Department of Oncology and Pathology

CANCER RISKS AND PROGNOSIS IN FAMILIAL MELANOMA KINDREDS

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FAMILIAL MELANOMA KINDREDS

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Meðalsnotr
skyli manna hverr
æva til snotr sé
því at snotrs manns hjarta
verðr sjaldan glatt,
ef sá er alsnotr er á
(Original text)

Each man must be
moderately wise,
but never too wise;
because the wise man's heart
is seldom glad,
if he who owns it is completely wise.
(English translation)

Hávamál, author unknown, ca 900 AD

To my family
ABSTRACT

Malignant melanoma of the skin is one of the most rapidly increasing cancers in many western countries, including Sweden. This incidence rise is mainly attributed to sun-seeking habits with increased intermittent UVR exposure, a major risk factor for melanoma. Family history is another important risk factor for melanoma, approximately 10% of all cases occur in melanoma families. Germline mutations in the tumor suppressor gene *CDKN2A* occur in 5–25% of familial melanoma cases. A single founder mutation, p.Arg112dup, accounts for the majority of *CDKN2A* mutations in Swedish carriers. Individuals with p.Arg112dup and several other *CDKN2A* mutations also have an increased risk of developing pancreatic carcinoma, but less has been known about carriers’ risks of other cancers. High-risk melanoma associated mutations, other than *CDKN2A* have yet only been identified in a small number of families, in the majority of melanoma families, the cause for heredity still remains unsolved. So far, there have been no studies investigating cancer risks in *CDKN2A* wild type (wt) melanoma families. Also research addressing survival functions in melanoma families have until now been lacking. Compared to cutaneous melanoma, uveal melanoma is a much rarer disease, where no incidence rise or any strong association with UVR exposure has been observed. Familial uveal melanoma cases exist, but are rare. Until 2-3 years ago, there was no germline gene mutation known to be associated with uveal melanoma.

In papers I-III cancer risks and prognosis in familial melanoma kindreds, depending on *CDKN2A* mutation status is estimated by linkage of personal identity numbers of familial melanoma kindreds to several Swedish Registries, including the Multi-generation Registry and the Cancer Registry. Paper IV is a family-based association study employing whole-exome sequencing to identify a disease associated mutation in a rare uveal melanoma family.

Carriers of the Swedish founder mutation in *CDKN2A* and also carriers’ un-genotyped first- and second-degree relatives were found to have significantly increased risks of melanoma, pancreatic cancer, and cancers in respiratory and upper digestive tissues. Ever-smoking carriers had, compared to never-smoking carriers, significantly higher risks of these non-melanoma cancers. Familial melanoma cases with no *CDKN2A* mutation and their first-degree relatives had significant increased risk of melanoma and of squamous cell skin cancer, but not of other cancers. *CDKN2A* mutated melanoma cases had compared to *CDKN2A* wt cases, after adjusting for age, sex and tumor thickness, significantly increased mortality from melanoma and from non-melanoma cancers. Compared to matched sporadic melanoma cases, *CDKN2A* mutated cases had significantly increased mortality from both melanoma and non-melanoma cancers, while *CDKN2A* wt cases had no mortality increase compared to sporadic cases. In the uveal melanoma family, a disease segregating mutation was found in the *BAP1* tumor suppressor gene on chromosome 3p21.

These studies demonstrate different risk spectra among familial melanoma kindreds. *CDKN2A* mutation carriers have besides from melanoma high risks of tobacco-related cancers and have worse survival from both melanoma and other cancers compared to non-carriers. Familial melanoma cases with no *CDKN2A* mutation have increased risks only of skin cancers and have survival comparable to sporadic melanoma cases. *BAP1* mutation carriers have high risks of uveal melanoma and also of cutaneous melanoma and of other cancers. These findings further justify *CDKN2A* mutation testing of melanoma family members in the clinical setting where the mutation status should determine the follow-up routines in affected families. Members of *CDKN2A* wt melanoma families require counseling and screening aimed at prevention and earlier detection of skin cancers while *CDKN2A* mutation carriers require in addition to dermatologic surveillance, follow-up for non-skin cancers and also close follow-up for melanoma recurrences. *BAP1* mutation carriers require ophthalmologic, oncologic and dermatologic surveillance.
LIST OF SCIENTIFIC PAPERS

High risk of tobacco-related cancers in CDKN2A mutation-positive melanoma families.
*Journal of Medical Genetics, 51*:545-552, 2014.

II. **Hildur Helgadottir**, Veronica Höiom, Rainer Tuominen, Göran Jönsson, Eva Månsson-Brahme, Håkan Olsson, Johan Hansson.
*CDKN2A* mutation-negative melanoma families have increased risk exclusively for skin cancers but not for other malignancies.

III. **Hildur Helgadottir**, Veronica Höiom, Rainer Tuominen, Karie Nielsen, Göran Jönsson, Håkan Olsson, Johan Hansson.
Survival in familial melanoma cases carrying germline CDKN2A mutations:
Increased mortality from melanoma and non-melanoma cancers compared to mutation-negative melanoma cases.
*Submitted for publication.*

IV. Veronica Höiom, Daniel Edsgärd, **Hildur Helgadottir**, Hanna Eriksson, Charlotta All-Ericsson, Rainer Tuominen, Ivayla Ivanova, Joakim Lundeborg, Olof Emanuelsson, Johan Hansson.
Hereditary uveal melanoma: a report of a germline mutation in BAP1.

**Additional paper:**

The common Scandinavian human leucocyte antigen ancestral haplotype 62.1 as prognostic factor in patients with advanced malignant melanoma.
*Cancer Immunology, Immunotherapy, 58*:1599-608, 2009.
LIST OF ABBREVIATIONS

**ACD** Adrenocortical dysplasia protein homolog gene, encodes for a shelterin complex protein, TPP1

**AJCC** American Joint Committee on Cancer

**Akt** RAC-alpha serine/threonine-protein kinase, also known as Protein kinase B

**ASIP** Agouti-signaling protein gene

**BAD** Bcl-2-associated death promoter protein

**BAP1** BRCA1 associated protein-1 gene

**BCL2** B-cell lymphoma 2 protein

**BRAF** v-raf murine sarcoma viral oncogene homolog B1 gene coding for B-Raf proto-oncogene

**BRCA1** Breast cancer 1, early onset gene

**BRCA2** Breast cancer 2, early onset gene

**CDK4** Cyclin dependent kinase 4 gene

**CDK6** Cyclin dependent kinase 6 gene

**CDKN2A** Cyclin-Dependent Kinase Inhibitor 2A gene coding for tumor supressor proteins p16 and p14ARF

**CDKN2A\textsuperscript{mut}** Familial melanoma case with germline mutation in the CDKN2A gene

**CDKN2A\textsuperscript{wt}** Familial melanoma case with no germline mutation in the CDKN2A gene

**CI** Confidence Interval

**CM** Cutaneous melanoma

**CTLA-4** Cytotoxic T-lymphocyte-associated protein 4

**EGFR** Epidermal growth factor receptor

**FDA** Food and Drug Administration

**FDR** First degree relative

**GenoMEL** The melanoma genetics consortium

**GNA11** Guanine nucleotide-binding protein (G protein) subunit alpha-11

**GNAQ** Guanine nucleotide binding protein (G protein) q polypeptide

**Hdm2** Human double minute 2 homolog protein, also known as MDM2

**HLA** Human leukocyte antigen

**HR** Hazard ratio

**IARC** International Agency for Research on Cancer

**ICD** International Classification of Disease

**KIT** Stem cell growth factor receptor (SCFR) gene coding for c-Kit proto-oncogene

**LOH** Loss of heterozogosity
MAPK  Mitogen-activated protein kinases
MC1R  Melanocortin 1 Receptor gene
MEK  Map-ERK kinase/Mitogen-activated protein kinase kinase
Melanoma  Melanoma of the skin (if not otherwise specified)
MHC  Major histocompatibility complex
MITF  Microphthalmia-associated transcription factor gene
Mut  Mutated gene
NRAS  Neuroblastoma RAS Viral Oncogene Homolog gene coding for N-ras oncogene
OR  Odds ratio
P13K  Phosphatidylinositol 3-kinase
PD-1  Programmed cell death-receptor 1
PD-L1  Programmed cell death-ligand 1
POT1  Protection of telomeres protein 1 gene
PTEN  Phosphatase and tensin homolog gene
RB1  Retinoblastoma 1 gene coding for tumor supressor protein pRB
RR  Relative risk
SDR  Second degree relative
SNP  Single-nucleotide polymorphism
SweFam  Swedish network on familial melanoma
TERF2IP  Telomeric repeat-binding factor 2-interacting protein 1 gene
TERT  Telomerase reverse transcriptase gene
TNM  TNM Classification of Malignant Tumours (T; primary tumor, N; lymph node, M; distant metastasis)
TP53  Tumor protein p53 gene coding for tumor supressor protein p53
TYR  Tyr gene coding for Tyrosinase
TYRP1  Tyrosinase-related protein 1 gene
UM  Uveal melanoma
UVR  Ulte violet radiation
WHO  Worls Health Organization
Wt  Wild type (non-mutated) gene
XPF  Xeroderma pigmentosum, complementation group F gene, also known as ERCC4
XPG  Xeroderma pigmentosum, complementation group G gene, also known as ERCC5
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1 MALIGNANT MELANOMA OF THE SKIN

1.1 HISTORICAL PERSPECTIVE

1.1.1 Early observations

Although melanoma is not a new disease, written or archeological evidence for its occurrence before the 19th century is scarce. At a lecture in 1804 in Paris, the French physician René Leannec (1781-1826) was the first to describe melanoma as a disease entity. He described dark tumors in lungs, lymph nodes, liver, brain, stomach and peritoneum. He referred to the condition as *melanosis*, from the Greek word melas, which means black. He further noted that melanosis of the lungs was not associated with the same hectic fever as tuberculosis, that was a common condition at the time. Laennec is also renowned as the inventor of the stethoscope. The English general practitioner William Norris (1792-1877) was the first to study melanoma in depth, and made several principal observations on the pathology, epidemiology and management of melanoma. He described a correlation between moles, primary melanomas and disseminated melanoma. He noted that the degree of pigmentation varied and some lesions could be amelanotic. Norris observed that his patients had fair complexions and light colored hair. He also noted cases with family history of melanoma and multiple moles and suggested a probable hereditary predisposition. He also advocated wide excisions of the tumor and surrounding tissues.

1.1.2 Establishment of surgical management principles

The first known formal statement of advanced melanoma as untreatable was published in a book written in 1840 by the English surgeon Samuel Cooper (1780-1848) who remarked “no remedy is known for melanosis. The only chance for benefit depends upon the early removal of the disease by operation, when the situation of the part affected will admit of it.” The surgeon William Sampson Handley (1872-1962) advocated in 1907 at lectures for the Royal College of Surgeons of England, the importance of a wide local excision of the primary melanoma with a circular >10 cm incision of the skin and excision of underlying deep fascia and muscle in combination with regional lymph node dissection and amputation in selected cases. Handley’s recommendations formed the basis for melanoma treatment well into the 1980s when trials offered further refinement of the surgical approaches, such as defining proper surgical margins for primary melanoma and the utility of lymph node dissection.

1.1.3 Naissance of histological staging criteria predicting prognosis

A pioneer in the study on appropriate surgical margins for primary melanomas was the American pathologist Alexander Breslow (1928-1980). He had also, in a series of papers, the first published in 1970, showed that the most important single prognostic parameter of primary melanoma was tumor thickness, a better predictor of metastasis and survival than any other parameter, such as growth pattern, surgical margins or level of invasion. The Breslow tumor thickness became an important stratification criterion, and is since 2002 the primary criterion for primary tumor (T) classification in the TNM classification of the
American Joint Committee on Cancer (AJCC) melanoma staging system. The American pathologist Wallace H Clark, Jr (1924-1997) had in 1969, together with colleagues delineated the “Clark levels” of invasion, which were the primary stratification criterion in earlier AJCC staging schemae for primary melanoma. However, it became evident that Clark's level has a lower predictive value, is less reproducible, and is more operator-dependent as compared with Breslow's depth. Thus, in the current (2010) AJCC staging system, Clark level is no longer recommended as a staging criterion.

Clark also described 3 major histological types in melanoma, superficial spreading melanoma (SSM), nodular melanoma (NM) and lentigo maligna melanoma (LMM). Moreover, Clark described the dysplastic nevus, also known as the clinically atypical mole, Clark’s nevus or B-K mole (B and K; first initial in last names of melanoma families described by Clark et al. 1978) as a precursor and a marker of increased risk of melanoma among familial melanoma kindreds. The Australian pathologist Vincent J McGovern (1915-1983) wrote landmark papers on the significance of tumor thickness, ulceration, mitotic rate and regression and their relationship to prognosis. He had an important role in the implementation of classification systems and melanoma nomenclature. Tumor thickness, mitotic rate and ulceration are today all considered significant staging criteria and are included in the current AJCC staging system.

McGovern was further, in the late 1950s, one of the first to call attention to the role of sunlight in the development of melanoma.

1.1.4 Emergence of systemic therapies

During the 1970s chemotherapy began to make inroads in the treatment of disseminated melanoma. Studies of the alkylating agent dacarbazine (dimethyl traozeno imidazole carboxamide; DTIC) showed response rates up to 30%, which lead to the 1976 Food and Drug Administration (FDA) approval of this drug as the first systemic therapy for metastatic melanoma. Since other single or polychemotherapy agents have failed to show additional clinical benefit, dacarbazine, is still, together with its oral analog, temozolamide, the only FDA approved chemotherapeutic agent for the treatment of metastatic melanoma.

In parallel with the entrance of melanoma chemotherapy treatments, important observations on immunological responses to melanoma were made, such as the description of melanoma antigens in 1974. This marked the beginning of an elongated quest to identify immune based antitumoral regimens, leading to the FDA approval of adjuvant therapy in stage III melanoma with high-dose interferon in 1996 and of high-dose bolus IL-2 for advanced melanoma in 1998. Today, these drugs are in many countries, including Sweden, not considered part of standard melanoma treatment, but the translational discoveries on tumor immunology paved the way for subsequent discovery of targeted immune modulating therapies that, compared to standard chemotherapy, showed superior outcomes in randomized studies. This has lead to the FDA approval of the immune checkpoint inhibitors ipilimumab in 2011 and of pembrolizumab and nivolumab in 2014. The Human Genome project, that initiated in 1990 and was declared complete in 2003, significantly contributed to the ability to perform large-scale DNA studies. One of many results from this was the identification of high-frequency mutations in the BRAF and NRAS oncogenes.
that has subsequently lead to the discovery of selective inhibitors targeting the Ras-Raf-MEK-ERK pathway that is often constitutionally activated in melanoma\textsuperscript{30,31}. The BRAF inhibitors vemurafenib and dabrafenib were FDA approved in 2011 and 2013, respectively and the MEK-1 inhibitor trametinib in 2013\textsuperscript{27,32}. Currently, there are numerous ongoing studies investigating various targeted therapy regimens for metastatic melanoma\textsuperscript{33}.

1.2 EPIDEMIOLOGY

1.2.1 Population trends

The incidence of melanoma of the skin has been increasing in most Caucasian populations in the last decades\textsuperscript{34}. The increase in melanoma incidence is mainly ascribed to changes in attitudes toward sun bathing and tanning in westernized countries, but ageing populations as well as higher detection rates are also contributing factors\textsuperscript{34}. The highest melanoma incidences are observed in countries predominated by fair-skinned populations and sunny climates such as in Australia and New Zealand\textsuperscript{35} (Table 1). Countries predominated by African, Asian and Hispanic populations generally have much lower incidence rates\textsuperscript{35}. In Sweden there has been a steep increase in melanoma incidence since the 1970s (Figure 1), with close to 5\% yearly increase in the last decade (there are differences in the Swedish incidence numbers displayed, in Table 1 where the age standard incidence is based on the world standard population, whereas in Figure 1, the age-standardized incidence is based on the Swedish population, that is considerably older than the world population)\textsuperscript{36,37}. In recent years, several high-incidence countries have seen a leveling off in the melanoma incidence, implying a possible beginning of a decline\textsuperscript{34,36}. In Sweden, there is yet no sign of such a turn in the incidence, in 2014 there were over 3.723 melanomas diagnosed, compared to 3.358 in 2013 (C. Ingvär, personal communication, October 1\textsuperscript{st} 2015). The massive increase in melanoma incidence has not been followed by the same increase in mortality (Figure 1), only a subtle increase has been observed, probably explained mostly by increased preventive measures leading to earlier detection of tumors, but also by improvements in the management of the disease.

1.2.2 Age and sex

In 2013, 3.358 invasive melanomas (1.663 in women and 1.695 in men) were diagnosed in Sweden (current population 9.8 million inhabitants), which represents 5.5\% of all diagnosed cancers\textsuperscript{36}. Melanoma is the 5\textsuperscript{th} most common cancer among women and 6\textsuperscript{th} most common among males in Sweden. In both males and females, melanoma incidence increases with increasing age. Although the total numbers of melanomas, diagnosed in men and women, are almost equal, the age curves are differing, with earlier onset in women and higher incidences in older males (Figure 2)\textsuperscript{36}.
Table 1. Melanoma incidence* in different countries in 2012 per 100,000 inhabitants**

<table>
<thead>
<tr>
<th>EUROPE</th>
<th>AFRICA/ASIA</th>
<th>AMERICA/OCEANIA</th>
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<tbody>
<tr>
<td>Netherlands</td>
<td>19.4</td>
<td>South Africa</td>
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<tr>
<td>Denmark</td>
<td>19.2</td>
<td>Russia</td>
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<tr>
<td>Norway</td>
<td>18.8</td>
<td>Turkey</td>
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<td>Sweden</td>
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<td>Turkmenistan</td>
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<td>UK</td>
<td>14.6</td>
<td>Iran</td>
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<td>Ireland</td>
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<td>Iceland</td>
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<tr>
<td>Finland</td>
<td>12.6</td>
<td>China</td>
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<tr>
<td>Germany</td>
<td>11.4</td>
<td>Morocco</td>
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<tr>
<td>Italy</td>
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<td>Saudi Arabia</td>
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<tr>
<td>France</td>
<td>10.2</td>
<td>India</td>
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<tr>
<td>Spain</td>
<td>6.9</td>
<td>Ethiopia</td>
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<tr>
<td>Greece</td>
<td>2.4</td>
<td>Sri Lanka</td>
</tr>
</tbody>
</table>

*Based on data from WHO, IARC, Globocan 2012 (http://globocan.iarc.fr/Pages/Map.aspx)

**Age-standardized incidence rates based on world standard population

**Figure 1.** Age-standardized melanoma incidence and mortality per 100,000 inhabitants in Sweden 1970-2013. Reproduced from Swedish National Board of Health and Welfare.

