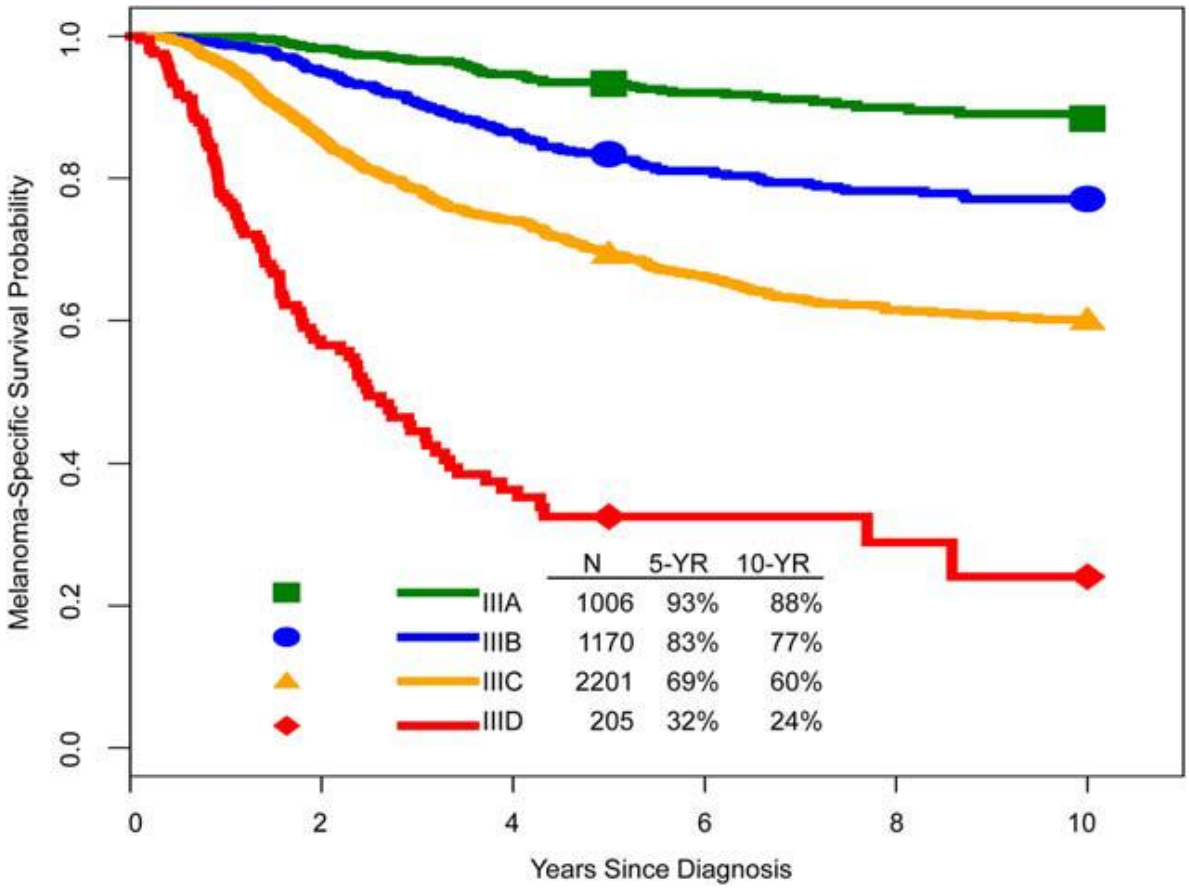


Figure 3. Melanoma-specific survival curves according to stage III subgroups from the Eighth Edition International Melanoma Database. Adapted from Gershenwald et al. CA Cancer J Clin 2017.

Reported rates of SN metastases in T2, T3 and T4 CMMs are 12-20%, 28-33% and 28-44% respectively [42]. In clinically node negative CMM a positive SNB is the best predictor of clinical outcome [6]. There is no evident documentation of overall survival benefits with SNB compared with nodal observation, although the regional disease control has been improved [5]. Two randomized trials have compared complete lymph node dissection in patients with positive SNB to including ultrasound examination of the affected lymph node basin and found no difference in CMM specific survival [6,43]. Hence SNB is used as a diagnostic staging tool to gain further prognostic information.

### 2.3.4 **Distant metastatic CMM**

Presence of distant metastatic disease (M-level/stage IV) is classified into four subcategories based on the anatomical location due to prognostic differences. Metastatic involvement to soft tissue (distant skin, nodes, subcutaneous tissue and muscle) defines a prognostic favorable subcategory (M1a) compared to other distant metastases. The following subcategories with metastatic disease to lung (M1b), other visceral organs excluding CNS (M1c) and CNS (M1d) define a significant increase in unfavorable prognosis. Elevated lactate dehydrogenase (LDH) in serum is a significant factor associated with adverse clinical outcome and has in the current 8$^{th}$ AJCC edition been revised to be an additional subcategory of each four M1-levels.

The clinical outcome is in general poor in stage IV CMM with a 5-year survival rate of approximately 10-30% [44]. However there has been a dramatic improvement with an increasing subset of patients with long-term survival due to more efficient systemic therapies developed during the last years. Subsequent survival data from larger cohorts will hopefully demonstrate improved survival rates.

## 2.4 MOLECULAR PROGNOSTIC FACTORS FOR CUTANEOUS MELANOMA

### 2.4.1 **Proliferation markers**

Mitotic rate is regarded as an adverse prognostic factor in CMMs, with worsening prognosis with increasing mitotic rates [3,4]. There is a threshold for increased risk for metastasis with a mitotic rate $\geq 1$ mitosis/mm² and most significant correlation is found in thin melanomas, T1-tumors with Breslow thickness $\leq 1.0$ mm [4]. Hence mitotic rate was included in the previous 7$^{th}$ AJCC edition in T1-tumors, however excluded in the revised 8$^{th}$ edition when the threshold for T1b at 0.8 mm tumor thickness was introduced and resulted in non-significant correlation in multivariate analyses. Nevertheless mitotic rate is still considered to be an important determinant in CMM, especially in cases where there is no nodal metastases. There is an increased risk of SN metastasis in T1 tumors with high mitotic rate and therefore guidelines recommend to perform SNB in this T1 subcategory.

