From Department of Oncology and Pathology
Karolinska Institutet, Stockholm, Sweden

CLINICAL AND EPIDEMIOLOGICAL STUDIES ON PROGNOSTIC AND PREDICTIVE FACTORS IN CUTANEOUS MELANOMA

Hanna Eriksson

Karolinska Institutet

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Front page: Photo by Dr Ismini Vassilaki, Karolinska University Hospital Solna:
Invasive cutaneous melanoma stained with alkaline phosphatase.

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Till Bruno och Valter

...The new
always happens against
the overwhelming odds of
statistical laws and their
probability, which for all
practical, everyday,
purposes amounts to
certainty; the new
therefore always appears
in the guise of a miracle...
H. Arendt

ABSTRACT

Background Cutaneous malignant melanoma (CMM) is one of the most rapidly increasing cancers in Sweden, as in many other western countries. In clinical practice, the histopathological evaluation has remained the basis for staging the CMMs and, thus, providing important information on prognosis and on therapeutic recommendations. Interobserver variation regarding the histopathological evaluation is known to exist. However, few studies have investigated how clinical, biological and social mechanisms interact and influence the prognosis in CMM. For patients with advanced disease progress of the treatment options has been achieved for tumours carrying $BRAF^{V600}$ mutations by the development of specific small-molecule BRAF inhibitors. The development of these targeted therapies thus mandates determination of *BRAF* mutation status. This thesis aims to describe the interobserver variability in evaluating histopathological prognostic factors, to assess the association between education as well as cohabitation status and prognosis in patients with a primary CMM, and also to analyze the patterns of $BRAF^{V600E}$ protein expression in primary and metastatic CMMs.

Methods In Paper I, a total of 234 cases of invasive CMMs from the Stockholm–Gotland Healthcare Region in Sweden were included in the study**.** In Papers II and III, 27,235 patients diagnosed with a primary invasive CMM between 1990 and 2007 were identified from the Swedish Melanoma Register. Data were linked to nationwide, population based, health and census registers with a follow-up through 2010. In Paper IV, a total of 200 CMMs were stained by immunohistochemistry (IHC) using a $BRAF^{V600E}$ specific monoclonal antibody (VE1).

Results Overall, interobserver variability between a general pathologist and an expert review was 79% in Paper I. The best agreement was found for tumour thickness, but 15.5% of the tumours were re-classified after review in a sub-set of thin (≤ 1 mm) CMMs 15.5% were re-classified.
In Paper II, we found significantly elevated odds ratios (OR) of higher disease stage at diagnosis among lower education groups after adjustments for other prognostic factors (OR stage II vs. I = 1.6; 95% confidence interval (CI) = 1.5-1.7. OR stage III–IV vs. I = 2.3; 95% CI = 1.8-2.9). The risk of dying of CMM, was significantly increased in patients with low education after the final adjustments for all clinical and histopathological prognostic factors (hazard ratio (HR) low vs. high = 1.13; 95% CI=1.01-1.27; $p = 0.04$).
In Paper III, after adjustments for established prognosticators and education, the ORs of higher clinical stage at diagnosis were significantly increased among men living alone vs. men living with a partner (OR stage II vs. stage I = 1.42; 95% CI = 1.29-1.57. OR stage III-IV vs. stage I = 1.43; 95% CI = 1.14-1.79). The ORs for higher stage among women living alone were also increased (OR stage II vs. stage I = 1.15; 95% CI = 1.04-1.28. OR stage III-IV vs. stage I = 1.04; 95% CI = 0.79-1.37). After adjustments for all potential and established prognostic factors, HR for CMM death for men living alone vs. living with a partner was 1.33 (95% CI = 1.19-1.49; $p<0.0001$), indicating a residual adverse effect on survival not accounted for by these parameters.
In Paper IV, the VE1 IHC staining intensity varied between primary CMMs and matched metastases in 47% of the cases, as well as between separate metastases. We found a sensitivity of the VE1 antibody of 97% and a specificity of 80% for detection of $BRAF^{V600E}$ mutations. A comparable sensitivity was obtained for primary CMMs and metastases when analyzed separately. However, the specificity was lower among primary CMMs (71%) compared to metastases (93%).

Conclusions Our results imply that the recommendation of surgical excision margins and/or sentinel node biopsy changed in subgroups of thin CMMs after a CMM-expert pathology review. Moreover, the results emphasize the need for improved early detection strategies directed towards specific patient groups to improve the CMM-specific survival. IHC using the VE1 antibody should be used in combination with genomic testing in primary CMMs specifically in cases with weak/moderate staining to accurately predict $BRAF^{V600E}$ status, whereas in metastases with strong VE1 staining no further mutation testing seems to be required.
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LIST OF PUBLICATIONS

The following papers are referred to in Roman numerals in the thesis:

I. **Eriksson H**, Frohm-Nilsson M, Hedblad MA, Hellborg H, Kanter-Lewensohn L, Krawiec K, Lundh Rozell B, Månsson-Brahme E, Hansson J. *Interobserver variability of histopathological prognostic parameters in cutaneous malignant melanoma: impact on patient management.* Acta Derm Venereol. 2013 Jul 6;93(4):411-6.

II. **Eriksson H**, Lyth J, Månsson-Brahme E, Frohm-Nilsson M, Ingvar C, Lindholm C, Naredi P, Stierner U, Wagenius G, Carstensen J, Hansson J. *Low level of education is associated with later stage at diagnosis and reduced survival in cutaneous malignant melanoma: A nationwide population-based study in Sweden.* Eur J Cancer. 2013 Aug;49(12):2705-16.

III. **Eriksson H**, Lyth J, Månsson-Brahme E, Frohm-Nilsson M, Ingvar C, Lindholm C, Naredi P, Stierner U, Carstensen J, Hansson J. *Later stage at diagnosis and worse survival in cutaneous malignant melanoma among men and older women living alone: a nationwide population based study from Sweden.*
Manuscript submitted for publication, under revision.

IV. **Eriksson H**, Zebary A, Vassilaki I, Omholt K, Gadheri M, Hansson J. *$BRAF^{V600E}$ protein expression in primary cutaneous melanoma and paired metastases***.**
Manuscript submitted for publication, under revision.

CONTENTS

LIST OF ABBREVIATIONS

In the order the abbreviations appear in the text:

CMM	Cutaneous malignant melanoma
UVR	Ultra violet radiation
T-stage	Tumour stage (T1-4)
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
PD-L1	Programmed cell death-1 ligand
PD-1	Programmed cell death-1 receptor
CSE	Clinical skin examination
SSE	Skin self-examination
SES	Socioeconomic status
RAS	Mitogen-activated protein kinase
MAPK	Mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinase
AKT	Also known as Protein kinase B
NRAS	Neuroblastoma Ras viral oncogene homolog
PTEN	Phosphatase and tensin homologue
GTPase	Guanosine triphosphate hydrolytic enzyme
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GEF	Guanine nucleotide exchange factors
GAP	GTPase activating proteins
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
ERK	Extracellular-signal-regulated kinases
CRAF	Normal cellular homolog of v-Raf
MEK	Map-ERK kinase
CR	Conserved region in the
N-terminal	The start of an amino acid chain terminated by an amino acid with a free positively charged amine group (-NH_2).
C-terminal	The end of an amino acid chain terminated by a free negatively charged carboxyl group (-COOH).
ATP	Adenosine triphosphate
Amino acids V, E, D, K, R	Valine, Glycine, Aspartate, Lysine, Arginine
A, G	The purine bases (adenine, guanine)
C, T	The pyrmidine bases (cytosine, thymine)
UVA	Ultra violet A, (315–400 nm)
UVB	Ultra violet B, (280-315 nm)
PIP3	Phosphatidylinositol triphosphate
CDK	Cyclin-dependent kinase
G1/S phase	Gap 1/synthesis phase
Rb	Retinoblastoma protein
Hdm2	Human double minute 2
G2/M phase	Gap 2, pre-mitotic phase/mitotic phase
RGP	Radial growth phase

VGF	Vertical growth phase
SSM	Superficial spreading melanoma
NM	Nodular melanoma
LMM	Lentigo maligna melanoma
ALM	Acral lentiginous melanoma
MMM	Mucosal malignant melanoma
UMM	Uveal malignant melanoma
MBN	Malignant blue nevus
BN	Benign nevus
AJCC	The American Joint Committee on Cancer
T/N/M	Tumour thickness/number of metastatic nodes/site of metastatic nodes
LDH	Lactate dehydrogenase
GNA11/GNAQ	Guanine nucleotide binding protein (G protein), alpha 11/ Guanine nucleotide-binding protein G(q)
KIT	A receptor tyrosine kinase
BANS	CMMs located at the back, upper arm, neck and scalp
TIL	Tumour infiltrating lymphocyte
OS	Overall survival
RT	Radiotherapy
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
VEGF	Vascular endothelial growth factor
PDGF	Platelet-derived growth factor
HR	Hazard ratio
KA	Keratoacanthoma
SCR	The Swedish Cancer Register
RCC	The Regional Cancer Center
SCDR	The Swedish Cause of Death Registry
SMSG	The Swedish Melanoma Study Group
RMR	The Stockholm-Gotland Regional Melanoma Registry
SMR	The Swedish Melanoma Register
LISA	The Longitudinal Integration Database for Health Insurance and Labour Market Studies
PIN	The Swedish Personal Identity Number
SNB	Sentinel node biopsy
FFPE	Formalin fixed and paraffin embedded
PCR	Polymerase chain reaction
IHC	Immunohistochemsitry
K-M	Kaplan Meier
CI	Confidence interval
OR	Odds ratio

1 INTRODUCTION

Cutaneous malignant melanoma (CMM) as an entity was first described by René Laennec in "Sur les Melanoses" in the beginning of the 19th century.[1] More than a century later the major prognostic criteria for CMM, a standardised nomenclature and a form of reporting of the histopathologic features of CMM were defined.[2]

CMMs arise from the malignant transformation of the neural crest derived melanocytes located in the basal layer of the epidermis where the melanocytes and keratinocytes in the epidermis form the melanin unit.[3] The melanocyte contains the melanosome that produces the pigment, melanin. The melanosomes are transferred via the dendrites to surrounding keratinocytes as a defense to ultra violet radiation (UVR), where the melanin absorbs and dissipates ultraviolet energy.[3, 4] It is well established that UVR is the major risk factor for development of skin cancer, including CMM, by causing direct DNA damage, impairment of the cutaneous immune function, increased local production of growth factors, and formation of DNA-damaging reactive oxygen species.[4-6] It has, however, become clear that CMM is a genetically heterogeneous disease comprising of biologically distinct genetic subsets.[7] The progression from a common acquired nevus to a malignant tumour involves multiple steps including several genetic changes.[8] Still, in clinical practice, the histopathological evaluation of localized invasive CMMs has over the years remained the basis for staging the tumour which provides critical information on the prognosis and on therapeutic recommendations.[2, 9]

Since several decades, CMM is one of the most rapidly increasing cancers among Caucasian populations world-wide.[10-13] The increase of invasive CMMs has previously been most prominent in thin tumours (T1 tumours) with a low risk of recurrence and death, a trend possibly related to educational campaigns resulting in a higher proportion of cases with early diagnosis.[14-16] While CMM incidence and mortality in Sweden, specifically in women, seemed to have leveled-off during the 1990´s, unfortunately a continuous increase of both incidence and also in the proportion of high risk CMMs (T2-T4 tumours) is now observed, accompanied by a slight rise of the mortality.[16-19]This thus represents an increasing public health burden and the trend in Sweden is consistent with recent data from Europe, Australia and the U.S.[10, 13, 19, 20] Socioeconomic differences in cancer are often associated with life-style factors, knowledge or access to health-care and health seeking behavior, but this is still insufficiently investigated for CMM in a population-based nation-wide setting.

Recently improved therapeutic effects have been achieved in patients with metastatic CMM with novel targeted therapies perturbing both tumour cell- and immune system targets. For example, improved response rates and increased overall survival, compared to treatment

with conventional cytotoxic chemotherapy, have been achieved for patients with tumours carrying *BRAF*^{V600E} mutations by the development of specific small-molecule BRAF inhibitors.[21-23] The development of targeted therapies thus mandates determination of *BRAF* mutation status in patients with advanced CMM. There is also a need for identifying novel predictive markers for durable response in existing targeted therapies. Moreover, another challenge is to find novel therapeutic targets and pathways in the large proportion of tumours where existing targeted therapy is not applicable or has become inactive due to resistance.

The understanding of CMM has progressed considerably, but this process has also generated novel questions of clinical importance. This thesis includes four papers approaching some of these complex issues in CMM, with the aim to increase our understanding of the disease in different settings of patients.

2 BACKGROUND

2.1 EPIDEMIOLOGY

Increasing incidence of CMM has been observed in fair-skinned populations world-wide during the last decades. In a recent report on international trends in the incidence of CMM between 1953 and 2008 by the International Agency for Research on Cancer, the authors conclude that the incidence rates continue to rise in most European countries, whereas in Australia, New Zealand, the U.S., Canada, Iceland and Norway, rates have become rather stable in recent years mainly in the youngest age groups (25–44 years).[24]

Invasive CMM constitutes 5.8% of all cancers in Sweden and approximately 30,500 individuals have a diagnosis of CMM.[13] In 2011, 3323 new cases were registered and the tumour was thus the 6th most common cancer among men and the 5th among women. Incidence rates, standardised according to the Swedish population per 100,000 for the year 2011, was 36.9 per 100,000 males and 32.6 per 100,000 females.[13] There are pronounced geographical differences in incidence in Sweden with higher rates especially in the Southern and Western parts compared to the Northern areas.[13, 18] In Sweden, the CMM incidence seemed to level off for short period of time during the 1990´s with a lower annual increase of 1.1% for men and 0.4% for women, followed by a significant decrease in CMM incidence in the male population in the Stockholm-Gotland region.[16] The adverse trend has, however, strengthened and the average increase in incidence per year was 5.2% for males and 5.3% for females during the past decade in Sweden.[13] Compared to the corresponding increase based on the past two decades (3.5 and 3.6 % for men and women, respectively), it is clear that the increase in incidence rate is accelerating.[13]

The annual increase of CMM incidence varies between populations but has been estimated between 3% and 7% with the highest incidence world-wide in New Zealand and Australia.[10, 12, 20, 25, 26] In Europe the most pronounced increase in incidence is found in the Nordic populations, but also in Switzerland, the Netherlands and the Czech Republic.[10, 12, 24] The annual increase has been 4.3% in the Nordic countries over the past decade with the highest rates in Denmark.[12] In the US the increase in incidence has been approximately 3% per year the last decade.[20] However, recent data suggest the incidence rates are leveling off or slightly declining with significant trends in Australia (at ages 25–44 years) and in Iceland (among women, all ages).[24]

The CMM-specific survival has improved over the decades in many countries. Recently also a stabilization of the mortality rates has been observed in both Australia and the U.S.[10,

[20, 27-30] However, a trend of an increasing CMM related mortality has been shown in Sweden.[19] In 1999, 348 individuals died due to CMM and 499 persons in 2009. The standardised mortality rate according to the Swedish population for the year 2011, is 6.4 per 100,000 males and 3.8 per 100,000 females. The incidence trends vary greatly within Europe but mortality rates show less variation. The 5-year relative survival has increased from around 50% in the 1960´s to over 95% mainly due early detection and treatment. Consistently over time, women have a better CMM-specific survival compared to men.[31]

The increasing trends have been correlated to lifestyle and socioeconomic factors such as a change from sun-avoidance toward sun-seeking behavior and indoor tanning. However, the rise in incidence is most prominent in thin invasive tumours (T1 tumours) with a low risk of recurrence and death.[16, 32-34 35] This might be associated with both a higher awareness of CMM in the population and improved early detection by the health-care. However, a worrying trend of an increasing proportion of thicker high-risk tumours (T2-4; with a tumour thickness >1.0 mm) in Sweden ,as well as of the presence of ulceration, has been found in recent years as compared to the 1990´s.[18, 29, 34-37] Since the incidence rates of all Breslow thickness categories have increased in Sweden, as well as the mortality rates, the CMM epidemic appears not to be caused only by over-diagnosis.