**Figure 2.** Age specific melanoma incidence in 1991-93 and 2011-13 per 100,000 male and female inhabitants, 3-year mean value. Reproduced from Swedish National Board of Health and Welfare.
1.3 RISK FACTORS FOR MELANOMA OF THE SKIN

1.3.1 Ultraviolet radiation

Today, there is a strong consensus regarding ultraviolet radiation (UVR) being the most significant environmental risk factor for malignant melanoma of the skin\textsuperscript{38}. UVR is divided into the longer wavelength UV-A, the intermediate wavelength UV-B, and the shorter wavelength UV-C, which is completely absorbed by the atmospheric ozone layer and does not reach the surface of the earth. UV-A radiation reaches deeply into the skin causing tanning and ageing of the skin, while UV-B radiation is absorbed by the superficial epidermis causing skin reddening and sunburn\textsuperscript{39}. The main carcinogenic effect is believed to be from the UV-B radiation, but UV-A has also been shown to have a carcinogenic effect\textsuperscript{40}. The carcinogenic effects of UVR is believed to be due to DNA damage and mutations in the melanocytes, and also due to immunologic and inflammatory processes, growth stimulation and oxidative stress\textsuperscript{41}. The main source of UVR is the sun, where UV-A radiation accounts for \textasciitilde 95\% of the radiation reaching the earth’s surface. Sun beds are also a significant source of UVR in the population, before the 1980s such lamps could emit up to 40\% of the highly carcinogenic UV-B radiation, while modern lamps have much lower percentages of UV-B, down to \textasciitilde 0.1\%, but this can vary greatly\textsuperscript{42}. Sun lamps are generally believed to be a significant melanoma-causing carcinogen, particularly in young users. Sun lamp use is forbidden by law before the age of 18 years in many countries, but not yet in Sweden. Different patterns of UVR exposures during lifetime affects the histological melanoma subtypes that arise. Intermittent UVR exposures and sunburns, occurring for example at beach holidays in sunny countries, increase the risk of superficial spreading melanoma (SSM), which is the subtype of melanoma with the fastest growing incidence. Chronic UVR exposure, often occurring in outdoor workers, increases the risk of lentigo maligna melanoma (LMM) and also of non-melanoma skin cancers on chronically exposed sun-damaged skin\textsuperscript{39}.

UVR is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC)\textsuperscript{43}. In the IARC Group 1, there are 117 listed agents that, beyond doubt, are carcinogenic to humans. There are many different types of carcinogenic agents such as chemicals, hormones, radiation, radioactive substances, chemotherapeutic agents, viruses and bacteria. The single largest environmental source of carcinogens in Group 1 is tobacco smoke with at least 20 substances listed as group 1 carcinogens, including polycyclic aromatic hydrocarbons (PAHs), aza-arenes, aromatic amines, N-nitrosamines and aldehydes\textsuperscript{43,44}. While UVR and tobacco smoke derivates are both mutagenic, the mechanism of mutational processes differ and result in different signature mutations. UVR causes signature base pair C\textrightarrow T transitions, while tobacco smoke signatures can be recognized by C\textrightarrow A transitions\textsuperscript{45}. Tobacco smoke is strongly associated with many cancers, but not with melanoma\textsuperscript{46}.

1.3.2 Pigmentation traits

Pigmentation traits are important risk factors for melanoma, best illustrated by the high incidence differences between individuals of north-European descent compared to individuals
of African or Asian descent (Table 1)\textsuperscript{35}. In a large meta-analysis, where most participating centers were in countries dominated by Caucasian populations, it was found that blue or green eye color, red, blond or light hair color, high density of freckles, light skin color and photosensitivity where all associated with significantly increased risk for melanoma. The highest relative risk was seen for red vs. dark hair color, with a relative risk of 3.6\textsuperscript{47}.

Pigmentation traits are determined by genetic variants in a set of different genes. Many of such pigmentation genes, such as the Melanocortin-1 Receptor (MC1R) and the Tyrosine (TYR) genes have variants, single nuclear polymorphisms (SNPs) that are known to be both indirectly (through pigmentation traits) and independently associated with ~1.1 - 5 fold risk increase for melanoma \textsuperscript{48-50}. These genes are therefore called low-risk melanoma genes (in contrast to high-risk melanoma associated genes, such as CDKN2A). Other known pigmentation involved low-risk melanoma genes are ASIP and TYRP. SNPs in pigmentation genes are not only associated with increased risks of melanoma, but also with increased risks of non-melanoma skin cancers, in particular squamous cell skin cancer and basal cell skin cancer, while such SNPs are not associated with increased risks of non-skin cancers\textsuperscript{49}.

1.3.3 Familial predisposition

It was early described that cutaneous melanoma sometimes occurs in blood related individuals\textsuperscript{4}. It was noted that members of such families often had multiple atypical looking nevi. This condition was previously described by names such as dysplastic nevus syndrome (DNS), familial atypical multiple mole melanoma (FAMMM) or B-K mole syndrome\textsuperscript{16}. However, it has become apparent that in some families with multiple cases of melanoma, members do not have the characteristic multiple nevi, and also there are individuals and families that have many nevi without an association with melanoma\textsuperscript{51,52}. The best predictor of melanomas risk is previous melanomas in two or more closely related family members\textsuperscript{53,54}. Today the condition is commonly described simply as “familial melanoma”. It is estimated that approximately 10% of all cases of cutaneous malignant melanoma occur in melanoma families\textsuperscript{55-57}. Familial predisposition is among the strongest known risk factors for melanoma, where affected members can have up to 90% life time risk to develop melanoma\textsuperscript{58}. Mutations in the tumor suppressor gene CDKN2A are found in 5-25% of melanoma kindreds, where mutation frequency varies between regions and selection criteria used (Table 2)\textsuperscript{53,59}. In countries, such as Australia, the fraction of mutation positive families is lower, probably explained by the larger impact of UVR exposures on high melanoma incidences in such regions. Families with more melanomas diagnosed and more affected individuals, as well as younger ages of onset have higher incidences of CDKN2A mutations.

| Table 2. Percentage of CDKN2A mutation carrying families, depending on different features of the families* |
| Numbers of diagnosed melanoma tumors within a family, 2 vs. ≥3 | 7% vs. 17% |
| Numbers of melanoma affected individuals in family, 2 vs. ≥3 | 8% vs. 27% |
| Numbers of melanoma and pancreatic cancer diagnoses in family, 2 vs. ≥3** | 10% vs. 65% |
| Origin of families, Australia vs. North-America vs. Europe*** | 20% vs. 45% vs. 57% |
| Median age at diagnosis of melanoma, >50 vs. 40-50 vs. <40 *** | 12% vs. 32% vs. 54% |

**Only families with pancreatic cancer. ***Only families with ≥3 melanoma cases
1.4 STAGING, CLASSIFICATION AND PROGNOSIS

1.4.1 AJCC melanoma staging system

The most recent AJCC melanoma staging system was published in the 7th edition of the AJCC Cancer Staging Manual in 2010\textsuperscript{14}. The staging criteria are based on prospective data on 30,946 patients with stages I, II, and III melanoma and 7,972 patients with stage IV melanoma. For classification of the primary melanoma, tumor thickness, ulceration and mitotic grade are staging criteria (Table 3). Staging criteria for nodal metastasis is the number of affected lymph nodes and presence of micro- or macrometastases or in transit/satellite metastases. Staging criteria for distant metastases are distant skin or nodal metastasis (M1a), lung metastasis (M1b), other visceral metastasis (M1c) and/or elevated lactate dehydrogenase (M1c). Figure 1 shows how the defined staging criteria (Table 3-4) correlate with prognosis in the AJCC cohort. In stage I melanoma the five year survival rate is high, 92-97%. In stage II melanoma the five year survival goes down to 53-81% and in stage III melanoma, to 40-78%. The five year survival rate in stage IV melanoma is extremely low, only 10-20%.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Class & Thickness (mm) & Ulceration Status/ Mitoses \\
\hline
T1 & \leq 1.00 & a: Without ulceration and mitosis < 1/mm\textsuperscript{2}  \\
\hline
T2 & 1.01-2.00 & a: Without ulceration \\
\hline
T3 & 2.01-4.00 & a: Without ulceration \\
\hline
T4 & > 4.00 & a: Without ulceration \\
\hline
\end{tabular}
\caption{TNM Staging Categories for Cutaneous Melanoma}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Clinical Staging & Pathologic Staging \\
T & N & M & T & N & M \\
\hline
0 & T1 & N0 & M0 & 0 & T1 & N0 & M0 \\
IA & T1a & N0 & M0 & IA & T1a & N0 & M0 \\
IB & T1b & N0 & M0 & IB & T1b & N0 & M0 \\
IIA & T1c & N0 & M0 & IIA & T1c & N0 & M0 \\
IIB & T2 & N0 & M0 & IIB & T2 & N0 & M0 \\
IIIA & T3 & N0 & M0 & IIIA & T3a & N0 & M0 \\
IIIB & T3b & N0 & M0 & IIIB & T3b & N0 & M0 \\
IIIC & T4 & N0 & M0 & IIIC & T4 & N0 & M0 \\
IV & Any T & Any N & M0 & IV & Any T & Any N & M0 \\
\hline
\end{tabular}
\caption{Anatomic Stage Groupings for Cutaneous Melanoma}
\end{table}

*Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

*Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial ile, sentinel node biopsy or complete lymphadenectomy. Pathologic stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes.

Figure 3. Survival curves of patients in the AJCC Staging Database, comparing the different T categories (A) and the stage groupings for stages I and II melanoma (B). For patients with stage III disease, survival curves are shown comparing the different N categories (C) and the stage groupings (D). Survival curves of patients with metastatic melanomas at distant sites, subgrouped by the site of metastatic disease (E) and by serum lactate dehydrogenase (LDH) levels (F). Reproduced, with permission, from Balch et al. J Clin Oncol 2009.

1.4.2 Histologic subtypes of melanoma

For melanoma of the skin, the following main growth patterns have been described; superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM), nodular melanoma (NM) and acral lentiginous melanoma (ALM) (Table 5)\textsuperscript{15,60}. In addition, rarer subtypes exist, such as desmoplastic, verrucous, Spitzoid melanoma and malignant blue nevus\textsuperscript{39}. SMM tumors, characterized by their initial radial growth pattern, often arising in preexisting nevi, typically occur in somewhat younger individuals on intermittently UVR
exposed locations, such as on the trunk, arms and legs\textsuperscript{39,61}. NM tumors are characterized by their vertical growth pattern that is not preceded by a radial growth phase, as in SSM tumors. Mean age of diagnosis of NM tumors is higher than of SSM, but lower than of LMM. NMs can occur at any location, often in the head and neck area. LMM are slowly evolving tumors, often occurring in older individuals in chronically sun exposed areas on the face, ears and back of hands. ALMs are prominent in black and Asian populations and occur in the palms and soles, fingers, nail bed and also in mucosal membranes. Survival is considered similar for patients with SSM and NM tumors, while outcomes are more beneficial in LMM patients, but poorer in ALM patients\textsuperscript{39}. In SSM and NM tumors, mutations in the \textit{BRAF} gene are seen in 50-60\% and in \textit{NRAS} in 20-30\% of cases\textsuperscript{62,63}. In LMM and ALM tumors, \textit{KIT} mutations are seen in 10-30\% of cases\textsuperscript{62,64}. \textit{CDKN2A} mutations are seen in 10-15\% of tumors, irrespectively of histologic subtype\textsuperscript{62}.

Table 5. Main melanoma growth patterns: Epidemiological, clinical, molecular and prognostic features.

<table>
<thead>
<tr>
<th>Incidence in Sweden</th>
<th>Main patient groups</th>
<th>Typical anatomical Location</th>
<th>UVR induced</th>
<th>Prevalent mutations</th>
<th>Prognostic Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSM 61%</td>
<td>Younger</td>
<td>Trunk, arms, legs</td>
<td>+++ (Intermittent exposure)</td>
<td>\textit{BRAF/NRAS, CDKN2A}</td>
<td>↔</td>
</tr>
<tr>
<td>NM 16%</td>
<td>Old/young</td>
<td>Any, head and neck area</td>
<td>++ (Intermittent exposure)</td>
<td>\textit{BRAF/NRAS, CDKN2A}</td>
<td>↔</td>
</tr>
<tr>
<td>LMM 7%</td>
<td>Older</td>
<td>Face, ears, back of hands</td>
<td>+++ (Chronic exposure)</td>
<td>\textit{KIT, CDKN2A}</td>
<td>↑</td>
</tr>
<tr>
<td>ALM 1%</td>
<td>Non-Caucasians</td>
<td>Palms/soles, fingers, nail beds, mucosa</td>
<td>(+)</td>
<td>\textit{KIT, CDKN2A}</td>
<td>↓</td>
</tr>
</tbody>
</table>

\subsection*{1.4.3 Other tumor specific prognostic factors}

Of tumor specific prognostic factors, the most relevant known today are included in the AJCC staging system. Anatomic site of the melanoma has also been shown to be an independent prognostic marker, with worse survival in patients with melanoma in the head and neck area, trunk, palms/soles and nailbeds\textsuperscript{65}. The presence of tumor-infiltrating lymphocytes, which are believed to represent the immune reaction/response to the melanoma, has both been supported and refuted as a positive prognostic marker\textsuperscript{66,67}. Melanoma regression with replacement of tumor tissue with fibrosis, degenerated melanoma cells, lymphocytic proliferation, and telangiectasia formation, is generally considered an adverse prognostic factor, but as with TILs, this has both been supported and rebutted\textsuperscript{68,69}.

\subsection*{1.4.4 Tumor-based genetic and molecular prognostic factors}

In the 1980s DNA flow cytometric analyses showed that aneuploidy correlated with poor prognosis in melanoma\textsuperscript{70}. Melanomas are genetically instable tumors and have been demonstrated to be the tumor type that harbors the highest mutational load\textsuperscript{45}. \textit{BRAF} and \textit{NRAS} mutations are common early and mostly mutually exclusive mutations in melanoma tumors\textsuperscript{63}. Mutations in these genes are generally considered markers of poor prognosis, but it remains unresolved if they are independent prognostic factors\textsuperscript{71}. Somatic mutations and deletions of the \textit{CDKN2A} gene have also been associated with worse outcomes in melanoma patients\textsuperscript{72,73}. Further, expression analyses have shown that expression levels of \(|1,500\) distinctive genes reveals “low- and high-grade” signatures that predict survival in all stages of melanoma\textsuperscript{74,75}. 
1.4.5 Host related prognostic factors

In several studies, older age and male sex have been shown to be independent predictors of poor prognosis. The intrinsic or extrinsic causality behind this remains unresolved, but hormonal and immunologic differences have been suggested, as well as social factors affecting the course of diagnosis and treatment in different groups. Lower socioeconomic status as well as living alone are associated with later diagnosis of melanoma tumors, but also independently predict poorer prognosis. On a molecular level, certain haplotypes of human leukocyte antigens (HLA) of the human major histocompatibility complex (MHC) have been shown to affect prognosis, probably reflecting different host-dependent immune responses to tumors. Germline mutations in the tumor suppressor gene CDKN2A are among the strongest known inherited genetic risk factors of cutaneous melanoma, but until now it has been unresolved whether such mutations also affect prognosis.

1.5 MELANOMA PREVENTION

1.5.1 Primary prevention: Education campaigns

Primary prevention aims to avoid the development of disease. In the early 1960s increasing knowledge began to emerge on the role of sunlight and ultraviolet radiation (UVR) in the development of melanoma of the skin. In Australia, where very high incidence rates for melanoma were observed, preventive actions were initiated in the 1960s. In the 1980s coordinated regional or nationwide campaigns were introduced in Australia, the U.S. and many European countries, including Sweden. The focus of such campaigns was, and is still, to increase awareness of skin cancer in the population and promote habits to diminishing UVR exposure and sunburns, such as wearing protective clothing, avoidance of sun exposure in the middle of the day, staying in the shade, use of sunscreen, avoidance of tanning parlors etc. In spite of these preventive efforts, melanoma incidence has continued to rise steeply. Recent leveling off, observed in some high incidence countries, hopefully marks the beginning of a turn in this trend.

1.5.2 Secondary prevention: Skin cancer screening

Secondary prevention aims at detecting and treating diseases early. Among the most extensive secondary preventive measures carried out so far, are the population based screening programs for breast and cervical cancer that are available in most developed countries. In most western countries, including Sweden, there are no organized nationwide screening programs for skin cancers. In many countries, national or regional health care services instead provide public information or campaigns to promote self skin exams. In this sense, the ABCDE criteria (Asymmetry, irregular Border, multiple Colors, Diameter >5-6 mm, Evolving) have been used to aid the public to identify and seek medical assessment of suspicious lesions.