In proliferating cells an expression of Ki67 is found in every phase of the cell cycle, except from G0 phase [45]. Hence it is often used as a marker for cell proliferation in tumor samples. The Ki67-index based on immunohistochemistry (IHC) has been correlated with clinical

outcome in several different tumor types, for example in breast cancer, lung cancer and mesothelioma [46,47].

The Ki67-index is used as a diagnostic and staging tool in some malignancies, for example in neuroendocrine tumors [48,49], but not in CMM. Elevated Ki67-index has been correlated to poor clinical outcome in several studies on CMM [50,51].

Although Ki67 is a positive marker for proliferation it is uncertain how many of the cells expressing Ki67 that will actually undergo mitosis. Other biomarkers involved in cell cycle regulation like Cyclin A and Cyclin D3 are correlated with tumor thickness, progression and adverse prognosis in CMM [52,53]. Wee1 is involved in regulating G2/M checkpoint and has been identified to be correlated with worse prognosis in CMM [54].

### 2.4.2 **Metabolism**

Several important hallmarks of cancer have been identified and one of those is reprogramming of metabolism, which is also of importance in CMM [55-57]. The Warburg effect, predominant production of energy by a high rate of aerobic glycolysis, is a well known biological phenomenon in cancer cells. Upregulation of glycolysis generating an accumulation of lactate has been associated with worse prognosis [58].
The increased tumor metabolism in CMM can be used in clinical application by using fluorodeoxyglucose positron emission tomography (FDG-PET) for detection of tumor lesions [59]. Elevated serum levels of LDH are associated with worse clinical outcome in stage IV CMM and are used in the AJCC staging to classify distant metastatic disease (the M-classification) [3]. It has also been demonstrated that an inverse correlation between LDHA expression and T cell activation in CMM may lead to tumor immune escape [60].
High expression of the glycolytic regulators PKM2 and GAPDH have been demonstrated to be adverse prognostic factors in different cancers and to promote the Warburg effect [61,62].

### 2.4.3 **Pigmentation**

At the time of skin exposure to UV radiation, keratinocytes stimulate the melanocytes to produce melanin pigment which is distributed to the keratinocytes to protect their nuclei from damaging effect of radiation. The synthesis of melanin pigments depends on expression of several genes, such as *tyrosinase* (*TYR*) and *tyrosinase-related protein* 1 (*TYRP1*) [63].

These pigment genes are regulated by melanogenesis associated transcription factor (MITF) which also induces transcription of other genes involved in melanocyte differentiation, apoptosis and proliferation, such as *CDK2, HIF1α* and *CDKN2A* [64]. They also play an important role in the pathogenesis of CMM [65].
*MITF* is regarded to be a CMM oncogene. However the expression of MITF is heterogeneous in CMM, an amplification of *MITF* is found in about 20% of metastatic CMM lesions [66]. A strong amplification of *MITF* is a prognostic marker associated with a reduced survival in CMM [66]. Sporadic mutations in *MITF* are rarely seen. TYRP1 has been associated with a poor prognosis in stage III-IV CMM [67].

### 2.4.4 **MAPK and PI3K signaling pathways**

There are two major pathways commonly activated in CMM, the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway. Both pathways are involved in the initiation and progression of CMM [68].
*NRAS* and *BRAF* mutations activate MAPK pathway. About 50% of CMM patients harbor *BRAF* mutations and approximately 20 % harbor *NRAS* mutations [69].

*BRAF* mutations have been detected in exons 11 and 15 [70].The most common *BRAF* mutation is V600E (around 75 %), located in exon 15. Another common subtype of *BRAF* V600 mutation is V600K (around 17%) while V600D, V600R and V600E2 are more rare (< 5%) [71].

*NRAS* mutation activates both pathways of MAPK and PI3K. *BRAF* and *NRAS* mutations almost never occur simultaneously in the same CMM. Downstream activation results in increased proliferation by unregulated expression of Cyclin D1. Mutation in *BRAF* or *NRAS* alone is not sufficient for malignant transformation of melanocytes. Thus additional genetic events are required for tumor progression [72].

Both *BRAF* and *NRAS* mutations are early somatic events in malignant transformation as many benign and dysplastic nevi harbor such mutations [73]. Some studies have demonstrated that *BRAF* or *NRAS* mutations are adverse prognostic factors, whereas others could not confirm these findings [32,74,75]. However, these studies differed regarding type of patient cohorts and whether the study was prospective or retrospective.

The PI3K pathway is activated by loss of the tumor suppressor PTEN activity or amplification and overexpression of AKT. Loss of PTEN activity by deletion or mutation is

observed in 25-50% of the CMM patients. In *BRAF* mutated CMMs approximately 50% have an inactivation of PTEN [76].

BRAF activation regulates metabolism in CMM by decreasing oxidative phosphorylation and contributing to increased glycolysis [77]. This is consistent with reported higher levels of serum lactate in *BRAF* mutated patients [78]. An *in vitro* study has shown that overexpression of GAPDH rescued the *BRAF* mutated cells from cell death after treatment with BRAF inhibitor [77,79]. This supports the importance of glycolysis in BRAF activated CMMs and implicates it as a possible therapeutic target to overcome resistance to BRAF inhibitors.