2.2 PREVENTION

2.2.1 Primary and secondary prevention

Primary and secondary prevention are the major efforts in the reduction of the incidence and mortality from CMM. Substantial activities concerning primary prevention have been carried out over the past decades, yet the incidence of CMM continues to increase in many countries. Secondary prevention directed at early detection has been followed by a marked improvement of the 5-year relative CMM survival with an increase from 80% in mid-seventies to over 95% today.

Prevention activities were first initiated in Australia from the 1960´s, and coordinated primary prevention campaigns were introduced from the 1980´s in Australia, the U.S. and also in several European countries including Sweden.[16, 38, 39] The objective was to encourage reduction of UVR exposure. The Sun Smart project is still on-going in Australia since the late 1980´s. In Sweden, the prevention campaigns started in the Stockholm-Gotland health-care region in the mid 80´s and some years later the preventive work was initiated also in the other health-care regions.[40]

It has been discussed whether the stabilizing or decreasing incidence rates among cohorts born in the 1960´s and 1970´s are a result of public health campaigns. However, since

primary prevention interventions take considerable time until effects are found at the population level, the interval after the campaigns might be too short for the observed reduced rates to be the direct result of such interventions.[41, 42] The prevention efforts in Queensland in Australia have emphasized primary prevention whereas prevention for example in Scotland has largely been directed towards improving early detection and treatment of CMM.[32, 42] The success of secondary prevention has been related to increased awareness of CCM and curative surgical treatment of early tumours.[41, 43] In future, primary prevention may be improved by molecular markers for CMM risk.

2.2.2 Skin examination

The early detection of CMM can be performed by health care professionals and at the individual level by self-examination. The detection guidance for the public has focused on ABCDE criteria: *A* for asymmetry, *B* for irregular border, *C* for multiple colors, and *D* for diameter >5-6 mm and *E* for evolving.[2] These rules have some limitations, though, for example when the tumours have other clinical features or are smaller than 6 mm, which is a growing group of tumours as the clinicians are improving early detection.[44, 45]

Clinical skin examinations (CSE) and skin self-examinations (SSE) have through the years been performed and taught in order to increase the detection of early low-risk, curable CMMs. In approximately 40-60% of all incident CMMs, the patients or family members initially discover the tumour.[46] Women are more likely to perform SSE as compared to men (69% vs. 47%, respectively), while men more often report that the CMM first was detected by the spouse.[46, 47] Other factors except for gender that also correlate with performance of SSE are age, socioeconomic status (SES) or level of education, a previous diagnosis of CMM, information about SSE from the health-care or a previous CSE and awareness as well as perceived risk of developing CMM.[34, 36, 37] Moreover, tumour thickness at diagnosis is associated with gender, educational level, other measures of SES and absence of CSE.[46, 48, 49] There have to date been no randomized controlled trials demonstrating that screening does reduce mortality from CMM. However, Berwick et al.[50] showed in 1998 that SSE may decrease CMM mortality by 63%.

The frequency of both CSE and SSE seem to vary between populations. In the Nordic countries, as compared to Australia and the U.S., the frequency of skin examinations is low.[51] As described above, male patients also in a Swedish setting were found to more seldom or never pay attention to bodily changes such as skin tumours.[52]

2.3 SOCIOECONOMIC STATUS

A socioeconomic gradient in incidence and survival rates has been reported in different populations for various health conditions, including cancer.[53] In most studies, SES is not a

strong independent prognostic factor, but the impact of SES on outcome is relevant in relation to biological and clinical markers of prognosis. SES may be associated with life-style factors, access to health-care, participation in cancer screening programs, adoption of health seeking behavior, residential area as well as exposure to biological and carcinogenic agents.[53] The time to diagnosis and the time from diagnosis to death could both be correlated to socioeconomic disparities in diagnostic procedures, screening, compliance and treatment discrepancies.

Income, occupation and level of education are the SES measures most often used. These measures are often correlated but are not always interchangeable in health outcome research.[53] Income describes access to material goods and services that may influence health, but this measure is considered less stable and is to a wider extent age-dependent. Occupation is the link between education and income.[53] The variable provides more specific information on environmental and working conditions, but the measurement lacks precision since it often comprises a heterogeneous grouping of occupation according to education, income and prestige. Education may therefore reflect the socioeconomic gradient in a more relevant way. Level of education is relatively easy to measure, excludes few groups in the population, is considered more stable than either income or occupation, is not affected by retirement and is mostly reached quite early in life.[53] There are several possible mechanisms thorough which education has an impact on health status. For example, persons with higher education might have developed better health-related knowledge as well as skills in navigating and interacting effectively within the healthcare system.[49, 53] However, birth cohort effects need to be taken into account in using all individual- or household level measures of SES. Also, low SES among cancer patients is associated with a significantly higher risk of comorbidity, for example cardiovascular disease, chronic obstructive pulmonary diseases, diabetes mellitus and cerebrovascular disease independently of the socioeconomic indicator used.[54] Survival differences between SES groups may therefore partly be explained by other diseases. Many countries have not registered SES data on the individual level and proxys on the household or community level are used instead. In most Nordic countries, including Sweden, SES measures on the individual level are registered in national databases which facilitate large-scale epidemiological research.

It is established that patients with high SES have an increased risk of CMM but there is an inverse correlation with a reduced survival for low-SES CMM patients.[55-64] The reduced survival is mainly due to thicker tumours and later stages at diagnosis among these patients.[48, 58, 59, 61, 63-66] Inequalities in survival and treatment intensity in patients with different SES have also been found for other cancers in Sweden.[67-69] Only a few studies have been conducted to explore the impact of cohabitation status on CMM survival. Unmarried and

widowed older patients have been found to have a higher risk of CMM-related death compared to married patients. [61, 70]

Cohabitation status is not included in the concept of SES, but marital or cohabitation status may be associated with survival, also in cancer patients.[71-73] Married individuals have a higher 5-year survival in cancer in comparison with unmarried, divorced and widowed persons.[71] These differences could be explained by later stages of disease at diagnosis and lower frequency of receiving potent treatment if unmarried/living alone, but also by social isolation and lack of social relationships.[71, 72]

2.4 SIGNAL PATHWAYS INVOLVED IN MELANOMA PROGRESSION

Malignancies are induced by accumulation of genetic changes affecting the function of oncogenes and tumour suppressor genes.[74] Tumour progression has been described to start from a single genetically changed cell, followed by malignant clonal expansion secondary to different biological capabilities acquired during tumour progression.[75] Inappropriate activation is found in several signaling pathways in CMM. Two of the most well described effector pathways downstream of RAS are frequently altered in CMM; the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinaseinhibitor PI3K/AKT (Figure 1).[76] Activating point mutations in *NRAS* and *BRAF* cause constitutive activation of the MAPK-pathway leading to increased proliferation and survival of the tumour. The PI3K-pathway is also crucial in CMM tumourgenesis by selective activation of the downstream AKT protein or by genetic changes that decrease the expression of PTEN, which also leads to a constitutive activation of the PI3K-pathway and increased AKT expression.

2.4.1 The MAPK-pathway

2.4.1.1 RAS

The RAS proteins belong to a family of GTPases located at the inside of the cell membrane, and are involved in signaling downstream from membrane bound receptors.[77] In normal cells, RAS has an inactivated GDP-bound state which is activated through upstream stimulatory signals. GDP is thereby replaced by GTP through the GEFs (guanine nucleotide exchange factors). The signal transduction is rapidly inactivated by the GAPs (GTPase activating proteins) which are stimulating GTP hydrolysis. Three distinct variants of the *RAS* gene have been identified that encode different proteins: the *NRAS* gene on chromosome 1, the *HRAS* gene on chromosome 11 and the *KRAS* gene on chromosome 12. However, CMMs carry almost exclusively *NRAS* mutations, which occur in approximately 20% of the tumours, while *HRAS* or *KRAS* are infrequently mutated.[7, 78-80] *NRAS* mutations occur predominantly at codon 61 (Q61R/K/L) and more rarely at codons 12-13. Activating

mutations in *NRAS* can lead to parallel activation of both the MAPK and the PI3K-pathways through extracellular-related kinase (ERK) kinases which phosphorylate targets in the cytoplasm of the cell and interact with other pathways including PI3K (Figure 1).

2.4.1.2 RAF

In normal cells, GTP bound RAS activates the *RAF* family of serine/threonine kinases by phosphorylation within the kinase domain.[8, 81] Except for *BRAF*, residing at chromosome 7q34, there are two more known isoforms of *RAF*.[82, 83] CRAF is also activated by RAS and stimulates the MAPK-pathway downstream including MEK and signaling events outside the MAPK pathway. ARAF is the third and least investigated RAF isoform. However, the isoforms are structurally related, but BRAF demonstrates the highest basal kinase activity. RAS binds to one of the conserved regions, CR1, at the N-terminal of RAF.[84] This region contains the RAS binding domain and a cysteine-rich domain whereas CR3 at the C-terminal contains the kinase domain. BRAF only requires phosphorylation of two sites (Thr598 and Ser601) within the activation segment of the kinase domain as compared to CRAF and ARAF that require phosphorylation of additional sites to become active.[82] MEK is the only known substrate of BRAF and exerts the effects of BRAF after being phosphorylated.[8] MEK1 and MEK2 activate ERK1 and ERK2 which either activate cytoplasmic targets or migrate to the nucleus, where they phosphorylate transcription factors (Figure 1).

Over 50% of patients with CMM harbor activating mutations in the oncogene *BRAF* which is highly expressed in tissues of neural crest origin such as melanocytes.[7] More than 50 distinct mutations of the *BRAF* gene have been identified. Several of these mutations are located in the glycine residues within the glycine rich loop of the kinase domain which anchors the beta- and gamma-phosphates of ATP causing the catalytic activity. In CMM, the substitution of valine (V) for glutamic acid (E) at codon 600 of the kinase domain in *BRAF* (*BRAF*^{V600E}) accounts for approximately 90% of mutations of *BRAF*.[85] This results in a constitutive activation in downstream signaling through the MAPK-pathway promoting proliferation and decreased cell death of the tumour.[85, 86] Alternative point mutations at codon 600 (*BRAF*$^{V600D/V600K/.V600R}$) contribute to 5–6% of the *BRAF* mutations.[79] However, the T>A nucleotide change in *BRAF*^{V600E} is distinct from the "UV-signature" changes CC>TT/C>T associated with pyrimidine dimer formation by UVB or the G>T change by UVA found in non-melanoma skin cancers.[85, 87] This suggests that the *BRAF* mutations may reflect a secondary effect of UVR damage.

Inter-and intratumoural variation in *BRAF* mutation status among primary and metastatic CMM specimens has been reported.[88, 89] Primary CMMs are often polyclonal and may

contain a heterogeneous mixture of *BRAF*^{V600E} and *BRAF*^{wt} tumour cells. This is of importance to optimize treatment with targeted therapies as well as to understand and overcome acquired resistance.

2.4.2 The PI3K/AKT pathway

2.4.2.1 PTEN/AKT

The tumour suppressor gene phosphatase and tensin homologue (*PTEN*), located on chromosome 10q23, has also been identified to be involved in CMM progression.[90] Phosphoinositide 3-kinase (PI3K) is a downstream effector of RAS. Under normal conditions, PI3K generates activation of phosphatidylinositol triphosphate (PIP3) leading to phosphorylation of AKT (Figure 1).[91] AKT inactivates proteins that suppress the cell cycle or stimulate apoptosis, thereby facilitating the proliferation and survival of cells. PTEN down-regulates the PI3K pathway signaling by dephosphorylating PIP3, which induces cell cycle arrest and apoptosis. PTEN also up-regulates the cyclin-dependent kinase p27 which arrest the cell-cycle progression at the G1/S phase, as well as inhibiting the focal adhesion formation, migration and growth factor-stimulated MAPK signaling. Loss of *PTEN* activity through deletions or mutations occurs in 25 to 50% of CMM.[91] This attenuates the levels of PIP3 and the activation through AKT.[79] AKT activity can also be increased in cells by gene amplification and overexpression of the AKT protein.[92]

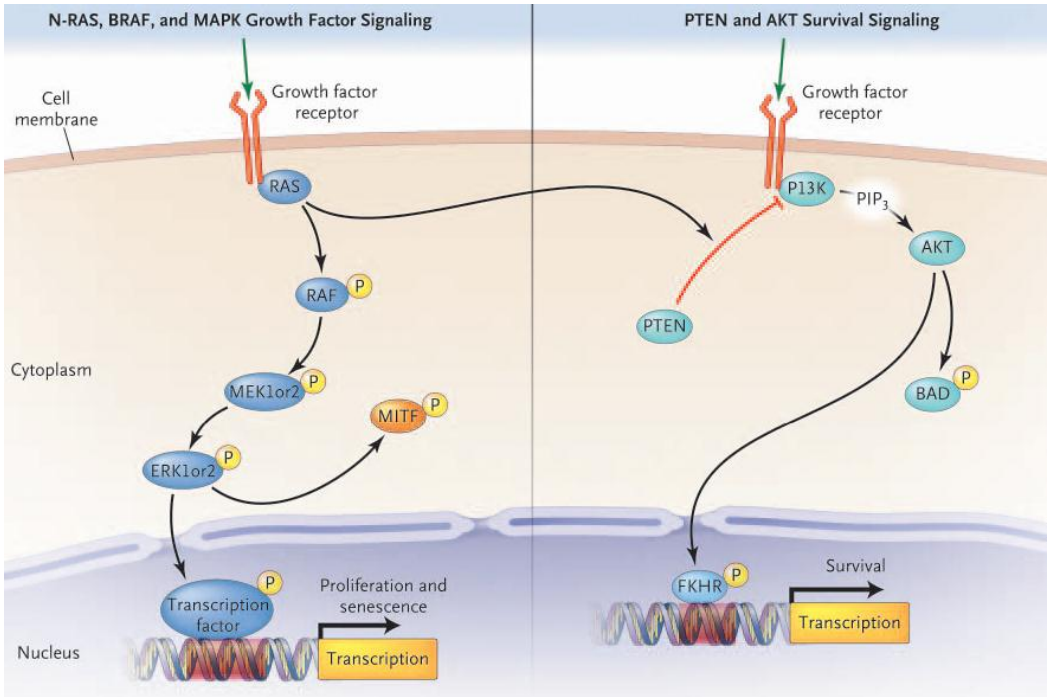

Figure 1. The MAPK- and PI3K/AKT pathway. Reproduced with permission from Miller et al. N Engl J Med 2006; 355:51-6; Copyright Massachusetts Medical Society.