In the Schleswig-Holstein region in Germany, a population-based study started in 2003 with whole-body inspection by general practitioner and dermatologists. This study showed that in the
following 5 years, melanoma and non-melanoma skin cancers were diagnosed at earlier stages and that the melanoma specific mortality significantly decreased compared to neighboring regions as well as in a nationwide comparison\(^\text{87}\). As a result of this, Germany was in 2008, the first (and is still the only) country to introduce skin cancer screening as a standard benefit of the general health insurances with biennial skin exams starting at age 35 years. A subsequent study was carried out to evaluate the outcomes in 2008-2012 of the Schleswig-Holstein intervention and the nationwide screening program\(^\text{88}\). In this period no decrease in melanoma mortality was seen compared to before the interventions and melanoma mortality in Germany did not differ from those observed in surrounding countries. While evaluation of this screening initiative will continue, as for now, the rationale for population-based programs is weak.

In many countries, including Sweden, there are preventive programs aimed at identifying high-risk individuals, in particular members of melanoma families or individuals with multiple primary melanomas. These individuals are subsequently enrolled in screening programs with regular dermatologic surveillance with total body photography and digital dermatoscopy. Such screening has been shown to result in high numbers of histopathologically dysplastic nevi being excised and a low incidence of melanomas, and that the melanomas that do arise have favorable prognostic characteristics\(^\text{54,89,90}\). The effect on survival outcomes from screening of high-risk groups has not yet been evaluated.

1.5.3 **Tertiary prevention: Melanoma follow-up**

Tertiary prevention aims at preventing progression and complications from a manifest disease. Follow-up of patients after the diagnosis of melanoma is, by definition, an example of this. Although follow-up is practiced at most oncology centers, there is no clear evidence of this resulting in a survival benefit\(^\text{91,92}\). Not either when blood tests or radiology exams are used as part of the follow-up program, does this seem to affect survival\(^\text{93,94}\). Of melanoma recurrences, over 80% manifest within the first three years after diagnosis of the primary tumor. In most cases it is, in fact, the patient him/herself that detects metastases or new primary melanomas\(^\text{95}\). As for now, the role of the follow-up is considered to be mainly psychosocial support for the patient by providing help and guidance to cope with the melanoma diagnosis. Also follow-up has a role to educate the patient to recognize symptoms and signs of melanoma recurrences and new primary melanomas. To enable quick medical assessments of such findings, the patient needs to have good access to the clinic where the follow-up is carried out. The follow-up schedules differ between countries, and are mostly dependent on local traditions and preferences. In the Swedish clinical guidelines for melanoma management, it is recommended that stage 0-I melanoma patients receive a follow-up visit within 6 weeks after the operation with general information on the disease and education on UVR protection and self-exams, no further routine controls are recommended thereafter\(^\text{61}\). Stage II melanoma patients receive, in addition, yearly follow-up visits with clinical exams and education for three years. Stage III patients receive such follow-up visits biannually for three years. To enable good access to the follow-up clinic, all patients get a contact-nurse. With better treatments options for advanced
disease, and if adjuvant therapies emerge in the melanoma management, is possible that a more active approach in follow-up routines will be implicated.

1.6 MANAGEMENT OF CUTANEOUS MELANOMA

1.6.1 Surgery of primary melanoma

Appropriate surgical management remains the most important life-saving treatment of cutaneous melanoma. As described earlier, before the extent of proper surgical margins had been determined, very large skin resections were common, requiring large skin grafts with substantial morbidity and poor cosmetic results. Today, multiple randomized studies have provided evidence for more limited surgical margins\(^{61}\). In the Swedish national guidelines for malignant melanoma, a wide local excision of skin and subcutaneous tissue is recommended. Surgical margins to a pigmented lesion should be at least 2 mm, to a melanoma in situ at least 5 mm and to a thin melanoma (≤1.0 mm), at least 10 mm and to a thick melanoma (>1.0 mm) at least 20 mm. In the head and neck area, due to the cosmetic and functional aspects, 10 mm are considered sufficient margins\(^{61}\). There is no evidence of a benefit of postoperative radiation at the operation site.

1.6.2 Management of regional lymph node involvement

Previously, prophylactic regional lymph node dissections were common, resulting in quite a degree of morbidity in many patients, mainly due to wound complications and lymphedema. Randomized studies have demonstrated that prophylactic elective lymphadenectomies do not have any positive effect on patient survival\(^{96}\). In most countries, including Sweden, sentinel node biopsies are performed in melanomas >1.0 mm as well as in all ulcerated tumors or if mitotic rates are high\(^{61}\). A radioactive colloid as well as a blue color substance is injected at the site of the primary melanoma to identify the nearest draining lymph node(s) that is subsequently excised. If pathology examination detects melanoma cells in an excised lymph node, local lymph node dissections are performed. A large randomized study has shown that this method is efficient to prevent disease recurrences, but does not seem to effect disease survival\(^{97}\). The method is nevertheless an important tool for melanoma staging and could also take on an important role, if adjuvant therapies become implicated in standard melanoma treatment schemas.

Today, there is no clear evidence of a benefit of studied systemic adjuvant regimens for high-risk melanomas. High-dose interferon as well as immune checkpoint blockade with the CTLA4-inhibitor ipilimumab has shown some effectiveness but also considerable morbidity and mortality, and there are no predictive markers for therapy gain\(^{98,99}\). Currently there are ongoing studies on the adjuvant use of the PD-1 inhibitors pembrolizumab and nivolumab, and of the BRAF and MEK inhibitors vemurafenib, dabrafenib and trametinib\(^{33}\).

Postoperative radiotherapy to metastatically involved regional lymph node stations has been shown to prevent local relapses but does not affect survival. In the Swedish national melanoma guidelines, postoperative radiotherapy is recommended after non-radical lymph
13

node dissections, if there is involvement of >3 lymph nodes, if size of lymph node metastasis is >3 cm or if there is periglandular involvement.

1.6.3 Local management of melanoma metastasis

Local recurrences (satellite tumors) in or adjacent to the previously operated primary melanoma or in transit metastasis should always be treated by radical surgery.\textsuperscript{61,96} Metastatic survey with PET-CT, CT or MRI is recommended, as the risk for distant metastasis is substantial. If radical operation is not possible, postoperative radiation should be considered. In patients with frequent multiple local recurrences in a limb, but no signs of distant spread, isolated hyperthermic perfusion of the limb, with melaphalan has shown objective response in majority of cases and complete remissions in 60% of cases.\textsuperscript{100}

For limited, solitary distant metastasis there is some evidence of a survival benefit of radical surgery of such solitary metastases, e.g. in distant lymph nodes and subcutis and also in the liver, brain and other visceral organs. There is no evidence of a benefit of debulking non-radical surgery of disseminated melanoma.\textsuperscript{96}

Brain metastases are common distant sites of melanoma. As mentioned previously, surgery is an option for solitary metastases. If the number of brain metastasis does not exceed 4-5 and lesions are small (≤2 cm), stereotactic gamma radiation is recommended. If there are more brain metastases, whole brain radiation can be considered. Glucocorticoid steroids, such as betamethason, should be used to improve symptoms of brain edema.\textsuperscript{96}

Palliative radiotherapy can also be considered for painful bone metastasis or for metastasis with threatening perforation and ulceration of the skin.

1.6.4 Systemic therapies for stage IV melanoma

Disseminated melanoma has until recent years been considered one of the more therapy-resistant malignancies. Since dacarbazin was approved in the 1970s, no other chemotherapy agents have been approved for treatment of metastatic melanoma. Immunologic treatments involving interferons and interleukins have shown low response rates and considerable side effects, and are not recommended for melanoma treatment in many countries, including Sweden. While dacarbazine and its oral analog temozolamide, are still indicated in melanoma treatment, novel immunological and targeted therapies have entered the melanoma field with a vengeance. There are two different groups of new drugs that have been approved, the immune checkpoint inhibitors and inhibitors of the Ras-Raf-MEK-ERK pathway (Figure 5 and 7). The first approved checkpoint inhibitor was ipilimumab that had shown a response rate of about 15%, but sometimes remarkable durable remissions.\textsuperscript{24} The treatment has substantial, immune related, sometimes life-threatening side effects such as colitis, hepatitis, dermatitis and rarely also hypophysitis. The PD-1 blocking immune checkpoint inhibitors pembrolizumab and nivolumab were later shown to have higher response rates, improved progression-free and overall survival and milder side effects than Ipilimumab, and are currently considered the first line of immune-therapy in metastatic melanoma.\textsuperscript{25,26}
Activating BRAF mutations at codon 600 (mainly V600E) are found in approximately 50% of melanomas, particularly in nodular and superficial spreading melanomas\textsuperscript{62,63}. Vemurafenib was the first approved BRAF-inhibitory therapy for BRAF mutated melanoma. Objective, rapid and sometimes striking complete responses are seen in around 50% of metastatic melanoma cases, but therapy resistance and relapse usually develops after some months\textsuperscript{30}. Although the therapy, which is in tablet form, is generally well tolerated it is sometimes complicated by quite peculiar side-effects such as photo-sensitivity and fast evolving benign and sometimes malignant keratinocytic skin lesions. Later, the BRAF inhibitor dabrafenib was approved. Dabrafenib has similar antitumor effect as vemurafenib, but is not associated with the same photo-sensitivity, but instead pyrexia is more frequent with this therapy\textsuperscript{32}. Addition of the MEK-1 inhibitor trametinib has shown further improvements in survival, and less incidence of keratinocytic lesions, although pyrexia is more frequent\textsuperscript{31}. Currently, there are several ongoing phase II-III studies on novel agents and treatment combinations which will hopefully lead to further improvements in the treatment of stage IV melanoma in the coming years\textsuperscript{33}.

1.7 BIOLOGY OF MELANOMA SUSCEPTIBILITY AND PROGRESSION

1.7.1 UVR induced pigmentation and carcinogenesis

The epidermis consists of two main cell types, the epidermally derived keratinocytes and the neural crest derived melanocytes\textsuperscript{101}. Melanocytes are dendritic cells that are prominent in the skin, but are also found in other tissues, such as in mucous membranes, uveal tract and leptomeninges, in which primary melanocytic tumors also sometimes arise. The pigment melanin is produced in the melanocytes and is transferred to surrounding keratinocytes\textsuperscript{102}. In what is known as an epidermal melanin unit, melanocytes are, through dendritic extensions, in direct contact with on average 10, but up to as many as 50 neighboring keratinocytes. There are two types of melanin pigment, the dark eumelanin that is abundant in dark skinned individuals, and the light-colored pheomelanin. Both eumelanin and pheomelanin are synthesized in the melanocyte from the precursor amino acid tyrosine. Both dark and light skinned people have equal amounts of pheomelanin, while eumelanin is absent or low in individuals with fair complexions and high in individuals with dark complexions\textsuperscript{102}.

The deep penetrating UV-A radiation, directly stimulates melanocytes to release melanin and also causes oxidation and darkening of existing melanin, leading to immediate tanning\textsuperscript{102} (Figure 4). UV-B radiation causes inflammation, sunburn and apoptosis of keratinocytes, initiating a cascade of events leading to delayed tanning. The cellular damage of keratinocytes promotes production of α-Melanocyte stimulating hormone (α-MSH). α-MSH is a ligand of the Melanocortin-1 Receptor (MC1R). Activation of MC1R subsequently leads to activation of the transcription factor MITF with upregulation of tyrosinase (TYR), TYRP1 and other enzymes leading to increased synthesis of melanin which is packed into melanosomes that are distributed to neighboring keratinocytes to protect the skin against further UVR damage. The signaling protein ASIP is an antagonist of the MC1R receptor that blocks the downstream production of eumelanin, increasing synthesis of pheomelanin.
Figure 4. Mechanisms of the cutaneous response to UVR.

Figure 5. RAS-RAF-MEK-ERK and PTEN pathways (A). p53 and RB pathways (B). Reprinted, with permission, from Miller et al. N Eng J Med 2006.
Variants in many of the genes involved in skin pigmentation, including \textit{MC1R}, \textit{TYR}, \textit{TYRP1} and \textit{ASIP} are associated with pigment features in individuals, and also with melanoma susceptibility, were some variant are associated with 1.1–5 fold risk increase of melanoma\textsuperscript{48,49} (Table 6).

UVR (mainly UV-B, but also to some extent UV-A radiation) causes both directly and indirectly DNA damage and mutations. The indirect effect on DNA is mainly through the formation of free radicals that cause DNA mispairing and point mutations\textsuperscript{103}. In contrast to eumelanin, pheomelanin is especially prone to photodegradation, generating free radicals that can cause mutations\textsuperscript{104}. UVR is also directly absorbed in DNA causing typical UVR-signature mutations (C→T, CC→TT)\textsuperscript{105}. There are several DNA-repair proteins that recognize and repair DNA damage, but also in these genes there are polymorphisms and mutations that are associated with increased melanoma susceptibility, e.g. in the xeroderma pigmentosum (XP) genes XPG and XP\textit{F}\textsuperscript{50}. Individuals with certain polymorphisms in such genes only have low risk increases for melanoma (RR 1.1–1.7), while sufferers of xeroderma pigmentosum, an autosomal recessive disorder with biallelic mutations in nucleotide excision repair genes, including XP-A to XP-G, develop multiple skin lesions including melanomas and non-melanoma cancers\textsuperscript{106}. XP cancers have multiple characteristic UV signature mutations, demonstrating the role of DNA-repair to hold back UV induced mutations. \textit{BRAF} and \textit{NRAS} mutations are often early, mutually exclusive events in the formation of melanocytic tumors and are frequently seen already in benign melanocytic nevi\textsuperscript{41,107,108}. Although \textit{BRAF} and \textit{NRAS} mutations are mainly seen in melanomas arising on UVR-exposed areas, the mutations seen in these genes are mostly not typical UVR signature mutations. It has been suggested that in spite of the lacking classical UVR signature, these mutations could be secondary effects of UVR damage\textsuperscript{109}. Other, later occurring mutations in genes such as tumor suppressors \textit{CDKN2A}, \textit{PTEN} and \textit{RAC1} are essential for the transformation to malignant melanoma, and mutations in these genes are often UV signature mutations\textsuperscript{110,111}.

1.7.2 \textbf{RAS-RAF-MEK-ERK and PTEN-P13K-AKT pathways in melanoma}

The RAS-RAF-MEK-ERK (also known as MAPK pathway) and the PTEN-P13-AKT pathways are frequently activated in melanoma tumors (Figure 5a)\textsuperscript{41}. Both pathways can be physiologically activated through receptor tyrosine kinases such as epidermal growth factor (EGFR) or c-Kit. The RAS proteins belong to a family of GTPases (including NRAS, H\textit{RAS} and \textit{KRAS}) located on the inside of the cell membrane. In melanoma, activating \textit{NRAS} mutations (mainly at codon 61 and more rarely on codon 12-13) are found in approximately 20\% of tumors\textsuperscript{63,112}. Activating mutations in \textit{NRAS} can cause parallel activation of both the RAS-RAF-MEK-ERK and the PTEN-P13-AKT pathways. RAS proteins phosphorylate and activate proteins of the RAF family of serine/threonine kinases (including \textit{BRAF} and \textit{CRAF}). In melanoma, activating \textit{BRAF} mutations (mainly at codon 600) are found in approximately 50\% of tumors\textsuperscript{63,112}. Oncogenic activation through mutations in \textit{NRAS} or \textit{BRAF} triggers downstream signaling through MEK and ERK kinases with expression of Cyclin D1 which promotes cell proliferation\textsuperscript{41}. 
The PTEN-P13K-AKT pathway is regulated through PTEN, an inhibitor of the P13K kinase (Figure 5a). Inactivation of PTEN promotes cell survival by downregulating pro-apoptotic signaling through proteins AKT and BAD. The tumor suppressor gene PTEN is, after BRAF and NRAS, among the most frequently mutated genes in melanoma tumors. PTEN mutations are hence believed to have an important role in the malignant dysregulation and malignant transformation of melanocytes. In a rare inherited condition, Cowden syndrome, germline mutations in PTEN are found. Carriers of germline mutations in PTEN predominantly have increased risks of breast, thyroid and endometrial cancer, but also elevated risks of other cancers, including melanoma (Table 6).

1.7.3 Pathways involving CDKN2A encoded tumor suppressor proteins p16 and P14ARF

The CDKN2A gene encodes two distinct proteins, p16 (INK4A) and p14ARF that are tumor suppressors and cell cycle inhibitors (Figure 5b). The gene has 4 exons, E1B, E1A, E2 and E3. Separate first exons that are spliced into alternate reading frames of the second and third exons permit the expression of two different proteins, p16 and p14ARF, from the same genetic locus. Transcription can be initiated at either E1B or E1A which determines which gene will be expressed. The E1A containing transcript encodes the p16 protein which is structurally an ankyrin repeat protein, consisting of 4 separate ankyrin repeats that fold into the active protein that inhibits the cyclin dependent kinases CDK4 and CDK6. These two kinases drive the cell cycle by phosphorylating the retinoblastoma protein, pRB, releasing it from its inhibitory interaction with the E2F transcription factor, thereby allowing the expression of E2F-related genes and progression from G1 to S-phase. The cell cycle inhibitor p16 thus inhibits cell cycle progression while absence of p16 leads to unopposed CDK4 or CDK6 activity and increased cell cycle activity.

P14ARF, the other protein of the CDKN2A locus, transcribed in an alternate reading frame, works through yet another key cellular regulatory pathway. p14ARF sequesters the MDM2 protein which is a negative inhibitor of tumor suppressor p53, also called, “guardian of the genome” due to its central role in protecting the organism from DNA damage and harmful mutations. p53 either activates DNA repair and cell-cycle arrest or causes apoptosis. In the absence of p14-ARF, p53 levels are decreased resulting in uncontrolled proliferation.