The MAPK pathway is also linked to the turnover of the transcription factor MITF by ERK-mediated phosphorylation [80]. MITF degradation is promoted by a strong constitutive activation of MAPK pathway. *MITF* gene amplification is found in patients who relapsed during treatment with BRAF inhibitors, suggesting survival or growth advantages when the MAPK pathway is blocked, by restoration of signal activation downstream in the MAPK pathway [81]. Thus, MITF upregulation is a marker for acquired resistance to BRAF and MEK inhibitors.
BRAF inhibition is associated with increased melanoma antigen expression and increased CD8+ T cell infiltration of the tumor implicating that BRAF activation may lead to suppression of the immune response and a worse prognosis [82].

### 2.4.5 **CDKN2A**

*CDKN2A* is a tumor suppressor gene commonly inactivated in CMM. The *CDKN2A* gene encodes for two proteins, P16 (INK4A) and P14 (ARF), involved in two different essential pathways, the Retinoblastoma and the P53 pathway, respectively. Deletions occur frequently, but *CDKN2A* is also inactivated by mutations and transcriptionally by promoter methylation. Deletions are more frequent in metastatic CMM lesions than in primary tumors [83]. CMMs harbor less frequently P53 alterations compared to many other malignancies, probably due to frequent inactivation of P14 (ARF) in the P53 pathway [84]. We have previously found that biallelic losses of the *CDKN2A* gene correlate with a shorter overall survival in CMM [83]. A higher proliferation rate has been observed in stage IV CMMs where the proliferative subtype was characterized with a high frequency of biallelic *CDKN2A* deletions [85].

### 2.4.6 **Immunological host response**

Overexpression of immune related genes in stage III-IV CMMs has been correlated with a favorable prognosis in several gene expression studies [85-87].
Spontaneous complete regression is a well known, but rare phenomenon in CMM, and supports the concept that CMM is an immunogenic tumor [88]. Partial regression of the primary tumor is seen more frequently and is characterized by absence of tumor cells in dermis and epidermis together with a combination of fibrosis, melanophages and lymphocytes [89]. An immunological host response against the tumor explains the appearance of regression. During an early phase of regression the tumor infiltrating lymphocytes (TILs) appear. The lymphocytes infiltrate the tumor in different patterns and the TILs are categorized as brisk, non-brisk or absent [89]. Immunological host response as presence of CD4+ and CD8+ TILs is correlated with favorable prognosis [90-92].

However, the prognostic value of regression and TILs needs to be further explored since there are discordant results demonstrating that TILs may promote progression and metastasis [93]. Thus, TILs is not included in the current AJCC staging system.

The SN is normally the first site of metastasis and represents an immunological barrier against metastatic disease. Also tumor negative SNs have been detected with altered microenvironment, which may facilitate progression of CMM [94]. Thus, tumor immune microenvironment of the sentinel node (SN) may reflect the status of systemic immunity.
A recent study on patients with stage III CMM who later progressed into stage IV and were treated with checkpoint inhibitor anti-CTLA-4 has demonstrated better response and longer survival in patients with presence of TILs in their regional lymph node metastases [95].

Regulatory T-cells (Tregs), immunosuppressive cytokines and immune checkpoints (CTLA-4 and PD-1) are some examples of immunosuppressive factors to control excessive immune activation [96]. The Forkhead box P3 (FOXP3) is used as a marker for Tregs. However, also different tumors including CMM express FOXP3 [97]. The prognostic value of FOXP3 expression is still unclear [98].

The immune checkpoint receptor PD-1 is linked to PD-1 and PD-2 ligands (PD L1, PD-L2) and functions in the tumor microenvironment. Several different tumor types express PD-1 ligand and an overexpression is observed in CMM [99]. In some malignancies the expression of PD-L1 is a negative prognostic marker, including lung cancer and renal carcinoma [100,101]. Immune checkpoint-blockade with anti CTLA-4 and anti PD-1 antibodies has demonstrated significant survival benefits [9-11]. No predictive biomarker is available in

CMM, though it seems that expression of PD-L1 may predict responses to anti PD-1 [102]. Today there are several studies ongoing to identify suitable prognostic and predictive biomarkers for immune checkpoint-blockade in CMM.

## 2.5 MANAGEMENT OF CUTANEOUS MELANOMA

### 2.5.1 **Stage I-II**

For localized CMM, stage I-II, surgery with wide local excision is standard of care [103]. The recommended margin is determined by the T-level of primary tumor. According to international and Swedish guidelines for T1 tumors, i. e tumor thickness ≤ 1.0 mm, the recommended free lateral margin is 1 cm with an excision down to the underlying muscle fascia [104]. For T2-T4 tumors, i.e tumor thickness > 1.0 mm, there is a recommended margin of 2 cm. In head and neck locations a less margin is acceptable with respect to functional aspects. SNB is recommended as diagnostic staging tool for T2-T4 tumors, and should be considered for T1b, as previously described.

Follow-up is performed with clinical controls according to national guidelines.

### 2.5.2 **Stage III**

Due to the results from randomized trials demonstrating no outcome benefit with complete lymph node dissection after positive SNB compared to nodal observation, as previously described, the guidelines have recently been revised [6,43]. For SNB metastasis < 1 mm diameters there is support of nodal observation with ultrasound follow-up. Complete nodal dissection should be considered for SNB metastasis ≥ 1 mm and for clinically detected lymph node metastases (macroscopic). Postoperative radiotherapy may be considered in cases of non radical surgery, periglandular tumor involvement, large size of macroscopic metastasis and multiple lymph nodes affected, to decrease the risk of local recurrence [105].