2.4.3 The p16-CDK4-PRb pathway

Loss of tumour suppressor genes occurs in CMM, usually accompanied by mutated oncogenes within the same tumour. *CDKN2A*, located on chromosome 9p21, is a high-susceptibility gene with germline mutations in a minor proportion of familial CMM.[93] More rarely, CMM kindreds may carry germline genetic changes in CDK4 disrupting the cell-cycle control.[93] Somatic mutations in CDKN2A are relatively common in cell-lines, but are not as frequent in primary CMMs.[94] However, deletions of *CDKN2A* are more frequently occurring in CMM metastases than primary tumours, indicating the importance of this tumour suppressor gene in CMM progression.[95] *CDKN2A* is encoding two different tumour suppressor proteins through alternative splicing, $p16^{INK4A}$ and $p14^{ARF}$. Activation of p16 blocks the phosphorylation of the retinoblastoma protein (Rb) by inhibiting cyclin-dependent kinases causing cell cycle arrest at the G1–S checkpoint.[8, 96] The tumour suppressor p14 binds human double minute 2 (hdm2) and thereby relieves the tumour suppressor p53.[8] Hdm2-mediated p53 degradation blocks cell proliferation by inducing cell-cycle arrest at the G2–M site. This allows for repair of damaged DNA or the induction of apoptosis. Inactivation of either p16 or p14 thus results in uncontrolled cell proliferation.[8, 97] The relatively low frequency of mutations in p53 could therefore be explained by alterations upstream either in hdm2 or p14. *CDKN2A* and *CDK4* alterations are generally reciprocal as are mutations in the tumour suppressor genes *p14* and *p53*.

2.4.4 The relation between different signal pathways

The relation of genetic changes in *PTEN*, *RAS* and *RAF*, in the context of the PI3K-AKT and RAS-MAPK pathways is important to understand in CMM progression. In CMM, *BRAF* mutations are rarely found in isolation, but occur in relation to other somatic genetic alterations.[98] Mutations in *NRAS* and *BRAF* are nearly always mutually exclusive.[78, 99, 100] Approximately 50% of *BRAF* mutated CMMs harbor *PTEN* mutations or have deletions or epigenetic silencing that results in significantly reduced PTEN expression.[79, 91] On the contrary, mutations in *NRAS* and *PTEN* seem not to coincide. It has therefore been suggested that concurrent *BRAF/PTEN* mutations function like *NRAS* mutations. Both PI3K and AKT may in turn directly alter RAF kinase activity. Activation of *BRAF* alone results in benign nevus formation, while malignant transformation seem to require concurrent loss of *p53* and inactivation of *p16*.[8] Both genetic changes of *NRAS* and *PTEN* appear to cooperate with *CDKN2A* loss which is contributing to CMM tumourgenesis.[91, 102]

2.5 TUMOUR PROGRESSION

2.5.1 Histological model of progression

Tumour progression from the formation of a benign nevus (BN) to metastatic CMM includes clinical, histopathologic and genetic changes. Clark et al.[103] have defined a five step model of progression based on clinical and histopathologic features within the tumour.

1. The first step is the formation of a BN from structurally normal melanocytes.
2. The DN progresses within a pre-existing BN or as a new lesion with sporadic cytologic atypical cells.
3. The distribution of melanocytes separates RGP from VGP. The tumour cells expand within the epidermal and superficial dermal parts during the early phase of CMM growth (RGP). Continuous atypia is found. If there are solitary or clusters of CMM cells in the papillary dermis, none are larger than any of the intraepidermal nests and none demonstrate mitoses. The RGP corresponds to Clark level I and II featuring only solitary or small nests of 5-10 tumour cells in the superficial stratum papillare.
4. In the VGP aggregates of tumour cells (15-25 CMM cells) invade and are identified in the dermis, and at least one nest in the dermis is larger than the largest intraepidermal nest, or mitoses are identified in any of the dermal CMM cells demonstrating proliferative activity. Ultimately the tumour may expand through the dermis to the subcutaneous fat.
5. The CMM metastasizes through invasion of tumours cells via the stroma, blood and lymphatic vessels to the skin, lymph nodes and distant organs.

Tumours are also considered to exhibit cancer stem-cell like properties which also could explain CMM growth and progression.[101]

2.5.2 Molecular model of progression

Tumour progression is a stepwise process reflecting genetic alterations that cause the malignant transformation of normal cells by selection and clonal expansion of tumour cells enabling tumour growth and metastatic dissemination.[75] Mutations in the *RAS* genes are major events in the tumour progression of CMM where MAPK activation is known to be an early event in tumour progression.[100, 104] The *BRAF* mutations, like the mutually exclusive *NRAS* mutations, have been reported to occur early in the disease and seem to be preserved throughout tumour progression.[99] Both *NRAS* and *BRAF* mutations have been shown to increase with tumour progression from superficial to invasive disease.[105] *BRAF* mutations are also present in common acquired BN as well as in dysplastic nevi (DN), primary and metastatic CMMs.[100, 106-108] However, only a minor proportion of nevi undergo malignant transformation most probably because mutated *BRAF* increases the expression of tumour suppressor genes causing cell-cycle arrest and senescence.[109] This suggests that additional molecular events must occur in the nevi to become malignant (Figure 2). The *BRAF*

mutated BN seem to transform into an invasive CMM if concomitant *CDKN2A* mutations inactivating *p16* or deletions in *PTEN/p53/CDKN2A* are present.[8, 110, 111] Loss of or somatic mutations of *PTEN* seem to be less frequent in primary CMMs as compared to more advanced CMMs suggesting that PTEN participates in the later stages of progression of CMM. *BRAF* mutations occur irrespective of primary Breslow thickness whereas *PTEN* loss has been correlated with increasing Breslow depth and tumour progression.[79] Loss of *p14* and *p53* increase survival of CMM and tumour transformation.[97] Several genes, for example for β3-integrin and angiogenic factors and cadherins, have been associated with the transition from radial to vertical growth causing invasion and spread.[8] However, the genetic alterations involved in the progression from nevi to invading CMM still remain not fully explored.

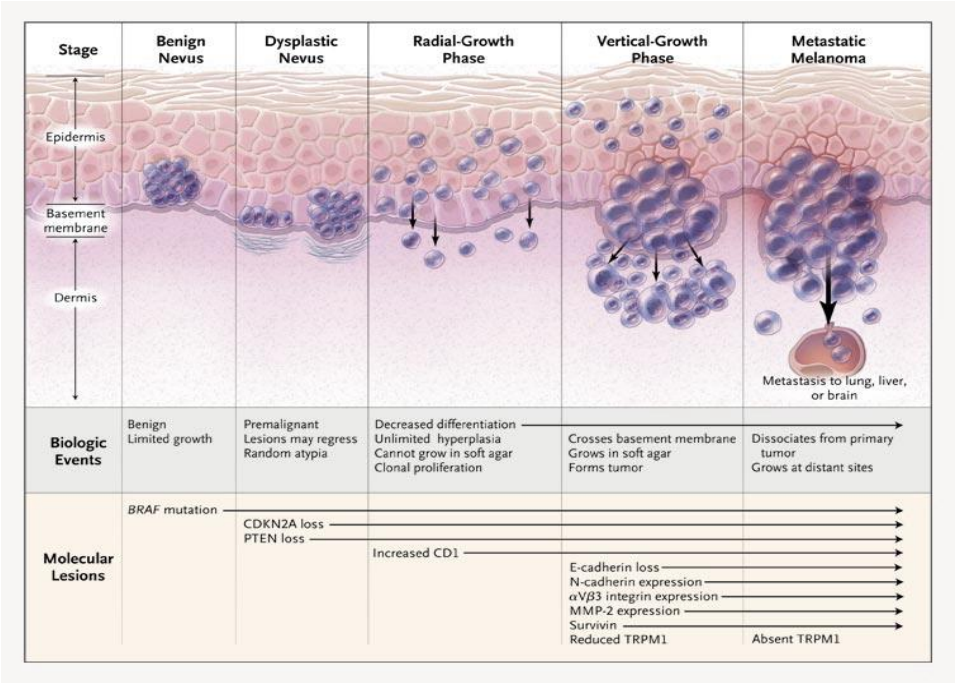

Figure 2. Biologic events and molecular changes in the progression of CMM.
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2.6 STAGING

2.6.1 The American Joint Committee on Cancer Staging System

The staging of CMM is the basis for the management and correlates with survival. The CMMs are categorized as local, regional and distant disease. The American Joint Committee on Cancer (AJCC) staging system is applied and the most recent revision was published in 2009.[9] The current system is based on the AJCC Melanoma Staging Database (data through 2008) containing prospective data on 30,946 patients with stages I, II, and III CMM and 7,972 patients with stage IV CMM treated at 17 medical centers in the U.S. Mitotic rate was included for the first time in this version and the mitoses are determined by

the “hot spot” technique and expressed as the number of mitoses per square millimeter of primary tumour.[112] Also the 2002 AJCC staging system was applied for the purpose of the thesis.[9, 113]

Clinical staging includes microstaging of the primary CMM and clinical/radiologic evaluation for metastases. In the thesis stage refers to clinical stage at diagnosis.[9] Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial (i.e. sentinel node biopsy) or complete lymphadenectomy.[9]

2.6.1.1 Localized disease, stages I and II (the T stage)

The primary criteria for localized tumours (stages I and II) is tumour thickness (T) measured in mm and the presence or absence of ulceration. In the AJCC 2009 Melanoma Staging and Classification (AJCC 2009) mitotic rate has recently replaced level of invasion according to Clark in defining T1 “a” and “b” sub-categories.[9, 114] In AJCC 2009 mitotic rate is treated as a dichotomized variable with a cut-off value of 1 mitosis/mm.[9] T1b CMMs are now defined as those CMMs for which the tumour thickness is ≤1.0 mm and for which there is at least 1 mitosis/mm^2 and/or tumour ulceration. There is a great variation in survival based on the TNM classification. The 5-year and 10-year survival rates range by sub-stage from 93 to 97% for patients with T1aN0M0 CMMs, respectively, to 39-53% and 39%for patients with T4bN0M0 CMMs.[9] T1 CMMs are considered to have a good prognosis, but for this group of tumours the 10-year survival varies between 85 and 99% depending sub-group.[9 115]

2.6.1.2 Regional metastatic disease, stage III (the N stage)

Stage III includes patients with regional metastases (N), either within the lymph node or as satellite or in transit metastases. There is a marked difference in 5-year survival rates from approximately 30 to 70% depending on the number of metastatic lymph nodes.[9]

2.6.1.3 Distant metastatic disease, stage IV (the M stage)

The sites of metastases and elevated serum levels of lactate dehydrogenase are used to classify the M1 stage into three M categories:
M1a (skin, subcutaneous tissue, or distant lymph nodes and a normal LDH level),
M1b (lung or with a combination of lung and M1a metastases and a normal LDH level) and
M1c (any other visceral sites or at any location with an elevated LDH level).
The general prognosis is low for stage IV CMMs and is further deceased by an elevation of LDH. The 2-year survival ranges from approximately 18 to 30%.

2.7 CLASSIFICATION

2.7.1 Classification by subtype

The clinical classification of CMM was first described by W. Clark from the 1960´s and included three types of CMMs: superficial spreading melanoma (SSM), nodular melanoma (NM) and lentigo maligna melanoma (LMM).[116] In the 1970´s acral lentiginous melanoma were added to the classification.[117] A major feature for classification is the distribution of melanocytes within the radial (RGP) and vertical growth phases (VGP), discussed in the Tumour progression section. The classification based on Clark et al.[116] expands to clinical, epidemiological and histopathologcial features of the CMMs.[18, 118, 119]

SSM is the most common histologic type of CMM accounting for about 70% of all cases and is associated with a RGP. The sites affected are on skin of intermittent sun exposure most frequently the back of men and the legs of women.

NM account for between 15 to 30% of all CMM cases according to age. The tumour type is more common among older men and is often located at the head-neck area or the trunk. NM, by definition, has no significant preceding RGP, suggesting an accelerated transition to the VGP.

LMM represents 4 to 15% of all CMMs and are found preferentially in the head-neck region associated with high levels of accumulated sun exposure. The progression from lentigo maligna type of CMM (in situ) to LMM may occur over a longer period of time compared to other types of CMMs. The tumour is thus characterized by a longer duration of the RGP.

ALMs constitute only 1.5% of all CMMs among patients with Caucasian origin and are located on palms, feet, soles and as subungual lesions. This type of CMM is the most common type among patients of Asian, Hispanic or African origin.