The CDKN2A gene is frequently mutated or deleted in melanoma tumors, leading to the disruption of p16 and/or p14ARF activity. In addition, germline CDKN2A mutations are among the strongest known inherited genetic risk factors for cutaneous melanoma, being mutated in 5-25% of melanoma kindreds. Germline mutations in CDK4, an oncogene, are also found in rare melanoma families. Somatic mutations in TP53 and RB1 are rather common in melanoma tumors, but usually mutually exclusive with CDKN2A mutations. Germline mutations in TP53 (Li Fraumeni syndrome) and RB1 (Retinoblastoma) are cancer syndromes that are dominated by non-melanoma cancer types, although increased melanoma risk has been described in both syndromes (Table 6).
**Table 6. Genes with germline variations associated with melanoma risk**

<table>
<thead>
<tr>
<th>Melanoma, medium to high-risk</th>
<th>Year identified</th>
<th>Role in cancer</th>
<th>Pathway involved</th>
<th>Population incidence</th>
<th>Incidence in melanoma families</th>
<th>Melanoma penetrance or relative risk (RR)</th>
<th>Main cancers</th>
<th>DNA alterations in melanoma tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDKN2A</strong></td>
<td>1994</td>
<td>Tumor suppr.</td>
<td>Cell cycle</td>
<td>&lt;0.1%</td>
<td>5-25%</td>
<td>60-90% penetrance</td>
<td>Melanoma, pancreatic, lung</td>
<td>Frequent (12-19%)</td>
</tr>
<tr>
<td><strong>CDK4</strong></td>
<td>1995</td>
<td>Oncogene</td>
<td>Cell cycle</td>
<td>&lt;0.1%</td>
<td>18 known families</td>
<td>&gt;70% penetrance</td>
<td>Melanoma, ND</td>
<td>Occur (4-7%)</td>
</tr>
<tr>
<td><strong>MITF</strong></td>
<td>2011</td>
<td>Oncogene</td>
<td>Pigmentation, proliferation</td>
<td>&lt;2%</td>
<td>~1%</td>
<td>RR 5-10</td>
<td>Melanoma, renal</td>
<td>Occur (1-8%)</td>
</tr>
<tr>
<td><strong>TER1- promoter</strong></td>
<td>2013</td>
<td>Tumor suppr.</td>
<td>Telomere maintenance</td>
<td>&lt;0.1%</td>
<td>1 known family</td>
<td>ND</td>
<td>Melanoma, ND</td>
<td>Frequent (&gt;30%)</td>
</tr>
<tr>
<td><strong>POT1</strong></td>
<td>2014</td>
<td>Tumor suppr.</td>
<td>Telomere maintenance</td>
<td>&lt;0.1%</td>
<td>~10 known families</td>
<td>Highly penetrant</td>
<td>Melanoma, glioma, ND</td>
<td>Occur (2-7%)</td>
</tr>
<tr>
<td><strong>ACD</strong></td>
<td>2014</td>
<td>Tumor suppr.</td>
<td>Telomere maintenance</td>
<td>&lt;0.1%</td>
<td>4 known families</td>
<td>ND</td>
<td>Melanoma, ND</td>
<td>Occur (2-4%)</td>
</tr>
<tr>
<td><strong>TERF2IP</strong></td>
<td>2014</td>
<td>Tumor suppr.</td>
<td>Telomere maintenance</td>
<td>&lt;0.1%</td>
<td>4 known families</td>
<td>ND</td>
<td>Melanoma, ND</td>
<td>Rare (0-1%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>High-risk cancer</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RB1 (Retinoblastoma)</strong></td>
<td>1986</td>
<td>Tumor suppr.</td>
<td>Cell cycle</td>
<td>&lt;0.1%</td>
<td>&lt;1%</td>
<td>RR 20</td>
<td>Retinoblastoma, sarcoma</td>
<td>Occur (4-5%)</td>
</tr>
<tr>
<td><strong>XP (Xeroderma Pigm.)</strong></td>
<td>1990s</td>
<td>Tumor suppr.</td>
<td>DNA repair (different genes)</td>
<td>&lt;0.001%</td>
<td>&lt;1%</td>
<td>RR 2,000</td>
<td>Skin cancers</td>
<td>Bialelic mut ND</td>
</tr>
<tr>
<td><strong>PTEN (Cowden)</strong></td>
<td>1998</td>
<td>Tumor suppr.</td>
<td>Survival signaling</td>
<td>&lt;0.1%</td>
<td>&lt;1%</td>
<td>RR 5-10</td>
<td>Breast, thyroid</td>
<td>Frequent (4-12%)</td>
</tr>
<tr>
<td><strong>BRCA2</strong></td>
<td>2004</td>
<td>Tumor suppr.</td>
<td>DNA repair</td>
<td>&lt;0.1%</td>
<td>&lt;1%</td>
<td>RR 2-3</td>
<td>Breast, ovarian</td>
<td>Frequent (7-12%)</td>
</tr>
<tr>
<td><strong>TP53 (Li Fraumeni)</strong></td>
<td>2006</td>
<td>Tumor suppr.</td>
<td>Preventing mutations</td>
<td>&lt;0.1%</td>
<td>&lt;1%</td>
<td>ND</td>
<td>Sarcoma, breast</td>
<td>Frequent (12-19%)</td>
</tr>
<tr>
<td><strong>BAP1</strong></td>
<td>2012</td>
<td>Tumor suppr.</td>
<td>e.g. chromatin regulation</td>
<td>&lt;0.1%</td>
<td>&lt;1%</td>
<td>~10% penetrance</td>
<td>Uveal, mesenchymoma, melanoma, renal</td>
<td>Occur (1-8%)</td>
</tr>
<tr>
<td><strong>BRCA1</strong></td>
<td>2015</td>
<td>Tumor suppr.</td>
<td>DNA repair</td>
<td>&lt;0.1%</td>
<td>&lt;1%</td>
<td>RR 2-3</td>
<td>Breast, ovarian</td>
<td>Occur (2-6%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Melanoma, low-risk</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MC1R</strong></td>
<td>1998</td>
<td>Risk modifier</td>
<td>Pigmentation</td>
<td>&gt;5%</td>
<td>2-3 fold popl. incidence</td>
<td>RR 1.1-5</td>
<td>Skin cancers</td>
<td>Rare (0-1%)</td>
</tr>
<tr>
<td><strong>TYR</strong></td>
<td>2008</td>
<td>Risk modifier</td>
<td>Pigmentation</td>
<td>&gt;5%</td>
<td>2-3 fold popl. incidence</td>
<td>RR 1.1-5</td>
<td>Skin cancers</td>
<td>Occur (0-5%)</td>
</tr>
<tr>
<td><strong>TYRP1</strong></td>
<td>1992</td>
<td>Risk modifier</td>
<td>Pigmentation</td>
<td>&gt;5%</td>
<td>2-3 fold popl. incidence</td>
<td>RR 1.1-5</td>
<td>Skin cancers</td>
<td>Occur (4-6%)</td>
</tr>
<tr>
<td><strong>ASIP</strong></td>
<td>2005</td>
<td>Risk modifier</td>
<td>Pigmentation</td>
<td>&gt;5%</td>
<td>2-3 fold popl. incidence</td>
<td>RR 1.1-5</td>
<td>Skin cancers</td>
<td>Occur (1-3%)</td>
</tr>
<tr>
<td><strong>XP genes SNPs</strong></td>
<td>2007</td>
<td>Tumor suppr.</td>
<td>DNA repair (different genes)</td>
<td>Varying</td>
<td>Varying</td>
<td>RR 1.1-1.7</td>
<td>Skin cancers</td>
<td>Occur (1-8%)</td>
</tr>
</tbody>
</table>

**ND:** Not determined

Year identified: Year when germline alterations in gene associated with melanoma risk (by first evidence based study on www.pubmed.com)

**RR:** Relative risk

Skin cancers: Melanoma and non-melanoma skin cancer

**NA:** Not applicable

The transcription factor MITF has a central role in the regulatory functions of the melanocyte (Figure 4-5). MITF is regulated both through pigmentation pathways and Ras-Raf-MEK-ERK signaling. MITF regulates various cell processes through the transcription of proteins involved in pigmentation (TYR, TYRP1 and MC1R), proliferation and cell cycle regulation (p16, p21, CDK2) and apoptosis (BCL2). MITF is considered to be an oncogen in melanoma where the gene locus is infrequently duplicated. A MITF gene variant, p.E318K has been shown to be associated with increased numbers of nevi and a moderate risk increase for melanoma. MITF is considered to be an intermediate melanoma risk gene (between the low-risk pigmentation genes and high-risk genes such as CDKN2A), with a 2-10 fold risk increase in p.E318K carriers (Table 6).

1.7.4 Telomere maintenance mechanisms in melanoma

A telomere is a region of repetitive nucleotide sequences at each end of a chromatid, which protects the end of the chromosome from degradation or from fusion with neighboring chromosomes. Progressive shortening of telomeres with each cell division is a characteristic of normal aging cells, and may be hastened by exposure to harmful environmental exposures such as UVR or tobacco smoke. A major determinant of cellular mortality is the telomere shortening that accompanies normal proliferation, so a key event in acquisition of cellular immortality is the up-regulation of a telomere length maintenance mechanism. Telomeric DNA is bound by a protein complex called shelterin, which contains six proteins, TRF1, TRF2, TIN2, TERF2IP, ACD and POT1 (Figure 6). These proteins protect the telomeres and prevent telomere shortening. The enzyme telomerase lengthens telomeres by synthesizing new telomeric DNA, to compensate for replication-associated telomere shortening. Telomerase is a complex molecule with several subunits, including the reverse transcriptase TERT (Figure 6). Telomerase activity can be detected in human embryonic stem cells at levels which are sufficient to prevent telomere shortening. In contrast, somatic tissues have very low levels of telomerase and undergo telomere shortening throughout life. A majority of human cancers have detectable levels of telomerase, sufficient to prevent telomere shortening. Mutations in the TERT gene promoter are found in many cancers, including melanoma, where it has been reported that UVR signature mutations are seen in 33% of primary melanomas and in 85% of melanoma metastases. In rare melanoma families, highly penetrant germline mutations have been found in the TERT promoter and also in shelterin complex genes POT1, ACD and TERF2IP (Table 6).

1.7.5 Role of immune surveillance in melanoma

One of the fundamental roles of the immune system is to distinguish self from non-self. In the late 1950s, the hypothesis of cancer immune surveillance emerged, suggesting that cancers can provoke an effective immunological reaction with regression of the tumor. This is illustrated in patients that are immune suppressed that have increased risks for cancers, including melanoma. Immune surveillance has been defined as having three key components: elimination (tumor eradication after antigen recognition), equilibrium (maintenance of tumor stability by immune control) and escape (tumor growth).
Figure 6. Components of telomere maintenance complexes. Reproduced, with permission, from Townsley et al. Blood 2014.

Figure 7. Immune checkpoint regulation through CTLA-4 and PD-1.
There are several clinical examples of melanomas being immunogenic tumors\textsuperscript{139}. An example of tumor elimination is metastatic melanoma with unknown primary tumor, sometimes with a patch of vitiligo at the postulated site of the original lesion that may represent immune recognition and elimination of the primary melanoma, supporting this hypothesis. Late recurrences of distant metastases (sometimes after decades) in patients with early-stage melanoma suggest prolonged periods of tumor equilibrium where tumor cells remain in a regional lymph node or at a distant site until a further event allows the tumor to escape. The mechanisms underlying increased immunogenicity of melanoma compared to many other tumors are unclear. One hypothesis relates to the high mutation rates seen within melanomas compared with other tumor types\textsuperscript{45}. While cells with low mutational burden will display mostly normal cellular protein antigens on their MHC surface molecules without any immune activation, melanoma cells will display mutant proteins (tumor antigens), initiating an activation of the immune system\textsuperscript{140}. A study on RNA expression profiles in melanoma metastases has shown inferior overall survival in tumors with a low expression of immune response genes\textsuperscript{74}. Tumors often create an immunosuppressive microenvironment by mechanisms of immune suppression that prevent effective antitumor immunity. One regulatory pathway is through the CTLA-4 protein that after T-cell activation is upregulated on its plasma membrane and inhibits the T-cell function and suppresses the immune response (\textbf{Figure 7}). Another regulatory pathway involves the PD-L1 and PD-L2 ligands that are expressed on tumors and other cells and bind to PD-1 receptors on T-cells and inhibit their function. As described earlier, active melanoma therapies have emerged in the last years that inhibit immune checkpoints molecules CTLA-4 (ipilimumab) and PD-1 (pembrolizumab and nivolumab)\textsuperscript{140}. 
2 UVEAL MELANOMA

2.1 INTRODUCTION

William Norris, a pioneer in the study of melanoma, described in his 1857 study “Eight Cases of Melanosis with Pathological and Therapeutical remarks on that Disease” one case of ocular melanoma in a 15 year old who died from the disease. Ocular melanomas are rare tumors that arise from melanocytes in the eye. Ocular melanomas are either conjunctival or uveal melanomas (see anatomy of the eye in Figure 8A). Conjunctival melanomas are extremely rare tumors (2-3 cases in Sweden yearly), that are more closely related to cutaneous and mucosal melanoma, but entirely distinct from uveal melanomas. Uveal melanoma is as a disease entity very distinct from cutaneous melanoma, with entirely different etiology, epidemiology, biology, genetics and clinical aspects. Uveal melanoma, although rare is the most common form of intraocular cancer in adults, while retinoblastoma is the most common eye neoplasm in children. More than 90% of uveal melanomas involve the choroid, the remainder are confined to the iris and ciliary body. In posterior uveal melanoma, the choroid and/or ciliary body are affected, while in anterior uveal melanoma the iris is affected (Figure 8).

2.2 EPIDEMIOLOGY AND RISK FACTORS

The incidence of uveal melanoma is 2-8 cases per million inhabitants, per years in most western countries. In Sweden there has been a stable incidence of 70-80 new cases yearly. Contrary to cutaneous melanoma, there has been no incidence rise in uveal melanomas in the last decades. The incidence of uveal melanoma increases with age and peaks around 60 years, males and females are affected in equal numbers. Fair skinned and blue, grey or green eyed individuals have higher risks for the disease than brown eyed individuals. Uveal melanomas can arise de-novo and also from uveal nevi. Uveal nevi are clusters of melanocytes seen in around 6% of the population where one in 5,000 undergo malignant transformation. Also congenital oculodermal melanocytosis and neurofibromatosis can predispose to uveal melanoma. Unlike cutaneous melanoma, the association between uveal melanoma and UVR is weak. Familial uveal melanoma cases exist, but are rare.

2.3 STAGING, CLASSIFICATION AND PROGNOSIS OF UVEAL MELANOMA

Iris melanomas have the best prognosis, whereas melanomas of the ciliary body have the least favorable prognosis. In the 2010 7th edition of the AJCC Cancer Staging Manual, the T categories for iris melanomas are different from the T categories for ciliary body and choroidal melanomas, while the N and M categories are the same for melanomas in all parts of the uvea. For iris melanomas, a T1 tumor is only in the iris, a T2 tumor has grown into the ciliary body or choroid, a T3 tumor has grown into the sclera and a T4 tumor extends outside the eyeball. For posterior uveal melanoma, T is categorized based on tumor basal dimension and thickness into four increasing size classes (Figure 9A). Secondarily, T is classified according to specific anatomic extent regarding ciliary body involvement and extrascleral extension, which are both poor prognostic indicators. Tumor stages I-III spans all
Figure 8. Anatomy of the eye with the uvea consisting of the iris, ciliary body and choroid (A). Patient with an iris melanoma (B).

Figure 9. AJCC tumor (T) classification of uveal melanoma (primary choroidal and ciliary body melanoma) defined by basal diameter and tumor thickness (A). AJCC anatomic stage of uveal melanoma defined by tumor, node, and metastasis values (B). Kaplan-Meier estimate of melanoma-related death from posterior uveal melanoma in 7731 patients, based on the AJCC tumor staging (C). Reproduced, with permission, from Phoebe et al. Oman J Ophthalmol 2013.

T categories without local lymph node involvement or metastasis. Tumor stage IV is not subgrouped and includes all patients with local lymph node involvement or any distant metastases, illustrating the unconditional poor prognosis of uveal melanoma that has spread beyond the eye (Figure 9B). In stage I uveal melanoma the five year survival rate is high, 97%. In stage II the five year survival is 90% and in stage III 75% (Figure 9C). A five year survival rate is not attained in stage IV uveal melanoma, since after one year survival rate is 0%. Metastatic uveal melanoma invariably spreads to the liver. The liver is the most common site for uveal melanoma metastases, with 50% of patients having liver-only disease, and 90% of those with metastases elsewhere (commonly in lungs, bone, or skin) also having liver metastases.

2.4 MOLECULAR BIOLOGY OF UVEAL MELANOMA

On a molecular level, uveal melanomas are distinctive from cutaneous melanomas. Uveal melanomas are characterized by frequent (>80%), mutually exclusive mutations in guanine nucleotide-binding protein G(q) subunits alpha (GNAQ) and alpha-11 (GNA11), two closely related large GTPases of the Gaq family. Guanine nucleotide-binding proteins are a family of heterotrimeric proteins that couple cell surface, 7-transmembrane domain receptors to intracellular signaling pathways. Oncogenic mutations in GNAQ and GNA11 render the GTPase constitutively active, triggering downstream signaling events leading to the activation of the MEK kinase (Figure 10A). This is similar to the consequence of mutations in the BRAF or NRAS oncogenes in cutaneous melanomas. A frequent second event in uveal melanoma progression, are mutations or deletions of the BRCA1 associated protein (BAP1) gene locus. The BAP1 gene maps to chromosome 3p21 and BAP1 mutations in uveal melanomas are often accompanied by somatic complete or partial loss of chromosome 3. This is consistent with a two hit model with activation of GNAQ/GNA11 and loss of activity of a tumor suppressor gene BAP1. Loss of heterozygosity in the BAP1 locus has been associated with higher risks of metastatic uveal melanomas. BAP1 is a deubiquitinase of histone H2A involved in chromatin remodeling. BAP1 forms multiprotein complexes with several chromatin-associated proteins, including BRCA1, BARD1 and HCF1. The exact role of BAP1 has not been entirely clear, earlier reports suggested that the BAP1 tumor suppressor function was through its deubiquitinating activity upon BRCA1, but later studies have indicated that BAP1 is an independent tumor suppressor. Further studies revealed its involvement in various biologic processes including chromatin dynamics, DNA damage response, cell cycle regulation and cell growth (Figure 10B).

