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CUTANEOUS MELANOMA RISK FACTORS AND SINGLE NUCLEOTIDE POLYMORPHISMS IN A REPRESENTATIVE PART OF A BELGIAN MELANOMA POPULATION

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Erat, est et semper erit de amore.

PREFACE

YOU WERE

I went

There

Where

You Were

But you were

Not There.

Nowhere

You Were.

- Soli Loquy

To the many souls that have come and gone, this thesis is my rage against the dying of your light.

SUMMARY

Cutaneous melanoma (CM) is the most common lethal skin cancer among Caucasians. CM incidence and mortality are projected to rise in most predominantly White populations including Belgium. Melanoma survival is best if diagnosed at an early stage. However, the prognosis of a percentage of melanoma patients cannot be accurately predicted by the AJCC staging criteria reflected by relatively wide ranges of survival. Furthermore, novel targeted and immunotherapies have been shown to increase survival in a set of CM patients but are disadvantaged by high costs, therapy resistance and toxic adverse effects. Hence, understanding risk factors for melanoma development and survival is of vital importance. Well known risk factors for melanoma susceptibility are a high common naevi count, presence of atypical naevi, sunburn, low phototype, freckles, actinic damage, personal and family history of melanoma and genetic variants linked with these clinical risk factors. These risk factors have not yet been extensively studied for their potential relation with melanoma outcome. Also, a potential protective effect of atopy on melanoma development and outcome is not fully elucidated. Finally, a substantial part of CM patients develop multiple CM and it remains to be fully determined whether risk factors for multiple primary melanoma (MPM) differ from risk factors for single primary melanoma (SPM).

In order to better understand risk factors associated with CM etiology and survival, we performed a series of investigations. First, we conducted a meta-analysis comparing risk factors for MPM versus SPM. This demonstrated that a positive family history, presence of dysplastic nevi and light hair colour were correlated with a higher risk for developing MPM. Second, in a case-control study, we found a significant negative association between personal atopy and melanoma development, while no significant associations were found between atopy and melanoma outcome. Third, we demonstrated that known clinical risk factors (sun damage, family history, presence of atypical nevi) for melanoma susceptibility affect melanoma outcome. After Bonferroni correction, the correlation between presence of solar lentigines on the back of hands and arms remained the most robust and significant prognosticator for relapse free survival in CM patients. Finally, we examined the association between melanoma susceptibility genetic variants and CM survival which showed a significant association between the rs869330 variant (in the *MTAP* gene) and progression free survival. To conclude, we have observed new evidence on the associations between clinical risk factors, genetics and melanoma risk and survival broadening our understanding of the disease and underlining the multiple pathways involved in the disease.

CONTENTS

Summary……5

Contents……6

Chapter 1: Introduction……9

1.1 Epidemiology of cutaneous melanoma……9

1.2 Risk factors for cutaneous melanoma development……10

1.2.1 Clinical risk factors ……10

1.2.2 Genetics and melanoma susceptibility……18

1.3 Clinical and histopathological presentation of cutaneous melanoma……27

1.4 Cutaneous melanoma staging……29

1.5 Treatment of cutaneous melanoma……30

1.6 Prognostic factors for cutaneous melanoma ……33

1.6.1 Clinical risk factors and cutaneous melanoma outcome……34

1.6.2 Histo-pathological risk factors for outcome……37

1.6.3 Immune system and melanoma outcome……39

1.6.4 Genetics and melanoma outcome……40

1.7 Methodological considerations……47

1.7.1 Study Population……47

1.7.2 Statistical Techniques……48

Chapter 2: Aims……50

Chapter 3: A meta-analysis on major risk factors of multiple primary cutaneous melanomas……51

3.1 Abstract……51

3.2 Introduction……52

3.3 Methods……52

3.4 Results……54

3.5 Discussion……60

Chapter 4: Inverse association between atopy and melanoma: a case-control study ……63

4.1 Abstract……63

4.2 Introduction……63

4.3 Methods……64

4.4 Results……67

4.5 Discussion……71

Chapter 5: Solar lentigines are associated with better outcome in cutaneous melanoma……73

5.1 Abstract……73

5.2 Background……73

5.3 Methods……74

5.4 Results……76

5.5 Discussion……76

Chapter 6: Melanoma susceptibility variant rs869330 in the *MTAP* gene is associated with melanoma outcome ……82

6.1 Abstract……82

6.2 Introduction……82

6.3 Patients and Methods……83

6.4 Results……85

6.5 Discussion……86

Chapter 7: General discussion……91

7.1 MPM: Genetic and/or environmental?……91

7.2 Atopy and melanoma risk: adaptive versus innate immunity or both?……94

7.3 Melanoma clinical and histopathological risk factors and outcome: genetics and UV exposure……103

7.4 Genetics and melanoma outcome……111

7.5 Conclusion……117

Appendix……120

Bibliography……124

Curriculum Vitae……143

Publications……144

Acknowledgements……145

Personal Contributions……146

Conflict of Interest……146

List of Figures and Tables……147

List of Abbreviations……149

CHAPTER 1: INTRODUCTION

1.1 EPIDEMIOLOGY OF CUTANEOUS MELANOMA

Cutaneous melanoma (CM) is the most common lethal skin disease affecting mainly Caucasians worldwide. Although less than 5% of all skin cancer types, melanoma accounts for 75% of all skin cancer mortality in adults. [1] Worldwide, the estimated number of new melanoma cases in 2018 is 287 723 with a crude rate of 3.8 per 100 000 and an age standardised rate (ASR) world of 3.1/100 000 whereby the highest ASR world were estimated for Australia with 33.6/100 000 closely followed by Norway, Denmark and the Netherlands. [2]

Melanoma was on the 7th place of the most common cancers in 2012 in the European Union (EU).[3] Approximately 82 000 new melanoma diagnosis were made in the EU in 2012 as shown by the latest figures by European Cancer Estimates (EUCAN). [4] In 2018, it is estimated to be the sixth most frequently diagnosed cancer type in Europe with approximately 144 209 new cases and a crude incidence rate (CIR) of 19.4/100 000 and age-standardized rate (ASR) (world) of 11.2/100 000. [2] Within Europe, the highest incidence rates were forecasted for Western Europe and the lowest for Central and Eastern Europe [2]. In the EU, the 5 year-prevalence was estimated to be 494 911 in 2018.[2]

Within Western Europe, Belgium is one of the top ten countries with the highest estimated 5-year prevalence at 12277 cases and a one-year prevalence of 3265 for 2018 (Appendix Fig.1). [2] The latest data from the Belgian Cancer Registry dating in 2016 showed CM to be the fourth most common invasive tumor with 1786 new cases among females and fifth in males with 1283 cases. [5] The same registry reported a rising incidence rate from 2004 until 2016 in Belgium (Appendix Fig.2). The trend seems to affect females more than men. The average annual percentage change over 2004-2016 period was 5.1 for males (95% CI:4.0-6.1) and 4.9 for females (95% CI:4.1-5.7). As for 2018, 3532 new cases have been projected for Belgium with a crude rate of 30.7/100 000 and ASR world of 19.9/100 000 placing Belgium on the 9th place in the world ranking and 4th place within Western Europe.[2]

Melanoma incidence has been rising in the last decades and is estimated to increase worldwide from approximately 288 000 incident cases in 2018 to 467 000 in 2040. [2] The Belgian Cancer Registry is expecting at least 60% increase in incidence by 2025. [5] Variations in incidence rates are possible between countries which can be attributed to differences in national registration processes and population risk factors. Moreover, underreporting in National Cancer Registries is also probable, implying that melanoma incidence might be greater than reported. [6]

With regard to mortality, approximately 16 000 deaths due to melanoma were reported in the EU in 2012. [4] Worldwide, approximately 60 172 deaths are anticipated to occur due to melanoma in 2018, 27 147 in Europe and 464 in Belgium. [2] CM is expected to be the sixth most frequent cause of death due to cancer for both males and females in Belgium under the age of 54 in 2018.[2] Melanoma mortality rate is 2.2/100 000 in Europe on average compared to 2/100 000 in Belgium in 2012. [7] For 2018, the estimated crude mortality rate for Europe is expected at 3.6/100 000, the second highest in the world after Oceania. Furthermore, the expected world (age-) standardized mortality rate (WSR) worldwide was 0.6/100 000, for Europe it was 1.7/100 000 for cutaneous melanoma and 1.9/100 000 for Belgium. [2] In 2014, the average 5 year survival rate 82.7% was in the EU countries and 83.6% in Belgium. [3]

Overall, melanoma incidence and mortality have been estimated to rise in 2018 compared to 2012 in Europe including Belgium. [4,8] The rising trend in CM incidence might be caused by an increase in detection of early stage melanomas and therefore better survival. [9,10] However, thick CM incidence rates are in the upward trend in some countries implying that the rise could not be explained by early detection alone. [11]

1.2 RISK FACTORS FOR CUTANEOUS MELANOMA DEVELOPMENT

1.2.1 Clinical Risk Factors

Several risk factors have been linked to melanoma susceptibility. Clinical risk factors can be grouped into either host-specific e.g. phenotypic/genetic traits or environmental risk factors. Host risk factors include age, gender and race. A series of meta-analyses reported an association between a high common naevi count, presence of atypical naevi, sunburn, low phototype, freckles or ephelides, actinic damage, personal and family history of melanoma with higher melanoma risk. [12–14] Immunological risk factors for melanoma consist of the well-established environmental determinant (i.e. iatrogenic immunosuppression) as well as host susceptibility (e.g. atopic diathesis) albeit the latter is still a subject of debate. Genetic risk factors, although host-specific, will be discussed in detail later in this chapter.

1.2.1.a Host-specific clinical risk factors

Age and gender

It has been observed in the past that worldwide melanoma incidence peaks at a higher age, at the seventh or eight

decades in most Western countries. Melanoma trends for Belgium between 2004 and 2007 showed melanoma as having one of the highest average annual incidence per 100 000 in those younger than 40 years versus other cancer types. [2] Within the melanoma population, the estimated crude and ASR incidence rates in 2018 are positively associated with increasing age worldwide. Incidence rates are highest in Oceania versus other world regions with a CIR of 203 and an ASR of 194/100 000 cases in those 70 years and older versus 7.5 and 6.7/100 000 cases in those aged 15-39. [2] Europe though has the highest estimated new melanoma cases in 2018 in absolute numbers with approximately 50000 in the 70 plus group compared to 17000 cases in those aged 15-39. [2] In the recent years, melanoma incidence seems to be predominantly increasing in the older age groups in Europe especially in Southern Europe, whilst a stabilising trend has been signalled among younger groups (<50 years). [15,16] Some authors postulate that the contrast in the age trends might be attributable to the change in sun exposure habits as consequences of primary prevention campaigns and a ban on sunbeds [17,18] which might have led to better awareness of sun exposure and its association with skin cancer. [16,18] In the case of Southern Europe, the authors hypothesized that this might be due to the migration of fair skinned individuals from Northern and Western Europe to Southern Europe or to longer working hours and subsequent greater sun exposure. Alternatively, the worse prognosis might be attributed to late diagnosis and therefore usually more advanced stage whereby treatment options are much more limited. [19]

Differences in melanoma incidence between men and women have also been reported. Arnold et al. showed a higher CM incidence in females overall in Europe. However, the increase in incidence was greater in men especially in Switzerland but the reverse was true in the Netherlands. [16] However, projections of incidence rates are greater in females. [16] Ferlay et al. reported melanoma as the 5th most common cancer in European females accounting for 3.9% of the new cancer cases in this group versus 3.5% in European male counterparts. [8] Melanoma seem to affect young women more than men after correcting for age which reverses after the fourth decade probably attributable to hormonal factors (i.e. menopause).[20,21] Gender disparity is not limited to melanoma but has been reported for other tumors as well e.g. liver, gastrointestinal tract, bladder and brain to name a few. [22–26]

A higher overall cancer incidence and mortality in males have been demonstrated. [23,27] A review by Rubin et al. tried to explain the sex discrepancies in cancer through circulating sex hormones and sexual differentiation that affect genetic and epigenetic processes which in turn influences immunity, growth, longevity, wound healing,

angiogenesis and metabolism. In turn these mechanisms may affect not only the development and progression of neoplasms but also treatment response and ultimately survival. [28]

Sex hormones e.g. estrogen, testosterone and progesterone have been shown to be part of the development and progression of tumors such as breast and prostate cancer. [29,30] Estrogen can inhibit liver and colon cancer which are more prevalent in males and can stimulate cancer development in female biased cancers such as meningiomas and thyroid cancer. [28] However, since circulating hormone levels, which are age dependent, do not match the degree of gender discrepancies in cancer incidence and severity, other factors might be involved such as sexual differentiation. Behavioural differences between genders regarding UV exposure habits and self-examination might also explain gender discrepancies in cancer. [31,32] In addition, smoking behaviors, BMI, adult height and clinicopathological subtypes may also contribute. [33–35] For example, smoking in females had increased in certain birth cohorts but in general, females started smoking later than men and at older ages. [36]