In Sweden adjuvant systemic therapy has to date not been standard of care in stage III CMM. Adjuvant high-dose interferon-alpha therapy may be used in high risk stage III but due to side effects and uncertain effectiveness it has not been widely used. The subcategory of patients with ulcerated primary tumors has been better benefited from adjuvant interferon-alpha therapy and ulceration may be a predictive marker [41].

Adjuvant therapy in stage III CMM with immune checkpoint inhibitor of anti-PD-1 nivolumab has recently been introduced in the US. Data from a randomized trial has demonstrated superior benefit with adjuvant anti-PD-1 nivolumab compared with anti-CTLA-4 ipilumumab with significantly improved reduced recurrence-free survival (RFS) [14]. An introduction of adjuvant anti-PD-1 inhibitors, nivolumab and pembrolizumab, is expected in Europe later during 2018.
Adjuvant combination therapy of BRAF and MEK inhibitors in stage III CMM, *BRAF* mutated patients, has shown significant benefit in RFS and is expected as well to be introduced [15].

There have been different local guidelines in Sweden regarding the follow-up of patients in stage III CMM with clinical controls and at some places also in combination with imaging methods. An ongoing national multicentre study, TRIM trial, with randomization between follow up with clinical controls and imaging controls has been initiated to get harmonized guidelines.

### 2.5.3 **Stage IV**

There has been a paradigm shift in treatment of disseminated stage IV disease since the introduction of novel systemic therapies at 2011-2012 with significant survival benefits with anti-CTLA-4 ipilimumab and BRAF inhibitor vemurafenib. Prior to 2011 gold standard therapy has been palliative chemotherapy, usually dacarbazine or temozolomide, with low response rates and median progression-free survival (PFS) about 1,5 months [106]. No significant survival benefit.
Ipilimumab is an antibody against CTLA-4 and was the first immune checkpoint inhibitor to be introduced. CTLA-4 is expressed on the surface of activated T-cells and regulatory T (Treg) cells. It functions as an immune checkpoint that inhibits T-cell activation during an early phase in lymphatic tissues with inhibitory feedback mechanism [107]. Thus, blockade of CTLA-4 enhances T-cell-mediated antitumor immunity by removing an inhibitory signal. Treatment with ipilimumab in stage IV CMM has in pooled analysis of phase II and phase III studies demonstrated a plateau in the survival curve at 22% at three years. Hence there is a subset of patients who may achieve prolonged survival with long-term remission [108]. There are immune related side effects which may be of severe grade and sometimes life-threatening.

Some years later, in 2014-2015, the anti-PD1-blocking antibodies nivolumab and pembrolizumab were introduced. They represent another type of T-cell activating immune checkpoint inhibitors. PD-1 is linked to PD-1 ligand (PD-L1) which may be expressed on tumor cells and immune cell infiltrates, hence anti-PD-1-blocking antibodies function in the tumor microenvironment, as previously described [107]. The responses to anti-PD-1 antibodies are impressive with higher response rates and less immune related side-effects compared to anti-CTLA-4 antibodies. The responses to nivolumab and prembrolizumab are similar with objective response rates of about 40% [109]. Today both therapies are used as first-line therapy in stage IV CMM, regardless of *BRAF* mutational status. Recently combination therapy of nivolumab and ipilimumab has been introduced, demonstrating higher objective response rate compared to monotherapy nivolumab or ipilumumab (57.6, 43.7, and 19.0%, respectively) [11]. A subcategory of patients with low expression of PD-L1 might benefit from combination therapy to a higher extent and therefore this combination is today in Sweden approved for patients with PD-L1 expression < 1% of tumor cells. The immune related side effects with combination therapy are substantially higher and of severe grade compared to monotherapy nivolumab.

Vemurafenib is a per oral protein kinase inhibitor and was the first selective BRAF inhibitor to be introduced in 2011-2012 for patients with *BRAF* V600E mutated tumors. The introduction of vemurafenib demonstrated impressive responses with often rapid shrinking of the tumor load [106]. Later dabrafenib, another BRAF inhibitor, with similar response rate was introduced [110]. The objective response rate with BRAF inhibitors is about 50 % with median progression-free survival of about 6 months. When relapses occur they might develop with rapid progression due to drug resistance. When combining BRAF inhibitor with MEK inhibitor the responses are in general prolonged with median PFS about 9 months [8]. Today BRAF and MEK inhibitors in combination are standard therapy for *BRAF* mutated patients in first or second line therapy post immunotherapy.

There is a rapid development of novel therapies with several ongoing trials in stage IV CMM with a focus on different combinations of immune checkpoint inhibitors and in combination with targeted drugs.

# 3 AIMS OF THE THESIS

The overall aim of the thesis is to identify prognostic biomarkers in stage I-III CMM to better define patients that may be suitable for adjuvant therapy or longer follow-up.

**Specific aims**

**Paper I:** To identify prognostic markers, based on gene expression profile, in stage III CMM patients.

**Paper II:** To investigate the impact on clinical outcome of immune cells (CD8+ and FOXP3+ T-cells), proliferation marker and *BRAF* mutational status in stage III CMM patients.

**Paper III:** To validate the prognostic impact of *BRAF* mutation on RFS in ulcerated stage I-II CMM in combination with expression of proliferation marker, the extent of ulceration and presence of TILs.

**Paper IV:** To validate the prognostic impact of the expression of CD8, FOXP3, GADPHS and presence of TILs on RFS in ulcerated stage I-II CMM.