2.5 IMMUNE SURVEILLANCE AND HOMING OF UVEAL MELANOMA CELLS

The eye is a site of “immune privilege” where immune responses to antigens (including tumor antigens) are modulated to protect ocular tissues that, if damaged by inflammation, would compromise vision. Anatomical constraints include the absence of afferent lymphatics within the eye and blood-ocular barriers that restrain traffic of immune cells to the eye and raise the threshold for priming of immune cells in secondary lymphoid organs. Immune cells that do enter the eye encounter significant biochemical barriers including
soluble immunosuppressive factors that limit T-cell functions. The immune suppressive mechanisms that maintain ocular ‘immune privilege’ are believed to be utilized by uveal melanomas to limit immune surveillance\(^ {147}\). Uveal melanomas are characterized by tumor dormancy, with many patients experiencing distant metastatic recurrence, mostly to the liver, more than 5 years after treatment of the primary lesion\(^ {147,154}\). The inclination of uveal melanoma to metastasize to the liver, has been attributed to hematogenous spread and homing to the liver through chemotactic factors, increased adherence to liver endothelium of uveal melanoma cells and thriving in the microenvironment of the liver\(^ {154,155}\). As local recurrences are uncommon it is believed that distant micrometastasis occurs before the treatment of the primary uveal melanoma. The molecular events involved in maintaining dormancy of micrometastasis are not known\(^ {154}\), but immunological control of metastatic cells, lack of angiogenesis within micrometastases and a low initial tumor cell proliferation has been suggested\(^ {154}\).

### 2.6 MANAGEMENT OF UVEAL MELANOMA

#### 2.6.1 Management of the primary tumor and follow-up

Most uveal melanoma patients present with symptoms, including blurred vision, visual field loss, distorted vision, flashing lights, visible tumor, red eye and pain. 30-40% of patients are asymptomatic, their tumor being detected on routine ophthalmic examination by an optometrist or ophthalmologist\(^ {142}\). Without timely treatment, uveal melanomas tend to make the eye blind, painful and unsightly as a result of retinal detachment, glaucoma and uveitis. The objectives of ocular treatment are to attempt to prevent metastatic disease and if possible to conserve the eye with as much useful vision as possible. The Collaborative Ocular melanoma study (COMS) from 1985 is the only evidence based study on the management of primary uveal melanomas\(^ {156}\). Uveal melanomas were grouped into small (0-3mm thick), medium (3.1-8 mm thick) or large (>8 mm thick). The five-year survival in patients with small to medium-sized choroidal melanoma was equally high (~90%) in patients randomized to either radiotherapy or to enucleation. In Sweden eye preserving brachytherapy is standard for small to medium sized tumors, while enucleation is standard care in large tumors\(^ {141}\). There is no evidence of a benefit of preoperative radiotherapy\(^ {156}\). There is no consensus regarding the follow-up of uveal melanoma, which relates to the fact that metastatic disease is generally untreatable with no evidence of a benefit of early detection of metastasis. Since uve melanomas are so rare, in Sweden the treatment of these tumors is centralized to one center, the St. Erik Eye Hospital in Stockholm were consensus is to offer biannual follow-up for 5 years after diagnosis, with abdominal ultrasound or CT scans\(^ {141}\).

#### 2.6.2 Management of distant metastasis of uveal melanoma

In patients with disseminated uveal melanoma, there are today no known therapies that effect survival outcomes\(^ {141,142}\). Dacarbazine and its oral analog temozolamide, can give rare responses, but do not affect survival rates\(^ {157}\). Among immune checkpoint inhibitors that are effective in metastatic cutaneous melanoma, ipilimumab has failed to show efficacy in uveal
melanomas, while occasional responses and clinical benefit has been reported with PD-1 inhibitors\textsuperscript{158}. BRAF or MEK inhibitors have not either been effective in the treatment of disseminated uveal melanoma\textsuperscript{159}. As uveal melanoma patients often have isolated liver metastasis, several local liver treatment strategies have been applied. Patients with unilobar liver metastases have undergone liver resection, where a retrospective review found a median survival of 14 months, but 27 months in patients when surgery was radical\textsuperscript{160}. In Gothenburg, Sweden, patients with liver metastasis only, receive isolated hyperthermic liver perfusion with melphalan. The phase II study of this method showed that patients had a promising median survival of 27 months and in 2013, the Scandium-study, a randomized phase 3 study was initiated and first interim analysis results from this study are expected in 2016\textsuperscript{161}. 
3 PRIOR KNOWLEDGE ON CANCER RISKS AND PROGNOSIS IN FAMILIAL MELANOMA KINDREDS

3.1 CANCER RISKS IN CDKN2A MUTATED MELANOMA KINDREDS

It is estimated that approximately 10% of all cases of cutaneous malignant melanoma occur in kindreds with hereditary predisposition for melanoma. Among melanoma families 5-25% carry a germline mutation of the CDKN2A gene. In Swedish melanoma families, occurrence of CDKN2A mutations has been analyzed in studies from Southern Sweden and from Stockholm and were identified in 19% and 8% of the families, respectively. In Sweden a single CDKN2A mutation, NM_000077.4: c.335_337dup, p.Arg112dup is the predominant mutation in melanoma families. The mutation inserts (duplicates) an arginine at codon 112 in one of the ankyrin repeats of p16-INK4A, disrupting its binding to CDK4/6. The mutation is located in CDKN2A exon 2 in a region that is also part of a second transcript with alternative reading frame, giving rise to a duplication of Ser-127 in p14ARF, still of unknown functional consequence. This mutation, which has only been detected in Sweden is a founder mutation estimated to have arisen in Sweden approximately 2,000 years ago. Individuals with p.Arg112dup and several other CDKN2A mutations also have an increased risk of developing pancreatic carcinoma. Several studies have reported an excess risk of other cancer types in CDKN2A mutated families, including gastrointestinal, breast, lung, neural system, gynecological, childhood, head and neck, non-melanoma skin cancers and uveal melanomas, but these cancer risks are not as well established, nor as consistently observed as the increased risks of melanoma and pancreatic cancer. Higher risk of breast cancer has previously been reported in p.Arg112dup carriers from Southern Sweden. In the study by Borg et al., nine p.Arg112dup families were analyzed, but it was later discovered that in one family with a high burden of breast cancer, family members had a germline mutation in the BRCA1 gene, in addition to the CDKN2A mutation, which may have contributed to the conclusion that there was a marked increase in breast cancer risk. In a study by de Snoo et al, specific cancer risks in melanoma families with the Dutch founder mutation (p16-Leiden) in CDKN2A were analyzed. In this study, the 221 mutation carriers from 22 melanoma families had a significantly increased risk of cancers of the pancreas, respiratory tissues, lip, mouth and pharynx, digestive tissues female genital tissues and eye/brain.

In CDKN2A carriers, melanoma risk has been positively associated with sun exposure, but apart from this there have been no studies so far investigating the association of exposures to other carcinogens, such as those in tobacco smoke, on cancer risk in CDKN2A mutation carriers from melanoma prone families. In three separate case reports of CDKN2A mutation carriers that were smokers and/or alcohol consumers, cancers of the tongue, oral cavity, pharynx and lung have been reported. Another study showed that among subjects that ever smoked, the risk of pancreatic cancer was higher in CDKN2A mutation carriers compared with non-carriers, but among non-smokers the risk for pancreatic cancer was not significantly different in carriers and non-carriers. Although interesting, this study was
limited by a low number of confirmed carriers (n=9), which were all carriers of different CDKN2A mutations 179.

3.2 CANCER RISKS IN CDKN2A WILD TYPE MELANOMA KINDREDS

High-risk melanoma associated mutations in genes other than CDKN2A have yet only been identified in a small number of families, in the majority of melanoma families, the cause for heredity still remains unsolved. In a few melanoma families, germline mutations in CDK4, POT1, ACD, TERF2IP and TERT genes have been found 132-135,180. Although several other cancers have been observed in some of the families carrying these mutations, the numbers of known carriers are too still too few for statistical risk estimation. In families with the MITF E318K variant, increased risks for melanoma and renal cell carcinoma have been observed 126,127. Also, in individuals with germline mutations in PTEN, RB1, TP53, BRCA1 and BRCA2, increased risks of melanoma have been described, although the cancer phenotypes are dominated by other tumor types (Table 6, p. 18) 116,122,181,182.

While germline mutations in high penetrance cancer predisposing genes are very rare in the normal population, single nucleotide polymorphisms (SNPs) in low penetrance genes like MC1R, ASIP, TYR and TYRP are relatively common 49. These low-risk melanoma genes are involved in cellular pigmentation pathways and population frequencies differ between countries and ethnic groups. Variants in low-risk melanoma genes have been associated with sporadic and familial melanoma, as well as with basal cell and squamous cell skin cancers, but no association has been made with increased risks of non-skin cancers 48,49. In a study by Höiom et al., on sporadic and familial melanomas in Stockholm, carrier frequencies of multiple MC1R variants were 21%, 30% and 40% in controls, sporadic and familial melanoma, respectively, indicating a gene dose effect 48.

Previously, there have been no studies investigating the cancer risk in CDKN2A wild type (wt) melanoma families. While extensive molecular research is needed to identify new mutations or genetic variants associated with hereditary melanoma, clinically orientated studies in cohorts of families negative for mutations in known melanoma high-risk genes are also needed for a better understanding of this group.

3.3 PROGNOSIS IN MELANOMA FAMILIES DEPENDING ON CDKN2A MUTATION STATUS

Several studies have compared patient and tumor specific factors among germline CDKN2A mutation carriers to non-carriers. In summary, the only consistent finding has been that CDKN2A mutation carriers are younger at melanoma diagnosis. Findings on differences in tumor specific features such as body site, ulceration, regression, presence of oncogenic BRAF or NRAS mutations have been non-significant or discordant (Table 7) 183-187. In melanomas from CDKN2A mutated individuals, there is a vague trend towards an increase in superficial spreading melanomas and lower Clark levels and Breslow thicknesses. Notably, in the reviewed studies, either it is not specified whether the first melanoma diagnosed or subsequent melanomas are used for comparative analysis or both first and later melanomas
are both used in the studies. This is of importance since multiple melanomas are very common among familial melanoma cases and the first melanoma is commonly the thickest, since after the first melanoma awareness increases, resulting in subsequent melanomas being thinner and less invasive. Also, in most of the studies, sporadic melanoma cases have been used as control groups and these have not received the same dermatologic follow-up as members of melanoma families that indeed has the goal to identify melanomas at earlier stages. The presumption that melanomas of CDKN2A mutation carriers have more favorable prognostic features has lead to speculations that mutation carriers might have similar or even more beneficial outcomes from their melanomas, but research addressing this specific question have until now been entirely lacking.

Table 7. Summary of previous studies on patient and tumor specific factors in CDKN2A mutated familial melanoma cases compared to controls.

<table>
<thead>
<tr>
<th>Control group</th>
<th>Zebary et al. 2013 n=89</th>
<th>Staaf et al. 2014 n=43</th>
<th>van der Rhee et al. 2010 n=182</th>
<th>Måsbäck et al. 2002 n=26</th>
<th>Sargen et al. 2015 n=123</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>↓ (NS)</td>
<td>↓ (S)</td>
<td>↓ (S)</td>
<td>↓ (S)</td>
<td>ND</td>
<td>CDKN2A mut significantly younger</td>
</tr>
<tr>
<td>Gender</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓ (S)</td>
<td>ND</td>
<td>No significant findings</td>
</tr>
<tr>
<td>Site</td>
<td>↔</td>
<td>↑ Extr. HN (NS)</td>
<td>←</td>
<td>↑ HN (NS)</td>
<td>ND</td>
<td>No significant findings</td>
</tr>
<tr>
<td>Histology type</td>
<td>↓ SSM (NS)</td>
<td>↑ SSM (NS)</td>
<td>↑ SSM (S)</td>
<td>↓ SSM (NS)</td>
<td>↑ SSM (NS)</td>
<td>Discordant findings, trend towards SSM in CDKN2A mut</td>
</tr>
<tr>
<td>Breslow</td>
<td>↓ (NS)</td>
<td>↓ (NS)</td>
<td>↓ (NS)</td>
<td>ND</td>
<td>ND</td>
<td>No sign. findings, trend towards ↓ Breslow in CDKN2A mut</td>
</tr>
<tr>
<td>Clark</td>
<td>↓ (NS)</td>
<td>↓ (S)</td>
<td>↓ (NS)</td>
<td>↓ (S)</td>
<td>ND</td>
<td>Trend towards ↓ Clark in CDKN2A mut</td>
</tr>
<tr>
<td>Ulceration</td>
<td>↓ (NS)</td>
<td>ND</td>
<td>ND</td>
<td>↑ (NS)</td>
<td>↔</td>
<td>No significant findings</td>
</tr>
<tr>
<td>Regression</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>↓ (NS)</td>
<td>ND</td>
<td>No significant findings</td>
</tr>
<tr>
<td>BRAF mutations</td>
<td>↑ (NS)</td>
<td>↑ (NS)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>No sign. findings, trend towards ↑ BRAF in CDKN2A mut</td>
</tr>
<tr>
<td>NRAS mutations</td>
<td>↓ (NS)</td>
<td>↓ (NS)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>No sign. findings, trend towards ↓ NRAS in CDKN2A mut</td>
</tr>
<tr>
<td>Mult. Primary mel.</td>
<td>ND</td>
<td>ND</td>
<td>↑ (S)</td>
<td>ND</td>
<td>ND</td>
<td>ND in 4/5 studies. Sign increased in one study</td>
</tr>
<tr>
<td>First or secondary mel in study</td>
<td>Both</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND in 3/5 studies. Both first and secondary mel in 2/5</td>
</tr>
</tbody>
</table>

S: Significant, NS: Not significant
HN: Head neck area
Extr: Extremities
Wt fam: Familial melanoma controls with wild type CDKN2A mutation status
ND: No data
Mel: Melanoma
CDKN2A mut: Familial melanoma cases with mutation in CDKN2A

3.4 GERMLINE MUTATIONS IN UVEAL MELANOMA FAMILIES

Familial predisposition to uveal melanoma is rare and has been suggested to account for less than 1% of all cases. However, families have been described with uveal melanoma cases along with several other malignancies, indicating that uveal melanoma may be a part of hereditary conditions with predisposition to other types of cancer in some kindreds. The proportion of patients with uveal melanoma with inherited susceptibility might therefore previously have been underestimated. The existence of an association between uveal melanoma, breast cancer, and ovarian cancer has been previously proposed. As described earlier, mutations and deletions in the BAP1 tumor suppressor gene is a common somatic event in uveal melanomas and associated with worse prognosis. It has been demonstrated that germline alterations in the BAP1 gene underlies inherited predisposition of cancer related syndromes characterized predominantly by mesothelioma or melanocytic tumors together with isolated cases of uveal melanoma and other tumors. It has also
been reported that *BAP1* germline mutations were found in families with uveal melanoma in combination with cutaneous melanoma and/or other cancer types and rarely in families without uveal melanomas\textsuperscript{194,195}. In a study by Jönsson et al. linkage-based evidence for the presence of a susceptibility gene was detected on 9q21 in three large Danish families with predisposition of both cutaneous and uveal melanomas. So far, no candidate gene has been identified; however, this substantiates the existence of several separate susceptibility genes for uveal melanoma\textsuperscript{196}.
4 SWEDISH HEALTH CARE SYSTEM AND REGISTRIES: TOPICS RELEVANT FOR THE THESIS

4.1 SWEDISH HEALTH CARE SYSTEM

As in the other Nordic countries, Swedish health care system is mainly government-funded and financed primarily through taxes. The responsibility for health and medical care in Sweden is shared by the central government, the 20 health care regions (landsting) and the 290 municipalities (kommuner). All Swedish residents have equal access to the public health care system. There is a limit on yearly health related costs paid by the patient, healthcare fees over 1,111 SEK (€120) and prescription medicine costs over 2,200 (€240) are paid by the government for the rest of the year. Primary health and emergency care is available directly, while a referral from a primary health care physician or another specialist is required for specialist appointments within the public health sector. In most specialty fields, the issuing of clinical guidelines as well as registration of patients (for quality assessments and research) is organized on a regional and national level. Management on different aspects of cancer prevention and care is coordinated by six Regional Cancer Centers (RCC) that work both individually and cooperatively.