Ethnicity

Race could also influence cancer statistics. Melanoma incidence has risen annually by ~5.5% between 1981 and 2011 among Caucasians in Australia. [37] Melanoma predominantly affects Caucasians but it also affects other races albeit in a substantially lower incidence such as African Americans and Asians. A US study also reported racial and ethnic differences in melanoma incidence and survival. [38] Melanoma incidence rate was shown to be higher among females in the Caucasian group and in the younger age bracket among Hispanics and Asian Pacific Islanders (API). In addition, melanoma patients with ethnicities other than White and Black (e.g. Hispanics, American Indians/Alaskan Natives and API) tend to have a younger age of diagnosis than their White and Black counterparts. Furthermore, disparities in melanoma characteristics were shown between different races. Blacks and Hispanics usually develop ALM as subtype whilst SSM is most prevalent in other races. [38] Among those younger than 40 years, melanoma tend to be localised on the trunk in males of all racial groups and female non-Hispanic whites. [39]

Nevus phenotype

It has been postulated that two major pathways exist in cutaneous melanoma pathogenesis. One major pathway is nevus susceptibility either influenced by host characteristics with or without intermittent solar exposure. This hypothesis is supported by studies that found ~30% of cutaneous melanomas have developed from nevi and the

association is higher, as high as 50%, in those with high nevi count. [40–42] Common nevi are typically < 5 mm with benign features and can either be raised or flat. [42] Other studies have demonstrated that nevi-related melanomas frequently form on body sites with low solar exposure. A meta-analysis conducted by Olsen et al. demonstrated a significant positive dose-dependent relation between common nevi count and melanoma risk. [43] Patients with high nevi count usually have early onset melanomas localized on the trunk whilst individuals with a lower count tend to develop melanomas at a later age on non-chronically sun exposed locations. [43] A large international study on primary cutaneous melanoma demonstrated an independent positive association between high nevus counts and early onset and male sex but a negative correlation with lentigo maligna (LM) subtype. [44] In Caucasians, nevi count tend to increase during childhood and decrease usually after 40 years of age through involution which is directed by senescence. [45] A high nevi count later in life has been linked with an unfavourable melanoma risk. [46]

Atypical nevi can be defined according to the presence of at least ≥3 of the following parameters: ill-defined borders, diameter ≥5 mm, variable pigmentation, irregular border and erythema. [47] Atypical nevi is sometimes referred to as dysplastic nevi although some authors posit that the term atypical nevi should be reserved for clinical classification whilst dysplastic nevi should be used for histopathological classification. [48]

The AMS (atypical mole syndrome) scoring system has been devised by British dermatologists consisting of 5 variables which aimed to increase interobserver agreement. The scoring system had been shown to be robust and can be easily implemented in practice. [47] Atypical mole syndrome is defined as having at least 3 of the following variables namely 100 or more naevi (at least 50 in 20 years or younger or older than 50) that are at least 2mm in size, 2 or more atypical nevi, at least 1 nevi on the anterior of scalp and/or buttocks and/or on the iris and/or at least 2 nevi on the feet dorsum. [49] It has been postulated by Bataille et al. that having an atypical mole or an atypical mole syndrome are associated with a higher chance of developing melanoma. [49] In addition, not only the presence but also a high number of clinically atypical naevi was shown to be associated with an increased melanoma risk. [50,51] A meta-analysis revealed a fourfold increased risk for melanoma in those who have at least 1 atypical nevus versus none. The population attributable fraction (PAF) of atypical nevi in melanoma development was estimated to be 0.25. [52]

Phototype

Host factors that play a role in an individual's susceptibility to sun exposure include skin/hair and eye color (specifically fair skin phenotype) and tanning ability. [14] Inherited phenotypic traits that were correlated with melanoma risk consist of light hair, skin or eye color, red hair and freckles. [14] Skin phenotype and tanning ability are usually grouped according to Fitzpatrick's skin type classification (see Appendix Table 1). [53] A meta-analysis estimated a statistically significant twofold melanoma risk in fair skin phenotypes (Fitzpatrick type 1 vs IV and fair versus dark skin color) and in those with high density of freckles. In addition, they observed a 47% increased risk between blue eye color vs dark and an almost fourfold risk between red vs dark hair color. [14]

Personal melanoma history

Another risk factor for melanoma is a positive melanoma history. A diagnosis of melanoma increases the chance of developing a second melanoma, with percentages ranging from 0.2 to 20%. [54] A meta-analysis reported a tenfold elevated risk of developing a subsequent melanoma in those who had melanoma history. [55] The risk of developing a second melanoma is the same between non-invasive and invasive first primary melanomas and is highest in the first year post diagnosis. [56,57] The 5-year risk of developing a second melanoma is estimated to be between 2 to 11%.[57] Factors associated with an increased risk of additional melanomas consist of a history of dysplastic nevi [58], age at diagnosis (<30 yrs) [59], history of head/neck melanoma [59], Caucasian ethnicity [60] and female gender [60]. However, other studies found that multiple primary melanoma (MPM) are more likely to present in males and in older age compared to single primary melanomas (SPM). [61] Other patient characteristics comprise of familial melanoma [58], fair skin, inability to tan, high nevus counts, and a lentigo maligna melanoma (LMM) or nodular melanoma at initial melanoma diagnosis which give approximately a two to threefold elevated risk for second primary lesions. [62] Conversely, it has been posited that a lower intentional UV exposure post melanoma diagnosis significantly diminishes the development of subsequent melanomas. [63] Interestingly, melanoma risk is approximately fourfold higher in those with pre-malignant and skin cancer lesions compared to those who lack these lesions. [14] Knowledge of which risk factors are more prevalent in MPM patients compared to SPM patients was scarce however when our study was construed.

Family history of melanoma

Familial melanoma is positive when a patient with CM has at least one first degree family member diagnosed with melanoma whilst others prefer two first degree relatives. First degree family members consist of parents, children and siblings whilst second degree relatives include grandchildren, aunts or uncles, nephews or nieces, grandparents and half-brothers/sisters.

Approximately 10% of melanoma cases have a positive family history. Melanoma risk increases with the number of first degree relatives with melanoma, ~1.7 times with one first degree family member and nine-fold with two relatives. [64] A meta-analysis also calculated a pooled estimate of a 74% elevated melanoma risk for melanoma if family history is positive. [14] Melanoma risk is higher in those with familial melanoma and atypical nevi compared to familial melanoma without atypical nevi [65] and in those that harbour dysplastic nevi or a high total nevi count in melanoma prone families. [66] Patients with a positive family history usually have an earlier melanoma onset compared to sporadic melanoma. [67] A higher cumulative melanoma risk that rises with age is more likely with familial CM compared to sporadic CM. [68] Of note, patients with familial melanoma are more likely to develop MPM compared to sporadic melanoma. [69]

Atopy

It has been hypothesized that a possible negative link between atopy and melanoma might exist. The logic behind this negative link is that chronic immune surveillance induced by atopy might lead to detection of neoplasms in an earlier stage. Moreover, the fact that melanoma is an immunogenic tumor might render this disease susceptible for the heightened immune surveillance in atopic patients. [70–72] On the other hand, atopy is also an inflammatory disease and inflammation has been linked with tumor development and has been postulated as one of the hallmarks of cancer. [70,71] It has been hypothesized that inflammation can contribute to genetic instability and might even sustain a pro-oncogenic environment. [70,71]

Studies that have been conducted on atopy and cancer are scarce. The results of these studies were non-consistent. [73] Mills et al. tried to explain these inconsistencies by postulating that specific types of atopy might be associated differently with distinct types of cancer. [74] Most did not conduct subgroup analysis for atopic dermatitis (AD) or the various kinds of cancer but rather looked into all types of atopy or cancer in general. AD affects

approximately 20% of children and 3% of the adults whereby 60% of the children with severe AD will develop asthma. [75] It has been shown that fewer nevi are present in patients with AD which implies that having the disease might protect against melanoma since a high nevi count is a well-known risk factor for melanoma. [76]

In the period that our atopy study was conceived, literature that have been published on the association between atopy and melanoma were scarce and contradictory. Three studies in large AD cohorts reported lower incidence ratios of melanoma cases in AD populations versus non-AD [70,77,78] while another study found a significant positive association between AD and melanoma . [79] These studies looked into the incidence of melanoma in AD vs non-AD subjects but did not take other melanoma risk factors into account. Other studies comparing the incidence of atopic dermatitis in melanoma cases versus healthy control population, demonstrated a significant negative [80] and non-significant positive association [81] between melanoma and atopic dermatitis but these studies also did not correct for other melanoma determinants. Interestingly, a Dutch study on atopy and melanoma found a borderline significant association between asthma and melanoma risk but not with AD. [82] The study was limited because no correction was performed for other melanoma risk confounders except for phototype.

1.2.1.b Environmental risk factors

Major environmental risk factors for melanoma include sun damage and immunosuppressive drugs. Other environmental risk factors (e.g. arsenic poisoning, diet etc.) will not be discussed in this chapter.

UV exposure and sun damage

One major pathway to melanoma development is the combination of solar exposure damage accumulation and sun sensitivity. UV radiation, especially UVB (280-315 nm) and UVA (315-400 nm), [83] from sunlight or tanning devices and/or PUVA (psoralens plus UVA) treatments for psoriasis, is known to be the main cause of photocarcinogenesis either through direct deoxyribonucleic acid (DNA) damage or indirect DNA damage via production of reactive oxygen species which induces DNA photoproducts and DNA damage. [84] Genetics play a role through inactivating mutations of tumor suppressor genes or gain of functions of oncogenes. Defective DNA repair, especially in the nucleotide excision repair pathway, can also contribute to photocarcinogenesis. UVA1 was recently shown to inhibit the DNA restoration of the genetic damage caused by UVB in melanocytes. [85]

Sun damage can accumulate through acute intermittent exposure with sunburn as clinical sign or through chronic cumulative UV exposure. Clinical signs of the latter include wrinkles, solar lentigines, solar elastosis, actinic

keratosis and hypomelanosis guttate. A meta-analysis reported the presence of actinic lesions to be associated with a twofold higher melanoma risk. [14] Melanomas associated with chronic sun damage have been correlated with later onset, location on chronic sun-exposed sites such as head and neck region and higher mutation load compared to melanomas without chronic solar damage. [86] A systematic review of case-control studies has shown that childhood sunburn and intermittent sun exposure were linked with elevated melanoma risk unlike occupational sun exposure. [87] As for childhood sun exposure, having had 5 or more sunburns in early life precipitates a twofold higher melanoma risk. [88] A meta-analysis reported a statistically significant increased relative risk (RR) for melanoma with both intermittent and total UV radiation even after adjusting for naevi. [13] In addition, geographic variation and UV levels were also shown to correlate with melanoma incidence, whereby melanoma risk seem to be highest along the equator and decreases in proportion to the distance from the equator. [89]

Hypomelanosis guttate (HG) has been described as benign multiple, round/oval hypopigmented/white macules on sun-exposed sites such as the extensor aspects of the limbs which are common with increasing age. [90] [91] [92] HG has been shown to be positively associated with a high count of solar lentigo [91] and UV exposure. [92] An Australian study on solar elastosis showed that marked or moderate solar elastosis adjacent to melanomas was present in 37% of trunk melanomas versus 77% of melanomas situated on the head/neck. [93] Interestingly, there was a significant positive association with age and marked elastosis in both melanoma sites and a negative association with contiguous nevi. Marked or moderate solar elastosis on trunk melanomas were both significantly positively associated with high levels of solar exposure, freckles on the face and negatively associated with tanning ability and high counts of nevi.

A systematic review in Western countries revealed that 36% of adults, 55% of university students and 19% of adolescents had used indoor tanning at least once. [94] A positive correlation was demonstrated between melanoma risk and total number of tanning sessions especially a starting age younger than 30. [95] A meta-analysis has calculated that ever users of tanning beds have a 16% higher risk of developing melanoma versus never users with a 61% elevated risk in those reporting at least one year of use, 34% in those with at least 10 sessions and 35% who used tanning beds before 25 years of age. [96] Interestingly, a study in a group of people who reported no sunburns in their lifetime, a fourfold prevalence of tanning bed use was observed in melanoma cases versus controls. [97] The population proportional attributable risk for melanoma due to indoor tanning in Western countries was estimated to be between 2.6% to 9.4%. [94] In 2009, the World Health Organization (WHO) classified ultraviolet

radiation and tanning beds as carcinogens [98] due to mounting evidence of association and causation between these risk factors and cutaneous cancer development. [99]

Immunosuppression

Early evidence supporting the association between immunosuppression and melanoma has been derived from the organ transplant population. Melanoma in organ transplant recipients (OTRs) can develop before (history of melanoma), during (melanoma through an organ donor) and after transplantation (de novo melanoma). Robbins et al. reported a statistically significant higher SIR (standardized incidence ratio) of 2.2 in transplant recipients for invasive melanoma compared to the general population. [100] The risk for melanoma is highest within 4 years post-transplant and are even more elevated with azathioprine and polyclonal antibody induction therapy. As for de novo melanoma, there are studies that have shown an elevated incidence risk for OTR groups compared with the non-immunosuppressed which seems to be lower than non-melanoma skin cancer (NMSC) risk. [101,102] Although melanoma risk factors are similar between OTRs and the healthy population, melanoma developing in a nevi was more common in the OTR group compared to the healthy control group. [103] Studies that further support that melanoma is an immunogenic tumor include a multivariate analysis whereby a decreased MM risk was shown after graft rejection versus during retained post-transplant function. [104] A meta-analysis reported an almost threefold increased overall risk for melanoma in transplant patients albeit with significant heterogeneity among the included studies. [105] As to be expected, the pooled RR for melanoma in liver and heart transplant patients was raised at 5.27 whilst renal transplant recipients had a 2.5 higher RR compared with the general population.