# 4 MATERIALS AND METHODS

## 4.1 **Tumor samples**

### 4.1.1 Papers I-II

A set of tumor samples of regional lymph node metastases was collected from patients with stage III CMM. Specimens from forty-two patients were included in the analyses performed in papers I-II. The patients were from two distinct different prognostic groups in stage III CMM; long-term survival ≥60 months versus short survival ≤13 months after lymph node dissection, respectively. There were 19 patients and 23 patients in each prognostic group, long-term respectively short-term survival. One fresh frozen biopsy of lymph node metastasis was collected from each patient at the time when radical lymph node dissection was performed. The biopsies were fresh frozen in liquid nitrogen and kept in a biobank at -70 C until analysis. All samples were defined to be the first regional relapse of each CMM patient. The regional relapses were all clinically detected, i.e. macroscopic lymph node metastases. The proportion of tumor cells in the specimen from the biopsies used in papers I-II was estimated by a pathologist. For further analyses there was an inclusion criteria of at least 50% tumor cells and for a majority (67%) of the samples there was >70% tumor cells.

### 4.1.2 Papers III-IV

A consecutive and retrospective cohort of ulcerated primary tumors was collected from patients with diagnosed stage I-II CMM. The patients were identified from the Stockholm-Gotland Regional Melanoma Registry and they had all undergone the primary excision in the Stockholm region during 2004-2006. In total 120 patients were identified from different pathology departments in the Stockholm County. However, we only received samples from 76 primary tumors, T1b-T4b, of the originally 120 identified tumors. All the samples were re-evaluated by a pathologist to confirm presence of ulceration for further analyses in papers III-IV. In total 71primary CMM tumors were included in the final cohort that consisted of formalin fixed and paraffin embedded specimens.

## 4.2 **Patient and pathological characteristics**

Data on follow-up information and patient characteristics such as gender, age, time of diagnosis, CMM related event, survival outcome and treatment history were collected from the Stockholm-Gotland Regional Melanoma Registry, patient records and pathology files.

## 4.3 **Methods**

### 4.3.1 Microarray

Gene expression profiling was performed in collaboration with Professor Lundeberg and his team at KTH (paper I). The platform was an in-house manufactured microarray consisting of >35 000 features representing almost 29 000 different genes. RNA was prepared and amplified followed by labeling and cDNA synthesis of the samples prior to hybridization to microarrays overnight. Then scanning and imaging of the microarrays was done and finally data analyses including Bayes moderated t-test to control for multiple testing.

### 4.3.2 Real-time PCR

Real-time PCR was performed in paper I to validate mRNA expression of selected genes based on identified gene expression profile by the oligonucleotide microarray. First-strand cDNA was made from total RNA followed by Real-time PCR analysis using the ABI7500 qPCR system (Applied Biosytems, Foster City, CA, USA). All results were normalized to two reference genes (HPRT1 and HMBS) and a reference sample. For each plate, a dissociation curve was generated, and a standard curve for both the test gene and reference genes was plotted to monitor PCR efficiency. The ΔΔCt method was used when analyzing data using the GenEx software (MultiD, Gothenburg, Sweden).

### 4.3.3 Immunohistochemistry

Immunohistochemistry (IHC) was performed in all studies, papers I-IV, to examine the protein expression of selected prognostic candidates.

IHC was performed on 4-µm-thick, formalin-fixed, paraffin-embedded sections. In brief, heat-induced antigen retrieval was performed in a decloaking chamber according to the manufacturer's instructions (Biocare, Concord, CA, USA). Sections were incubated overnight at 4°C with anti-human GAPDH (Santa Cruz Biotechnology, CA, USA), GAPDHS (Abcam, UK), PKM2 (Abcam), TYRP1 (Novocastra, UK), Ki67 (Dako, Denmark), CD8 (Dako), FOXP3 (eBioscience, AffymetrixCompany, USA). Secondary antibody incubation using streptavidin/peroxidase complex was done with Vectastain Universal Quick Kit (Vector Laboratories Inc, CA, USA), development with DAB Substrate Kit (Vector Laboratories Inc) and counterstaining with Mayers Hematoxylin (Histolab,Sweden).

### 4.3.4 Mutation analyses

In paper III mutation analyses were performed to assess the *BRAF* status of each tumor sample in the cohort of ulcerated primary tumors using three different methods; Cobas, pyrosequencing and Sanger sequencing.
Prior to DNA extraction the proportion of tumor cells was estimated and in some cases dissection was performed for tumor cells enrichment.

The Cobas test is designed to detect mutations in codon 600 in the *BRAF* gene, and will only report if a mutation is present without information about the type of mutation detected.

*BRAF* in codon 600 (exon 15) and 464-469 (exon 11) was also analyzed by pyrosequencing to detect mutations.

For the samples where no mutations were detected by Cobas or pyrosequencing, or only by one of these methods, Sanger sequencing of *BRAF* was performed to detect mutations at exons 11 and 15 of the *BRAF* gene.

# 5 RESULTS

## 5.1 Paper I

Gene expression profiling by oligonucleotide microarray revealed that several gene ontology (GO) categories were highly significantly differentially expressed between the two prognosis groups in stage III CMM. We did not detect any single genes as significantly differentially expressed. Two of the top GO categories were glycolysis (GO: 0006096; $p< 0.001$) and pigment biosynthetic process (GO: 0046148; $p<0.001$), in which overexpression was associated with short survival. Further validation was performed by using IHC and qRT-PCR. Candidate genes from the two top GO categories were selected based on the most differentially expressed genes between the prognostic groups and on documentation in literature. Three glycolytic genes, *GAPDHS*, *GAPDH* and *PKM2*, and two pigment-related genes, *TYRP1* and *OCA*, were selected. A significant difference in GAPDHS protein expression between short and long survivors ($p=0.021$) and a trend for PKM2 ($p=0.093$) was reported in univariate analysis. The best result was also observed for *GAPDHS* by qRT-PCR ($p=0.11$). High expression of at least two out of four proteins (GAPDHS, GAPDH, PKM2, TYRP1) was found to be an independent adverse prognostic factor when adjusting for ulceration and number of lymph node metastases in a multivariate analysis ($p=0.011$).