2.7.2 Molecular classification

Clinical and histopathologic features of the CMM have been shown to correlate with specific genetic alterations. Genetic changes of *BRAF, NRAS; AKT, PTEN, CDKN2A* and *CDK4* may occur in all histologic types of CMM. Activating mutations in *GNA11/GNAQ* are uniquely found in uveal CMMs.[120] CMMs from acral, mucosal and sun-damaged sites harbor mutations and/or amplifications of *KIT*.[121]

Several studies have found that *BRAF* mutations in CMMs are associated with younger age, truncal site, the SSM/NM subtypes and on intermittent sun-exposed skin.[7, 122, 123]. A worse prognosis has been reported in some studies for patients with *BRAF* mutated CMMs compared to $BRAF^{wt}$ tumours whereas others have not found survival differences.[122, 123]

There is not as clear results for phenotypic characteristics of *NRAS* mutated CMMs as for *BRAF* mutations. The *NRAS* mutated tumours are found in older individuals, in a higher

frequency on intermittently sun-exposed areas, are correlated with SSM/NM subtypes and relatively more often occur in the head-neck region compared to the limbs and trunk.[122, 124]

Table 1. Molecular classification of melanoma

Histogenetic type	Type of mutation	Frequency
SSM / NM	*BRAF*	50-60%
	NRAS	20%
LMM / ALM / MMM*	*CKIT*	10-15%
UMM** / MBN***	*GNAQ GNA11*	50%

* mucosal malignant melanoma
** uveal malignant melanoma
*** malignant blue nevus

Furthermore, *NRAS*-mutant CMMs are more frequently associated with thicker tumours and deeper level of invasion. p16 and $p14^{ARF}$ are both frequently inactivated in CMMs arising on chronically exposed skin.[97, 125]

2.8 PROGNOSTIC FACTORS

2.8.1 Localized disease

2.8.1.1 Clinical factors

Age and *gender* are independent prognostic factors.[113, 126-128] Older age is correlated with thicker tumours, but age is also independently associated with lower survival in CMM as is male gender.[18, 113, 31, 129]

The *anatomic site* of CMM varies between men and women. In men, the tumours are most often localized on the trunk and in women on the lower extremities.[18, 130] The site is also correlated with the prognosis where a worse outcome has been found for CMMs located at the back and in the head-neck region compared to the lower extremities.[126, 130] Although tumour site significantly affected survival in several studies[127, 128, 131], other studies suggest that there are no significant survival differences among CMMs located at the back, upper arm, neck and scalp (BANS) as compared to non-BANS areas, even when adjusted for other prognostic factors.[132]

2.8.1.2 Histopathologic factors

The histopathological evaluation provides critical prognostic and staging information. Tumour thickness according to Breslow[133] and ulceration[134] are considered the most powerful independent prognostic factors in localized invasive CMM.[114, 126-128, 135] Generally, both variables also have a high interobserver agreement.[136-144]

Tumour thickness according to Breslow is measured from the granular layer of the epidermis, or from the base of an ulcer, to the deepest point of invasion in the dermis.[133, 145, 146] The measurement sometimes involves detached nests of tumour cells but adventitial dermal invasion, within the perineural space or within vessels are not included in this measurement. Thin CMMs (≤1 mm in tumour thickness) are low risk tumours for recurrence or death. However, the current AJCC staging system uses a cut-off of 1 mm rather than the original Breslow cut-off of 0.76 mm.[9, 133] Recent data emphasizing the need to consider a lower threshold for tumour thickness in this group of CMMs since tumours with a thickness of around 0.7-1.0 mm appear to have a slightly reduced prognosis within the group of thin CMMs.[14, 115, 147, 148] Combining clinical and histopathologic prognostic factors may further help identify subgroups of patients with a worse prognosis.[14, 148]

Tumour ulceration is defined as the absence of an intact epidermis overlying a portion of the primary CMM based on pathologic microscopic observation.[134, 145] Presence of ulceration is correlated with a worse prognosis and the CMM is upstaged one category in the same thickness group.[9]

Mitotic rate is reflecting cellular proliferation within the primary tumour. The cut-off points for mitotic rate in CMM proposed by Clark et al.[147] were set to 0, 0.1–6, and >6 mitoses/mm^2. Other reports have identified other thresholds of mitotic rate in correlation with survival for CMM. In the 2009 AJCC staging of CMM the most significant correlation with survival was identified at a threshold of at least 1 mitosis/mm^2, resulting in upstaging of T1 CMMs from T1a to T1b. In several previous studies an increasing mitotic rate has been correlated to a worse prognosis in patients with localized primary CMMs.[9, 128, 149] Reports on the interobserver reproducibility of mitotic rate have revealed concordance varying from low to high.[112, 138, 146, 150].

Clark´s level of invasion[151] refers to the anatomical levels of tumour invasion representing epidermis (I), invasion of papillary dermis (II), expansion in papillary dermis (III), invasion of reticular dermis (IV) and invasion of the subcutaneous fat tissue[145, 151] A correlation between anatomic depth of the tumour and prognosis has previously been found for thin (≤1 mm) CMMs. Although Clark´s level of invasion has been considered a significant prognostic factors in T1 CMMs[114], many investigations have not confirmed this correlation and mitotic rate has recently replaced Clark´s level of invasion in staging T1 CMMs as already described above.[9, 128, 131] Previous studies have shown a low to intermediate agreement between pathologists.[136, 142]

Tumour infiltrating lymphocytes (TILs), an immunologic host response against the tumour appearing during the early phase of *regression*, are characterized by lymphocytes which are infiltrating tumour nests throughout the vertical growth phase or at the base of the vertical growth phase.[147] The TILs are distributed in different patterns and with varying density within the primary CMM and are sometimes categorized as brisk, non-brisk or absent. The

resulting regression may cause an entire disappearance of the CMM. The presence of TILs in CMM has been shown to be correlated with a favorable prognosis in some studies[152, 153] whereas other studies have failed to demonstrate such an association.[131, 154] Some studies have shown that the metastatic rate is higher in thin melanomas with extensive regression.[155]

Histogentic type is associated with prognosis, being worse in the case of NMs, but more favorable for SMMs. However, also the inverse relation has been reported and in most studies this variable is not an independent prognostic factor.[127, 131]

Radial and vertical growth phase are closely correlated to tumour thickness and are therefore not considered independent prognostic factors in the majority of studies.

2.8.2 Metastatic disease

In stage III CMM (regional metastatic disease), the number of tumour-bearing nodes, tumour burden at the time of staging (i.e., microscopic vs. macroscopic), presence or absence of primary tumour ulceration, and thickness of the primary melanoma are independent prognostic factors according to the 2009 AJCC staging of CMM.[9] The site of metastases and elevated serum levels of LDH are established prognostic markers in stage IV CMMs (distant metastatic disease).[9] However, the outcome is related to the clinical stage at diagnosis, and patients with metastatic disease have a survival between 8-18 months according to subgroup.[9] By the introduction of new therapies, the relapse-free and overall survivals have improved for patients with advanced tumours.[21-23]

2.9 MANAGEMENT

2.9.1 Surgery

Surgery of early CMMs is crucial for the prognosis, but local surgical excisions and lymphadenectomy are also appropriate as the initial treatment for recurrent or metastatic CMM.[2] The recommended surgical margins have changed over the years. There are few studies performed on surgical margins for tumours <1 mm and >2 mm of tumour thickness. Surgical margins of 1-2 cm have been analyzed for CMMs with a tumour thickness of 1-2 mm in the majority of cases and the results are similar according to overall survival (OS), CMM-specific survival and relapses.[17, 156] Recently a large randomized study conducted by the Swedish Melanoma Study Group showed that a 2 cm resection margin is sufficient and safe for patients with CMM thicker than 2 mm.[157] According to the newly revised national management guidelines in Sweden, surgical treatment of primary CMMs with a thickness of ≤1 mm (T1 CMMs) is performed with an excision of skin and subcutaneous tissue down to the underlying muscular fascia with one centimeter free lateral margins. In T1b CMMs also a sentinel node biopsy may be considered. Thicker CMMs

>1.0 mm (T2-T4 CMMs) are excised with a two centimeter margin and usually a sentinel biopsy is performed. In routine practice, margins of excision refer to the surgical margins measured by the operating physician, not to the pathology report. In stage III disease, surgical excision of all lymph nodes in the regional lymphatic draining area is the recommended surgical treatment, either electively or after SNB. Surgery is the first choice also for limited regional recurrences. Screening for distant metastases is performed before surgery in patients with recurrent or metastatic disease. All tissue samples should undergo a histopathological evaluation.

2.9.2 Radiotherapy

Radiotherapy (RT) is not used following radical surgery for primary CMMs, recurrent CMMs or regional cutaneous metatstases since the effect is limited. In Sweden, RT postoperatively is recommended after lymph node dissection if the excision margins are narrow, if 4 or more lymph nodes are involved, if extranodal invasion is present or if the metastatic nodal size is >3 cm.[158, 159]

2.9.3 Systemic treatment

Systemic treatment is the alternative for unresectable stage III and for stage IV disease. These patients are often enrolled in clinical trials. Major advancements in the treatment of metastatic CMM have recently been achieved with the approval of the Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) blocking monoclonal antibody ipilimumab (today approved as second line treatment in the European Union) and *BRAF*^{V600E} and/or MEK kinase inhibitors. However, cytotoxic chemotherapy continues to be an important treatment in disseminated CMM. Although not having a demonstrated OS benefit, chemotherapy is used for palliation of patients with CMM that are ineligible for treatment with ipilimumab, that do not harbor a *BRAF*^{V600E} mutation or with resistance to new treatment alternatives.

Below follows a description of BRAF inhibitors in more detail since this treatment is correlated to Paper IV.

By the development of selective BRAF inhibitors improved therapeutic responses have been obtained in patients with metastatic CMM carrying *BRAF*^{V600E} mutations, translating into prolongation of both progression-free and OS as compared to treatment with conventional cytotoxic chemotherapy.[21-23] The first small-molecule RAF inhibitor was sorafenib which inhibits multiple kinases, including BRAF, CRAF, and the VEGF and PDGF receptor tyrosine kinases.[160] This non-selective RAF inhibitor has not demonstrated survival benefits as monotherapy in patients with advanced CMM.[161] Vemurafenib was the first selective BRAF inhibitor that showed significant effects on survival in a clinical setting.[162] By binding to the kinase domain, the small-molecule BRAF inhibitor has its

effect on the active conformation, thus inhibiting MAPK pathway signaling and thereby also the proliferation in CMM.[162, 163] Median survival during treatment is approximately 16 months and median progression-free survival approximately 7 months [22] A reduction in Hazard ratios (HR) of >60% for death and >70% for death or progression has been demonstrated for vemurafenib as compared to DTIC.[21] A side effect found with vemurafenib is the development of keratoacanthomas (KA) and invasive squamous cell carcinomas of the skin. A stimulatory effect of selective BRAF inhibitors on the activity of the MAPK pathway seems to occur in cells lacking *BRAF* mutations, in which upstream activation of the MAPK pathway has occurred.[164 165]Therapy-induced KAs frequently harbour activating *RAS* mutations.[166] *HRAS* mutations have been found in 20–30% of cutaneous SCCs. It is plausible that similar mutations may predispose to KAs in the context of selective BRAF inhibitor therapy.[165, 167] Tumour cell resistance to treatment with BRAF inhibitors is a major problem, since complete responses are rare (5%) and the majority of treated patients relapse through secondary resistance within 7 months. Several potential resistance mechanisms have been demonstrated, including compensatory activation of NRAS or upregulation of PDGFR-b[168], activation of MEK1[169], ectopic expression of both CRAF and BRAF[165, 170] and amplification of BRAF[171]. Indeed, MEK inhibitors, targeting MEK kinases immediately downstream of BRAF, as single agents have shown efficacy in CMM with $BRAF^{V600E}$ mutations. Combination therapy with BRAF- and MEK inhibitors has shown promise as a therapeutic approach to delay resistance to BRAF inhibition and further prolong survival, compared with BRAF/MEK-inhibitors as monotherapy [172, 173].This combination also causes fewer therapy-induced keratinocytic skin tumours. Both the BRAF-inhibitor dabrafenib and the MEK-inhibitor trametinib have been approved as monotherapy by the US Food and Drug Administration (FDA) for use in patients with BRAF mutated metastatic or unresectable CMM and recently dabrafenib was approved in the European Union. Ipilimumab is a fully human monoclonal antibody (IgG1) that blocks cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) which otherwise down-regulates pathways of T-cell activation, thus promoting antitumour immunity. By the introduction of ipilimumab a significant effect on OS was seen for the first time in patients with disseminated CMM.[174] The median OS with ipilimumab alone was approximately 10 months. Also a significant improved OS was found in previously untreated patients with ipilimumab plus dacarbazine as compared with dacarbazine plus placebo.[175] Early attempts to combine anti-CTLA.4 antibodies with targeted drugs have not been successful since combination treatment with ipilimumab and concurrent vemurafenib has demonstrated a high incidence of severe hepatic adverse events.[176] However, several new therapeutic strategies are now possible with novel targeted therapies and combinations of treatments perturbing both tumour cell- and immune system targets.

2.9.4 Follow-up

Follow-up for CMM patients in Sweden is performed according to national management guidelines summarized in Table 2.

Table 2.Follow-up for cutaneous malignant melanoma in Sweden according to clinical stage. (From: Nationellt vårdprogram för malignt melanom, 2012)

Clinical stage	Follow-up
Stage I, SNB negative	Clinical examination post-operatively, no planned clinical follow-up
Stage I, high-risk patient*	As for Stage I, but continued clinical follow-up at a dermatologist
Stage I SNB positive Stage II SNB negative Stage III (lymph node metastasis, SNB positive, in transit metastasis)	Clinical controls twice a year for 2-3 years
Stage IV	Individual based follow-up or inclusion in clinical trials
Suspect hereditary CMM (CDKN2A)	Oncogenetic investigation
Clincial trial	According to study protocol

*Patients with heredity, multiple CMMs, multiple dysplastic or common nevi

3 AIMS

The overall aim with this thesis is to improve early detection strategies and management of patients with CMM by increasing our knowledge of

- the variability in primary histopathologic diagnosis of CMM;
- risk groups for adverse prognosis in CMM;
- treatment predictive factors, specifically by:

Investigating interobserver differences of the histopathological evaluation of CMM and whether such variability has an influence on patient management (Paper I).

Assessing the association between social, clinical and histopathological prognostic factors on the individual level. Also, by studying CMM-specific survival as well as differences in surgical management in sub-groups of patients diagnosed with CMM in Sweden (Papers II and III).

Characterizing the patterns of $BRAF^{V600E}$ protein expression in primary and metastatic CMMs including matched pairs of tumours by immunohistochemistry, using a $BRAF^{V600E}$ specific monoclonal antibody. (Paper IV).