1.2.2 Genetics And Melanoma Susceptibility

1.2.2.a Somatic mutation and melanoma susceptibility

Somatic mutations are DNA changes that occur after conception which can arise from any body cell other than a germ cell and can potentially lead to diseases including cancer. As these mutations do not develop in germ cells, they do not pass to the next generation. [106] Melanoma has the highest number of mutations of all cancers. [107] The most common mutation is the C>T nucleotide transition signature due to UV radiation which is not present in all melanomas. [107] Established melanoma genes such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*), mitogen-activated protein kinase (*MAPK*) mutations including V-raf murine sarcoma viral oncogene homolog B1 (*BRAF*)

and neuroblastoma RAS viral oncogene homolog (*NRAS*), tumor protein 53 (*TP53*) and phosphatase and tensin homolog (*PTEN*) have been reported to have a statistically elevated mutation burden in melanoma. [108] The earliest somatic mutation found for CM is the *CDKN2A* gene. This locus encodes the p14ARF and p16INK4A, which are both tumor suppressors. P14ARF blocks mouse double minute 2 (MDM2) ubiquitylation, which in turn halts p53 degradation. P16INK4A, which is much more prevalent than p14ARF aberrations regarding melanoma, prevents retinoblastoma associated protein activation and cyclin dependent kinase 4 (CDK4) or 6 phosphorylation. [109] Inactivation of these tumor suppressors leads to stimulation of the cell cycle and to oncogenesis. *CDK4* somatic mutations are very rare in melanoma. [110] *CDKN2A* and *CDK4* are cell cycle control genes. *CDKN2A* is also involved in DNA damage repair before cellular replication.

Another important melanoma somatic mutation can be found in the mitogen-activated protein kinase (MAPK) pathway which plays a preponderant role in cell growth and proliferation and therefore has an important role in melanoma development. Activating mutations of the *BRAF* oncogene, a part of the MAPK pathway, has been detected in about a third of melanoma cases [111] and are also present in approximately 80% of benign nevi. [112] However, germline *BRAF* mutations are not associated with elevated melanoma risk, indicating that *BRAF* mutations in CM are most probably somatic. [112] The most prevalent mutation is valine replacement with glutamic acid at codon 600 (*BRAFV600E*) [111] which activates MEK and ERK further down the pathway. [113] The rat sarcoma (RAS) family consisting of KRAS, HRAS and NRAS are upstream of the RAF family of protein kinases (i.e. ARAF, BRAF, CRAF). Induction of *BRAF-V600E* expression can result in melanoma. [114] The MAPK pathway has substantial therapeutic consequences for melanoma but most of its mutations lack UV damage signatures. The pathway include *BRAF* mutations which can be found in 50% of all melanomas . [115] *BRAF* and *NRAS* genes encode proteins belonging in the MAPK pathway which when mutated can lead to unbridled proliferation and tumor growth through extracellular signal-regulated kinases (ERK) phosphorylation. Among the three members of the rat sarcoma (*RAS*) gene family, *NRAS* is the second most common mutation in melanoma and activates the production of cytoplasmic proteins RAF and phosphatidylinositol 3 kinase (PI3K) .[116] The Harvey rat sarcoma viral oncogene homolog (*HRAS*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations were detected in 1 to 2% of melanoma cases whilst tyrosine protein kinase (*KIT*) mutations were found in less than 1% melanomas. [117] Benign melanocytic nevi commonly harbour *BRAF* and *NRAS* mutations implying that these mutations are not sufficient

for melanoma development and might be only the first step in overcoming senescence. [116] *BRAF* mutations were shown to be prevalent in SSM and NM subtypes unlike mucosal or acral melanoma sites of which only 10-20% harbor *MAPK* genetic aberrations. *NRAS* mutations on the other hand have a consistent prevalence rate of 15-20% in all cutaneous melanoma subtypes. [118] *KIT* mutations are infrequent in most melanoma subtypes but are abundant in mucosal (20%) and acral melanomas (20-30%). [119] However, due to overstimulation of lethal pathways, *MAPK* aberrations such as *BRAF* or *NRAS* do not coexist. The same applies to mutations in cell cycle control genes such as *CDKN2A* and *CDK4*. [117] But *MAPK* and cell cycle control mutations can co-exist. [108] Other mutations in the MAPK pathway are quite rare. [110] [120]

PTEN mutations have been detected in 5-20% of uncultured melanomas. [121] PTEN is a phosphatase that cleaves phosphate groups from tyrosine, serine and threonine. *PTEN* inhibits PI3 activity and has been shown to be a key driver gene in melanoma. [108,122] Combined *BRAF* and *PTEN* mutations have been shown in approximately forty percent of human melanomas. [123] A mouse model showed that *BRAFV600E* is responsible for benign melanocytic hyperplasia but a concurrent *PTEN* deletion is necessary for metastasis development. [123] Inhibitors of P13/AKT/PTEN are being investigated as of this moment. [120]

The *TP53* gene encodes the P53 protein. P53 has a major role in skin pigmentation and acts as a facilitator of the pigmentation signal in the keratinocyte and the regulation of skin pigmentation through keratinocyte-melanocyte signalling cycle. [124] *TP53* mutations can be found in 15-19% of melanomas. [110,125] P53 has anti- tumor activity which can be inactivated through *p53* mutations, interaction with inhibitors mouse double-minute 2 or 4 homolog (*Mdm2* or *Mdm4* respectively) and deletion/inhibition of activators *p14ARF* and *p16INK4a*. P53 dysregulation can result in overproliferation, ineffective apoptosis and cellular transformation. [126]

This list is not exhaustive but since somatic mutations are not the focus of this thesis, only a few of the important somatic mutations for melanoma risk have been discussed. Zhang et al. have reported 13 genes as significant driver genes in melanoma consisting of *BRAF, NRAS, TP53, NF1, CDKN2A, ARID2, PTEN, PPP6C, RAC1, IDH1, DDX3X, MAP2K1, and RB1*. [110] I would like to refer to this article for further reading.

1.2.2.b Inherited genetic factors and melanoma susceptibility

There is strong evidence that inherited genetic factors (e.g. germline mutations and single nucleotide polymorphisms (SNPs)) play a role in melanoma susceptibility. Germline mutations are genetic mutations present in germ cells (ovum or sperm) that are passed on to offspring. In general, they are prevalent in less than 1% of the population. [127] They can consist of many structural changes or quantity changes in the DNA and are consequences of mishaps in DNA replication or environmental exposure.

In contrast, SNPs are the most frequent type of inherited DNA changes and are due to single nucleotide replacements. SNPs are normal genome variation within a population whilst germline mutations are de novo alterations in the DNA that are also frequently inherited albeit rare at population level. [128] Changes in a single nucleotide may affect disease development, immune response and therapy response. These inherited variations can be characterized in the general population by their level of penetrance and frequency. [129] Gene penetrance in CM is a scale varying from relatively rare but highly penetrant alleles such as *CDKN2A* and *CDK4* to moderate risk genes (*MC1R*) and various lower risk but more frequent alleles e.g. *TYR, ASIP, TYRP1, OCA2, SLC45A2, VDR, XPC, PLA2G6* and numerous others. [129]

Familial melanoma, highly penetrant germline mutations and melanoma risk

An Australian twin study consisting of 12 000 cases reported a statistically significant difference in melanoma concordance between identical and non-identical twins. [130] Identical co-twins of melanoma patients were 10 times more likely to develop melanoma than by chance compared to twice more likely in non-identical co-twins. As previously mentioned, ~10% of melanoma cases have a positive family history. [64] These familial melanoma patients have a genetic predisposition to develop melanoma. Their CM risk is 30 to 70 times more compared to the general population. [69,131,132] Familial melanoma develop CM earlier and are prone to develop multiple melanomas. [133] The younger age of diagnosis can be explained by the genetic predisposition and/or the familial shared environmental exposure e.g. solar damage which can facilitate and accelerate oncogenesis. Alternatively, it can also be suggested that family members of CM patients are screened more often and earlier, potentially leading to earlier diagnosis. [13,66,134–136] Estimations of the attributable risk of genetic factors regarding cutaneous melanoma susceptibility varies from 21% [137] to 55%.[64] The heterogeneity of the aforementioned CM risk estimates

including CM risk in familial melanoma members may be explained by the differences in applied study methods as well as the selected populations e.g. patient characteristics and (the combination with/of) other risk factors).

Linkage analysis of family cases have identified highly penetrant mutations for melanoma susceptibility such as *CDKN2A*, which has been reported in 25 to 45 percent of the members of melanoma-prone families. [133,138] In families with more than 3 family members, germline *CDKN2A* mutations have been found in varying degrees averaging 40% with melanoma in different regions with values as high as 57% in Europe and as low as 20% in Australia with North America in the middle (45%).[139] CDKN2A mutation penetrance leading to melanoma development among the 50-80 age group varies between regions (i.e. 13-58% in Europe, 50-76% in the US and 30-91% in Australia). [140]

CDKN2A mutations are not only prevalent in familial melanoma but even more so in those with MPM, 22% and 57% respectively. [141] However, the CDKN2A mutation detection rate is lower if family history is not accounted for with rates as low as 1% for SPM and 3% for MPM. [142] Of note, CM risk is lower in those harboring CDKN2A mutation in non-familial melanoma cases but does accumulate with age with percentages ranging from 14% in 50-year olds to double the risk among 80-year olds implying less inheritance of additional melanoma risk genetic variants. *CDK4* is another high penetrant germline mutation prevalent in 2-7% of familial melanoma. [122] Further down the pathway is retinoblastoma 1 tumor suppressor (*RB1*) which is linked to an elevated melanoma risk and is prevalent in 5% of individuals with melanoma. [122] In melanoma families, high-penetrance mutations in ubiquitin carboxyl-terminal hydrolase BAP1 (*BAP1*) and the telomerase reverse transcriptase (*TERT*) pathway (e.g. *POT1, TERF21P, ACD, TERT* promoter) were demonstrated albeit a rare finding. [110] Other germline mutations in the *CDKN2A* pathway such as *TP53/14ARF* and *MDM2* are also quite few in familial melanomas. [110] Unlike sporadic melanoma, which will be discussed shortly hereafter, *TP53* is relatively rare in familial CM. It has been estimated that more than 50% of all familial melanomas have a genetic basis that are still undiscovered. [143]

All of these findings suggest risk factors in families i.e. genetic (e.g. high and/or lower penetrance germline mutations and/or inherited SNPs) or shared environmental factors that might contribute further to CM development. [133,134]

As of now, there is no consensus regarding the definition of hereditary melanoma with authors suggesting that at least 3 members of the same side of family should have a CM and/or pancreatic cancer diagnosis including the patient or one patient with at least 3 melanoma primaries which have been termed as the rule of 3's. [64] Another group proposed a '3 by 40' criterion whereby genetic risk is more probable if a patient younger than 40 years old had been diagnosed with at least 3 CM primaries. [133] These rules apply to CDKN2A testing. It is possible that these guidelines might not be appropriate for non-CDKN2A cancer syndromes involving melanoma. [134]

Familial cancer syndromes exist whereby an elevated occurrence of different tumors is observed in families including melanoma which has been named as mixed cancer syndromes. However, it has been proposed to use the term melanoma tumor syndromes instead to distinguish it from familial melanoma whereby CM is the main cancer type. [133] Indicators of hereditary melanoma syndrome consist of several family members diagnosed with CM, MPM in one patient, early age onset and presence of both pancreatic cancer and melanoma. [144]

Leachman et al. proposed to divide hereditary melanoma syndromes into two categories. [134] One type is the melanoma dominant type whereby melanoma is the main tumor type diagnosed with other cancers (e.g. renal cell carcinoma, uveal melanoma, mesothelioma, pancreas cancer and neurological tumors) having lower penetrance than melanoma. [134] Apart from well-established melanoma risk genes i.e. *CDKN2A, CDK4* mutations and breast cancer gene 1 associated protein 1 (*BAP1*), genetic defects have been detected in less established melanoma risk genes such as protection of telomeres 1 (*POT1*), telomerase reverse transcriptase (*TERT*), telomeric repeat-binding factor-2 interacting protein (*TERF2IP*), adrenocortical dysplasia (*ACD*) and microphthalmia-associated transcription factor (*MITF*) for this cancer syndrome type. Familial atypical multiple mole-melanoma (FAMMM), sometimes also known as familial melanoma and pancreatic cancer syndrome (FMPC) or familial atypical multiple mole melanoma-pancreatic carcinoma syndrome (FAMMPC), is an example of a melanoma dominant type. [134] It consists of several clinical attributes i.e. high nevi count, increased number of atypical nevi and positive family melanoma history. [134] Higher disease penetrance has been linked with age of onset of cancer in family members and skin phototype.[135] Interestingly, most melanomas in FAMMM individuals arise on normal skin instead of a (atypical) nevus. [145] After CM, pancreatic cancer is the most frequent cancer type in FAMMM patients with a confirmed *CDKN2A* mutation.[146]

The second type of hereditary melanoma syndrome is termed as melanoma subordinate syndrome whereby cancer forms other than melanoma such as prostate, colon and breast are typically more frequent although melanoma risk is still elevated albeit less so compared to other cancer types. Examples of the melanoma subordinate type include xeroderma pigmentosum (XP), breast and ovarian cancer syndrome, Li Fraumeni and Cowden syndrome which are associated with lower penetrance of melanoma genetic mutations in *XP, BRCA1, BRCA2, TP53* and *PTEN* genes respectively. However, these lists are not exhaustive and investigations are ongoing for other potential cancer syndromes and associated mutations.