## 5.2 Paper II

In this study we examined immune response related proteins in association with proliferation and *BRAF* mutation status and their impact on clinical outcome in stage III CMM. In our previous microarray study (paper I), a number of GO categories related to immune response showed a significantly higher expression among long survivors. The same 42 specimens were used as in paper I. To examine the impact of immune cells in stage III CMM we performed IHC using antibodies against CD8+ T-cells and FOXP3, which was used as a marker for Tregs. There was a higher proportion of CD8+ and FOXP3+ T-cells among long survivors but these differences were not statistically significant. There was, however, a significant correlation between CD8+ and FOXP3+ ($p=0.029$).

In this study we investigated the proliferation by Ki67 expression in the two tumor sets using IHC and found a significant difference between the two prognostic groups with a higher expression among short survivors ($p=0.013$). Although more short survivors (56%) carry *BRAF* mutations than long survivors (32%) no significant difference is observed ($p = 0.11$). By combining high expression of CD8+ and FOXP3+, low expression of Ki67 and *BRAF*

wildtype status a significant difference between the groups was found. A test of trend illustrating a significant association with favorable outcome when the four factors are increasing in presence from one to four factors (p=0.003). The best result is shown in univariate analysis when at least three of four factors are present resulting in an odds ratio (OR) of 8.1 for belonging to the group with long survival (p = 0.004). The result remains significant in the multivariate analysis when adjusting for ulceration and number of lymph node metastases (OR 19.4, 95% CI 1.9-197, p=0.012). Ki67 remained significant as well in multivariate but with a wider 95% CI (OR 26.1, 95% CI 2.0-344, p=0.013).
When adjusting for the previously identified panel in paper I the result remained significant. Hence the results on the same case series of samples in paper I and paper II are independent of each other.

### 5.3 Paper III

In this study we examined the adverse prognostic impact of ulceration in association with *BRAF* mutation, proliferation, presence of TILs and the extent of ulceration on RFS in a cohort of ulcerated primary CMM stage I-II.
We wanted to validate the results from a previous study [111] on two smaller cohorts demonstrating that ulceration is associated with poor outcome in tumors carrying *BRAF* mutation, in a larger and consecutive ulcerated cohort with different *BRAF* mutation analyses. Cobas test and pyrosequencing were performed on all samples and in cases of wildtype *BRAF* or a discrepancy between the two methods subsequent analyses with Sanger sequencing was performed (n=42). Six samples with a detected *BRAF* mutation by pyrosequencing were not detected by Cobas. Four of these six samples had a *BRAF* mutation in exon 11. The other two non-detected mutations by Cobas were one V600K and one rare mutation complex (V600E2). In total 69% of the detected *BRAF* mutations were of subtype V600E and 20% of subtype V600K. Sanger confirmed all *BRAF* mutations detected by pyrosequencing. However, Sanger also detected one rare *BRAF* deletion in VK600-601E. *BRAF* mutation was detected in 49% (35/71) of the samples, excluding the identified deletion. There was no correlation between *BRAF* mutation status and clinical outcome in this ulcerated cohort (p=0.71). However, when the samples were divided based on the proportion of *BRAF* mutated alleles, assessed by pyrosequencing, there was a significant association with RFS. There was a longer RFS among tumors with *BRAF* wildtype/low proportion of *BRAF* mutated alleles ≤35% (median) than among cases with a high proportion of *BRAF* mutated alleles >35% (HR 2.44, 95% CI 1.23-4.84, p=0.011).

There was a significantly higher presence of TILs in tumors from patients without recurrence (HR 0.48, 95% CI 0.23-0.98, p=0.045). The extent of ulceration was evaluated and grouped as minor, moderate and major. Minor ulceration was found to segregate from moderate and major ulceration with fewer recurrences in a Kaplan-Meier and trend analysis (p=0.015). There was a significant difference when combining moderate and major ulceration in comparison to minor ulceration regarding risk of recurrence (HR: 1.6, 95% CI: 1.02-2.50, p=0.039). The proliferation rate was investigated, like in the previous study (paper II) by analyzing Ki67 expression using IHC and we found in this cohort a significantly higher Ki67 expression among subjects with recurrence than no recurrence (HR 2.65, 95% CI 1.32-5.35; p=0.006). The combination of *BRAF* wildtype/low proportion of *BRAF* mutated alleles, low expression of Ki67, minor ulceration and presence of TILs in univariate analysis was highly significant with the best result correlating with favorable RFS when at least two out of four factors were present (HR 0.28, 95% CI 0.14-0.56, p<0.001). This result was supported by a test of trend showing a highly significant decrease in the proportion of recurrence by including all the four factors in the analysis (p= 0.0003). In multivariate analysis none of the significant factors alone in univariate remained significant when adjusting for Breslow thickness, although Ki67 was border-significant (p=0.056). However, the panel of four factors in combination when at least two out of four factors were present, remained significant in the multivariate analysis (HR 0.30, 95% CI 0.15-0.60, p = 0.001).