4 SUBJECT AND METHODS

4.1 SWEDISH REGISTERS, DATABASES AND THE PERSONAL IDENTITY NUMBER

4.1.1 The Swedish Cancer Register (SCR)

Papers II and III

The SCR was founded in 1958 and is managed by the Swedish National Board of Health and Welfare. The reporting of newly detected cancer cases is by law compulsory for the clinician, pathologist and cytologist diagnosing the malignant tumour. In cases of multiple primary tumours, each tumour is registered separately. Only Swedish citizens are included in the SCR. The Regional Cancer Centers (RCC) send information about newly registered cases and correction concerning those previously reported to the SCR on an annual basis. During 2011, nearly 60,000 cases of malignant tumours were diagnosed and reported to the SCR.[13] The information in SCR covers patient information (personal identity number, sex, age, place of residence), medical data (site of tumour, histological type, stage for certain cancers since 2004), basis/date of diagnosis and reporting institution. The overall coverage rate is estimated to approximately 96% (underreporting 4%).[177].About 99 % of all malignant tumours are morphologically verified. The underreporting is highly dependent on the cancer site.

4.1.2 The Swedish Cause of Death Registry (SCDR)

Papers II and III

The SCDR, founded in 1961, is annually compiled by the National Board of Health and Welfare.[178]. Computerized information on date of death, the cause of death and on age at death in all deceased individuals registered as Swedish citizens at the time of death is collected. The deaths must be certified by the attending physician. In 2011, 1.8 % of all deaths the Board of Health and Welfare was not able to obtain a death certificate, which is a decrease of 3.4 % compared to the previous year.[19] The causes of death are classified according to the English version of the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10). The quality of the data may vary due to the thoroughness and accuracy of the physician report and because of changes in diagnostic methods and classifications.

4.1.3 The Stockholm-Gotland Regional Melanoma Registry (RMR)

Papers I and IV

The Oncologic Centers (from 2012 called RCC) in each of the six health care regions in Sweden were established between 1976-1982 which was initiated by the National Board of Health and Welfare in 1974 to optimize the Swedish cancer care. The RCC in Stockholm-Gotland was founded in 1976. The same year, a collaborative group of Swedish physicians and scientists working in the field of CMM, the Swedish Melanoma Study Group (SMSG), was created. The aim of the group was to ensure a high quality of CMM management in Sweden, including prevention, diagnostic activities, treatment and care of CMM by issuing national guidelines for melanoma. The guidelines included recommendations about referral, diagnosis, staging, treatment, registration, and follow-up of all patients diagnosed with CMM in Sweden.

The responsibility of the RCC comprises cancer registration and coordination of the regional cancer care as well as the implementation of regional guidelines to ensure a uniform standard of care for all patients. Also, the RMR have been affiliated with the care programs. According to the care program, all patients diagnosed with a CMM are recorded in the RMR. The RCC in the Stockholm-Gotland region is covering a source population of approximately 2 million (representing about 23% of Sweden's total population in 2011). The completeness of the RMR was 96.5% in 2011 assessed by linkage to the population-based nationwide SCR. Data on clinical characteristics, histopathological variables, surgical treatment and follow-up are continuously and prospectively collected. Information on causes and date of death is collected annually by record linkage to SCDR.

4.1.4 The Swedish Melanoma Register (SMR)

Papers II and III

The SMR was founded in 2003 by annually compiling the prospectively collected regional data on CMM from each of the RMRs. Data was assembled since 1990 from the databases of the Stockholm-Gotland and Western regions; from 1991 from the Southeastern, Southern, and the Northern regions; and from 1996 from the Uppsala-Örebro region. In 2012 the SMR comprised information of more than 40,000 cases of invasive CMCs (Swedish population 2013: 9.6 millions). The main aim with the registry is to study and report data on prevention, diagnostic methods, treatment outcomes and survival in invasive CMM. The completeness of the SMR is overall high (97% in 2011 as assessed by cross linkage to SCR).

The SMR includes extensive information on the following parameters (coverage of histopathologic parameters within brackets during the study period 1990-2007 for Papers II and III):

- Patient data (age, sex, personal identity number, tumour identification number, date at diagnosis, living area, heredity, skin type).
- The primary tumour (tumour site, tumour diameter according to the reporting physician, TNM-classification).
- Clinical stage at diagnosis.
- Surgical treatment (including primary and secondary surgery, lymph node surgery).
- Histopathologic variables (histogentic type (98%), tumour thickness according to Breslow (96%), tumour ulceration (75%), level of invasion according to Clark (95%), tumour growth in the resection area, marked regression (63%)).
- Information on sentinel node biopsy since 2004 but only sporadic cases. initially
- Follow-up (cause of death, date of death, emigration status).

The histopathologic characteristics has been classified according to 2002 American Joint Committee on Cancer.[113] The registration was performed according to 2009 American Joint Committee on Cancer since 2011[9], which allows international comparisons of statistical data continuously published by SMSG.[18] Information on death status is obtained annually by record linkage to SCDR.

4.1.5 The Swedish Housing and Population Censuses

Papers II and III

The Swedish Housing and Population Censuses are based on mandatory questionnaires sent out to the households every five year in Sweden from 1960 to 1990.[179] The data contains information on demographic, occupational and SES such as marital status, household size, housing, employment, occupation, education (only in 1970 and 1990 Censuses) and income (not in the Census from 1980) for the whole population. The Census of 1990 had a non-participation rate of 2.5%.

4.1.6 The Longitudinal Integration Database for Health Insurance and Labour Market Studies (LISA)

Papers II and III

The LISA database, founded in 1990, is a nationwide database managed by Statistics Sweden including all Swedish citizens 16 years of age and older.[180] Data is retrieved and integrated into the LISA database from several registers at Statistics Sweden including the

Census database 1990, the Total Population Register, the Income and Assessment Register and the Swedish Register of Education. This enables integration of existing data from the labour market, educational and social sectors including information on occupation, employment status, highest attained education, sick leave, income and welfare dependency.

4.1.7 The Swedish Personal Identity Number (PIN)

The individually unique PIN is assigned to each Swedish resident at birth or from time of permanent residency by the National Tax Board. The first 6 (-8) digits encode information on the date of birth, and the last 4 digits are based on an algorithm that ensures a unique number, including information on gender and an algorithm generated check number.[181] The PIN is a crucial tool to cross-link national register data and data collected through charts and biobanks in Swedish medical research. The PIN was used for this purpose in Study I to III. The most common reasons for change of PIN are incorrect recording of date of birth or sex among immigrants or newborns. A re-use of PINs may occur when immigrants are assigned a PIN that has previously been assigned to someone else due to a shortage of certain PIN combinations.[181]

5 STUDY DESIGN

5.1 PAPER I

5.1.1 Material and Methods

Paper I was initially conducted as a quality control investigation of the RMR, since all CMM histopathologic slides primarily analyzed by a general pathologist have routinely been reviewed by a pathologist with CMM expertise in the Stockholm-Gotland region in Sweden. From January 1through December 31, 2006, 664 cases of CMMs were registered in the RMR. This covered 97.5% of all CMMs (n=681) diagnosed and reported to the SCR from the Stockholm-Gotland Health Care Region during the same period. In total, 234 cases of invasive CMMs primarily analysed by a general pathologist and then reviewed by a pathologist with CMM expertise were included in the study. Information on histopathologic prognostic factors, surgical management and sentinel node biopsy (SNB) was obtained from the RMR for all CMMs included. Concordance between pathologists was evaluated for the histopathologic parameters included. The staging at the time was performed according to the 2002 final version of the AJCC staging system for CMM.[182] Surgical margins (measured by the operating physician) and SNB were reported as recommended based on the primary report compared to the recommendation after the review.

5.2 PAPERS II AND III

5.2.1 The SMR Cohort

Papers II and III were based on a large population-based cohort including all cases of CMM in Sweden between1990-2007. All cases were verified CMMs by a histopathologic evaluation. From a total of 37,739 cases diagnosed with primary CMM between 1,990 and 2007, we excluded all in situ CMMs (n=9,292), and patients diagnosed with an invasive CMM before registration in the SMR and study start (n=512), while 700 patients with multiple CMMs were censored for follow-up at the time of a new CMM. The final cohort for analysis included 27,235 patients diagnosed with a primary invasive CMM. All patients were observed from date of diagnosis until the diagnosis of a new invasive CMM, death, emigration or common end of follow-up on December 31, 2010, whichever occurred first. No patients were lost to follow-up.

5.2.2 Study variables

Detailed information on clinical data, histopathologic variables and surgical management was accessed from the SMR. Staging according to the 2002 American Joint Committee on

Cancer was used during the study period[113]. *Living area* was classified as metropolitan: >200,000 inhabitants (the cities of Stockholm, Gothenburg and Malmö with suburban municipalities); urban: 50,000-200,000 inhabitants; rural: <50,000 inhabitants. *Geographical region at diagnosis* was defined by the six health care regions (Northern Region, Uppsala/Örebro Region, Stockholm-Gotland Region, Western Region, South-Eastern Region and Southern Region).

The SMR was cross-linked to several population-based nationwide registers and censuses managed by Statistics Sweden and The National Board of Health and Welfare (SCR, SCDR, the Swedish Housing and Population Censuses and the LISA database) to obtain data on the coverage of CMM in the SMR, date/cause of death and socioeconomic information on the individual level for each patient by use of the PIN. If information on the socioeconomic variables was missing in the LISA database, supplementary data were obtained from the Census database 1960-1990. The most recent historical data was used to assess information for retired patients.

Education was classified according to the highest educational level completed at the time of diagnosis: low (6-9 years), intermediate (10–12 years) and high (13 years or more), corresponding to mandatory school, high school and college/university in Sweden.[183] We also collected information on income and socioeconomic index[184] based on occupation, but for the purpose of Papers II and III, level of education was chosen as the main indicator of SES which is described above in the SES section.

Cohabitation status at diagnosis in Paper III was categorized as married or living with a partner vs. living alone. Information on cohabitation status for persons younger than 15 years of age (n=16 in the present cohort) was not registered in national databases

5.3 PAPER IV

5.3.1 Tumour samples

CMM samples from primary tumours and metastases from the same patients, when available, were tested for *BRAF* and also *NRAS* mutations, the mutation results were partially available from previous studies.[100, 185] The patients were followed-up at the Department of Oncology, Karolinska University Hospital. All tissue samples were obtained as surgical excision biopsies that had been formalin fixed and paraffin embedded (FFPE). Both primary localized CMM tumours and stage III-IV CMMs were analyzed. A total of 200 CMMs were included in the analyses (124 primary tumours and 76 metastases). In 63 cases of primary CMMs, matched metastases were available for analysis including 8 primary tumours with more than one metastasis from the same patents. A majority of the

primary CMMs displayed a histologic subtype of SSM and of ALM due to inclusion of tumours from another study of ALMs from our laboratory. Metastases mainly involved lymph nodes and skin.

5.3.2 Methods

5.3.2.1 DNA extraction

Laser capture microdissection and DNA extraction sections of 4 µm thickness were prepared from FFPE blocks and placed on plain slides.[186] The sections were deparaffinized in two washes of xylene, rehydrated in increasing concentrations of ethanol, rinsed with deionized water, shortly stained with hematoxylin, rinsed with deionized water and dehydrated in decreased concentrations of ethanol and two washes of xylene. Samples were incubated overnight with 30-60 µl of proteinase K-enriched digestion buffer to extract the DNA from the dissected cells. Proteinase K was then inactivated by heating samples at 950 C for 10 minutes.

5.3.2.2 Polymerase Chain Reaction (PCR)

In PCR, DNA polymerases are used to selectively replicate and amplify specific DNA sequences.[186] Oligonucelotides act as primers for new DNA synthesis. The three step process includes denaturation (heating of the hydrogen bounds of the complementary DNA strands to give single-stranded DNA), primer annealing by base pairing to a complementary sequence of the single-stranded DNA and DNA synthesis of new complementary DNA strands of the target DNA which is repeated 30-40 times. The reactions are initiated by the DNA polymerase in the presence of deoxynucleoside triphosphates. The synthesis of the new DNA strands is toward the other primer-binding site in order to serve as templates for new DNA to obtain an exponential increase of the DNA product. To ensure a highly selective amplification, the primers used are complementary to the bases at either side of the target DNA and the annealing temperature, depending on the base composition, is set as high as possible. Also semi-nested PCR could be performed after the first amplification using one original PCR primer and one internal primer. DNA sequencing has developed and been modified over the years to obtain long read-length (number of bases read per run), short analysis time, lower costs and high accuracy. Also the reagents used in the sequencing reaction have improved. Novel sequencing technologies "next generation sequencing" allows for deep sequencing which improves the sensitivity of detection of mutations in minor proportions of molecules in DNA samples.[186]

In Paper IV, the results from previous PCR analyses were used to identify *NRAS* (exon 1 and 2, respectively) and *BRAF* (exons 11 and 15) mutations for the 200 samples included in the study. These analyses were initially performed in the same research group by Omholt et

al.[80, 100] and by Zebary et al.[185]. All mutations were confirmed by independent PCR and either single-cell conformational analysis or sequencing. Sequence analyses were performed in both directions.

5.3.2.3 Sequencing

Pyrosequencing is used to examine short DNA sequences, but can resolve complicated gene aberrations.[186] Using a single, biotin-avidin captured strand from PCR product, the different nucleotides are built individually to form a double strand DNA using a DNA polymerase. Principally, all nucleotides, A, C, G and T are tested for incorporation. When incorporation is successful, the triphosphate of the nucleotide is dephosporylated to monophosphate. The released diphosphate is used as a substrate for added sulfurylase enzyme to produce ATP. This ATP then acts as a co-activator for the enzyme luciferase to produce light. The monophosphorylated nucleotide is inactivated using the enzyme apyrase. This process is repeated for all nucleotides in a cycle to produce a pyrogram where addition of a known nucleotide is plotted against light intensity observed, unsuccessful incorporations not producing signal and repeated incorporations (same nucleotide in a row) producing a multiple of the light intensity.

In Sanger sequencing, double-stranded PCR products are converted into single-stranded DNA, acting as templates for the sequencing, after denaturation and hybridizition using specific primers, DNA polymerase and deoxynucleotides.[186] A 20% pool of dideoxynucleotides of A, C, G and T each carrying a different fluorochrome and containing a hydrogen group at the 3´carbon instead of hydroxyl group, serves as a base-specific chain terminator preventing further integration of nucleotides in the sequence and the DNA chain elongation is then terminated. After capillary electrophoresis, length differences between the elongated chains of one base are detected with the help of the termination-specific fluorescence. The different nucleotides are presented in chromatograms where the peaks are compared to a reference sequence.

In Paper IV, a re-analysis by pyrosequencing was performed for 25 cases with discordant results between the first mutations analysis, serving as the golden standard, and VE1 IHC.