Sporadic melanoma, SNPs and melanoma susceptibility

Sporadic melanoma, also known as non-familial melanoma, is defined as having no family history of melanoma or an inherited DNA change that increases their cancer risk for melanoma. [147] Approximately 90% of CMs are sporadic and only 10% familial. [129,148] Normal germline variants or SNPs may confer increased susceptibility to sporadic melanoma. SNPs are a type of genetic variation which are analysed with genome wide association studies (GWAS). SNPs are small common genetic variations due to a single nucleotide substitution of one base for another with minor allele frequencies (MAF) greater than 1-5% within a population. It is general knowledge that DNA bases consist of thymine and cytosine which are pyrimidine bases while adenine and guanine are purine bases. Furthermore, two hydrogen bonds bind adenine and thymine whilst three hydrogen bonds tie guanine with cytosine (see Appendix Fig.3). Although SNPs typically exert a low to moderate effect on melanoma risk, they are much more frequent in the general population with a prevalence of >1% unlike highly penetrant mutations and could therefore affect more individuals.[149] In addition, other risk factors such as UV exposure may influence the penetrance of melanoma risk SNPs. As a consequence, SNPs are considered to have an important value for risk prediction. For example, in sporadic melanoma, *CDKN2A* somatic mutations only contribute minimally to melanoma risk with a population attributable fraction ranging from 1% in Europe to 6.4% in Australia. [150] Furthermore, several SNPs in *CDKN2A* and *CCND1* genes have been identified by GWAS [151] implying SNP association with melanoma predisposition in the general population.[122] Interestingly, somatic mutations of pigmentation genes e.g. *TYR, TYRP1, DCT, SLC24A5, SLC45A2, and PMEL* are uncommon making up for only approximately 21% of melanomas. [152]

GWAS have reported 12 genetic regions that have reached a genome wide significant association with CM. Other loci had been identified through candidate gene studies. [151] Many of these CM risk loci have been demonstrated to be related to CM clinical risk factors such as pigmentation, phototype and cutaneous nevi development. The most important SNP germline variants with regard to melanoma susceptibility are discussed below.

SNPs and Melanoma Susceptibility

A meta-analysis of CM risk GWAS confirmed associations between inherited variants in pigmentation genes (*SLC45A2, TYR, MC1R and ASIP*), genes associated with nevus count (*CDKN2A/MTAP, PLA2G6 and TERT*), and genes potentially involved in DNA repair mechanisms (*PARP1* and *ATM*) and SNPs with unknown functions *(ARNT/SETDB1, CASP8, FTO and MX2)*. The study also identified 5 new loci which reached genome wide significance. The novel loci are likely to be involved in melanocyte regulatory pathways and in telomere biology. [153] Meanwhile, another meta-analysis had confirmed 20 GWAS identified melanoma risk SNPs in 10 loci *(TYR, AFG3L1P, CDK10, MYH7B, SLC45A2, MTAP, ATM, CLPTM1L, FTO, and CASP8)*. A further 4 other loci were found to be borderline significant *(MITF, CCND1, MX2, and PLA2G6)* and one new locus in chromosome 1q21 (upstream of *ARNT*) was discovered. [154] Interestingly, although some studies demonstrated evidence of increased melanoma risk of *BRAF* germline variants [155], the evidence remains weak for *MAPK/P13K* pathway involvement in melanoma susceptibility. [153]

One of the most studied loci for sporadic melanomas is the melanocortin 1 receptor gene *(MC1R)* located in chromosome 16q4 with more than 100 variants and a medium-penetrance locus for melanoma predisposition. These variants have been associated with melanoma risk factors such as red hair, light skin color and poor tanning ability as well as a higher risk for developing melanoma when inactivated. [156] Specifically, the variants D84E (rs1805006), R142H (rs11547464) , R151C (rs1805007), R160W (rs1805008), and D294H (rs1805009), confer a higher melanoma risk compared to other *MC1R* variants. [157] *MC1R* variants have been shown to abrogate cell proliferation and confer less resistance to cell death. *MC1R* genes control the production of skin pigmentation which consists of eumelanin (brown pigment) and pheomelanin (red-yellow pigment). Murine models showed that pheomelanin regulates cystine cell delivery in normal cell survival which confers protection against reactive oxygen species. [157]

GWAS has also suggested a melanoma risk locus on the agouti signaling protein *(ASIP)* locus in chromosome 20q11 which regulates hair color during hair growth cycle and acts as a *MC1R* antagonist. [158] Other loci related to pigmentation that have been identified by GWAS consist of *OCA2* (oculocutaneous albinism II)[153], *SLC45A2* (solute carrier family 45 member 2) [159] and *TYR* (tyrosinase) [152]. The function of other pigmentation related genetic variants not limited to SNPs, for example copy number variations and others, still need to be discovered and fall outside the scope of this thesis.

Genes associated with telomeres especially telomere length (e.g. *OBFC1, TERT-CLPTM1L, RTEL1 and TERC*) have been linked with melanoma susceptibility. [153,160] Genes that regulate telomere function such as *RTEL1* and *TERF2*, have been found to be correlated with dysplastic nevi, nevi count and melanoma risk in a Mediterranean population but was not validated in US population suggesting difference in genetic melanoma susceptibility. [161] Barrett et al. fine mapped most of the SNPs that had been identified by GWAS studies to be related to melanoma susceptibility and are depicted in the Appendix (Table 2). [151]

Melanoma susceptibility risk factors within the same ethnic group such as tanning ability, skin type and sunburn susceptibility have been linked with melanoma risk. [162] Notably, most SNPs studies have been conducted in Caucasian populations. Nevertheless, some SNP studies have been carried out in other ethnic groups. Pigmentation SNPs in other races had been correlated with fair skin phenotype and other clinical MM risk factors. For example, a Japanese study showed a correlation between *MC1R* SNPs and light skin tone and freckles.[163] A GWAS in Latin Americans for cutaneous and ocular pigmentation found 18 SNPs to be associated with 12 genomic regions including *GRM5/TYR* and *HERC/OCA2* genes.[164] However, the strongest link with skin tone was seen in a variant in *MFSD12* which is most prevalent not only in East Asians and Native Americans but also in Africans. The same SNP was linked with UV radiation intensity. Furthermore, associations between *MC1R* SNPs and melanocytic nevus phenotypes were observed in Han Chinese.[165] In a Spanish population, a SNP in the pigmentation gene *OCA2* (rs1800407) was found to be increased in CM and the association seems to be stronger in those with solar lentigines or more than 50 nevi. Also, variants in *S1666C* (rs2276288) in the *MYO7A* gene were found to be linked with fair skin and childhood sunburns in this South European population.[166] All of these illustrates the association of clinical CM risk factors and SNPs as well as the variety of SNPs involved in melanoma susceptibility in different populations.

1.3 CLINICAL AND HISTOPATHOLOGICAL PRESENTATION OF CUTANEOUS MELANOMA

Melanoma is a type of tumor that arises from melanocytes. The melanocyte originates from the pluripotent neural crest cell which separates itself from the neural tube and ectoderm in the early development stages. A part of these neural cells undergoes changes to become melanoblasts, further proliferating and differentiating as they migrate along the dorsal-lateral path beneath the ectoderm. Ultimately, they settle in the epidermis and into hair follicles where they transform into melanin producing melanocytes. Melanocytes can be found in different places of the body (e.g. on the skin, mucosae, inner ear, ocular structures of vertebrates, lymph nodes and meninges). [167] Cutaneous melanoma is the most frequently diagnosed type of melanoma. Clinically, a pigmented lesion becomes suspect for melanoma, when it acquires features according to the ABCDE mnemonic: A for asymmetry (shape or color), B for borders (irregular), C for color (more than 2 colors), D for diameter(>6mm), and E for evolution.[168] Some types of melanoma might not fit in the ABCD criteria e.g. nodular melanoma or amelanotic melanoma, therefore the E criteria was added for Evolution whereby growth and changing lesions should raise suspicion of malignancy. Additional signs that might help diagnosis include the presence of pain, itch, burning sensation, irregular surface, ulceration, bleeding, crust formations, inflammatory or depigmented halos. [169]

Haematoxylin and eosin staining (HE staining) remains the gold standard for diagnosing melanoma and experienced dermatopathologists make the diagnosis. Breslow (thickness in mm), ulceration status, mitotic rate[170], presence of microsatellites, and excision margins are also assessed and reported. [171] Growth phase, status of regression, tumor infiltrating lymphocytes (TIL), vascular or perineural expansion and lymphatic emboli may also be included in the histopathology report. Immunohistochemical (IC) staining is conducted frequently together with HE staining. S100 IC staining is the most sensitive marker for melanocytic lesions compared to other immunohistochemical markers such as HMB-45, MART-1/Melan-A, tyrosinase, and MITF. [172] Advanced techniques include genetic and genomic analysis such as fluorescence in situ hybridization (FISH) or comparative genomic hybridization (CGH). [173] [174]

Subtypes of melanoma

There are different subtypes of cutaneous melanomas with variable clinical and histopathological features. [175] The most common histological subtypes are superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo

maligna melanoma (LMM) and acral lentiginous melanoma (ALM) (see Appendix Table 4). Other infrequent subtypes consist of Spitz(oid) melanoma, nevoid melanoma, desmoplastic melanoma, amelanotic melanoma and melanoma arising in congenital or blue nevus but this list is not complete. [176]

SSM is the most frequent cutaneous melanoma subtype in Caucasians accounting for ~70% of melanomas. [177] SSM has been associated with nevi count [178] and at least thirty percent arises from an existing nevi. [40–42] They typically present on the legs in females and the trunk in males. NM is the second most frequent type after SSM and accounts for 15-30% of all melanomas. [179] This subtype usually presents in men and can be often found on the trunk. It is not the typical ABCD melanoma lesion and is frequently missed leading to poor prognosis. [180] LMM is the third most common type and accounts for 10-15% of the cases and is usually seen in the elderly. [179] It is associated with chronic cumulative sun damage and typically develops slowly in chronically sun exposed areas e.g. the face, head etc. ALM is the next most frequent subtype and is responsible for 2-3% of melanoma cases worldwide [179] but accounts for 70% of melanomas in darker skin types. It usually develops on typically non-sun exposed sites such as palms, soles, wrists, heels and nail beds. [181] This subtype is often diagnosed at a late stage with associated worse prognosis.

A genomic classification of CM has been proposed consisting of different molecular subtypes. [122] It should be noted however that histopathologic subtypes as well as genomic subtypes have not been implemented yet in the AJCC staging criteria for melanoma. A number of molecular subtypes of melanoma have been reported and is currently an intense topic of research. The three clinically relative molecular subtypes are *BRAF, NRAS* and *C-KIT* mutations which will be discussed further in the Genetics section, specifically in the subchapter Somatic Mutations and Melanoma Outcome. For a more comprehensive overview of histological and melanoma molecular subtypes, an article by Garbe et al. is highly recommended. [176]

Body sites and melanoma

As for site, the most common site for melanoma are the backs and shoulders in men and legs in women. The intermittent UV exposure theory has been suggested as an explanation whereby intermittent, intense sun exposures might potentially elevate melanoma risk in low solar exposure body sites. [182] In contrast, locations with high solar exposure such as the head and neck has been shown to be the most common site for melanoma in both

genders in Australia. [183] One study calculated the melanoma per unit area of skin which considered the surface areas of the body sites. The face was reported to be the predominant site followed by shoulders and backs for both genders. [183] The gluteal region was the least common site of melanoma. [184] Some studies have found that trunk melanomas usually occur in the fifth to sixth decades of life whilst the head and neck are more frequent in later age. [185–188]

1.4 CUTANEOUS MELANOMA STAGING

In 1969, Dr. Clark and Dr. Breslow laid out the foundations for the current prognostic models for melanoma which is being implemented in clinical practice as recommended by the American Joint Committee on Cancer (AJCC) criteria. [189,190] The criteria consist of stage 0 to IV which is based on the prognosis. Stage 0 is defined as in situ melanoma where tumor cells are limited in the epidermis, stages I and II are localized invasive disease whilst stage III consist of regional metastasis to the nodes and stage IV is distant metastatic disease. Stages I and II have relatively good prognosis whilst stages 3 and 4 have graver outcomes. [171] Tumor characteristics, regional node metastasis and distant metastasis status form the TNM classification from which the staging is derived. [171] Approximately 4% of new melanoma cases has distant metastasis at initial diagnosis. [191] One third of those diagnosed in earlier stage will either recur or metastasize. [192] A distribution of the different stages in 2016 by the Belgian Cancer Registry is depicted in the Appendix (Figure 4).

The 7th AJCC prognostic algorithms produced the following 5 year survival rates: >90 (stage I), 53-81% (stage II), 40-78%(stage III), 6-25% (stage IV). [170] The 8th and latest version of AJCC staging criteria was introduced in 2017 with the following 5 year survival rates: stage I: 98% (range 96-99%), stage II: 90%(range 82-93%), stage III: 77%(range 52-84%) and stage IV remained the same as current data for this group was limited and prior to the introduction of novel treatments for advanced melanoma, outcome differences within this stage was minimal. [171] A recent review found that the 5 year stage specific overall survival varied in 9 countries in Europe: 95%-100% (stage I thinner than 1 mm), 65%–92.8% (stage II), 41%–71% (stage III), and 9%–28% (stage IV). [193] Hence, large ranges and variability are not sensitive enough for personalised prognosis. Although based on a huge number of patient data, the melanoma prognostic model as has been suggested by the AJCC was limited according to some authors because prognostic markers were identified by using archival tumor tissues and samples from single institutions.