### 5.4 Paper IV

In this study we examined the adverse prognostic impact of ulceration in association with presence of TILs and the expression of CD8, FOXP3 and GAPDHS in a cohort of ulcerated primary CMM stage I-II. The same 71 specimens were used as in paper III.
The presence of TILs is known from the previous results in paper III. The expression of CD8 and FOXP3 in immune cells was investigated, like in the previous paper II by using IHC. The expression of the glyolytic protein GAPDHS in tumor cells was investigated by using IHC as in paper I. The expression of CD8, FOXP3 and GAPDHS as single factors were not associated with a difference in recurrence in univariate analyses. However, when combining the presence of TILs with positive expression of either CD8 or GAPDHS there was a more significant correlation with fewer recurrences (p=0.030 respectively p=0.018) compared to TILs as a single factor (p=0.045). A similar test of trend as in the previous studies (papers II-III) was performed and demonstrated a significantly improved RFS by including TILs, CD8, FOXP3 and GAPDHS (p=0.024). When combining presence of TILs with the expression of CD8, FOXP3 and GAPDHS the best result was found when at least three out of four factors

are present/expressed, both in univariate and multivariate adjusting for Breslow thickness (HR 0.26, 95% CI 0.11-0.62, p=0.002 respectively HR 0.27, 95% CI 0.12-0.63, p=0.003). The result with GAPDHS as being prognostically favorable in combination with the other factors in this cohort of stage I-II ulcerated CMM opposed the previous results from paper I where GAPDHS was identified to be an unfavorable prognostic factor in stage III CMM.

# 6 DISCUSSION

## 6.1 Paper 1

We did not detect any single genes as significantly differentially expressed but glycolysis and pigment related GO categories were significant, illustrating the documented heterogeneity in CMM. Our observations on the stage III CMM points at the importance to focus on the activity of multiple pathways and biological functions (GO groups) rather than single genes or proteins. These results are in agreement with other gene expression studies and the statement of metabolism being an important hallmark of cancer [55,85-87]. Pigment related proteins have been associated with worse prognosis and are involved in proliferation and apoptosis through MITF [64,66,67]. However, GAPDHS is an interesting protein as a potential prognostic marker. So far there is little reported in the literature on GAPDHS in relation to cancer and further studies need to be performed. The study design with two patient groups of extremely different clinical outcome in stage III CMM is an appealing strategy to identify candidate prognostic factors which needs to be further validated in other patient cohorts.

## 6.2 Paper II

The gene expression results in paper I demonstrated a significant overexpression of immune related GO categories in the group of patients with long survival. In paper II we have shown that presence of CD8+ and FOXP3+ T-cells are prognostically favorable in combination with other factors. Surprisingly we did not find that high expression of FOXP3 was associated with an unfavorable prognosis as Tregs are in general associated with an unfavorable clinical outcome [112]. There are opposing/conflicting data regarding the prognostic impact of the expression of FOXP3 in several malignancies. In recent years several studies have demonstrated that FOXP3 can sometimes be a favorable prognostic marker and there is an indication that FOXP3 may be a favorable prognostic factor due to its association with CD8 T-cells [98,112], which is supported by our result in paper II.

The proliferative marker Ki67 remained significant in the multivariate analysis as a single factor, thus superior to the other prognostic factors in this study. The results in paper II support the assumption that Ki67 is a strong negative prognostic marker in CMM, which may be important to take into consideration when risk for relapse is assessed in stage III CMM.

*BRAF* mutation has also been suggested to be an adverse prognostic factor but this is still controversial. We found the *BRAF* mutations status to be of prognostic impact in combination with other factors, but not as a single factor.

We have had access to limited case series of samples, which could be considered as a limitation, but the results may still indicate the prognostic relevance of CD8+ TILs in combination with Tregs, proliferation rate and *BRAF* status and support the statement that several factors in combination are better to predict the prognosis. These results are in concordance with the results in paper I supporting the role of heterogeneity in metastatic CMM. The results on the same case series of samples in paper I and paper II are independent of each other. Hence the prognostic information may increase by combining the different prognostic factors of glycolytic-pigment related proteins in paper I and the panel of immune cells, proliferation rate and *BRAF* status in paper II in stage III CMM.

## 6.3 Paper III

*BRAF* mutation has been suggested to be an adverse prognostic factor but this is still controversial. The primary aim of this study was to validate previous results from our research group showing that ulcerated primary tumors are associated with a worse outcome in combination with presence of *BRAF* mutation [111]. We did not confirm this result regarding *BRAF* mutation versus wildtype status, but we could demonstrate that having a high proportion of *BRAF* mutated alleles in the tumors is of prognostic relevance in univariate analysis and contributes to be a significant prognisticator in combination with the expression of Ki67, presence of TILs and minor extent of ulceration in multivariate analysis. These results are concordant with the results in paper II which demonstrated the *BRAF* mutation status to be of prognostic impact in combination with other factors, but not as a single factor.

To our knowledge the impact of the proportion of *BRAF* mutational alleles on clinical outcome in primary CMM is a novel finding. It needs to be validated in further studies. We have compared different methods to detect *BRAF* mutations and our result supports the relevance of using pyrosequencing to gain knowledge about the proportion of *BRAF* mutated alleles in primary tumors at diagnosis. In addition, we have confirmed previous findings from other publications that the extent of ulceration [37] and presence of TILs [113] are of prognostic relevance in ulcerated CMM. In this study the expression of Ki67 was border-

significant in multivariate analysis implicating that Ki67 is a superior prognostic factor compared with the other factors in the evaluated panel, in accordance with paper II**.**

## 6.4 Paper IV

High expression of GAPDHS was in paper I identified to be an adverse prognostic factor in stage III CMM. Surprisingly we found GAPDHS to have favorable prognostic impact in the cohort of ulcerated stage I-II CMM in presence of TILs. Our differing result may be related to the published data on the related glycolytic protein GAPDH presenting it as a multifunctional protein, a so called moonlighting protein [114,115]. The glycolytic protein GAPDH has been shown to be an immunomodulator which may also be the case for GAPDHS. The specific immune microenvironment in ulcerated primary tumors with inflammatory cells supports the role of GAPDHS as an immunomodulator in this feature. However, a limitation is that we have not used a control group of non-ulcerated primary tumors. We cannot exclude that the known reprogramming of tumor metabolism during transition to a more malignant phenotype in CMM might explain the difference between primary tumors and more advanced stages of disease, i.e stage I-II versus stage III-IV. The combination of using cytomorphological evaluation of TILs with IHC of CD8/FOXP3 expression is an interesting concept to improve the prognostic prediction at diagnosis of ulcerated stage I-II CMM.