5.3.2.4 Immunohistochemsitry (IHC)

IHC is a multi-step technique which enables detection and visualization of antigens in tissue sections by using specific antibodies binding to the antigen in this case through a secondary antibody to the primary.[186] The technique enables investigations of the distribution and localization of proteins in different tissue samples. An enzyme is usually conjugating the antibody complex and the chromogen allows for a visible reaction. Antibodies detecting a

specific protein can be raised in several ways. The *BRAF*^{V600E} mutation specific monoclonal antibody clone VE1 in Paper IV was prepared by Capper et al.[187] from an 11-amino-acid synthetic peptide representing the *BRAF*^{V600E} mutated amino acid sequence from amino acid 596 to 606 (GLATEKSRWSG). Mice were immunized with the coupled peptide. After the immunoreaction, lymph node cells of anti-BRAF^{V600E} seropositive mice were fused with mouse myeloma SP2/O cells. In summary, immortal cell lines are established from which a hybridoma of the particular antibody was selected.

In Paper IV, we evaluated tumours by IHC using the VE1 antibody described above, developed by Capper et al, to characterize the patterns of BRAF^{V600E} protein expression within and between CMMs with emphasis on primary CMMs and their metastases in routinely processed FFPE tissue.

6 STATISTICAL ANALYSES

6.1 PAPERS I AND IV

In Papers I and IV, agreement between pathologists and between mutation analysis vs. IHC, respectively, was analysed and reported in percentages. In Paper I, all cases with not reported data on any of the variables tumour thickness, tumour ulceration or level of invasion (and also on histologic type) were excluded from the analyses of the histopathologic review of 234 cases of CMM compared to the primary histopathologic report.

6.1.1 Kappa (κ) statistics

Proportions of agreement between observers do not discriminate between actual agreement and agreement which arises due to chance. In Paper I, we also performed a Kappa (κ) statistics. κ-statistics is used as a quantitative measure of the agreement when comparing different types of readers (general pathologists and experts), defined as the agreement beyond what might be expected by chance divided by the amount of possible agreement beyond chance.[188] κ statistics gives an overall measure of agreement but depends on the prevalence of the factor being measured.

$$\text{Kappa} = \frac{\text{Observed} - \text{Expected Proportion in Agreement}}{1 - \text{Expected Proportion in Agreement}}$$

κ values are often used when considering data arising from nominal or ordinal scales and the value varies from perfect agreement (+1) to disagreement (-1) (Table 3). Values equal to 0 indicate agreement equivalent to that expected by chance. Negative κ-values describe agreement that are less than that expected by chance alone.

Table 3. Interpretation of kappa values proposed by Byrt et al.[189]

Kappa value	Agreement
0.93-1.00	Excellent
0.81-0.92	Very good
0.61-0.80	Good
0.41-0.60	Fair
0.21-0.40	Slight
0.01-0.20	Poor
≤ 0.00	No agreement

6.2 PAPERS II AND III

All statistical analyses were performed using SAS 9.2.

6.2.1 The Kaplan-Meier method

The non-parametric method of Kaplan and Meier (K-M) was used to estimate cumulative CMM-specific survival probabilities and confidence intervals over time.[190] Survival is estimated each time a patient die and withdrawals are adjusted for. The exact survival times and survival proportions are therefore given by the K-M analysis. However, the analysis is not adjusted for potential confounders at base-line. As the sample size decreases over time, the confidence interval (CI) and the standard deviation become larger.

The log rank test was performed to compare survival curves or the estimates of the hazard functions of the samples.[191] The analysis is used if observations are censored and compares in each stratum the number of observed CMM-deaths among the exposed with the expected number of CMM-deaths in both the combined groups using equal weights as if there were no difference in hazards between exposed and unexposed.

For presentation in Paper III, survival curves were summarized in three age groups: 15-54 (age 15-39 and 40-54), 55-69 and ≥70 years (70-79 and ≥80) using direct standardization.[191]

6.2.2 Cox proportional Hazard Model

The Cox proportional hazard model is used to analyze a time-dependent censored outcome by producing HRs adjusted for confounding variables.[191] The hazard function describes the probability of the event (here CMM-death) at a specific time interval, given that the patient has survived until the time of the event. The model requires that the ratio of the probability of dying (HRs) remains constant over time. This is the proportional hazards assumption. The Wald chi^2 test was used to evaluate the significance of independent variables. The independent variables can be both nominal and numerical observations.

Cox proportional hazard regression model was used in Papers II and III to assess the independent prognostic contribution of variables, either alone, or after adjustment for other established and potential prognostic variables by using the maximum partial likelihood method for model selection. The prognostic impact of the included variables was presented as HRs with 95% CIs. Associations were considered significant at a two sided *p*-value of <0.05. In Paper II, 18,531 patients were included in the multivariate analysis and 18,271 patients in Paper III. All prognostic factors were treated as categorical variables except for tumour thickness that displayed a skew distribution. Tumour regression did not add any prognostic information ($p = 0.09$). Almost 5,000 patients missed information on this variable that was, thus, not included in the final model. In all Cox analyses the regression

models were stratified by health care region to adjust for any potential differences in treatment and diagnostic procedures.[191] The non-proportional assumption (time-dependence) was tested for each prognostic variable comparing ⩽5 years after diagnosis and >5 years after diagnosis.

For subgroup analysis, the *p*-values for interaction terms from multivariate Cox-analysis are presented in Paper II and were also calculated in Paper III. In the interaction test in the sub-group analysis, education level, age-group and period of diagnosis were treated as continuous variables to increase the power of the test in Paper II.

In all analyses of CMM-specific survival, date of death from other causes than CMM, date of emigration, date of a new invasive CMM or the end of follow-up on December 31, 2010, were time points of censoring.

When analysing median tumour thickness according to educational level or cohabitation status, the median test was used.

6.2.3 Logistic regression

A logistic regression model is used to estimate the probability of a dichotomous outcome as a function of one or several exposures while adjusting for other variables.[191] A chi^2-test is performed to determine whether a variable adds significantly to the prediction. [191] The independent variables include both numerical and nominal measures. The exponential function of the regression coefficient may be interpreted as an odds ratio.

A multiple binary logistic regression is used for a binominal dependent variable where the explanatory variables are continuous, discrete or both.[191] This model was used for assessing the Odds ratio (OR) of education or cohabitation status, respectively, and time for surgical treatment. Patients only receiving primary surgery were counted as 0 days. A cut-off value based on the median time between primary surgery and secondary excision was used to create a response with two categories. In the adjusted model, all significant prognostic factors from the final multivariate Cox-regression and health care region were included.

A multinomial logistic regression is used to analyze exact relationships between a categorical dependent variable and several binary or numerical independent variables.[191] This model is similar to a logistic regression model, except that the probability distribution of the response is multinomial instead of binomial. The method was used evaluate the association between education or cohabitation status, respectively, and clinical stage at

diagnosis (three levels). In the analysis, stage III and IV at diagnosis were grouped because of the small numbers in stage IV.

7 RESULTS

7.1 PAPER 1

Overall, we found an agreement of 79% between general pathologists and pathologist with CMM expertise after review of reported tumour parameters tumour thickness, ulceration and Clark´s level of invasion. The best agreement was found for tumour thickness (86.5%; $\kappa = 0.806$), but in a sub-set of thin CMMs (≤1.0 mm) a lower agreement was shown between pathologists. In this group of CMMs, 15.5% (16 of 103 cases) were re-classified either as in situ or invasive CMMs >1mm. Among in situ CMMs a re-classification to invasive CMM was found in 17 of 83 cases (20.5%) following the expert review which was more frequent compared to the re-classification of invasive CMMs to in situ tumours (7.3%).

For ulceration when reported and Clark´s level of invasion the overall agreement was good (85.6%; $\kappa = 0.690$) and fair (68.8%, $\kappa = 0.561$), respectively, although with a lower agreement within invasion level II and III, respectively.

The review resulted in a change from 1 to 2 cm margin in almost 8% of the invasive tumours as well as a change to a recommendation of SNB in nearly 16% of the cases without a recommendation of SNB according to the results from the initial histopathological report.

7.2 PAPERS II AND III

A total of 27,235 patients with primary invasive CMMs were identified with a median age of 62 years at diagnosis and a median follow-up time of 6.9 years (range 0–21 years). The cohort was almost evenly distributed between men (49%) and women (51%). Almost 14% of the patients died due to CMM during follow-up. Differences in survival between men and women were found throughout the study with a higher 5-year CMM-specific survival among women compared to men (91.5% compared to 85.0%).

The main findings in Paper II were:

- The ORs of more advanced stage of disease at diagnosis was increased for lower levels of education compared to high education, for metastatic disease in particular (stage III-IV vs. I), after adjustments for age, gender, tumour site, living area, period of diagnosis and health care region (OR low education, stage II vs. I = 1.6; 95% CI = 1.5-1.7. OR low education, stage III-IV vs. I = 2.3; 95% CI = 1.8-2.9).
- The relative impact of level of education on the risk of CMM-related death was higher for lower levels of education vs. high education in the unadjusted Cox multivariate analysis (HR low = 2.02; 95% CI = 1.80-2.26. HR intermediate = 1.35;

95% CI 1.20-1.51). These differences remained significant after adjustments for age, gender and stage at diagnosis (HR low vs. high education = 1.19; 95% CI = 1.06-1.34; $p < 0.001$. HR intermediate vs. high education = 1.13; 95% CI =

- 1.01-1.27; $p = 0.01$), but further adjustments including tumour site, histogenetic type, tumour ulceration, tumour thickness, Clark's level of invasion, living area and period of diagnosis, only changed the HRs marginally. The relative effect of low education on HR was significantly associated with younger age (<55 years), female gender and CMMs located to the trunk.

The main findings in Paper III were:

- After adjustment for age, education, living area, year of diagnosis, and, tumour site, the ORs of higher clinical stage (stage) at diagnosis were significantly increased among men living alone vs. men living with a partner (OR stage II vs. stage I =1.42; 95% CI = 1.29-1.57. OR stage III-IV vs. stage I, 1.43; 95% CI = 1.14-1.79). The ORs for higher stage among women living alone were also increased (OR stage II vs. stage I = 1.15; 95% CI = 1.04-1.28.OR stage III-IV vs. stage I = 1.04; 95% CI = 0.79-1.37).
- After adjustments described above, the CMM-specific survival was significantly decreased among men living alone (HR = 1.51; 95% CI =
- 1.35-1.68; p<0.0001). After adjustments for all potential and established prognostic factors (HR men living alone vs. men living with a partner = 1.33; 95% CI = 1.19-1.49; p<0.0001), indicating a residual adverse effect on survival not accounted for by these parameters.

7.3 PAPER IV

The VE1 IHC staining intensity varied between primary tumours and matched metastases in 47% of the cases, as well as between separate metastases. In six tumours, the mutation status changed between the primary CMM and its matched metastasis, but the IHC staining corresponded to the change. Interestingly, we found changes in mutation status both of $BRAF^{V600E}$ to $BRAF^{wt}$ but also of NRAS mutation status. The sensitivity of the VE1 antibody was 97% and the specificity 80% for detection of $BRAF^{V600E}$ mutations (Table 4). A comparable sensitivity was obtained for primary CMMs and metastases separately. The specificity was lower among primary CMMs (71%) compared to metastases (93%). Discordant results between the mutation analysis and IHC showed in general weak staining.

Table 4. Sensitivity, specificity, positive and negative predictive values for all tumour samples as well as primary CMMs and CMM metastases, respectively**.**

Test charachteristics	**All tumours % (n)**	**Primary CMMs % (n)**	**Metastases % (n)**
Sensitivity	98 (88/91)	97 (56/58)	97 (32/33)
Specificity	80 (87/109)	71 (47/66)	93 (40/43)
Positive predictive value	80 (88/110)	75 (56/75)	91 (32/35)
Negative predictive value	97 (87/90),	96 (47/49)	98 (40/41)

8 METHODOLOGICAL CONSIDERATIONS

8.1 PAPER I

Paper I was initiated and also conducted as a quality registry control analyzing all incident cases of CMM diagnosed in the Stockholm-Gotland region during 2006. Still, a major strength of this study is the prospectively and continuously registered information of high quality on tumour characteristics collected from the RMR which covers almost 97% of the cases in the region.

Difficulties in interpreting the κ value may arise because κ is affected by the prevalence of the variable being measured. Low values of κ may therefore not reflect low rates of overall agreement in the case of rare findings.[188] Likewise, a high level of agreement might be due to a high prevalence. Moreover, the κ statistics does not consider or separately identify the impact of bias which itself is a form of disagreement.[188] Therefore, the analysis of observer variation should take both agreement and bias under consideration.

We considered the total number in each group to be sufficient for analyses, but the number of cases decreased with increasing tumour thickness and higher levels of invasion. An alternative would have been weighted statistics to adjust for the seriousness of different levels of disagreement for observations when using more than two categories. In Paper I this approach would have generated a weighted κ of 0.88 for tumour thickness using standard weights of 1; 0.75; 0.50; 0.25 and 0.0. However, the choice of weights which largely determines the result is subjective. Adding CIs to the results might not show the extent of any agreement just if the agreement exists or not.

Paper I was limited by the fact that the definition of a pathologist with special CMM expertise in Sweden is informal and a certification of pathologists specialized in CMMs is lacking. On the other hand, Stockholm–Gotland is the only region in Sweden where it is possible to analyse the effect of a review in clinical practice, as this is not routinely performed in other parts of Sweden. This gives the pathologists reviewing the CMMs more experience of evaluating difficult cases which have not been studied extensively previously. We could not compare our results to the 2009 AJCC staging of CMMs since mitoses were not reported in 2006 and data are thus not registered.[9] Another limitation is the fact that CMMs re-classified as BN after the review disappear from all registries if not registered specifically for the purpose of a study.

8.2 PAPERS II AND III

8.2.1 Study Design

Cohort studies are a group or groups of individuals classified with an exposure of interest and then followed in time from exposure to a defined outcome.[192, 193] The direction of the study is forward in time, from a possible cause to an outcome. Both exposed and unexposed participants must be at risk of developing the specific outcome measured. Exposure data may be collected prospectively. However, in retrospective cohorts, the events being evaluated have already occurred before the onset of the study and information is recorded in the past after the outcome has taken place by using medical records, databases or registries. Cohort studies are useful for studying rare exposures, evaluating multiple effects of an exposure and for diseases with long induction or latent periods.[192, 193] The design is not appropriate for investigating rare outcomes or outcomes that take time to develop.