In addition, validation through prospective studies is lacking in most cases. [194–196] This calls for other factors that can potentially aid in a more sensitive prognostic stratification.

As stated earlier, melanoma incidence is rising and is being diagnosed in an early stage whereby the absolute number of patients in early stage who progresses is increasing. [197] Notably, the prognosis of approximately twenty percent of melanoma patients can't be accurately predicted by the AJCC staging criteria as reflected by relatively wide ranges of survival. [198]

The Belgian Cancer Registry conducted a study on disability-adjusted life year (DALY) related to melanoma stratified by stage. The burden of melanoma mortality in years of life lost (YLLs) were higher in stages 1-3 (28%, 33%, 26.2% repectively) compared to stage IV (13%). The proportion of contribution to years lived with disability (YLDs) is highest in node positive cases (~50%), followed by localised lesions (35%) with distant metastases as least (13%). [199] In the past decade, deaths due to metastatic melanoma has reached approximately 48,000 per year with a 5-year survival rate of less than 15%. [148] Understanding the etiology of CM and potential risk factors contributing to CM survival is therefore a pressing matter.

1.5 TREATMENT OF CUTANEOUS MELANOMA

Surgical treatment is necessary for the diagnosis, staging and treatment of melanoma. In a systematic review of the current management of early stage melanoma, surgical excision remains the standard, whereby the margins are based on the Breslow depth, minimizing local recurrence and SNB for nodal staging and treatment guidance. [200] When a pigmented lesion is suspected to be malignant, an excisional biopsy is always performed with a margin of 2mm. [201] Wide and deep local excision (up to the muscle fascia level) of the biopsy proven primary cutaneous melanoma is the recommended treatment with consideration of margins based on the Breslow depth. Sentinel node biopsy (SNB) is indicated in melanomas with a stage at least T1b for treatment guidance. [201] During our study period, when clinically occult stage 3 disease was detected by performing SNB, complete lymph node dissection (CLND) was considered depending on the tumor load which could yield 20% extra non sentinel lymph nodes (SLN) if SNB is positive. [202] However, there is lack of hard evidence for overall survival benefit of SNB staging. Two recent RCTs have demonstrated that CLND in SNB positive patients is not associated with better survival which had led to CLND not being routinely offered to this group of patients. [203] However, a therapeutic lymph node dissection can

still be considered in those with macroscopic nodal disease and in some cases with distant melanoma metastases. [203]

Higher melanoma stages necessitate other treatment modalities. Stages IIIB and IIIC were usually treated with surgery as well as adjuvant therapy. [204–206] Surgery can be considered either in combination with systemic therapy such as targeted therapy or immunotherapy for debulking purposes if the metastasis is limited. [207] Stage III melanomas with in transit metastasis are excised if possible otherwise isolated limb infusion or perfusion are considered. [201] For macroscopically invaded lymph nodes, CLND is the treatment standard. Stage III cases are eligible for adjuvant therapy with anti-PD1 or BRAF/mitogen-activated protein kinase (MEK) inhibitors. Systemic treatment is the standard of care for stage IV or unresectable stage IIIc disease. Interferon-gamma-2b and IL-2, were the first immunotherapies used as adjuvants for advanced melanoma. However, the survival benefits have been modest and was effective only in a subset of patients. [208] For a while, vaccination against melanoma specific antigens Mart1 and gp100, which stimulates T-cell responses, and adoptive T-cell immunotherapy were popular whereby response rates as high as 49% have been reported. [209] In the past, immunotherapies had the reputation of giving small clinical advantage coupled with major toxicities in advanced melanoma. Radiotherapy used to be given in stage IV melanoma for unresectable metastases, recurrence and in the last stage as palliative therapy. In-transit metastases and satellites can be treated with different modalities including excision, isolated limb perfusion, intra-lesional oncolytic virus therapy (not available in Belgium), CO2 laser and electrochemotherapy. As for unresectable metastatic melanoma, the earlier golden standard of chemotherapy has been replaced by targeted therapies (e.g. BRAF inhibitors usually combined with MEK inhibitors since recently) and immunotherapies. [210,211] In the follow-up period, melanoma patients undergo regular monitoring of the CM site and lymph nodes at each visit through clinical examination and ultrasound. Potential distant metastasis are checked through blood tests (including LDH), chest x-rays, CT-scans and PET-CTs when required depending on the initial disease stage and symptoms. [201] As for adjuvant therapy, interferon-alpha 2b is advised for ulcerated CMs in settings that lack access to novel adjuvant drug therapies. [212] If adjuvant therapies are available, then the choice is dependent on the *BRAF* mutation status. Anti-programmed death-1 (PD1) antibody is preferred in *BRAF* mutation negative patients whereas dabrafenib and trametinib combination is recommended if *BRAF* mutation is present. [212]

The Belgian Association of Dermato-Oncology (BADO) recommendations regarding stage IV melanoma are more or less aligned with the aforementioned albeit with some additional comments. [201] In some *BRAF* negative cases, first line therapy might also include either a combination of anti-PD1 and ipilimumab or surgery of gamma knife in limited metastases depending on the location. As for second line intervention in this group, ipilimumab can be considered if the disease is progressive despite anti-PD1. Otherwise, either chemotherapy or imatinib can be an option depending on whether *C-KIT* mutations are present, not only as second-line but also as third-line management. In *BRAF* positive patients, BADO recommends BRAF/MEK-inhibitors combination or the same first line management as the *BRAF* negative group. For second line therapy, anti-PD1 (with or without ipilimumab) can be considered should BRAF/MEK inhibitors be inefficient. Alternatively, BRAF/MEK inhibitors can be an option should the disease progress under immunotherapy. More detailed information on melanoma management can be found on the BADO website. [201] Of note, the melanoma protocol mentioned above was the version that was being implemented during the study period of this thesis. Nevertheless, melanoma protocols are constantly changing. The latest update on melanoma management was published by Garbe et al. in 2020 and is highly recommended to those who would like to read more on this subject. [176]

Novel Therapies of Stage IV Melanoma

Novel immunotherapies consist of immune checkpoint blockades. The first of its kind were cytotoxic T lymphocyte associated antigen 4 (CTLA4) inhibitors (e.g. ipilimumab and tremelimumab). Later, antibodies against programmed death 1 cell receptor (anti-PD1) (e.g. nivolumab and pembrolizumab) and its ligand (anti-PD-L1) (e.g. durvalumab, atezolizumab, avelumab and others) were discovered. Targeted therapies such as BRAF inhibitors or MEK inhibitors are useful in patients with *BRAFV600* mutations in stage IV melanomas. MEK inhibitors (eg binimetinib) seem to induce some response in patients with *NRAS* mutations. [213] KIT inhibitors have been tested on ALM and mucosal MM given their association with *KIT* mutations rather than *BRAF* mutations and 23% were shown to respond with imatinib.[214] The average 1-year survival results in stage IV melanoma were promising: 75% with BRAF/MEK inhibitors and 72% for anti-PD-1 therapy with or without anti-CTLA-4. [215] Ipilimumab, a CTLA-4 inhibitor, was shown by two RCTs to increase overall survival and has been approved for clinical use in the Western world.[216,217] Although durable remissions were seen, this was only the case in a small group of patients. Nivolumab has a high response rate of 31% in advanced melanoma patients and 26 to 51% with pembrolizumab.

[218] Immunotherapy is being more actively utilised in early adjuvant setting. A meta-analysis showed that PD-1/PD-L1 inhibitors confer a better OS as a first-line therapy compared to later treatments. [219] Anti-PD1 treatments also have a lower toxicity profile compared to chemotherapy or CTLA-4 inhibitors. Conversely, recent studies demonstrated higher response with combined CTLA-4 and PD-1 blockade albeit with more toxic side-effects. [220]

With the arrival of immunotherapies and targeted therapies, the survival of melanoma patients has much improved compared to the relatively poor response with chemotherapy. Nevertheless, a maximum of 51% of advanced melanoma patients respond to the latest systemic therapies. In addition, resistance to BRAF inhibitors and PD1 antibodies has been reported. Other disadvantages of the therapies mentioned are adverse events that can affect the quality of everyday living but the quality of life is hardly studied. [221] [222] The most common immune related adverse events (irAEs) consist of dermatologic (40%) and gastrointestinal (9-17%) toxicities usually occurring within the first weeks of therapy whereby the less frequent side effects such as pulmonary (2-13%) or endocrine (5-10%) side effects tend to present later.[223] In addition, more severe toxicities such as cardiotoxicity due to PD1 and PD-L1 expression in human cardiomyocytes might have been underestimated. [224] Another major disadvantage of the novel therapies are the high costs involved.

Clinical and pathologic data are most readily available and has therefore been used in building prognostic models. [198] Before the advent of targeted and immunotherapies, the median survival of advanced stage melanoma was 6-12 months. [221] With the advent of new therapies, survival has improved after its introduction. As mentioned earlier, survival rates are highest in early stages whereas stage IV has 6-25% rates of survival. [170] Hence, detection of risk factors for melanoma development and progression are of diagnostic and therapeutical importance.

1.6 PROGNOSTIC FACTORS FOR CUTANEOUS MELANOMA

Melanoma survival was shown to be associated with clinical risk factors such as age, gender, phototype, ethnicity, sun exposure and geography. Other factors include nevi count, family history, and personal history of melanoma. However, only age, anatomic site and gender were demonstrated to be independent clinical risk factors by the AJCC. [170,225] Independent histopathological predictive factors will be discussed later in this chapter.

1.6.1 Clinical Risk Factors And Cutaneous Melanoma Outcome

Age and gender

Age and gender have been shown to influence melanoma outcome. Published literature have demonstrated an association between older age and increased melanoma mortality. [226–229] Older patients tend to present less with a sentinel node metastasis but the prognostic value of SLN is positively associated with increasing age. Moreover, age can affect treatment response in stage IV melanoma and therefore outcome. Discrepancies in age had been signalled by Zeng et al. in the period 1990 to 2010 with regard to survival of the most common cancers. Survival seem to have improved in younger patients probably due to novel therapies. [230] Some authors posit that variances in tumor biology, immune system and host characteristics might underlie the survival differences between age groups.[227,229] Furthermore, it has also been proposed that age stratification of the AJCC survival rates might lead to a more precise melanoma survival prediction.

Across different populations, men have been shown to fare worse than their female counterparts. [2,231] In a pooled analysis of RCTs, gender was shown to be an independent risk factor for outcome in advanced melanoma. [232]The same trend has been reported in Belgium (see Appendix Table 3). The latest 5-year relative survival in Belgium in the period 2012-2016 is 91.2% for males and 95% for females (see Fig. 2 in Appendix).[5]

Notably, primary melanomas in males often have worse clinical and histological characteristics such as deeper Breslow, ulceration and located in head/neck/trunk sites. Better female survival is highest in early stage melanomas but the difference in survival with men diminishes in advanced stages. [233] Males and individuals older than 65 at the time of diagnosis do worse compared to females and younger people, respectively. [5] In addition, a German study reported a better outcome for females attributable to more locoregional metastasis and a higher metastatic rate in men. [25]

As for sex disparities specifically in relation to melanoma outcomes, a review by Nosrati et al. gave the following possible explanations. [234] Genders may differ in their behaviour regarding UV exposure and the use of health care services. Other processes that have been suggested are biological processes e.g. hormonal regulation, immune system, vitamin D metabolism, oxidative stress response and sex chromosome gene expression e.g. non-random X chromosome inactivation and oncogenes on the Y chromosome. Moreover, sex discrepancies seem to affect immunotherapy response. Ye et al. observed that 6 out of 7 clinical immunotherapy trials revealed a better overall

survival (OS) in male melanoma patients. [235] Their meta-analysis of molecular biomarkers in the immunotherapy trials showed a significantly elevated tumor mutation burden, neoantigen load and PD-L1 in the same demographic. Discrepancies in immunotherapy response were also seen in other tumors such as colorectal cancer, bladder cancer and glioblastoma multiforme to name a few. Male biased immune features were observed in kidney renal papillary cell carcinoma including tumor mutation burden, increased immune cells and mRNA expression of immune checkpoints. [235] Female-biased immune features such as stimulatory as well as inhibitory immune checkpoints were seen in other cancers such as lung squamous cell carcinoma. [235]

Geography and ethnicity

Melanoma death rates has been increasing worldwide and are highest in Oceania and in low latitude areas of Europe and America which are regions with predominantly Caucasian populations. [226] Racial disparities regarding melanoma outcome have been reported. An American study demonstrated the best survival in Whites and the worst in Blacks with other ethnicities in the intermediate risk (i.e. Hispanic/Asian American/Native American/Pacific Islander). [236] Interestingly, melanoma specific mortality (MSS) is lower in non-Hispanic African Americans compared to non-Hispanic Caucasians but the reverse is true after adjustment which has been postulated however to be partly caused by social inequality. [237]