4.2 FOLLOW-UP OF MELANOMA FAMILIES IN SWEDEN

The Swedish Melanoma Study group (SMSG), formed in 1977, is a multidisciplinary network of melanoma health care professionals and scientists from all health care regions. The aim of the SMSG is to ensure a high quality and equality of melanoma health-care in Sweden. SMSG formulates national clinical guidelines on the prevention and management of melanoma, coordinates academic melanoma studies and registration of melanoma patients in the National Melanoma Quality Registry. In 1985, SMSG initiated a working group on familial melanoma. At the time, familial melanoma was regarded as a “dysplastic nevus syndrome” (DNS) and the group was called the DNS group. In 2014 the name of this group was changed to the Swedish Network on Familial Melanoma (SweFaM), reflecting the current knowledge, that familial melanoma is not necessarily associated with dysplastic nevi. The task of the group was to, on a national level, identify and register melanoma families, formulate clinical guidelines, organize primary and secondary preventive measures and to be a platform for research. In 1987 the first clinical guidelines were issued and enrollment of melanoma family members in a preventive program started. The initial criteria for participation were cutaneous melanomas (invasive or in situ) in at least two blood relatives (first-, second- or third-degree relatives) as well as clinical dysplastic nevi in two or more relatives (“D-2 kindreds). Familial melanoma kindreds were identified through questioning of newly diagnosed melanoma patients regarding family history of melanoma. Melanomas in relatives were verified by pathology reports and/or clinical records. A pedigree was established and blood relatives, contacted by the proband, were invited to participate in the program. At the initial visit, participants received written and oral information regarding protection from damaging sunlight and skin self-examination. Skin examination and photo documentations were performed. Individuals who were diagnosed with melanoma and
unaffected individuals with dysplastic nevi were followed-up at 6-month intervals. Since mid-1990s, melanoma family members have also been invited, for the purpose of study, to undergo CDKN2A mutation testing. This program was reviewed and evaluated in a 2007 paper by Hansson et al. From 280 melanoma families, 2,080 family members had been enrolled, 614 with melanoma and 866 relatives with dysplastic nevi only and 600 relatives without melanoma or dysplastic nevi. Between 1987-2001, 26 invasive and 15 in situ melanomas, as well as 766 histopathologically dysplastic nevi, were excised. The majority of melanomas (66%) were identified in individuals previously diagnosed with melanoma. In addition all melanomas, except one, were diagnosed in families with at least two first-degree relatives with melanoma. As a result of this study and also of other studies with similar findings, the definition of melanoma has become more stringently focused on the numbers of melanomas and degree of relation between the melanoma cases in families rather than on dysplastic nevi. The more melanomas in a family and the closer the blood-relations is between affected individuals, the higher is the risk of new melanomas evolving and the higher is the proportion of families that carry high-risk melanoma associated mutations such as in the CDKN2A gene (Table 5, p. 9).  

4.3 REGISTRIES EMPLOYED IN THE THESIS

4.3.1 Personal identification number and the Swedish Population Registry

The Swedish 10 digit personal identity number (personnummer) was introduced in 1947 and was first of its kind covering the total resident population of a country. Numbers are issued by the Swedish Tax Agency (Skatteverket) as part of population registration. The individually unique personal identity number is assigned to each Swedish resident at birth or from the time of permanent residency. The first 8 digit encode information on the date of birth (year-month-day) and the last 4 digits are based on algorithms that ensures a unique number, including information on gender. The personal identity number is used by authorities, employers, banks, health care, registries etc. The personal identity number is crucial to cross link national register data for the purpose of study. The Population Registry, kept by the Tax Agency, is the civil registration of vital events (e.g. births, deaths, and marriages). Until 1991, the Population Registry was under the church, and although not complete, goes back many centuries. In Papers I-III, the Swedish personal identity number was used for registry linkages and the Population Registry was used to attain census data on all subjects, and in Papers I-II to identify control subjects.

4.3.2 The Swedish Multi-generation Registry

Swedish Multi-generation Registry contains connections between all individuals born after 1931 (index persons) and their biological parents that have been registered in Sweden after 1960. The Multi-generation Registry is held by Statistics Sweden (Statistiska Centralbyrån) that retrieves information on the biological mother and father of index persons from the Population Registry system, which identifies the relationship between mother and child and between father and child. There are about 10 million index persons in the registry. If an index
person’s parent was dead or emigrated before 1961, then data on that parent is missing from the registry. For index persons who were adopted, there is also information on their adoptive parents. The Swedish Multi-generation Registry was used in Papers I-III.

4.3.3 The Swedish Cancer Registry

The Swedish Cancer Registry was founded in 1958 and covers the whole population. The registry is held by the Swedish National Board of Health and Welfare. Reporting to this registry is by law, compulsory for clinicians and pathologists or cytologists diagnosing a cancer. Approximately 60,000 malignant cases of cancer are now registered every year in Sweden. In the registry, there is patient specific information such as personal identity number, age, sex and date of death and tumor specific data such as site of tumor, histological type, and stage. Site of the tumor is coded according to ICD, but there have been different versions used, depending on the time span each version was in use. For unison registration, all cancers are still coded by ICD-7 codes, while tumors diagnosed from 1987 are also coded by ICD-9 codes. Cancers diagnosed after 1993 are also coded by International Classification of Diseases for Oncology codes (ICD-O). Since 1993 by ICD-O-2 and since 2005 by ICD-O-3. Histological types have since 1993 been coded according to ICD-O/2 and since 2005, ICD-O/3. From 1958 and onward, the codes are also available as the old histology code WHO/HS/CANC/24.1. For most tumors, stage has been collected since 2004 according to the AJCC TNM classification system. Completeness of the registry has been estimated to 97% \(^{201}\). The Swedish Cancer Registry was used in Papers I-III. It should be noted that the registry does not systematically record basal cell carcinomas of the skin, therefore these tumors are not included in the present studies.

4.3.4 The Stockholm-Gotland Regional Melanoma Registry

The Regional Cancer Center (RCC) in Stockholm-Gotland was founded in 1976. One of its tasks was to register cancer patients; data has been collected on melanoma patients in the Stockholm-Gotland Regional Melanoma Registry since 1976. Data is registered on patient and tumor characteristics, including TNM stage, treatments and follow-up. Since 2003 data is passed on to the Swedish Melanoma Registry that collects data from all regions for purpose of quality control. The Stockholm-Gotland Regional Melanoma Registry was used in Paper III.

4.3.5 The Swedish Cause of Death Registry

For every death in Sweden, a physician issues, within 24h a death certificate to the Tax Agency and, within 3 weeks a cause of death certificate to the National Board of Health and Welfare (Socialstyrelsen)\(^{199,202}\). The Swedish Cause of Death Registry, held by the National Board of Health and Welfare has data from 1961, the year when the registry started. The physician reports the underlying cause (e.g melanoma) that subsequently leads to death. The underlying causes of death is coded in the registry by ICD codes, but there have been different versions used, depending on the time span each version was in use. Registration is almost complete, with <1% missing certificates yearly. The quality of the data depends on the
thoroughness and accuracy of the physician report, which can vary. In a study of reporting to
the registry in 1995, there was a discrepancy in 23% of cases between hospital discharge note
and the cause of death certificate\textsuperscript{203}. The highest discrepancy was seen in elderly with
multiple morbidities, while the lowest discrepancy was seen in cancer patients (8%). The
Swedish Cause of Death Registry was used in Paper III.
5 AIMS OF THE THESIS

The main aim of this thesis is to gain more knowledge on the effect of mutation status in melanoma-associated genes on cancer risks and prognosis in familial melanoma kindreds.

More specifically:

I. To study the risk of melanoma and non-melanoma cancers in germline CDKN2A mutation carriers identified in Swedish melanoma families. Swedish Registries, including the Multi-generation Registry and the Cancer Registry are used for this purpose.

II. Also, with the aid of Swedish registries, to estimate the risk of melanoma and non-melanoma cancers in CDKN2A mutation-negative familial melanoma kindreds.

III. To compare survival from all causes, from melanoma and from non-melanoma cancers in CDKN2A mutation-positive or -negative familial melanoma kindreds.

IV. To identify germline gene mutations associated with hereditary uveal melanoma
6 MATERIALS AND METHODS

6.1 STUDY DESIGN

Papers I-III are cohort studies of cancer risks and prognosis in familial melanoma kindreds and controls. Although these studies were planned and carried out in 2012-2015, the register data used on cancer diagnoses and deaths is prospectively registered from the date when the families were identified. Paper IV is a case study with a family-based association analysis. The main feature of this type of association analysis is that controls are family-based rather than population-based.

6.2 ACCRUAL OF CASES

6.2.1 Identification of familial cutaneous melanoma kindreds: Papers I-III

As described earlier (paragraph 4.2) Swedish melanoma families have since 1987 systematically been enrolled in a nationwide preventive program. Since 1995, CDKN2A mutation analysis has been offered to family members, for the purpose of study. Mutation analysis has mainly been carried out at the Lund University and at Karolinska Institutet in Stockholm. Totally, 861 family members from 321 melanoma families that have undergone CDKN2A mutation testing were included in the register studies. Informed consent was obtained before family members underwent mutation analysis and the study was approved by Research Ethical Review Boards in Lund and Stockholm.

6.2.2 Identification of familial uveal melanoma kindreds: Paper IV

At the Oncology clinic at Karolinska University hospital in Stockholm a rare uveal melanoma family was identified in 2010. The proband, a female, had at age 16 years been diagnosed with a choroidal melanoma, with tumor stage T4N0M0. The patient underwent enucleation of the right eye but six months later she was diagnosed with multiple liver metastasis and later bone metastasis and subsequently died from the disease. When the patient and her parents were asked about other cancers in the family, it became known that the patient had two relatives with uveal melanoma, her paternal grandfather and his brother had been treated for the disease at 39 and 44 years, respectively, and both later died from disseminated disease. The patient’s father, at the time in his early forties, had no cancer diagnosis. No unusual cancer predisposition was seen on the patient’s maternal side. Due to the three cases of uveal melanoma in a family, all diagnosed at earlier ages than is normally seen, in particular in the proband, this was considered a hereditary case of uveal melanoma. A pedigree was established using information from the proband’s parents and from medical records. Informed consent from living family members was obtained, and the study was approved by the Research Ethics Board of Karolinska Institutet. Blood samples for extraction of germline DNA from the proband, her parents and a healthy younger sister for exome sequencing. From the extended pedigree, six family members donated a blood sample for follow-up genetic analysis. One of them was a dizygotic twin brother of the proband’s paternal grandfather who was diagnosed with prostate cancer at 67 years. Paraffin-embedded archival material from
primary uveal tumor surgical specimens were collected from the affected proband, her grandfather, and his brother.

Additional screening of candidate predisposing genes was done in three patients with uveal melanoma from three other families using DNA from archival tumor material from all the individuals and germline DNA from blood for two of the three individuals. The investigated families all had two first- or second-degree relatives with uveal melanoma, and one of the families also had one case of cutaneous melanoma and one case of mesothelioma.

6.3 MUTATION AND GENE VARIANT ANALYSES

6.3.1 Genotyping of the CDKN2A gene: Papers I-III

From familial melanoma kindreds, 8 ml of venous blood were drawn and peripheral blood mononuclear cells were isolated, from which DNA was extracted with FlexiGene DNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Polymerase chain reaction (PCR) was performed on exons 1α, 1β, 2 and 3 of CDKN2A to give PCR fragments of 340 bp, 678 bp, 576 bp and 319 bp for exons 1α, 1β, 2 and 3, respectively. PCR conditions were: initial denaturation and DNA polymerase activation at 95°C for 6 min followed by 40 cycles of 95°C for 10 sec, 61°C, 59°C, 60°C or 62°C (for exons 1α, 2 and 3, respectively) for 20 sec and 72°C for 30 sec. The cycling was followed by 5 min. incubation at 72°C then soak at 4°C. The PCR products consisted of 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 1 U of Platinum Taq polymerase (all reagents from Invitrogen, Carlsbad, CA), 1 M of betaine (exons 1α and 2) or 5% of DMSO (exon 3) (both Sigma-Aldrich Chemie GmbH, Steinheim, Germany), or no additives with 20 pmol of each primer (Eurofins-MWG GmbH, Ebersberg, Germany) and 50 ng of genomic DNA in a total volume of 20 µl. Exon 1β PCR was run using the PCRx Enhancer System™ (Invitrogen, Carlsbad, CA) at final PCRx Enhancer solution concentration of 1X, in 1X PCRx Amplification buffer, 0.2 mM dNTPs, 1.5 mM MgSO4, 30 pmol of each primer and 3 U of Platinum Taq polymerase (all reagents Invitrogen, Carlsbad, CA) with PCR conditions as above except with an annealing temperature at 56°C. 3 µl of each PCR product was run on a 1.6 % agarose gel to confirm PCR specificity. Ten µl of the PCR product was purified using 2 U of exonuclease I and 1 U of FastAP alkaline phosphatase (both Thermo Fisher Scientific, Gothenburg, Sweden). The purification conditions were 50 min at 37°C followed by 20 min at 80°C then soak at 4°C. 0.5 to1.0 µl of the purified PCR corresponding to approximately 25 to 50 ng of PCR product was used in a sequencing reaction utilizing Applied Biosystems BigDye Terminator Cycle Sequencing Kit version 1.1 according to a 1:4 protocol with 1 µl of BigDye Terminator™ in a final 0.75X BigDye Terminator sequencing buffer (reagents Applied Biosystems, Foster City, CA) and 4 pmol of each primer (Eurofins-MWG GmbH, Ebersberg, Germany) in total volume of 10 µl. The sequencing reactions were analyzed in ABI Prism® 3700 genetic analyzer (Applied Biosystems, Foster City, CA) All PCR products were sequenced bi-directionally, with analyses of electropherograms using Mutation Surveyor v.3.97 software (Softgenetics LLC, State College, PA).
6.3.2 Genotyping of the MC1R gene (Paper III)

Using direct sequencing the whole coding region and 5′proximal part of the MC1R gene starting at position-325 was analyzed in DNA from 100 subjects. Ten variants were found to be present in DNA from sporadic melanoma patients and control individuals. These variants and an additional 11 MC1R variants chosen from the literature were then screened for using DNA from control subjects and patients. The literature based MC1R variants were selected to have an elevated allele frequency in populations of European ancestry. DNA from sporadic and familial melanoma cases and control subjects was genotyped for the 21 identified MC1R variants using Protease mediated Allele-Specific Extension (PrASE). Shortly, this is a multiplex, chip-based SNP genotyping method utilizing allele-specific oligonucleotides with unique (for the specific allele), non-cross hybridizing 5′-ends. These oligonucleotides are annealed to a single-strand immobilized PCR product and elongated with fluorescence labelled nucleotides. The time for elongation of an oligonucleotide with matching 3′-end in relation to an oligonucleotide with non-matching 3′-end gives the allele specificity: elongation of the non-matching oligonucleotide is interrupted by protease degradation of the DNA polymerase. The labelled oligonucleotides are then hybridized to a chip with immobilized complementary anti-tag oligonucleotides to give signal.

6.3.3 Whole-Exome sequencing and Sanger sequencing of the BAP1 gene: Paper IV

From familial uveal melanoma kindreds, 8 ml of venous blood and DNA extracted. 10 μg of genomic DNA was extracted and paired-end libraries were created according to standard protocols (Illumina, San Diego, CA). Whole-exome enrichment was performed using the TrueSeq Exome Enrichment Kit (Illumina). Enriched libraries were sequenced using Illumina HiSeq 2000 generating 2 x 100 bp reads. Before mapping, quality control was performed by 30-trimming of reads, removing bases with a Phred score encoded by ‘B,’ which is an Illumina quality-control indicator, indicating that the read end should not be used in further analyses. If a read after trimming was less than 40 bases long it was removed. Furthermore, reads having five or more bases with a Phred score of 10 or lower or 10 or more bases with a Phred score of 20 or lower were discarded. In addition, reads with four or more uncalled bases were also discarded. Overall quality after quality-control filtering was verified by manual inspection of FastQC reports. Reads were mapped using Mosaik to the hg19 reference genome allowing a maximum of four mismatches, and thereafter PCR duplicates were removed (MosaikDupSnoop). Variants [single-nucleotide variants (SNVs) and indels] were mapped using SAMtools with default parameters and thereafter prefiltered with vcfutils using default parameters apart from the maximum read-depth parameter, which was set to maximum three times the average consensus coding DNA sequence (CCDS) coverage, as to avoid regions with abnormal mapping behavior. Identified variants were annotated using Annovar and custom Perl and R scripts. To retrieve a subset of candidates, variants were filtered based on the annotations. Different sets of filters were used, generating candidate lists of variants: (1) nonsynonymous SNVs (2) frameshift indels, and (3) variants affecting splicing and miRNA/miRNA targets. Gene variants were also filtered based on their putative
effect (on the protein, frameshift, splicing, etc) and if they were in conserved regions. Variants were excluded if they were located in segmental duplicated regions and if they had a minor allele frequency more than 1% (1000 Genome Project dataset, release November 2010). Finally, only variants shared between the proband and her father but not present in the mother were included.

Germline DNA was extracted from peripheral blood using FlexiGeneVR DNA kit (QiagenVR, Hilden, Germany). Tumor DNA was extracted from archival material using QIAampVR DNA FFPE Tissue kit (Qiagen). Validation of the mutation identified by exome sequencing was performed by bidirectional sequencing of a fragment obtained by PCR. From germline DNA, a fragment covering exons 1–3 of BAP1 was amplified and sequenced using primers designed for targeted resequencing provided by the NCBI Probe database. Screening of all BAP1 exons was done using resequencing amplicon probe sets from NCBI Probe. From paraffin-embedded archival tumor DNA, a shorter fragment of 150 bp covering the position c.75 in exon 3 of BAP1 was amplified. Two informative microsatellite markers (D3S1578 and D3S3026) flanking the BAP1 gene locus were used for genotyping.

6.4 REGISTER LINKAGES AND FOLLOW-UP (PAPERS I-III)

The national 10-digit personal identity number of each CDKN2A tested familial melanoma kindred was linked with the Swedish Multi-generation Registry. This allowed identification of familial melanoma kindreds’ first-degree relatives (parents, siblings and children) and second-degree relatives (grandparents, uncles/aunts, nieces/nephews, half-siblings and grandchildren). Age and sex matched controls were identified from the Swedish Population Registry. The personal identity numbers of identified family members and controls were linked with the Swedish Cancer Registry to obtain data on all registered cancers (types, stages and dates) and with the Cause of Death Registry to obtain data on all deaths (causes and dates). In Paper III, sporadic melanoma cases, matched for age, sex, tumor thickness and year of diagnosis in familial melanoma cases, were obtained from the Regional Melanoma Registry of the Stockholm-Gotland health care region.