Papers II and III include nationwide prospectively collected information on clinical and histopathological variables as well on surgical data from the population based the SMR covering 97% of the diagnosed CMMs at the time. We included patients diagnosed with a first invasive CMM 1990 to 2007 to investigate the risk of CMM-related death in relation to education level (Paper II) and cohabitation status (Paper III), respectively. By the use of the PIN, cross-linkage to high quality national health care registries and censuses with almost complete follow-up on all individuals was performed to obtain information on date/cause of death and information on socioeconomic variables. In these studies we could analyze and present prognostic results on the individual-level even for sub-groups among the patients which, to our knowledge, has rarely been shown in such detail before.

8.2.2 Systematic errors

Systematic errors are categorized as selection bias, information bias and confounding.

8.2.2.1 Selection bias

Selection bias occurs when systematic differences in selecting and following study groups exist.[193, 194] In a (retrospective) cohort study selection bias most often arises when the selection or follow-up of exposed and unexposed participants is systematically related to disease status/outcome.

In the present cohort (Papers II and III), selection bias was reduced by the collection of disease related data and outcome from high-quality registries with an almost complete coverage of prospectively collected information (SMR) .The individual level data from nationwide health and census registers were collected independently of the other parameters included in the cohort.

The registries allowed for long-term follow-up also with respect to survival. The rate of non-reported cases has decreased in the SCR but autopsies are also still decreasing.[19]

8.2.2.2 Information bias

Information bias arises when the information on exposure and disease is assessed in systematic different ways between study groups.[193, 194] In cohort studies, information on outcome should be obtained similarly for exposed and unexposed, if not the participants may incorrectly be classified as having or not having the outcome.[192] The effect of information bias may be differential when the measurement the outcome is systematically different for one group than for another leading to higher or lower results than expected. The non-differential misclassification is random (not systematically different between groups) which might obscure or underestimate real differences between groups. Information bias can also occur because of losses to follow-up between the groups.[192] Under- or overestimation of the results may be introduced because of differential or non-differential losses to follow-up.

In Papers II and III data were recorded prospectively before the outcome had occurred, but the information was not recorded for the specific purpose of research. We had no patients lost to follow-up. Also, the pathologic evaluation of the histopathological prognostic factors has been shown in previous studies to be subject to interobserver variability, discussed in detail in Paper I. This is a fact that we could not exclude even in the obtained nationwide data from the SMR. Most likely information bias to some extent has been introduced in these studies, but any misclassification should be non-differential and rather dilute the effect of the findings.

The main indicator of SES in Paper II, education, and also cohabitation status in Paper III were obtained from the LISA database. Information on education was mainly collected from the Education register within the LISA databases. This register has recently been updated and has a high coverage of the Swedish population. The majority of subjects in Paper II had finished their highest level of education by the time of diagnosis since the median age was 62 years. The risk of misclassification bias should therefore be low. However, the school system has changed in Sweden over the years and in older birth cohorts a shorter education could result in similar living conditions as a longer education in the current system. Information on cohabitation status is not registered in the LISA database for individuals younger than 15 years of age, but this affected only16 individuals in the study cohort. Individuals not registered at the tax agency as living together are not registered as couples in the national databases which may cause a mix of single persons and individuals living as couples in the same category of cohabitation status (“living alone”).

This implies that such misclassification, if present, should be non-differential leading to a slight underestimation of the association between living alone and adverse outcome.

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8.2.2.3 Confounding

Confounding is a third factor that is related to both the exposure and outcome without being an intermediate step in the causal pathway.[193, 194]This systemic error could be preventable or removed by different measures during the design of the study or at the phase of the analysis. Mostly randomization, restriction, matching, stratification and multivariate statistical techniques are used. Subjects with the same value on a specific variable which might be a potential confounder are selected or excluded if restriction is used. This could potentially hinder recruitment and thus decrease the power of the study if the study cohort is not large enough. The internal validity might thus increase but with a lower external validity instead.

Restriction to only primary invasive CMMs and by assessing the independent prognostic contribution of variables, either alone, or after adjustment for other variables thorough a multivariate Cox proportional Hazard method, were used to minimize the risk for confounding in Papers II and III.

Several clinical and histoptahological prognostic factors have been identified for CMM. The information on almost all prognostic factors registered in the SMR and national health care registries allowed for adjustments for nearly all known important prognosticators in the logistic and multivariate analyses in Papers II and III. Mitotic rate was not recorded in the SMR during the study period. We also adjusted for health care region to correct for potential differences in reporting and diagnostic procedures in different parts of Sweden.

The impact of cohabitation status for men on CMM-specific survival was still evident in Paper III, even after adjustments for age, gender, stage at diagnosis, tumour site, histologic type, tumour ulceration, tumour thickness, Clark´s level of invasion, living area, period of diagnosis and education in the final model. This indicates a currently unexplained residual adverse effect by single living in men.[64, 114, 195] It is possible that the adjustments performed do not completely correct for more advanced CMMs within each stage of disease among men living alone. Differences in the further management of CMM after initial surgical treatment, such as intensity of follow-up and early detection and treatment of recurrent disease could differ between individuals living alone and living with a partner. Since such information is not available from the SMR this could not be addressed in the present investigation. Behavioral data such as information of sun protection, CSE or SSE were not collected for the purpose of this study since this information was not included in the SMR.

Also, one may speculate that the worse survival among men living alone may be influenced by a generally lower health status in this group of patients.

We did not include information on comorbidity which has been demonstrated to be more pronounced among patients with lower SES compared to high SES groups.[54] This could possibly have an impact on treatment intensity and thus affecting survival but no treatment, apart from early primary surgery, has been shown to impact survival in CMM until recently by the introduction of novel immunological and targeted therapies prolonging the survival in unresectable stage III and stage IV CMMs.[22, 172, 174]

8.2.3 Random error and precision

The probability that a result has been produced due to chance in an unsystematic way is called random error. This error arise from measurement errors or from sampling variability. Precision is a lack of random error. The precision can be increased and the random error reduced by increasing the sample size, by repeating a measurement, by repeating the whole study or by using an efficient study design.

The random error is reflected in the p-values and CIs.[193, 194] The p-value is defined as the probability of obtaining the observed result and more extreme results by chance alone, given that there is not a relationship between exposure and disease (that the null hypothesis is true). In our studies a p-value of ≤ 0.05 was considered significant and the null hypothesis was thereby rejected meaning that in such case the association was with 95% certainty or more not caused by chance alone. Random error could explain the discrepancy when p-values are greater than 0.05 and the null hypothesis is not rejected. The 95 % CI is defined as " if a study is repeated 100 times and 100 point estimates and 100 CIs were calculated, 95 out of 100 CIs would contain the true measure of association".

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Papers II and III were performed in large population based cohorts which ought to have decreased findings by chance and instead increased the precision reflected in the relatively narrow CIs.

8.2.4 Validity

The internal validity of a study is depending on that confounding, bias and associations by chance are minimized or eliminated. The external validity or the generalizability shows the probability that the results apply to other populations and therefore the internal validity has to be met.

The internal validity of Papers II and III depends mainly on the quality of the parameters in the SMR and the LISA database both of which are considered to be of high quality with a low amount of missing data on the variables included.
The large population based setting with nationwide data on the individual level including all patients from both in- and outpatient care with no losses to follow-up should have generate a high external validity, given that the same diagnostic/staging criteria are used and that the education as well as health-care systems are similar to the Swedish system. The results from the multivariate Cox analyses in this study did not change significantly if restricted to patients aged 15-89 years (n total=20,196) or after excluding immigrants (n total=18,161).

8.3 PAPER IV

An advantage of using IHC is that the method requires a small amount of tumour tissue (4 um thick sections from FFPE blocks of CMMs) compared to DNA-based analyses. IHC is both described as being less expensive and more rapid to perform compared to standard molecular techniques. In contrast to DNA-based methods, IHC allows visualization of individual antigen-bearing tumour cells and the IHC technology is established as a routine in most pathology departments. Monoclonal antibodies are immunochemically identical and react with a specific epitope on the antigen. The advantages of monoclonal antibodies are a high rate of homogeneity, absence of non-specific antibodies and a lack of variability. The antibodies are often characterized using frozen or FFPE tissue and fixation has therefore to be optimal to assure its survival. The specificity of the antibody is lost if the epitope is not unique to a certain antigen.

PCR, used in the initial analyzes by Omholt et al.[80, 100] and for the ALMs[185], is considered to be rapid, sensitive and robust for amplification of small amounts of DNA from diverse tissues.[186] The method might be both expensive and time consuming for larger amounts of DNA. Nested and semi-nested PCR increases the specificity and sensitivity of the PCR reaction since it is unlikely that amplification will proceed if the first reaction is amplified nonspecifically. The use of several primers increases the risk of unspecific contamination. The polymerases may be affected if the tumour is heavily pigmented.

In the initial IHC analyses, both performed manually and by the automated immunostainer, positive staining of all $BRAF^{V600E}$ tumours was generally too weak and therefore difficult to evaluate. Following pretreatment, the incubation with undiluted VE1 hybridoma at 37°C was performed for 60 minutes instead for 32 minutes as described by Capper et al[187] resulting in a clear improvement of the staining. We have no obvious explanation for these results, which was not correlated to the age of the sections or the FFPE blocks, the tumour thickness or the tumour subtype.

Several obstacles were experienced when evaluating the samples. All three separate investigators were indicating difficulties in separating unspecific background staining from very weak VE1 staining. Long et al [196] mention that the IHC was more easily assessed in metastatic lesions, although both *V600E*-mutated primary CMMs and metastases stained positively with VE1. In the present study, the intensity varied between tumours, in particular for primary $BRAF^{wt}$ CMMs where the positive staining frequently was evaluated as weak. In this group of CMMs we also found a large proportion of false positive cases. Vice versa, strong positive staining had to be interpreted with caution in highly pigmented CMMs and metastases. This could have been avoided by using a red staining instead. Another important issue is that the tumour sections for IHC only represents a small part of the tumour and might therefore not be representative and could even differ in adjacent areas.

9 DISCUSSION

9.1 PAPER I

It is established that interobserver variability exists for the histopathological prognostic markers in CMM.[136-144, 146, 150, 197-199] Previous studies vary in methodology and only a few were performed in a similar clinical setting to that of our study. Assessing differences between pathologists is important for interpretation of data derived and the observer variation could be inherent in the method itself.[188] For CMM, the classification usually determines the extent of primary surgery and whether sentinel node biopsy is required. The histopathologic classification of the primary CMM is also of importance when comparing trends in incidence and survival in epidemiologic studies.

Paper I shows that a major factor to consider is that even an apparently small change in the histopathological assessment of tumour thickness may result in a change in surgical management and performance of SNB, particularly among T1 lesions (tumour thickness ≤ 1.0 mm). Hence, in this subgroup of we found a variability of evaluating the tumour thickness of 15.5% between general and CMM pathologists, but the concordance was overall high (86.5%) corroborating previous data.[136-144 146]

Although a high concordance for tumour ulceration (84.8%) if reported was found in the present study, one of the most striking findings was that over 52% of the included tumours were not classified for ulceration in the primary pathological report. Concordance according to ulceration status is reported to be high to excellent in previous studies, but our result of missing reports on ulceration status among general pathologists is not comparable due to differences in information of study data and study design in previous studies.[138, 141-144, 146]. Low experience of CMM pathology could also explain that ulceration status in fact in the majority of cases was not reported by the general pathologists. The results may also reflect an inconsistency in reporting all of the pathological information required for making management decisions. Information concerning presence or absence of ulceration is crucial both for management of T1 CMMs and for the prognostic information to the patient. The lower concordance for Clark's level is consistent with previous studies showing a low to intermediate agreement between pathologists.[136-139, 141, 142, 144, 146, 200] and this could also have an influence on the management of T1 CMMs.

We could compare our results with a previous study where pathologists at the Sydney Melanoma Unit reviewed CMMs from referring pathology services.[146] The κ –values

reported for tumour thickness was κ = 0.883 and for ulceration κ = 0.832. This corresponds to κ = 0.806 and κ = 0.690, respectively, in our study. However, κ-values from different populations are not easy to compare since for example the prevalence differs between populations. There is always a risk of some degree of systematic bias which could in this study be for example that one observer may more easily evaluate DN as invasive tumours.

A shortcoming of our study is that we were not able to compare the proportion of CMMs re-classified as dysplastic nevi since theses would not be registered in the RMR. This would be possible to analyse within another study design.

In summary our results show overall a good interobserver concordance between general pathologists and pathologists with expertise in CMM in clinical practice. Tumour thickness was altered by the review in subgroups of T1 tumours and in situ CMMs to a larger extent, under consideration for a different surgical approach after the review of tumour thickness, ulceration and level of invasion according to Clark.

9.2 PAPERS II AND III

A socioeconomic gradient in cancer survival has been reported in different settings.[53, 201] This might be explained by differences in life-style, knowledge of and/or access to health-care resources, participation in cancer screening programs, adoption of health seeking behavior, all of which could have an impact on prognosis in cancer patients.[53] Also for CMM, several previous studies have reported a correlation between low SES and an inferior prognosis in the disease.[55, 56, 58, 59, 61-64, 202] However, the impact of cohabitations status or social isolation on specific health conditions and behaviors are restricted to certain conditions such as cardiovascular disease and cancer where a protective effect of a social context has been found. In contrast, for CMM only a few studies have been conducted to explore the impact of cohabitation status on CMM survival. Unmarried and widowed older patients have been found to have a higher risk of CMM-related death compared to married patients. [61, 70]

In Sweden, the national health care system aims to provide care on equal terms to all citizens. National guidelines for CMM diagnosis, treatment and follow-up have been implemented to minimize the variation of the primary management. Despite this, our results could demonstrate that the CMM-specific survival was significantly reduced for patients with low education (Paper II) and for men living alone (Paper III). By the use of detailed information from national registers and databases including the SMR, we could in detail further analyze these findings.

More advanced stage of disease at diagnosis was demonstrated for patients with lower education but only for men and older women living alone. Previous studies have found similar results of later stages or thicker tumours at diagnosis.[48, 58, 59, 61, 63-66, 70, 203-206] However, in our analyses we found that lower education was in particular associated with more advanced stages of CMM (III-IV vs. I) which was not found for single households in Paper III. The median age at diagnosis showed an inverse relation to level of education in Papaer II which could be explained by lower level of education among older patients or earlier diagnosis among high educated groups of patients.