A large US cohort study conducted before novel immunotherapies were introduced, also observed statistically significant differences in prognosis by ethnicity in the univariate analysis which disappeared in the multivariate analysis. [238] Notably, Caucasians in high ambient solar environments such as South Africa had been found to be associated with higher fatal melanoma risk. Compared to other non-white groups of the population, melanoma mortality rates adjusted for age are not only the highest in white South Africans but have also statistically significantly increased in this group in the period 1999-2014.[239] Interestingly, among Caucasians in Australia, the crude mortality rate (CMR) rose by 4% annually between 1981 and 2011. Moreover, melanoma CMR have risen by at least twofold amongst Whites compared to 77% for the whole (ethnically diverse) Australian population. [37]

UVR exposure and damage

Some published literature suggest a favorable link between sun exposure and sunny holidays with melanoma survival [240,241] and an opposite correlation between mean yearly environmental sunlight in different countries and melanoma deaths. [242] Clinical signs of chronic solar damage have been suggested to be associated with melanoma

outcome although published literature is scarce. Two studies have reported an inverse association between elastosis, a sign of chronic photodamage, and melanoma survival. [243,244] One study group however showed contradictory results and first demonstrated an inverse association between solar elastosis and melanoma related death (MRD) after correction for other prognostic factors but a later analysis was unable to confirm their results. [245,246]

Nevi count

Risk factors associated with melanoma development have been scarcely studied with regard to their association with melanoma survival. Nevertheless, a UK study showed high nevus counts to be linked with an increase in 5 and 10 year survival rate. [247] They postulated that biological factors that are associated with large nevi numbers might explain their findings and can be a potential therapeutic target. In contrast, Taylor et al. and Berwick et al. did not find a significant correlation between a high nevus count and melanoma mortality. [44,245]

Family history

Family history is an established risk factor for melanoma risk and has been associated with genetic mutations, both somatic and germline, predominantly involving the *CDKN2A* gene. [248] Published literature, albeit scarce, have indicated *CDKN2A* variants to be not only linked with melanoma development but is also correlated with melanoma outcome. [249]

Multiple primary melanomas

Multiple primary melanomas (MPM) has been shown to be an important risk factor for melanoma development. Its association with melanoma outcome is hardly studied. An Australian cohort study reported MPM to be at increased risk, approximately threefold higher, for melanoma specific death compared to single primary melanoma (SPM), independent of age, sex and Breslow depth, when the last invasive melanoma is taken as index melanoma. [61] Moreover, subsequent melanomas have generally thinner Breslows than the first primary melanomas probably attributable to regular check-ups after diagnosis or increased awareness post diagnosis by patients. [59]

Fair phenotype

Low phototype, usually described as Fitzpatrick skin type 1 and/or 2, has been previously shown to be associated with melanoma susceptibility but few studies have assessed the link between phototype and melanoma survival. A large population based study however did not find any correlation between phototype and melanoma prognosis. [44]

1.6.2 Histopathological Prognostic Factors For Melanoma Outcome

The current guidelines regarding histopathological reporting of melanoma tissues include many features. However, only a few have been considered in the AJCC staging criteria. According to the 7th AJCC version, independent histopathological risk factors for outcome consist of Breslow depth, mitosis, ulceration, sentinel node status, regional macro metastatic disease including the number of positive nodes [250] and distant metastatic disease. [170] Most of the aforementioned prognostic factors were also considered in the 8th AJCC version and are discussed hereafter. A multivariate analysis comprising of 7568 patients with T1NO showed that deeper Breslow thickness conferred a worse melanoma specific survival. [171] Furthermore, ulcerated melanomas have a threefold higher hazard rate melanoma specific survival versus non-ulcerated lesions. Although mitotic rate was shown in some studies to be associated with worse outcome, it was statistically non-significant in the same multivariate analysis. Less than 5% of those with lesions <0.8 mm depth had a positive sentinel node compared to 5-12% in those with Breslow between 0.8-1 mm. A prospective cohort study of approximately 27 000 patients with melanoma thickness up to 1 mm showed a Breslow depth >0.75 versus <0.25 mm to be linked with a fourfold higher HR and a threefold increased HR for age at diagnosis (>65 vs <25). [251] Of note, the risk of undetected lymph node metastasis is <5% in those with <0.75 mm thick melanomas and >50% in >4 mm ulcerated melanomas.

Mitotic rate in the primary tumor has been previously reported to be important for melanoma staging and was part of the 7th AJCC staging system. [252] However, the 8th AJCC version made several adjustments within the stages which included removing mitosis in the staging system. Of note, the committee still strongly recommends reporting mitosis due to the expectation that this will be re-implemented again in future staging recommendations. [171] Although definitions of ulceration vary, AJCC has defined ulceration as “the absence of an intact epidermis overlying a major portion of the primary melanoma based on microscopic examination of the histologic sections”. [253] The AJCC melanoma staging criteria has recognised ulceration as an independent prognostic factor which influences

the tumour (T)-stage. Melanomas with ulceration confer a shorter relapse free survival (RFS) and worse overall survival (OS) compared with non-ulcerated lesions. [170,252] Moreover, ulceration was shown to be linked with better survival and treatment response to immunotherapy versus observation only as management. [204] Clark level was reported to be a prognostic risk factor for localised melanoma and used to be part of the melanoma staging criteria. [170] However, it has been decided not to include Clark level in the newer staging versions. [171] Interestingly, tumor infiltrating lymphocytes (TILs) were linked with better survival in melanoma patients. [254–256] However, TIL has not been incorporated yet in the AJCC staging criteria.

The advantages of the 8th version vs 7th AJCC version are the higher reproducibility and concordance with consensus reference diagnosis although these two metrics remain relatively low. [257] Of note, our study was conducted using the 7th AJCC version as the 8th version was only recently implemented.

Site and subtype

Melanoma site is sixth in ranking as prognostic factor according to the AJCC 2008 for stage 1 and 2 melanoma. [258] Published literature often divides melanoma locations into two groups, axial (including melanomas on trunk, head and neck) and extremities (legs, arms). Axial melanoma has a shorter survival compared to locations in extremities. When further broken down, there seem to be no difference in MSS between axial melanoma located on the face, ears or trunk and extremities. [170,225] However, a poorer survival was observed for scalp and neck compared to extremities. [238] Scalp/neck melanomas were mainly observed in men and have statistically significant worse clinical and histopathological features than aforementioned sites, specifically their association with older age, deeper Breslow, presence of ulceration, positive lymph nodes and a predilection for a nodular or lentigo maligna melanoma subtype. The mortality rate for melanoma is almost twofold higher for scalp/neck melanoma and approximately 30% higher for trunk melanomas compared with those located on extremities. Strikingly, there was no statistically significant difference regarding the mortality rates between face/ear melanomas and extremities which are body sites prone to sun exposure. [238]

A large population based study reported a worse 5-year prognosis for NM and ALM compared to other subtypes, with ALM having the worst prognosis. [238] Moreover, NM is correlated with increased relapse risk in patients with negative sentinel node biopsies. [259] In a group of patients with NM, SLN status and presence of ulceration were

shown to have a significant association with RFS and OS. [260] Other population cohorts have confirmed these positive associations. An American cohort reported SSM and NM as the two major contributors to fatal melanomas in a time period before the introduction of novel immunotherapies. [261] A recent Norwegian study also reported nodular melanoma as the predominant subtype associated with a fatal outcome. [262] Explanations for these findings have been attributed to suboptimal public awareness of nodular [180] and ALM features and subsequent late presentation to medical professionals. [263]

1.6.3 Immune System And Melanoma Outcome

Most information on the effect of immune system on melanoma outcome comes from studies comparing the normal population with the iatrogenic immunosuppressed population, of which organ transplant recipients (OTR) have been most extensively studied. Studies in the past on OTRs with melanoma histories and survival outcome were conflicting. The earliest study reported a worse survival in OTR patients with a (pre-transplant) melanoma history. [264] However, this study had been criticised for their small patient numbers, incomplete data on Breslow score and lack of control group. [265] Later studies did not show any difference in survival with regard to OTR patients, who developed melanoma before transplantation and the general population but a potential selection bias might exist for transplantation of patients with thinner Breslows and hence a more advantageous survival outcome. [103,266,267]

Melanoma-specific deaths are increased by at least 3 times among transplant patients, who developed melanoma after transplantation (OTR with de novo melanoma), compared to non-transplants. Melanomas were found to be more lethal in the transplant population which can probably be partially attributed to azathioprine induced sun sensitivity or promotion of advanced melanomas by T cell depleting induction therapies which underlines the immunogenic nature of melanoma. [100]

According to a recent American study which utilised a large transplant-cancer registry database consisting of 140000 patients found that mortality due to skin cancer was highest for malignant melanoma, approximately 12 times higher, compared to non-melanoma skin cancers in OTRs. Furthermore, patients who are more immunosuppressed (e.g. older patients and thoracic transplant recipients) had elevated skin tumor mortality. [268] Studies conducted by the SCOPE group demonstrated a worse overall and melanoma specific outcome in OTRs than the immunocompetent group especially in higher Breslows. [103,267] Reduction of immunosuppression did not seem to affect mortality.

1.6.4 Genetics And Melanoma Outcome

Several somatic and germline mutations have been identified to be associated with melanoma survival. A non-exhaustive list of the most important genetic mutations is reviewed here.

Somatic mutations and melanoma outcome

As has been mentioned earlier in the Subtypes of Melanoma section, three molecular subtypes based on somatic mutations have clinical impact in melanoma disease i.e. *BRAF*, *NRAS and C-KIT. BRAF* and *NRAS* somatic mutations have been studied intensely in the past years. Approximately 74% of *BRAF* mutations were of the V600E type and 20% of the V600K type. [269] Comprehensive genetic analyses of melanoma tissue samples have shown *BRAF* and *NRAS* somatic mutations to be correlated with melanoma survival in higher tumor stages after correction for other risk factors for prognosis. [270] However, results between studies on this particular subject are still conflicting. Some studies did not show any link between *NRAS* and *BRAF* mutations and overall survival in a group with advanced melanoma [271] and in a clinical trial. [272] In contrast, Bucheit et al. found that *BRAF-V600E* mutations give a worse overall survival outcome versus *BRAF-V600K* mutations in advanced melanoma patients. [273] Barbour et al. found an increased twofold risk for recurrence and a shorter disease specific survival (DSS) in *BRAF* mutant patients compared to *BRAF* wildtype patients in surgically treated stage 3 melanoma unlike *NRAS* mutations. [250] On the other hand, *NRAS* mutations versus *BRAF-V600E* were shown to be associated with shorter melanoma specific survival in some studies [274] [115] and with better survival in others. [275]

Conversely, patients with *BRAF* mutated melanoma were statistically significantly younger than their *BRAF* wildtype counterparts not only in the metastatic melanoma group but in all stages. [269,270,276] *BRAF* mutation was also associated with truncal melanoma site, higher mitotic activity [269,270,277], melanoma sites of intermittent solar exposure [270,276] , deeper Breslow [277] and SSM subtype. [270,276] Pracht et al. confirmed the associations mentioned earlier and *BRAF* correlation with lymphatic metastasis at primary diagnosis. [276] However, relapse-free period were found to be similar between *BRAF* mutant versus *BRAF* wildtype patients. [269] Furthermore, a positive *BRAF* mutation status is statistically significant predictor of BRAF inhibition therapy response versus wildtype. [276] Of note, melanomas harboring *NRAS* mutations are mainly observed on chronic sun damage sites. [277] *NRAS* positive melanomas showed correlation with mitotic activity, decreased tumor infiltrating lymphocyte activity and preference

for trunk/pelvis site. [270] Correlations between *NRAS* gene alterations and nodular melanoma had been reported as well. [276]

C-KIT mutations compromise 1 to 3 % of melanoma cases. Typical CM locations for this molecular subtype consist of acral and chronically sun damaged (CSD) body sites. Only when *BRAF* and *NRAS* genetic tests are negative in acral or CSD melanomas would a *C-KIT* test be considered due to its low frequency. [176] KIT is a transmembrane receptor tyrosine kinase which regulates cell development, differentiation and growth. C-KIT inhibitors such as dasatinib, nilotinib, sunitib and imatinib were demonstrated to be beneficial to patients harboring *C-KIT* somatic mutations especially those with *L576P* and *K642E* mutations. [278]

Other somatic mutations in melanoma, which are technically molecular subtypes, have been reported but lack therapeutic implications for now. Some of these subtypes are discussed below.

CDKN2A, an established tumor suppressor gene in melanoma, is another one of the major players in melanoma somatic mutation. [279] *CDKN2A* mutations frequently dysregulate *p16INK4A* function and usually do not specifically target *p14ARF*. [280] A high level of p16 CDKN2A protein expression has been associated to be statistically significant with favorable overall survival in melanoma patients which is probably due to the mitigation of tumor proliferation by *p16 CDKN2A*. [194,281] Aligned to this, deletions in *p16 CDKN2A* gene and dysregulation of its protein expression was shown to stimulate melanoma progression. [194,281] In addition, the increased expression of *p16 CDKN2A* was also demonstrated to be linked with longer melanoma specific survival whilst an impaired expression was shown to be linked with worse overall survival not only in melanoma [194] but also in other cancer types. [282] Of note, the CDKN2A pathway, which acts in melanoma through suppression of *p16INK4A* expression, can also be affected through many processes such as germline mutations including polymorphisms, somatic mutations and epigenetic routes. [109]

Neurofibromatosis 1 (*NF1*) mutations ranks third as most commonly observed MAPK pathway mutation. A prevalence of 14% was observed in one study. [122] *NF1* inhibits RAS function through its intrinsic signaling pathway. *NF1* deletion is another track to stimulate the MAPK pathway. [122] *NF1* had been reported to be significantly associated with older onset compared to *BRAF* mutant patients. With regard to overall survival, no significant difference was observed between patients that harbour *NF1, BRAF, RAS* and those that lack these three mutations.