In Papers I-II, follow-up started the date the first blood sample was taken for CDKN2A analysis in each family (same date in family members and corresponding controls) and ended at the date of death, emigration or census date of December 31st, 2011. In Paper III, follow-up started at the date when the first invasive melanoma was diagnosed in each case and ended at the date of death, emigration or census date of December 31st 2011.

6.5 STATISTICAL ANALYSES

6.5.1 Basic statistical analyses (Paper I-III)

In the CDKN2A genotyped familial melanoma kindreds, median ages and ranges were calculated (Papers I-III). Relative risks (RR) for cancers were calculated from incidence rates (number of cancers/person years) (Papers I-II). To estimate age specific cumulative cancer incidence in CDKN2A mutation carriers, the incidence of cancer was analyzed in 10 year
intervals from 0-80 years of age (number of cancers/persons alive in each interval) (Paper I). Odds ratio (OR) was calculated for smoking status (ever/never) and having been diagnosed with cancers in pancreas, respiratory and upper digestive tissues (yes/no) (Paper I) and also in calculations on allele frequencies of red hair color (RHC) variants in the \textit{MC1R} gene in familial melanoma kindreds and controls (Paper II). Two sided 95\% confidence intervals (95\% CI) were calculated for all relative risks (RR and OR). In Paper III, student T-test was used to calculate \textit{p}-values for continuous variables (age) and chi-square test to calculate \textit{p}-values of categorical variables (sex; female/male, multiple primary melanoma; yes/no, etc.). A \textit{p}-value <0.05 was considered significant.

\subsection{6.5.2 Survival analysis (Paper III)}

Survival from all causes, from melanoma and from non-melanoma cancer was studied. For overall survival, all deaths were considered as events. For melanoma-specific survival, only deaths from melanoma were counted as events, deaths from other causes were labeled as censored and follow-up ended on the date of death. In the same way, for non-melanoma cancer-specific survival, only deaths from non-melanoma cancers were counted as events, deaths from other causes were labeled as censored and follow-up ended on the date of death. For survival analysis, the Kaplan-Meier method was used along with Log-rank test to estimate the hazard function of groups at each observed event time. The Cox proportional hazards regression model was used to analyze survival outcome by producing hazard ratios (HR) unadjusted or adjusted for age, sex and tumor thickness. Statistical analyses were done in StatSoft Statistica 10.
7 RESULTS

7.1 RESULTS FROM PAPER I

“High Risk of Tobacco-Related Cancers in CDKN2A Mutation-Positive Melanoma Families"

In carriers of the Swedish founder mutation in CDKN2A (p.Arg112dup) (n=120), the prospective relative risks (RRs) of all non-melanoma cancers was 5.0 (95% CI 3.7-7.3) compared to controls and 4.8 (95% CI 2.4-10.1) compared to their related non-carriers. Compared to controls, the RR of melanoma was 64.8 (95% CI 36.9-117.9), of pancreatic cancer 43.8 (95% CI 13.8-139.0), of cancers in upper digestive tissues 17.1 (95% CI 6.3-46.5), of cancers in respiratory tissues (lung, bronchi, larynx) 15.6 (95% CI 5.4-46.0), of gynecological cancers 8.8 (95% CI 3.8-20.4) and of non-melanoma skin cancer 3.3 (95% CI 1.0-10.7). In non-carriers there were no cases of pancreatic cancers or cancers in upper digestive tissues and one case of lung cancer, indicating a marked excess risk of these tumors in carriers compared to non-carriers from the same families.

RRs of all non-melanoma cancers was significantly elevated in CDKN2A mutation carriers’ non-genotyped first-degree relatives (FDRs), 2.1 (95% CI 1.6-2.7) but not in second-degree relatives (SDRs), 1.0 (95% CI 0.8-1.4). In FDRs and SDRs, the relative risks were significantly elevated for pancreatic cancer and cancers in respiratory and upper digestive tissues. For these tumors, lower RRs were seen in SDRs compared to FDRs that in turn had lower RRs for these tumors than the genotyped carriers.

When all p.Arg112dup family members (carriers, FDRs and SDRs) were compared to all controls, the following cancers had significantly higher RRs; melanoma, (RR 24.6), larynx (RR 21.8), pancreas (RR 13.9), esophagus (RR 5.4), tongue and oral cavity (RR 4.7), lung (RR 4.0), stomach (RR 2.5), breast (RR 1.7) and cervix (RR 1.5).

Age specific cumulative incidence in carriers showed that at age 50, 20% had been diagnosed with non-melanoma cancers and 7% with cancers in pancreas, upper digestive and respiratory and tissues. At age 80, 76% had been diagnosed with non-melanoma cancers and 53% with tumors in pancreas, upper digestive and respiratory tissues. Of all 28 families in the study, 16 (57%) families had cases of pancreatic cancer, 12 (43%) families had cases of cancers in respiratory tissues and 12 (43%) families had cases of cancers in upper digestive tissues.

In ever-smoking carriers compared with never-smoking carriers, the odds ratio of cancers in pancreas, respiratory or upper digestive tissues was 9.3 (95% CI 1.9-44.7). The median age at end of follow-up of smokers that had been diagnosed with cancers in pancreas, upper digestive or respiratory tissues was 70 years while in smokers that had none of these diagnoses the median age at end of follow-up was 50 years.
7.2 RESULTS FROM PAPER II

“CDKN2A mutation-negative melanoma families have increased risk exclusively for skin cancers but not for other malignancies”

In CDKN2A wild type (wt) familial melanoma index cases (n=224) and their first-degree relatives (n=944), the prospective RR for melanoma was 56.9 (95% CI 31.4-102.1) and 7.0 (95% CI 4.2-11.4) respectively. For all non-skin cancers combined, there were no significantly elevated risks in index cases or their relatives. Squamous cell skin cancers were the only non-melanoma tumors with significantly increased risks in either index cases (RR 9.1, 95% CI 6.0-13.7) or first-degree relatives (RR 3.4, 95% CI 2.2-5.2). As in the previous study, basal cell carcinomas were not included since these tumors are not registered in the Swedish cancer Registry. To investigate if the increased risk seen for these cancers was a result of the participation in the preventive program with regular skin examination, a retrospective analysis of cancer risks before inclusion also was performed. This analysis showed that in both index cases and first-degree relatives the risks for squamous cell skin cancers were significantly elevated even before inclusion.

Since no increases risks of non-skin cancers were found in the CDKN2A wt families, additional analysis was performed to explore if increased risks were present in families with young melanoma cases (<40 years old), multiple primary melanoma cases or >2 melanoma cases per family. It was found that families with young melanoma cases had a modest but statistically significant risk increase for non-skin cancers, RR 1.5, 95% CI 1.0-1.5, overall, but no individual tumor diagnosis was significantly increased. In families with cases of multiple primary melanomas or >2 melanoma cases per family, no significant risk increase was found for non-skin cancers.

A subset of the CDKN2A wt index cases (n=136) were analyzed for red hair color (RHC) variants in the MC1R gene. Allele frequencies were compared to a healthy Swedish control population described in an earlier study (n=663). At least one MC1R gene RHC variant (Asp84Glu, Arg151Cys, Arg160Trp or Asp294His) was found to be present in 33% of controls and in 54% of the CDKN2A wt melanoma index cases (OR 2.4, 95% CI 1.6-3.4). Within the cohort of melanoma index cases, at least one RHC variant was present in 67% of cases with squamous cell skin cancers (OR 1.8, 95% CI 0.7-4.9), in 59% of multiple primary melanoma cases (OR 1.3, 95% CI 0.6-2.9) and in 54% of non-skin cancer cases (OR 1.0, 95% CI 0.4-2.2).
7.3 RESULTS FROM PAPER III

“Survival in familial melanoma cases carrying germline CDKN2A mutations: Increased mortality from melanoma and non-melanoma cancers compared to mutation-negative melanoma cases”

Familial $CDKN2A^{\text{mut}}$ (n=104) and $CDKN2A^{\text{wt}}$ (n=444) melanoma cases were identified from 31 $CDKN2A^{\text{mut}}$ and 238 $CDKN2A^{\text{wt}}$ melanoma families, respectively. $CDKN2A^{\text{mut}}$ cases were significantly younger than $CDKN2A^{\text{wt}}$ cases at the age of the diagnosis of first melanoma (40 years vs. 50 years). Multiple primary melanomas were significantly increased in mutation carriers, 40% had multiple primary melanomas and 18% had 3 or more melanomas, compared to 15% and 4% in $CDKN2A^{\text{wt}}$ melanoma cases. There were no significant differences in body site, invasiveness or tumor thickness of melanomas between $CDKN2A^{\text{mut}}$ and $CDKN2A^{\text{wt}}$ melanoma cases. Non-skin cancers were significantly overrepresented among the $CDKN2A^{\text{mut}}$ cases; 38% were diagnosed with other tumors compared to 20% of the $CDKN2A^{\text{wt}}$ melanoma cases.

At the censor date, 22% of $CDKN2A^{\text{mut}}$ and 13% of $CDKN2A^{\text{wt}}$ cases were deceased from melanoma and 17% of $CDKN2A^{\text{mut}}$ and 5% of $CDKN2A^{\text{wt}}$ cases were deceased from non-melanoma cancers. Of the 31 mutated families, deaths from melanoma where seen in 18 families (58%) and deaths from non-melanoma cancers in 12 families (39%). Of the 238 non-mutated families, deaths from melanoma where seen in 53 families (22%) and deaths from non-melanoma cancers in 24 families (10%).

All families except two have the Swedish founder mutation in $CDKN2A$ (p.Arg112dup). A family with a p.Pro48Leu mutation in $CDKN2A$ had deaths from both melanoma and non-melanoma cancer. A family with a p.delAla60_Gly67 mutation in $CDKN2A$ had no deaths from any cause among its two melanoma cases, but a death from pancreatic cancer in a carrier with no previous diagnosis of melanoma (and hence not included in this particular study where only melanoma cases were included).

After adjusting for age, sex and tumor thickness, $CDKN2A^{\text{mut}}$ familial melanoma cases had, compared to $CDKN2A^{\text{wt}}$ cases, an increased mortality from all causes (HR 2.59, 95% CI 1.76-3.78), from melanoma (HR 2.50, 95% CI 1.49-4.21) and from non-melanoma cancers (HR 7.77, 95% CI 3.65-16.51). Also, when compared to the matched sporadic cases, $CDKN2A^{\text{mut}}$ cases had significantly worse survival outcomes, while no significant differences were found between $CDKN2A^{\text{wt}}$ and sporadic cases.

To assess if death rates from melanoma were affected by multiple primary melanomas or from diagnoses of non melanoma cancers, analyses were done by excluding all cases with 1) multiple invasive primary melanomas and 2) diagnoses of non-melanoma cancers. Both $CDKN2A^{\text{mut}}$ single melanoma cases and $CDKN2A^{\text{mut}}$ cases without any non-skin cancer diagnosis had significantly increased mortality rates from all causes, from non-melanoma cancers and from melanoma compared to $CDKN2A^{\text{wt}}$ single melanoma cases, both in the adjusted and in the unadjusted hazards models.
7.4 RESULTS FROM PAPER IV

“Hereditary Uveal Melanoma: A Report of a Germline Mutation in *BAP1*”

Next-generation exome sequencing was performed using peripheral blood derived DNA from the uveal melanoma proband, her younger sister and both parents (of which father was the obligate carrier). After mapping, and filtering of the variants likely to be artefactual, the remaining sequence variants were filtered to remove common SNPs and variants present in the mother. A frame-shift, truncating insertion, c.75insC in the *BAP1* gene on chromosome 3p21 was observed in the DNA from the proband and her father, but not in her mother or her sister. This frame-shift insertion results in a premature termination of the *BAP1* protein in amino acid 43. In the uveal melanoma tumor of the proband, a microsatellite-based loss of heterozygosity (LOH) analysis was performed confirmed corresponding loss of the wild-type *BAP1* locus allele.

Sequencing of DNA from the uveal melanoma tumors from the proband’s paternal grandfather and his brother revealed the same *BAP1* mutation in both tumors. Because of the very fragmented DNA, it was not possible to determine the presence or absence of LOH in the tumors.

Among other family members, the mutation was also found in the grandfather’s dizygotic twin brother who was diagnosed with prostate cancer. As the prostatic carcinoma was an early minimal T1 lesion, analysis of *BAP1* loss of heterozygosity (LOH) was not feasible. His children also carried the mutation, whereas the other tested non-affected family members did not have the mutation. The children of the twin brother with prostate cancer were found to have a high density of melanocytic nevi ranging from clinically benign to atypical. The atypical melanocytic nevi demonstrated a mild to moderate dyplasia histopathologically. One of the carriers was diagnosed with a preinvasive superficial spreading melanoma, which clinically appeared as an oval-shaped orange-red plaque with slight pigmentation only visualized in the dermoscope. Histopathologically, the melanoma harbored atypical melanocytes throughout epidermis lacking features of a Spitz-like tumor, as well as regression of 0.5 mm but no ulceration.

Individuals belonging to three other kindreds with uveal melanoma were screened in the complete coding region of *BAP1* without any findings of mutations.
8 METHODOLOGICAL CONSIDERATIONS

8.1 SELECTION BIAS

Selection bias is a systematic error in a study that stems from the procedures used to select subjects and from factors that influence study participation. Studies of hereditary disease can be subject to ascertainment (sampling) bias, a form of selection bias. Ascertainment bias can occur when ascertainment of the most severely affected individuals and families is favored, leading to the interpretation that the condition studied, is more severe than it actually is. In Papers I-III, CDKN2A mutation testing has been performed in familial melanoma kindreds, identified in families with multiple cases of melanoma and not sampled from the general population. For this reason, estimations of effects of the CDKN2A mutation could be biased compared to if CDKN2A mutation carriers had been sampled from the general population. Phenocopy is sometimes observed in families carrying a disease associated mutation and occurs when a disease that is associated with a certain mutation is also seen among non-carriers of the mutation in the same family. In CDKN2A mutated families, a certain risk increase of melanoma is seen, also among non-carriers of the CDKN2A mutation, indicating that some degree of ascertainment bias could be present. The families with CDKN2A mutations that are identified could have other melanoma risk modifying factors, such as pigmentation traits, UV exposures and other risk modifying gene variants that increase their melanoma risk. Ascertainment bias could result in CDKN2A mutation carriers’ risks for melanoma being overestimated. Paper IV, which essentially is a case study of a novel gene variant in a rare uveal melanoma family is by its nature subject to selection bias. However, in the study of inherited disease, reports of novel genetic associations often involve few cases where later studies will corroborate or challenge such associations. In the case of the BAP1 mutation, concurrent and later studies have indeed supported the association of BAP1 and hereditary uveal melanoma.

Another concern regarding selection bias is the accrual of controls and familial cases. While controls are sampled from the Population Registry, familial cases are sampled from preventive programs with the aim to prevent and detect skin cancers at earlier stages. The effect of this preventive program is that skin cancers are more likely to be detected than in those that are not in a preventive program. Also, participants in preventive programs could be more likely to take other preventive measures, such as reducing their exposures from known carcinogens and participate in screening of other cancers. In Papers I-III the following factors are likely to reduce the occurrence or the effect of a selection bias.

I. The frequency of CDKN2A mutations in the normal population is low, in a Swedish study, the mutation was found in none of 663 healthy control subjects and in one of 526 sporadic melanoma cases and in nine of 200 familial melanoma cases. This indicates that CDKN2A mutations are indeed mostly confined to familial melanoma cases.
II. In Sweden, the accrual of melanoma families is population based, since all new melanoma cases in the population should, according to national clinical guidelines, be questioned regarding relatives with melanoma and if this is the case, be offered participation in a preventive program. The accrual of subjects for CDKN2A mutation testing that has been done for the purpose of study, has been more biased, i.e. with highest participation among cases from Lund and Stockholm.

III. Melanoma risk is always shown prospectively from a date when the first blood sample was taken for CDKN2A analysis, which is the date when the family was identified for study.

IV. In CDKN2A wt kindreds, a high risk for squamous-cell skin cancers was found. This could possibly be related to the fact that melanoma kindreds are enrolled in a preventive program with regular skin exams, resulting in higher rates of detected skin cancers. For this reason, a retrospective analysis was also done to evaluate the risk of squamous cell skin cancers, before families were identified. This retrospective analysis showed that the risk increase for non-melanoma skin cancers was also present before inclusion. Interestingly, in CDKN2A mutation carriers, high risks of non-melanoma cancer were seen, but not of squamous-cell skin cancers, despite the fact that all family members had participated in the same preventive program.

V. The main aim of Paper I-II was to assess the non-melanoma cancer risk. In the preventive program or for the CDKN2A mutation analysis, there has been no inclusion criteria regarding other cancers than melanoma, but families with other cancer cases could have been more inclined to participate in preventive programs and donate blood for study. Also for other cancers, prospective risks are shown.

VI. In the study of cancer risk in CDKN2A mutation carriers, cancer incidences were compared, both to population controls and also to non-carriers from the same families, that also participated in the preventive program. Compared to both control groups the prospective risk for melanoma and other cancers was significantly increased.

VII. In Paper III, the aim was to evaluate whether CDKN2A mutation carriers had different survival following a melanoma diagnosis, compared to non-mutation carriers. To avoid selection bias, CDKN2A wt familial melanoma cases were selected as controls, since they had the same follow-up within a preventive program as CDKN2A mutation carriers. However, if they were suitable controls could be questioned since they have unknown underlying causes for increased melanoma susceptibility. For this reason, also, matched sporadic melanoma cases from The Stockholm-Gotland Melnoma Registry were used as controls, but with the awareness that this group has not had the same follow-up.