# 7 CONCLUSIONS

- The common findings in all four papers of the thesis, on stage I-III CMM, support using a panel of biomarkers instead of single biomarkers to identify patients with high risk for relapse. The known heterogeniety in CMM is demonstrated in the gene expression profiling in paper I by showing highly significantly differentially expressed GO but no detection of any significant single genes.

- The identified panel of different prognostic factors of glycolytic-pigment related proteins in paper I and the panel of immune cells, proliferation rate and *BRAF* status in paper II are independent from each other in stage III CMM.

- GAPDHS has been discovered to be a potential prognostic factor in stage I-III CMM that may have different functions, a so called moonlighting protein.

- Ki67 is supported to be used as an adverse prognostic factor in stage I-III CMM and becomes stronger when used in combination with other prognostic factors.

- The proportion of *BRAF* mutated alleles is an identified potential adverse prognostic factor in ulcerated stage I-II CMM and is overall an interesting concept of prognostic prediction. The *BRAF* status is of prognostic relevance also in stage III CMM together with other candidates.

- Minor extent of ulceration (minor versus moderate-major) and presence of TILs have been validated as favorable prognostic factors in ulcerated stage I-II CMM.

# 8 FUTURE PERSPECTIVES

It is of great importance to identify patients with high risk of relapse in ulcerated stage I-II CMM as well as in stage III CMM. Both presence of ulceration in primary tumors and the detection of regional lymph node metastases define in general an increased risk for relapse. With identification of patients with a higher probability of a good versus a poor clinical outcome at diagnosis it would be possible to improve the follow-up or adjuvant therapy. The rapid development of new and effective treatments during the last years has resulted in an introduction of adjuvant therapy in stage III CMM and we will most likely see more of different adjuvant therapies in the future. In the different panels of prognostic factors used in the thesis, papers II-IV, there are both immune related factors and presence of *BRAF* mutation. Hence it would be interesting to investigate the different panels in relation to a predictive context with immunotherapy or targeted BRAF blocking therapy.

However, the next step would firstly be to validate our results in papers I-II in a prospective and consecutive cohort of stage III CMM patients, including the intermediary prognostic group and validate the results in papers III-IV in a control cohort of non-ulcerated tumors.

# 9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Malignt melanom är den dödligaste formen av hudcancer, men vid en tidig upptäckt utan tecken på spridning är prognosen i allmänhet god. De senaste åren har det skett stora framsteg i behandlingen av spritt malignt melanom genom nya immunterapier och målsökande behandlingar. Numera finns det tack vare dessa behandlingar en möjlighet till långtidsöverlevnad även vid spridd sjukdom. Dessa behandlingar har nyligen även introducerats som tilläggsbehandling för vissa högriskgrupper för att förhindra återfall efter kirurgi, sk adjuvant behandling. Därmed finns det fler möjligheter till att kunna motverka spridning, men samtidigt ett större behov av att kunna identifiera och selektera rätt patienter till adjuvant behandling eller en anpassad uppföljning pga prognosen. Därför finns ett behov av ytterligare prognostiska faktorer då vissa högrisk patienter inte identifieras när man tar hänsyn till de för närvarande använda prognostiska faktorerna.

Syftet med avhandlingen är att finna prognostiska biomarkörer i tumörmaterial hos melanompatienter utan tecken på fjärrspridning (stadie I-III melanom) för att bättre kunna identifiera patienter som tillhör en särskild riskkategori.

I delarbete I och II undersöktes olika prognostiska faktorer hos patienter som blivit radikalt opererade för spridning till regionala lymfkörtlar, sk stadie III melanom. Körtelmetastaser från två olika grupper studerades, 19 patienter med lång överlevnad ≥ 60 månader respektive 23 patienter med kort överlevnad ≤ 13 månader efter körtelutrymning.
I delarbete I har en panel bestående av tre metaboliska proteiner (GAPDHS, GAPDH och PKM2) och ett pigmentrelaterat protein (TYRP1) visat att ett starkt uttryck av minst två av dessa fyra proteiner korrelerar till korttidsöverlevnad. GAPDHS är ett relativt okänt protein och har i avhandlingen identifierats som en ny prognostisk markör.
I delarbete II har en panel bestående av immuninfiltrerande T-celler med uttryck av CD8 och FOXP3, lågt uttryck av proliferationsmarkören Ki67 samt frånvaro av *BRAF* mutation visat en korrelation till långtidsöverlevnad när minst tre av fyra faktorer förekommer.

I delarbete III och IV har en kohort bestående av primärtumörer med ulceration från 71 patienter motsvarande stadie I-II melanom analyserats.
I delarbete III var en panel med avsaknad av *BRAF* mutation/låg andel av *BRAF* mutation, mindre grad av ulceration, låg tumörproliferation och förekomst av tumörinfiltrerande T-celler (TILs) korrelerat till färre återfall vid förekomst av minst två av fyra faktorer.
Vid undersökning i delarbete IV av TILs förekomst vid samtidigt uttryck av CD8, FOXP3 och GAPDHS+ tumörer förstärktes den prognostiskt gynnsamma bilden vid närvaro av minst tre av fyra faktorer. GAPDHS visade sig ha en motsatt prognostisk betydelse jämfört med resultaten i delarbete I på stadie III patienter, vilket ger stöd åt att proteinet kan ha flera funktioner.

Samtliga delarbeten visar ett ökat mervärde av att använda flera prognostiska markörer i kombination, jämfört med att använda en prognostisk markör enskilt, vid stadie I-III melanom.

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