In melanoma, *NF1* mutation is often present together with *RAS* and *BRAF* mutations. [283] *NF1* mutation has been shown to be *MEK* dependent and to confer resistance to BRAF inhibitors but not MEK inhibitors. [283]

Unlike familial melanoma, *TP53* is one of the most frequent mutations identified in sporadic CMM. [125] The p53 (*TP53*) tumor suppressor gene plays a pivotal role in cellular damage via induction of cell cycle arrest and cell death. Mutations in *p53* gene has been found in less than 10% of melanoma cases. [284] Alterations in *TP53* were linked with clinical characteristics such as older onset and a predilection for head and neck location. Among metastatic disease, a favorable overall survival was observed for tumors with *TP53* mutation after adjusting for other prognostic factors. [125]

Somatic mutations in the telomere maintenance pathway have been implicated in melanoma progression with somatic mutations in the *TERT* gene as most prevalent. [285,286] *TERT* promoter mutations were reported to occur in primary melanomas and more often in metastases whilst no *TERT* mutations were seen in benign nevi implying the role of *TERT* mutations in later tumor stages of progression. [285] These results were somewhat contradicted by Shain et al. in that increased *TERT* mutations frequency were also seen in localised and intermediate melanoma lesions and a low frequency in benign nevi indicating that *TERT* mutations are involved in early melanoma development. [287] Interestingly, co-occurrence has been reported between *TERT* gene alterations and *BRAF* mutations more often than expected by chance suggesting cooperation between the genes. [285]

Zhao et al. recently confirmed that somatic mutations mentioned earlier as well as in other genes (*BRAF, NRAS, NF1, TP53, PTEN, PPP6C, CTNNB1, PDGFRA, IDH1, and KIT*) are implicated in melanoma. [288] Approximately 22 genes were proposed to have potential correlation with melanoma prognosis. Only less than 10 % of the specimens harbour any one of the 22 genes. Moreover, only 9 of these genes were confirmed in RNA sequencing. Interestingly, individuals with *SPEN* gene mutations and high *SPEN* expression levels fare better than those without this mutation and low *SPEN* expression levels but only the high *SPEN* expression levels reached statistical significance in a Cox analysis. Also, *CIITA* mutations are more common in melanoma tissue specimens of individuals with favourable outcome. In contrast, *RAC1, FGFR1, AFF3,* and *NTRK1* gene alterations were more common in tissue samples of melanoma patients with worse 5-year survival. For more details, please see the article by Zhao et al. [288]

Germline variants and melanoma outcome

Germline genetic variants had been associated with survival which makes them potential biomarkers for disease outcomes. SNPs related to melanoma risk and/or host phenotypes that are linked with melanoma survival consist of variants located in *PARP1* [153], *MC1R, ASIP* [157], *CDKN2A* [289], *VDBP* [290],*RXRA* [290] , *VDR* [291]and *EGF* [292] genes. A non-exhaustive overview of the major SNP players in melanoma outcome are presented below.

MC1R variants have been reported to be linked with melanoma risk. Absence of *MC1R* consensus alleles were shown by Taylor et al. to be associated with 40% better melanoma specific survival compared to carriers of at least one risk variant allele. [157] However, there was no association with overall survival. The *MC1R* gene plays a major role in skin pigmentation but the function of most *MC1R* variants are yet to be discovered. Red-hair SNPs 'R' and 'r' which are located in the *MC1R* gene have been demonstrated to be linked with better overall survival. [293] Other presumed functions of the *MC1R* gene comprise of cell apoptosis, DNA repair and invasion through MITF transcription factor. [294,295] Taylor et al also reported an elevated risk for melanoma specific mortality and overall survival for Agouti signalling protein gene, *ASIP**TG versus GG genotype. [157]

CDKN2A variants (rs2811710/rs2518720/rs2811708*TT) were reported by Casula et al. to be associated with increased melanoma predisposition and deeper Breslow. [289] Helgadottir et al. reported a statistically significantly worse survival for melanoma in *CDKN2A* mutation carriers compared with *CDKN2A* wildtype patients within a familial melanoma population after correcting for other prognostic factors. Moreover, *CDKN2A* mutated familial melanoma patients fare worse compared with matched sporadic melanoma cases. However, melanoma outcome were not statistically significantly different between *CDKN2A* wildtype melanoma patients and sporadic melanoma cases. [249] Another study reported that within a MPM population, patients that harbour *CDKN2A* mutations had a threefold higher risk for worse survival versus familial *CDKN2A* negative and sporadic wild type melanoma cases. [296] In contrast, an Italian study found no statistically significant difference with regard to melanoma outcome between *CDKN2A* mutation positive and negative cases that were matched by age, gender and stage. [297] Although these last 3 articles by 2 study groups used germline variants in their titles, they seem to be testing mutations technically and not specific SNP markers. To our knowledge, no study had yet tested *CDKN2A* single nucleotide

polymorphisms in relation to melanoma survival at the time our SNPs study was conducted except the haplotype reported by Casula et al. [289]

PARP gene variants located at 1q21.3 was previously identified to be associated with melanoma susceptibility. [298] Melanoma patients harboring a variant in the *PARP1* gene (either rs2249844*A or rs3219090*A) were found to fare worse in two different studies [299,300] even though both SNPs were shown to be linked with lower melanoma risk. [300–302] Of note, these two SNPs are strongly linked.

Vitamin D receptor (*VDR*) polymorphisms had been shown to affect melanoma risk. Orlow et al. analysed 38 *VDR* SNPs and found only one SNP (rs2239182*AA) to have a statistically significant elevated risk for melanoma specific survival after adjusting for multiple testing. [303] This particular SNP plays a role in *VDR* expression. However, *VDR* SNPs were not associated with histopathological prognostic factors of melanoma implying other processes to be potentially involved. In a different melanoma population, vitamin D binding protein (*VDBP*) rs12512631*CC and *RXRA* rs7850212*AA were both found to reach the significant threshold for a favorable melanoma specific survival. These variants were shown to influence gene methylation status on further testing. [290]

Other melanoma prognostic variants have been identified through candidate gene analysis without being associated with melanoma risk first. Genes related to melanoma pathways have been studied and comprises of immunological pathways (*TLR3, IL-10, CCR5, CTLA4, IRF5*), DNA maintenance (*ERCC1, ERCC2, ERCC5, XRCC1*), pathways involved in metastasis (*EGF, MMP1, PDGFD*) and metabolism. Since melanoma is a highly immunogenic tumor, tumor growth might be suppressed. Furthermore, these immune modulation capability can differ in each person implying genetic susceptibility. [304] Several SNPs in genes involved in immune modulation such as *CCR5, HLA-DQB1, IFNG, CD28* and others have been studied in their association with melanoma survival. High expression levels of interleukin 10 (IL10) has been shown to correspond with mitigation of the anti-tumor response in melanoma patients stimulating disease progression. [305] Variants associated with high IL10 expression (rs1800896*GG, rs1800871, rs1800872) has been demonstrated to be correlated with non-invasive CM growth implying protection [306] and has been confirmed to be associated with melanoma prognosis. [307] In contrast, patients with *IL-10* SNPs (rs1800896*AA) linked with low IL-10 levels tend to present with deeper Breslow therefore suggesting worse prognosis which has been supported by other studies. [307,308] Another *IL-10* polymorphism,

rs1518111, was also reported to be related with higher melanoma specific mortality. [307] The same study investigating polymorphisms in angiogenesis and lymphangiogenesis has demonstrated significant correlations between 17 SNPs and melanoma mortality. However, only 4 SNPs have been confirmed in another dataset and meta-analysis to be significantly associated with nodal metastasis (*EGFR* rs723526 and *TLR3* rs3775292) and melanoma-specific death (*TLR3* rs7668666 and *PDGFD* rs2220377). Of note, *EGFR* and *PDGFD* were both implicated in angiogenesis. [307]

Rendleman et al. demonstrated an association between two melanoma risk variants (i.e. variants originally associated with pigmentation and blue eye color) and a better prognosis. [309] They investigated the association between 108 SNPs associated with skin cancer and prognostic outcomes in 891 CM patients. The study reported two SNPs, rs7538876 (AA genotype, located in *RCC2/PADI6*) and rs9960018 (TT genotype, located in *DLGAP1*), to be associated with better 3-year relapse free survival and the associations are even stronger in the multivariate analysis.

Variants in DNA repair pathways were not only shown to predispose to higher cancer risk [129] but also seem to have an effect on chemotherapy response by promoting resistance to alkylating agents such as cisplatin and dacarbazine. [310] Vogelsang et al. postulated that since melanoma progression is a multi-stepped process, germline alterations in genes controlling metastasis might be potential prognostic markers for melanoma. [311] For these reasons, these variants have been investigated in relation to melanoma outcome.

Variants in DNA repair genes and immunomodulatory genes were associated not only with melanoma survival but also with treatment response. A SNP (rs25487*AA) in *XRCC1,* a base-excision repair gene, was shown to confer a survival benefit versus the other genotypes. However, a second SNP in *XRCC1*, rs3213245*CC is associated with worse survival after the first metastasis. [312] The SNP rs11615*T located in the *ERCC1* gene which also regulates DNA repair was reported to be significantly correlated with favourable biochemotherapy response and longer survival period in stage IV melanoma patients compared to those with at least one C allele. The latter allele type seem to stimulate *ERCC1* gene expression. [313] The minor allele homozygotes of variants in *ERCC2* (rs13181) and *ERCC5* (rs17655) have been demonstrated to be correlated with worse disease outcome. [314]

Metastasis does not only confer an unfavourable prognosis but germline variants have been identified that facilitate the process. A variant in Epithelial Growth Factor (*EGF*) gene, rs4444903*GG, has been linked with deeper Breslow and reduced survival in several studies [311] which can probably be attributed to the role EGF plays in epithelial growth and tumor progression. [315] Matrix metalloproteinases (MMPs) are involved in tumor progression and spread. [316] A polymorphism in *MMP1*, rs1799750, was implicated with CM prognostic factors especially in deep invasive melanomas. [317] Other *MMP1* variants such as rs475007*T were associated with ulceration, thinner melanoma and axial site whilst rs498186*G is correlated with presence of ulceration and axial tumor site after adjusting for covariates. [318] Although these results suggest that *MMP1* might influence melanoma progression, no significant association has emerged between these variants and melanoma overall survival as of yet.

Immunomodulatory genes have been postulated to have a potential role in response to immunotherapy and thus disease outcome. The deletion of *CCR5* was shown to be associated with worse survival in metastatic melanoma patients undergoing immunotherapy [319] but was not confirmed in another study which reported an opposite effect. [320] Variants in *CTLA4* gene (rs4553808*G, rs11571317*T) respond better with anti *CTLA4* therapy whilst rs231775*G responds worse. [321] *IRF5* variant (rs10954213*GG) were shown to be significantly more common in non-responders of TIL adoptive therapy. [322]

Furthermore, susceptibility variants of other cancers such as breast cancer, prostate and colorectal cancer have been linked with disease prognosis and have the potential to be prognostic biomarkers. [323–325] It can therefore be inferred that the same might apply to melanoma. In addition, with the arrival of new technologies for genomic analysis, genetic biomarkers have the potential for a more accurate and personalised prognostication.

1.7 METHODOLOGICAL CONSIDERATIONS

Some of the methods utilised in our study including population selection are addressed in this section.

1.7.1 Study Population

Cutaneous Melanoma Risk Factors and Single Nucleotide Polymorphisms in A Representative Part of a Belgian Melanoma Population

Melanoma risk factors and melanoma development and/or survival had been hardly studied in the Belgian population. Most studies on melanoma risk factors in other populations had often made use of national registry databases, which although may contain data of a substantial number of people regarding melanoma deaths, usually lack data on progression. Furthermore, melanoma histopathologic diagnosis can sometimes be challenging and dependent on the level of expertise in melanoma histopathology which may vary in different centers. In addition, national registry databases usually lack data on clinical risk factors (e.g. phototype, CM family history, ethnic ancestry, etc.), histopathology details, disease stage, genetic factors and CM treatment. In University Hospital Leuven, a database containing all the aforementioned parameters exists. A multidisciplinary team, consisting of the departments of Dermatology, Surgery, Oncology, Pathology and Radiology, attend regular group meetings and together conduct a special melanoma clinic to facilitate interdepartmental referral as well as further disease management. All CM patients undergo standard clinical follow-up protocol depending on the disease stage whereby interinstitutional heterogeneity of this metric is mitigated.

As stated earlier, race might affect CM development and survival. The patient group that attended the University Hospitals Leuven and that were included in the hospital database were predominantly Caucasian with more than 90 percent self-reported having Caucasian/Belgian grandparents. Therefore, although (differences in) race might influence results of cancer studies, this is not the case in our predominantly ethnically homogenous study population. Hence, race was not included as a confounder in our investigations.