8.2 INFORMATION BIAS

Information bias (misclassification) is a systemic error in a study that can arise if the information collected about or from study subjects is erroneous. Misclassification can occur when data on subjects and controls are collected directly from the study participants.
themselves or gathered by the researcher. Individuals from melanoma families might, compared to individuals with no family history, be more prone to report other cancers diagnosed among themselves or their relatives. If cancers in relatives only would have been gathered from index persons, information on existing relatives and their diagnoses could have been left out. Also, researchers could have more clinical or pathology reports available for family members that are followed-up at their clinic, than in a healthy control population. All these scenarios would result in differential misclassification that could lead to an over- or underestimation of an effect-outcome association. In Papers I-III, the effect of misclassification has been reduced by the usage of nation-wide, high quality registries, with the identification of all first- and second-degree relatives attained from the Swedish Multi-generation Registry and all cancer diagnoses attained from the Swedish Cancer Registry and all vital events (births, deaths) from the Population Registry.

8.3 CONFOUNDING

Confounding is a systemic error that arises when an association between an exposure and an outcome is being studied, but the exposure and outcomes are both associated with a third variable, that is not an intermediate link between the two. There are different methods used to prevent confounding, such as matching, randomization, stratification or restriction of study cases. Regression analyses can be applied if confounders are known and accounted for. For cancer risks, age and gender are strongly associated variables. In Papers I-II, confounding due to age and gender was prevented by matching for these variables. For prognosis, age, gender as well as tumor stage are strongly associated variables. In Paper III, confounding due to these factors was prevented by the utilization of adjustments in the Cox proportional hazards regression model. Residual confounding occurs when there is remaining confounding after matching and adjustments have been performed, i.e. due to unknown confounding factors. In none of the papers there was any data included on factors that affect cancer risks and/or prognosis, such as socio-economic status, UVR exposures or smoking that are all factors that can differ between population based controls and familial melanoma kindreds participating in a preventive program.

8.4 VALIDITY

The internal validity refers to the quality of the study design and depends on that sources of systematic error, as those mentioned above are minimized. The external validity is the extent to which the results of a study can be generalized to other situations and to other populations. The external validity is highly dependent on the internal validity. In Paper I, all CDKN2A mutation carriers are carriers of the Swedish founder mutation p.Arg112dup. Findings from previous studies have demonstrated that cancer risks can differ between different mutations in the CDKN2A gene, hence the validity of the finding for carriers of other CDKN2A mutations is uncertain. Sweden and the Netherlands are the two countries with the largest known CDKN2A founder mutation populations. Both the Swedish and the Dutch (Leiden) CDKN2A mutations are located in the exon 2 of the gene, and both are set in ankyrin repeats 3–4 of the gene. Previous study of the Leiden mutation have shown similar spectra of tumors as in the
Swedish founder mutation carriers and a later study also showed an association with tobacco smoke\textsuperscript{166}.

8.5 RANDOM ERROR AND PRECISION

The error that remains after systematic errors are eliminated is called random error. Random error arises due to measurement errors or from sampling variability. By increasing the sample size, by repeating measurements or repeating the study, random error is reduced and precision increases. Random errors are reflected in \textit{p}-values and confidence intervals. In Papers I-III the precision is affected by the fact that familial melanoma cohorts are rather small, in particular the cohort of \textit{CDKN2A} mutation carriers. This, of course has to be regarded in the light of the rarity of \textit{CDKN2A} mutations in the population. Also the occurrences of cancers are highly increased in mutation carriers compared to the normal population, with increased numbers of outcome events and in turn increasing precision. In Paper I, the precision of the estimated cancer risks in mutation carriers, is increased by repeating analyses in different cohorts of carriers, first in confirmed carriers, then in first-degree relatives (approximately 50% carrier frequency) and next in second-degree relatives (approximately 25% carrier frequency). In \textit{CDKN2A} mutation carriers, significant risk increase for cancers in respiratory and upper digestive tissues were seen in all cohorts, being highest in carriers, followed by first- and second-degree relatives, indicating a gene-dose effect.

8.6 POWER

Statistical power is the likelihood that a study will detect an effect when there is an effect there to be detected. Statistical power is affected chiefly by the size of the effect and the size of the sample used to detect it. Bigger effects are easier to detect than smaller effects, while large samples offer greater test sensitivity than small samples. In the planning of Study I, it was estimated that the optimal size of an age- and sex-matched control group for the \textit{CDKN2A} mutation carriers would be three controls for every carrier. When data analysis started it became clear that in the controls, for several cancer diagnoses, there were none or only rare occurrences. This had the consequence that relative risks of several cancer types were non-calculable and interpretations of risks uncertain. To increase power of the analysis, the control groups of carriers, their first- and second-degree relatives were combined. This was done at the cost of the matching being disturbed, possibly increasing confounding, however in the prespecified control groups the age and sex distributions were similar to the combined control group.
9 CONCLUSIONS, DISCUSSION AND IMPLICATIONS

9.1 CONCLUSIONS, DISCUSSION AND IMPLICATIONS OF PAPER I

"High Risk of Tobacco-Related Cancers in CDKN2A Mutation-Positive Melanoma Families"

The main finding of this study is that the Swedish founder mutation in CDKN2A (p.Arg112dup) is associated with high risks of tobacco-related cancers in respiratory and upper digestive tissues, including pancreas. Upper digestive tissues and respiratory tissues have a common origin from foregut endoderm\textsuperscript{101} and are known to be sensitive to exposures from certain carcinogens. In particular strong associations with cancers in these tissues and tobacco smoke and/or alcohol have been established\textsuperscript{46,207}. In a study of mutational processes in multiple human cancers, it was reported that of the 30 different tumor types analyzed, melanoma, lung, esophageal, head and neck and gastric cancers were all among the tumor types with the highest numbers of acquired mutations\textsuperscript{45}. Digestive and respiratory organs are highly exposed to multiple carcinogens in our environment in analogy to the skin being exposed to ultraviolet (UV) radiation. It has been shown that melanoma penetrance in CDKN2A mutation carriers is associated with the environmental UVR exposure in the country of residence, supporting the impact of environmental factors in genetically predisposed individuals\textsuperscript{58}. In melanoma tumors, UVB signature DNA changes are commonly observed in the CDKN2A gene\textsuperscript{110}. Somatic CDKN2A alterations are frequently observed in pancreatic, lung, head and neck, esophageal and gastric cancers, where they are believed to be driver mutations\textsuperscript{208-211}. In lung and head and neck cancers it has been shown that somatic alterations in the CDKN2A gene are associated with tobacco smoke and/or alcohol exposure\textsuperscript{212-214}. Further, in a CDKN2A knockout mouse model, where both p16 and the murine homolog of p14ARF are eliminated, it was demonstrated that the cancer risk that was already very high in the knock-out compared to wt mice, was multiplied by adding the potent carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA). Notably, no such multiplicity effect was seen in the wt mice by adding the carcinogen\textsuperscript{215}. Germline mutations in both TP53 and RB1 gene are associated with high risks of lung cancer\textsuperscript{122,123}. In both RB1 and TP53 mutation carriers, the elevated risks for lung cancers are mainly observed in smokers\textsuperscript{123,216}. Thus, it seems that carriers not only of CDKN2A mutations, but also of mutations in tumor suppressors in the same pathways as p16 and p14-ARF, are at elevated risks of smoking-induced cancers.

In upcoming, updated Swedish guidelines on familial melanoma there are several novel recommendations that are based on the findings from this study. In particular, CDKN2A mutation carriers are recommended follow-up, not only for melanoma, but also oncologic follow-up for non-skin cancers. Carriers should at an early age be informed about the very high risks associated with smoking and offered cessation aids if they are already smokers. Further, it is recommended that CDKN2A mutation carriers should biennially undergo abdominal MRI and low-dose thoracic CT scans aimed at detection of premalignant or earlier stages of cancer.
9.2 CONCLUSIONS, DISCUSSION AND IMPLICATIONS OF PAPER II

“CDKN2A mutation-negative melanoma families have increased risk exclusively for skin cancers but not for other malignancies”

The main finding of this paper is that familial melanoma cases with no mutation in the CDKN2A gene have high risks for melanoma and squamous cell skin cancers, but no increased risks of other cancers. Our findings of high risks of skin cancers and no increased risks of other cancers in CDKN2A wt melanoma families, in combination with the increase observed in RHC variants in the MC1R gene, are consistent with the possibility that in the majority of these families, there are not mutations in high-risk cancer predisposing genes, but rather a segregation of variants in low-risk genes. Melanomas and squamous cell skin cancers are highly associated with increased UV-exposure and it is possible that shared environmental exposures, i.e. similar sun habits, also contributes to increased risks of both classes of skin tumors within families. Importantly, although no increased risk of non-skin cancers were observed in CDKN2A wt melanoma families, there may be families carrying high-risk cancer predisposing gene mutations that are masked under the majority of families that do not carry such mutations. Further genetic studies are essential to identify new melanoma associated genes. Our study shows that families with young melanoma cases have higher risks for non-skin cancers, indicating that such families could be more likely to carry high-risk mutations.

This study has, together with Paper I several clinical implications. Presently, the international melanoma genetics consortium (GenoMEL) and most national clinical practice guidelines recommend that CDKN2A testing should be conducted merely in a research setting, however, strong argumentation for clinical CDKN2A testing has been put forward. Our findings further justify CDKN2A mutation testing of melanoma family members in the clinical setting where the mutation status should determine the follow-up routines in affected families. Members of CDKN2A wt melanoma families require counseling and screening aimed at prevention and earlier detection of skin cancers while CDKN2A mutation carriers require in addition to dermatologic surveillance, also follow-up for non-skin cancers. In the upcoming, updated Swedish guidelines on familial melanoma these recommendations are included.
9.3 CONCLUSIONS, DISCUSSION AND IMPLICATIONS OF PAPER III

“Survival in familial melanoma cases carrying germline CDKN2A mutations: Increased mortality from melanoma and non-melanoma cancers compared to mutation-negative melanoma cases”

The main finding of this paper is that CDKN2A mutation carriers have, compared to non-carriers, significantly worse melanoma-specific survival, which is independent of age, gender, tumor thickness and diagnoses of multiple primary melanomas or of non-melanoma cancers. These results indicate that the CDKN2A germline mutation is directly associated with a more aggressive melanoma phenotype, but the mechanism for this is unclear. In a recent study, it was demonstrated that primary melanomas from CDKN2A mutation carriers do not exhibit a distinct gene expression signature compared to sporadic melanomas. Also frequencies of BRAF or NRAS mutations do not seem to differ in CDKN2A mutation carriers and non-carriers. Still, somatic losses of CDKN2A have been associated with worse outcomes in melanoma and also in various other cancers (gliomas, sarcomas, certain leukemias and lymphomas, lung, oral, gastroesophageal, renal cell, pancreatic, breast, bladder, hepatocellular cancers). As mentioned previously, CDKN2A mutation carriers appear to be more sensitive to carcinogenic/mutagenic exposures. It is possible, that germline and somatic mutations in the CDKN2A gene that lead to a disruption of proper cell cycle inhibition functions of the p16/p14ARF proteins permit cells with additional acquired mutations to progress through the cell cycle. This may result in an accumulation of carcinogen-induced mutation in tumor cells, contributing to greater tumor aggressiveness.

This study shows that CDKN2A carriers, not only need thorough surveillance to prevent and detect melanomas at earlier stages, carries also need close follow-up for melanoma recurrences. Earlier detection of stage III-IV melanoma will hopefully lead to better survival in this group, especially considering the landscape of effective melanoma regimens emerging. Activating BRAF mutations are at least as common in melanomas from CDKN2A mutation carriers as in sporadic melanomas, and such tumors may therefore be candidates for BRAF inhibitor-based therapies. Among BRAF mutation positive melanoma cases receiving BRAF inhibitor therapy, somatic loss of CDKN2A has been associated with worse outcomes, but this might reflect the generally worse prognosis associated with CDKN2A loss, rather than a specific association with worse response to BRAF inhibitors. Also, preclinical studies indicate that CDKN2A mutations may predict sensitivity in melanoma patients to CDK4/6 inhibitors that are emerging as promising novel kind of agents in various cancers. Further, a recent study demonstrated that increased mutational load and specifically presentation of neo-antigens was associated with better response to CTLA-4 immune checkpoint inhibition. If melanomas in CDKN2A carriers have, in line with our findings, increased mutational load, such tumors might have a beneficial response to immune checkpoint blockade.
9.4 CONCLUSIONS, DISCUSSION AND IMPLICATIONS OF PAPER IV

“Hereditary Uveal Melanoma: A Report of a Germline Mutation in BAP1”

The main finding of this study is the identification of a novel mutation in the BAP1 gene segregating with familial cases of uveal and cutaneous melanoma. Loss of heterozygosity in the tumor material further supports the role of BAP1 germline mutation as a causative factor for uveal melanoma. The identified mutation truncates the gene, obliterating most of the protein, including the binding domains to BRCA1, BARD1 and HCF1 and the active nuclear ubiquitin carboxy-terminal hydrolase domain. In Sweden, additional three families, all with different mutations in the gene (two truncating and one non-synonymous) have been identified.

Germline mutations in the tumor suppressor gene BAP1 has been associated with the development of various malignancies in predisposed families and the condition has been named the BAP1 tumor predisposition syndrome (BAP1-TPDS). In a recently published comprehensive review of 27 reports of germline BAP1 mutation families, mutations were seen at different gene loci and mutation site or type was not associated with any particular cancer type\textsuperscript{148}. In this study 31% of carriers had a diagnosis of uveal melanoma, 22% of mesothelioma, 13% of melanoma, 10% of renal cell cancer and 6% of basal cell cancers. The penetrance of cancers among BAP1 mutation carriers was estimated to 85%. Notably, 72% were identified with distinct subset of benign skin lesions. A range of names has been given to these lesions, including melanocytic BAP1-mutated atypical intradermal tumors, atypical Spitz tumors, BAPomas or Wiesner nevi. These lesions are well-circumscribed dome shaped, skin-colored or reddish-brown nodules, with average sizes of 5 mm, and range widely in number. Morphologically, the lesions are mostly intradermal with occasional involvement of the junctional epidermis, and show cytological features resembling atypical Spitz nevi. They are characterized by biallelic inactivation of BAP1 and frequent BRAFV600E mutation. These lesions are believed to be potential precursors of melanomas in carriers. Accordingly, verified BAP1 carriers require close follow-up for uveal and cutaneous melanoma as well as for other cancers.

The findings in Paper IV are based on the identification of one single family with an unusual hereditary disposition for uveal melanoma and on the availability of next-generation sequencing techniques. To conclude, this study shows that collaboration between clinicians and laboratory researchers has the potential to generate novel translational findings.
10 FUTURE PERSPECTIVES

10.1 BIOLOGICAL DIFFERENCES IN TUMORS FROM CDKN2A MUTATION CARRIERS AND NON-CARRIERS

The findings of sensitivity to carcinogens as well as worse prognosis in CDKN2A mutation carriers raises the question if tumors from CDKN2A mutation carriers have biological differences compared to tumors from non-carriers. Previous studies have found no differences in frequencies of BRAF or NRAS mutations or in RNA expression signatures, depending on germline CDKN2A mutation status\(^{185,186}\). In this aspect it would be of interest to explore other potential biological differences, such as mutational load in melanomas and other tumors from germline CDKN2A mutation carriers and non-carriers respectively, in particular mutations with carcinogen signatures. In addition to genetic changes, epigenetic events have emerged as key mechanisms in the development and progression of human cancer and could also potentially differ between CDKN2A mutation carrier’s and non-carrier’s tumors. In the study on expression signatures by Jönsson et al. it was found that the more beneficial “high-immune” signature was present in 27% of melanomas from sporadic cases, but only in 9% of melanomas from CDKN2A mutation carriers. Hence, it would also be interesting to investigate if immunohistochemistry specified immunophenotypes differ in tumors from CDKN2A mutation carriers and non-carriers.

10.2 PROSPECTIVE STUDY OF OUTCOMES IN CDKN2A MUTATION CARRIERS

In Sweden, the CDKN2A mutation test will soon be implicated as a clinical test in melanoma families, where melanoma families are defined as families with at least two first-degree relatives with melanoma or tree or more diagnoses of melanoma or pancreatic cancer in blood related individuals. In upcoming Swedish guidelines, CDKN2A mutation carriers will receive, besides from dermatologic surveillance, also oncologic follow-up and information to promote abstinence from tobacco smoke and smokers will get professional help to quit. Carriers will also be offered MRI and CT screening for pancreatic and lung cancers, respectively. Optimally, these interventions should be followed-up in a prospective study, i.e. by offering intervention to both members of CDKN2A mutated and non-mutated melanoma families. Bearing in mind that known carriers are few (in Sweden ~100 known alive carriers) such study could take many years to complete. It would therefore be more efficient if such a study would be conducted as a multi-center study i.e. by the GenoMEL consortium. Also a multi-center approach should be applied to assess therapy responses of CDKN2A mutation carriers, in particular to the novel immune based and targeted therapies.

10.3 SCREENING OF MUTATIONS IN FAMILIAL MELANOMA CASES

In melanoma families, mutations other than CDKN2A have so far only been identified in rare families. As no gene, other than CDKN2A, has been identified in any substantial portion of families, it is likely that in the CDKN2A wt families, there are quite many different genetic underlying causes for melanoma heredity. A recent study identifies 20 different melanoma...
susceptibility loci, whereof five have not been described previously. In our group we plan to initiate a next generation sequencing gene panel, involving both known high- and low-risk melanoma genes (Table 6, p. 18), that is continually updated along with new findings of melanoma predisposing genes. Families where no mutations or variants are found in known genes should be enrolled in exome or genome sequencing studies and to increase power, multi-center studies are preferable.
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