Gender differences with an increased CMM-specific survival among women compared to men are well-known.[31, 114]. Women consistently had a CMM-specific survival advantage over men in all educational groups but the relative risk of dying of CMM in the multivariate analysis after adjusting for all other available variables was increased for women compared to men as well as for patients <55 years and in individuals with tumours located to trunk, indicating that the effect of education was most pronounced in these groups. In Paper III, living alone was significantly associated with an increased relative risk of dying of CMM among male patients, in particular, independent of age, education, living area, period of diagnosis and tumour site, in the fully adjusted multivariate analysis. A trend of an association between living alone and increasing HRs for older women most likely reflects the findings of higher risks for more advanced stages of disease at diagnosis. The impact of cohabitation status on CMM-specific survival for men was still evident after adjustment for several potential parameters which previously have been correlated to a worse prognosis, indicting a residual adverse effect by single living in men, which is currently unexplained.[64, 114, 195] It is not inconceivable that the worse survival among men living alone is influenced by a generally worse health and by differences in the further management of CMM. Since such information is not available from the SMR this could not be addressed in the present investigation.

Complex combinations of factors may contribute to the observed results in Papers II-III, such as performance of SSE, awareness of the disease, access to health care and early detection as well as living with someone which could indicate a good social network and support. Patients or family members discover approximately 40-60% of all incident CMMs.[46] Women and high educated individual are more likely to perform SSE while men more often report that the CMM first was detected by the spouse.[46-48, 207] This could contribute to the marked educational effect in women in Paper II. Gender and SES differences are also related to awareness of CMM risk factors, information seeking behavior and usage of sunscreen.[47] Women seem more likely to perform SSE compared to men (69% vs. 47%, respectively), while men more often report that their CMM was

first detected by the spouse.[46, 47] Information to younger patients may result in a higher awareness of early warning-signs of the disease and could increase the likelihood of earlier detection of CMMs. Information concerning the disease as well as a higher level of education might result in more efficient SSE of the whole body leading to earlier diagnosis of tumours otherwise related with poor prognosis on the trunk.[114] In Paper III, individuals that were not living with a partner but with children in the household were classified as living alone since we did not consider young children to contribute to the early detection and practical help in the sense as living with an adult partner. This grouping may be discussed as the psychological importance for patients being surrounded by family members of any kind compared to living all alone could improve survival in other ways. However, single patients living with children constituted only a small proportion of the cohort in all age-groups (range: 0.5-3%).

Strengths and limitation of these studies have been described above in the section of Methodological Considerations. Papers II and III are limited by lack of information on SNB and mitotic rate as these parameters were progressively implemented and registered in Sweden during the study period. We also lack information on comorbidity, information on time from initial referral and more than 5,000 patients registered in the SMR had unknown stage of disease at diagnosis.

In conclusion, our results of more advanced stages of disease and reduced CMM-specific survival among patients with lower level of education and men living alone indicate that improved strategies for early detection directed to this group of patients may improve the prognosis in CMM.

9.3 PAPER IV

The implementation of IHC in histopathology has over the years developed and provides supportive information in diagnosing CMMs. The rapid development of targeted therapies mandates determination of BRAF mutation status in patients with advanced CMM to select patients who may benefit from treatment with BRAF inhibitors. The main findings in this study, based on tissue samples from 200 CMMs with a large number of primary tumours with matched metastases, was that the method had a high sensitivity and specificity overall comparable with previous studies. [187, 196, 208-214]. However, a lower specificity for the VE1 IHC specifically in primary wt CMMs tumours as compared to metastases was detected, which has not previously been shown. Also, the $BRAF^{V600E}$ protein was heterogeneously expressed between matched tumours in 47% of the cases.

We found that the majority of false positive *BRAF*^{wt} primary CMMs were ALMs. Our findings could be explained by an unspecific cross reaction of VE1 antibody to other epitopes present more frequently in primary tumours compared to metastases as well as differences in preparing and handling the tissues, such as sampling methods, fixation and storage conditions between different pathology laboratories. Finally, the change of incubation time from 32 to 60 minutes could have contributed, but the IHC would otherwise have resulted in false negative cases. If patients with false positive results are treated with BRAF-inhibitors, the therapy is inefficient as well as a paradoxal activation of the MAPK-pathway in *BRAF*^{wt} tumours may occur resulting in other tumours such as squamous cell carcinomas, keratoacanthomas and even newly developed CMMs.[215, 216]

Primary CMMs are often polyclonal and may contain a heterogeneous mixture of *BRAF*^{V600E} and *BRAF*^{wt} tumour cells which could explain the variation in VE1 IHC intensity found in our material. [89, 217]. The BRAF^{V6OOE} protein expression between primary tumours and their metastases changed in almost 50% of the cases suggesting an activation of other signal pathways during progression. However, a smaller proportion (9%) of matched pairs of primary and metastatic CMM tumours demonstrated an occurrence of intertumoural heterogeneity with respect to mutation status. The VE1 IHC staining was consistent with the genetic variation. We found changes in mutation status both of *BRAF*^{V600E} to *BRAF*^{wt} but also of *NRAS* mutation status (Table 5). Tumour progression in CMM is thought to be driven by several genetic changes in addition to the *BRAF* mutations and we speculate that *BRAF*^{V600E} might in rare tumors lose its role as a driver of tumor progression which might help explain our findings of a change from *BRAF*^{V600E} in some primary CMMs to *BRAF*^{wt} in their matched metastases in line with previous results by Yancovitz et al.[217]

Table 5. Discordant mutation status in primary cutaneous melanomas as compared to their matched metastases

Primary tumour	**Corresponding metastasis**
BRAF^{V600E}/NRAS^{wt}	BRAFwt/NRAS^{Q61R}
BRAF^{V600E}/NRAS^{wt}	BRAF^{wt}/NRAS^{wt}
BRAF^{V600E}/NRAS^{wt}	BRAF c.1797_1798insACTACG
BRAF^{wt}/NRAS^{Q61R}	BRAF^{wt}/NRAS^{wt}
BRAFwt/NRAS^{Q61K}	BRAF^{wt}/NRAS^{wt}
BRAF^{V600E}/NRAS^{wt}	BRAF^{wt}/NRAS^{Gly468Ser}

The strength of this study is that the results are based on a large number of CMMs analyzed, including 124 primary tumours, and also the inclusion of matched primary tumours and metastases. A limitation is that the CMMs tumour samples were prepared at several different pathology laboratories, which may have contributed to variability of results.

In conclusion, in primary CMMs or in cases with weak/moderate staining the IHC method should be used in combination with genomic testing to accurately predict $BRAF^{V600E}$ status, whereas in metastases with strong staining no further mutation testing may be required.

10 CONCLUSIONS

Paper I

The interobserver concordance between general pathologists and pathologists with expertise in CMM is overall good in clinical practice.

Tumour thickness was altered by the review in subgroups of T1 tumours and in situ CMMs, which may also be of concern for CMMs with a thickness just above 1 mm.

T1 tumours were to a larger extent under consideration for a different surgical approach after the review, due to increased T-classification.

Even a small change in tumour thickness and assessment of ulceration status, could result in altered management and prognosis in T1 CMMs

Papers II and III

There was a significant association between education and stage at diagnosis both among men and women in all age-groups

The (overall) association in Paper II between education and stage at diagnosis was significant in the low education group where the risk of stage III and IV vs. stage I at diagnosis was even higher compared to being diagnosed with stage II vs. stage I.

In Paper III, the corresponding findings were found for men living alone for all stages of disease as well as for women living alone for stage II vs. I.

The risk of CMM-related death was increased overall among patients with low compared to high education with an altered relative risk also in particular sub-groups of patients (women, younger patients aged <55 years and patients with tumours located to the trunk).

The HR for CMM-specific death was significantly reduced for men living alone independent of age, education, living area, period of diagnosis and tumour site.

The effect of education on survival was highest the first five years after diagnosis. Stage at diagnosis, level of invasion and gender were the only significant prognostic factors five years after diagnosis. No time-dependence was found for cohabitation status**.**

No clinically important delay of the secondary excision was seen after the initial contact with the health care system among patients with primary localized invasive CMMs.

These results indicate that improved strategies for early detection directed to sub-groups of patients may improve the prognosis in CMM

Paper IV

Heterogeneity of $BRAF^{V600E}$ expression within and between tumours could be visualized with VE1 IHC.

Overall, using mutation analysis as the golden standard, a high proportion of ^{BRAFV600E} mutant cases (both primary CMMs and metastases) stained positively with the VE1 antibody.

The sensitivity was high for the method overall, but a lower specificity for VE1 IHC was found specifically for primary $BRAF^{wt}$ tumours as compared to metastases**.**

In primary CMMs or in cases with weak/moderate staining the IHC method should be used in combination with genomic testing to accurately predict $BRAF^{V600E}$ status, whereas in metastases with strong staining no further mutation testing may be required

11 IMPLICATIONS AND FUTURE PERSPECTIVES

CMM has rapidly been increasing in Sweden during the last decades and also a lesser rise in mortality has been reported. The majority of the CMMs being diagnosed are thin, low-risk tumours but thicker, high risk CMMs, are still increasing mainly among men and older individuals. Hence, no clear effect of primary and secondary prevention efforts is observed indicating a need for improved prevention strategies. Although new markers are needed to improve the specificity and predictive value of the already established prognostic characteristics of CMMs in the selection of treatment or in stratification for therapeutic trials, the histopathologic prognostic markers still play a significant role and are the basics for prognostic and management decisions.

Study I, planned as a quality control, changed the routines the Stockholm-Gotland region from reviewing all CMMs initially analyzed by a general pathologist to focusing on T1 CMMs. However, this study describes the interobserver concordance, but variability in measurement and classification may also arise from a lack of consistency within an individual observer when carrying out successive recordings. Such findings of disagreement between observers might be followed by an investigation of intraobserver variability to explain further disconcordances. Since the majority of pigmented lesions are dysplastic nevi or early diagnosed in situ CMMs, investigating the diagnostic variability in these groups would be of interest. In particular to investigate the minority of tumours originally classified as nevi/in situ melanomas but resulting in metastases. The dysplastic nevi and in situ CMMs can be traced back in the SCR.

Moreover, since thin CMMs are considered low-risk tumours with respect to recurrence and death, the majority of patients are not followed after the surgical treatment. A small proportion of patients have a worse outcome but today we cannot distinguish whom to follow with clinical controls. Future efforts should be invested in investigating factors related to prognosis specifically for this group of patients. Apart from being followed-up at frequent controls, these patients could be offered specific treatment interventions as mutation testing and adjuvant therapies.

Our finding that patients with lower level of education or living alone are more likely to be diagnosed with CMM at a more advanced stage of disease and, thus, have a higher risk of CMM-specific death is of particular concern. The results suggest that differences in CMM survival might be reduced by improved strategies for early detection for these groups of individuals. The exact mechanisms explaining this are still not completely known.

Additional studies should include information on differences in access to health care and life-style factors that may be relevant. Studies from the U.S. have indicated an increasing incidence of CMMs in the Hispanic population. Also in Sweden, further studies are warranted to analyze the outcome in CMM among groups with different ethnic backgrounds. Another future topic is the correlation between outcome in CMM and different medical treatments for other diseases.

The recent development of new therapies for CMM makes this disease a suitable prototype for investigation of novel targets and biomarkers. There is a need for identifying novel predictive markers for durable response in existing targeted therapies, as well as to find novel targeted therapies based on biomarkers in the large proportion of tumours where targeted therapy is not applicable or has become inactive due to resistance.

12 SVENSK SAMMANFATTNING

Hudmelanom (MM) är idag en av de snabbast ökande tumörformerna i Sverige. Prognosen i sjukdomen är beroende av i vilket stadium tumören diagnotiseras. Det finns flera etablerade kliniska och histopatologiska prognostiska faktorer vid MM, i första hand vid lokaliserad sjukdom. Patologens bedömning av tumören är därför av stor betydelse men kan variera mellan patologer med eller utan erfarenhet av MM-bedömning. Tidiga, tunna, MM har mycket bra överlevnad, men prognosen försämras kraftig vid mer avancerad, spridd, sjukdom. De senaste åren har flera nya målsökande behandlingar etablerats vilket medför att patienter med svår sjukdom måste mutationstestas för val av behandling. Detta medför större krav på snabbare och mer känsliga analysmetoder. Målet med denna avhandling var att analysera skillnader i den histopatologiska bedömningen av MM mellan patologer, att analysera sambandet mellan utbildning respektive sammanboende och överlevnad i MM samt att med hjälp av immunohistokemi (IHC) bedöma uttrycket av $BRAF^{V600E}$ i primära tumörer och metastaser.

För att analysera skillnader mellan patologer avseende den histopatologiska bedömningen av MM, så inkluderades 234 MM från patienter som fått sin diagnos i Stockholm-Gotland regionen under 2006. För att analysera betydelsen för prognosen av så kallade socioekonomiska skillnader samt effekten av att leva med en partner, så inkluderades 27,235 patienter med ett första invasivt MM från det Nationella Melanomregistret. Data kopplades till olika nationella register på Statistiska Centralbyrån och Socialstyrelsen för att erhålla information om demografiska variabler. Slutligen analyserades 200 tumörer, diagnostiserade på Karolinska Universitetssjukhuset Solna, med IHC med en monoklonal antikropp specifik för $BRAF^{V600E}$.

Vi fann att den histopatologiska bedömningen mellan allmän- och melanompatologer överenstämde i hög grad, men att det fanns mer uttalade skillnader i bedömningen av de tunna melanomen. Vidare visade våra studier att patienter med låg utbildning diagnostiseras med senare stadier av MM och har sämre överlevnad i sjukdomen jämfört med patienter med hög utbildning. Både män och äldre kvinnor som lever ensamma får sin MM-diagnos senare jämfört med sammanboende män och kvinnor. Män som lever ensamma har även en högre risk att avlida i sjukdomen jämfört med sammanboende män, vilket inte återfanns lika tydligt för kvinnor. IHC hade hög känslighet generellt för att bedöma uttrycket av $BRAF^{V600E}$, men i primära tumörer fanns en högre andel MM som visade falsk-positiv immunfärgning. Immunfärgningen varierade i nästan hälften av fallen i styrka mellan primära tumörer och matchade metastaser.

Sammanfattningsvis, den histopatologiska bedömningen, som ligger till grund för den vidare handläggningen, kan påverka behandlingen främst av tunna MM beroende på om bedömningen utförs av en melanompatolog eller ej. Riktade preventiva insatser avseende särskilda patientgrupper för att finna och åtgärda MM i tid kan öka överlevnaden i MM. IHC skulle kunna användas som enda analys av uttrycket av $BRAF^{V600E}$ för metastaser och tumörer med stark immunfärgning för att snabbare påbörja behandling.

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