Furthermore, studies conducted in other regions on race and melanoma development/survival attributed the variation to social inequality and access to healthcare. [237] In Belgium, health care insurance is compulsory whereby 99% of the whole population is covered and 75% of all health care expenses is covered. [326] In addition, it is

mandatory for all working people to pay social security contributions and every entitled person should be affiliated with a sickness fund. [326] The Belgian health system is established on the principles of equal access and freedom of choice which mitigates social inequality regarding healthcare access and facilitates follow-up and treatment compliance. [326]

Our studies were conducted in the University Hospitals Leuven, a large tertiary hospital in Belgium. Taken together, the extremely high percentage of individuals with third generation Belgian ancestry, the multidisciplinary melanoma expert approach in University Hospitals Leuven, the standardized clinical follow-up protocol, a comprehensive database and the excellent accessibility of the Belgian healthcare system make our cohort representative of the Belgian melanoma population.

1.7.2 Statistical Techniques

Bayesian Random Effects Model

Two of the statistical methods used in our studies will be discussed here. Bayesian random effects model (BREM) was utilised in our meta-analysis study. However, the most frequently used form of statistical inference is the classical or frequentist method which hypothesizes that probabilities are the frequency of random events in a long run of repeated trials. The frequentist method strives to eliminate uncertainty by producing estimates. [327]

In contrast, BREM utilizes Bayesian inference which aims to incorporate prior beliefs using any available evidence to produce posterior beliefs. These posterior beliefs can then function as a priori beliefs under the generation of new data. Bayesian inference allows for continuous and dynamic adjustment of beliefs with each new evidence. The goal of Bayesian statistics is to preserve and refine uncertainty through adjustment of individual beliefs via incorporation of novel data. Likelihood-based or the default random effects method which is commonly used in meta-analysis performs poorly if the number of studies included are few. [328] In addition, traditional fixed-effect models (FEM) and random-effect models (REM) require a normal effect-size population distribution depending on parameters and covariates. These models fall short if the effect-size is not normally distributed. [329]
Seide et al. compared different methods for the meta-analysis and came to the following conclusions. [328] If studies are few, heterogeneity is present and especially with non-normally distributed effect sizes between studies then the

coverage probability is below the nominal level for all frequentist methods including classic FEM and REM unlike Bayesian methods. The confidence intervals in frequentist methods in such setting might also be inconclusively wide. A non-informative prior is used for the Bayesian method to estimate the overall mean and standard deviation between studies which takes study heterogeneity into account solves this problem. The Bayesian method is a non-parametric meta-analysis model which has the ability to describe a wider range of non-normally distributed effect-sizes and produces narrower and more accurate interval estimates compared to conventional frequentist procedures. [329,330]

Cox Proportional Models

Another statistical method that was used for our studies was the Cox proportional model. The purpose of Cox proportional model is to link the survival time of an individual or a patient to covariates. [331] In other words, Cox proportional model intends to analyze which explanatory variables have an effect on patient survival time. The model can provide an estimate of the effect of the main variable of interest given as risk of outcome being analyzed after correcting for other prognostic factors. [331]

Cox model utilizes the concept of likelihood for estimation and significance testing i.e. the likelihood or probability that a statistical model can explain the obtained data. Cox model estimated parameters (i.e. hazard and coefficients) have been defined as maximum likelihood estimates. The hazard function is the risk of an event such as dying at time t. If the regression coefficient is positive for an explanatory variable then this implies that the risk on the event of interest is high whilst a negative one indicates a lower risk of the event and therefore a favorable prognosis. The method does not depend on a specific survival time distribution. It does rely on the proportional hazard assumption being satisfied meaning that different survival variables are stable over time and additive in a certain scale. One of the methods to check this would be with a Kaplan-Meier curve which would demonstrate parallel curves in the graph of the survival function vs survival time. [332] Study findings necessitate validation through an independent population prior to being considered proven. [333]

CHAPTER 2: AIMS

Cutaneous melanoma (CM) is the most frequent deadly skin cancer among Whites. CM incidence and mortality are projected to increase in the coming years in most predominantly Caucasian populations including Belgium. Melanoma outcome is highest if diagnosed at an early stage. Furthermore, novel targeted and immunotherapies have been shown to increase survival in a set of CM patients but are disadvantaged by high costs, therapy resostance and toxic adverse effects. Hence, understanding risk factors for melanoma development and survival is of vital importance.

Well known risk factors for melanoma susceptibility are a high common naevi count, presence of atypical naevi, sunburn, low phototype, freckles, actinic damage, personal and family history of melanoma and genetic variants linked with these clinical risk factors. These risk factors and other potential determinants such as atopy, multiple primary melanomas (MPM) and genetic variants have not yet been extensively studied for their (potential) relation with melanoma development and outcome in the Belgian population. Moreover, genetic biomarkers such as SNPs have the potential for a more accurate and personalised prognostication. Hence, in this PhD project, we conducted a series of analyses with the aim to better understand risk factors associated with CM etiology and survival in a large Belgian melanoma population well-characterized for melanoma determinants.

In a meta-analysis of studies comparing risk factors for single versus multiple primary melanoma, we investigated which factors increase the susceptibility of developing MPM (AIM 1). In a subpopulation of our Belgian melanoma cohort, we analysed in a case-control study the effect of atopic dermatitis on melanoma susceptibility as well as the impact of atopic dermatitis on melanoma outcome (AIM 2). Subsequently, we investigated whether actinic damage, host melanoma phenotypes (e.g. phototype, nevus phenotype, personal and familial CM history) (AIM 3) and melanoma SNPs (AIM 4) affect melanoma outcome. Our aims are briefly outlined once more below.

AIM 1: To conduct a meta-analysis on major risk factors of multiple primary cutaneous melanomas

AIM 2: To investigate the effect of atopic dermatitis on melanoma susceptibility and outcome

AIM 3: To analyse the association between known clinical risk factors for melanoma susceptibility and melanoma outcome

AIM 4: To study the association between melanoma susceptibility SNPs and melanoma outcome

CHAPTER 3: A META-ANALYSIS ON MAJOR RISK FACTORS OF MULTIPLE PRIMARY CUTANEOUS MELANOMAS

Derived from: Marasigan V., Schmitt E., Garmyn M., Van den Noortgate W. A meta-analysis on major risk factors of multiple primary cutaneous melanomas. Clin Derm. 2014 April-June; 84 – 92. Published online 2014 July 28. doi: 10.11138/cderm/2014.2.2.084. ISSN: 2282-4103

3.1 ABSTRACT

Background: Identifying multiple primary melanoma (MPM) risk factors is an important step towards detecting secondary primaries at an early stage and optimizing follow-up of melanoma patients. Therefore, we examined the risk factors for MPM versus single primary melanomas (SPM) in a meta-analysis.

Methods: Published literature comparing risk factors for MPM versus SPM up until May 13, 2013 were retrieved from Embase and MEDLINE databases. Effect sizes were aggregated and analysed using random effects models (REM) using Bayesian methods.

Results: 16 articles comparing risk factors between MPM versus SPM, comprising of 2442 MPM and 24895 SPM individuals were included. Positive family history (OR 2.0,95% HPD: 1.6-2.7), presence of dysplastic nevi (OR 4.4,95% HPD: 2.6-8.9) and light hair color (OR 1.4,95% HPD: 1.1-1.7) were shown to be significantly linked with MPM. Bayesian estimates of other factors, including benign nevi count, sunburn history, freckles and phototype were not significantly raised.

Limitations: Heterogeneity between studies and the small number of eligible papers limited our meta-analysis.

Conclusion: This meta-analysis confirms that a positive family history, presence of dysplastic nevi and light hair color correlate with higher risk for developing MPM. Patients with these characteristics should be carefully monitored.

3.2 INTRODUCTION

Cutaneous malignant melanoma is the most frequent cause of skin cancer related death. With the incidence increasing steadily [334], understanding its etiology is a pressing issue. Major risk factors associated with development of melanoma are: high common naevi count, presence of dysplastic naevi (DN), a positive family history, actinic damage, fair skin type, intermittent sun exposure and sunburn history. [12–14] Although most patients do not develop more than one melanoma, a first melanoma is positively correlated with developing a second melanoma, with percentages ranging from 0.2 to 20%. [54,62,335] However, the incidence of multiple primary melanomas (MPM) is relatively low in the general population, making it difficult to study. Begg et al. proposed a solution to this problem by comparing multiple primaries (cases) to single primaries (controls), thereby increasing statistical efficiency. [336] Knowledge of which risk factors is more prevalent in multiple primary melanoma patients compared to single primary melanomas (SPM) patients might not only be useful for secondary prevention but might also lead to greater understanding of the etiology of multiple primary melanoma.

For this purpose, we investigated the risk factors associated with multiple primary melanomas versus single primary melanomas through a meta-analysis.

3.3 METHODS

Search strategy

Relevant literature in the Embase and MEDLINE (using PubMed) databases published from inception through May 13, 2013 was investigated with the aid of experienced librarians. The following keywords were used for Pubmed: (Secondary melanoma* OR second melanoma* OR multiple melanoma OR multiple melanomas OR mulitiple primary melanoma*) AND ("Risk Factors"[Mesh] OR "Risk Assessment"[Mesh] OR risk OR risks OR predictors OR "Sunlight"[Mesh] OR "Sunburn"[Mesh] OR sunburn* OR sunlight OR Sunshine OR "sun exposure" OR "Keratosis, Actinic"[Mesh] OR actinic keratos* OR ((atypical OR dys plastic OR common) AND ("Nevus"[Mesh] OR naevi OR nevi)) OR "family history" OR "Phenotype"[Mesh] OR Phenotype*[tiab] OR "solar lentigo" OR "solar lentigines"). For EMBASE: secondary NEAR/2 melanoma OR second NEAR/2 melanoma OR multiple NEAR/2 melanoma. The results were entered in Endnoteweb to facilitate an overview and to detect redundant articles. Relevant articles

from these sources and reference lists were chosen based on titles and abstracts. The Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines were used to guide the analysis. [337]

Included studies were: human observational and epidemiological studies in adults comparing clinical risk factors for cutaneous MPM versus SPM for which full length original research articles are available and which contain enough information for an estimation of an effect size and standard error.

Excluded studies were: non-human studies, reviews, case reports, editorials, or studies that focus only on specific types of melanoma or certain subgroups. The following information was collected from each study: bibliographical information, country of the study, study population, study design, type of effect size, definition of the risk factor, and quantitative data for effect size calculation. If two or more studies were based on the same/overlapping samples, the one with the largest sample or best definition of the risk factor and the estimates for a risk factor that were most adjusted by controlling for other covariates were used. Effect size estimates that were not explicitly available were calculated from information that was provided. Articles published in languages other than English were included provided that the title or the abstract was in English so that they could be detected. In the second step, only papers comparing melanoma risk factors in a multiple melanoma population against a single melanoma population were included.

Two reviewers independently extracted data from all included studies. Disagreements were resolved through discussions and arbitration of a third reviewer.

Statistical Methods

Most papers reviewed presented odds ratios (ORs), so we focused on presenting results for this metric. ORs and corresponding confidence intervals (CI) were retrieved, or calculated from summary or crude data. Subsequently, the natural logarithm of the ORs (log OR) and sampling variances were calculated. Bayesian estimates of the random effects model (REM) parameters were derived using the Openbugs software. [338] REMs have the advantage that they can account for heterogeneity between studies when estimating the size of the population effect. In contrast to maximum likelihood estimation (MLE), Bayesian estimation techniques do not rely on asymptotic assumptions, making them more suitable for small samples. We select priors that strongly assume there is no

significant effect for the random effects. In doing so, we estimated models that are unlikely to find significance if the sample is small and heterogeneous. Such a prior is known as a sceptical prior. This choice was made partly as a way to avoid spurious results given the small sample sizes of many of the risk factors as suggested by Wijeysundera et al. [339] Although incorrectly identifying a risk factor as significant might be preferable to ignoring an important one, a goal of this meta-analysis is to draw attention to risk factors that require further study. On the other hand, in spite of the small samples, if our meta-analysis finds a risk factor significant, it is likely that this factor genuinely affects the risk. Typically, for our specification, as the sample size grows, the Bayesian estimates quickly resemble those estimated using MLE.

This modelling approach produces estimates of a posterior density function for the mean log OR and for tau^2. A Bayesian alternative to a confidence interval is to consider highest posterior density interval (HPD). Log ORs are converted to ORs and the risk factor is considered significant if 1 is not included in the interval. To summarize the studies considered for the risk factors, we present the corresponding ORs and their confidence intervals in forest plots. [340]

For family history, a relatively high number of studies were available in contrast to other risk factors, therefore heterogeneity between studies was investigated by estimating and testing the estimates between study variance, tau^2, from the REM. Based on these results, the results of similar analyses for smaller sample sizes are not discussed.

3.4 RESULTS

Our initial broad search found 2993 articles in Embase and 1681 in MEDLINE, totalling 4674 articles (Figure 1). After deduplication, 3576 articles remained. Evaluation through scrutiny of titles and abstracts rendered 130 potential articles. A manual study of reference lists yielded an additional 20 articles. The inclusion/ exclusion criteria were further explored whereby 18 articles were identified. Marghoob et al. [341] presented cases with classic atypical mole syndrome only, and therefore cannot be compared with other papers. Siskind et al. [62] reported hazard ratios instead of ORs and contained no crude data. These two studies were excluded. In total, 16 studies had data on at least one of the aforementioned risk factors (Table 1).

Figure 1. Flow diagram of article selection process.

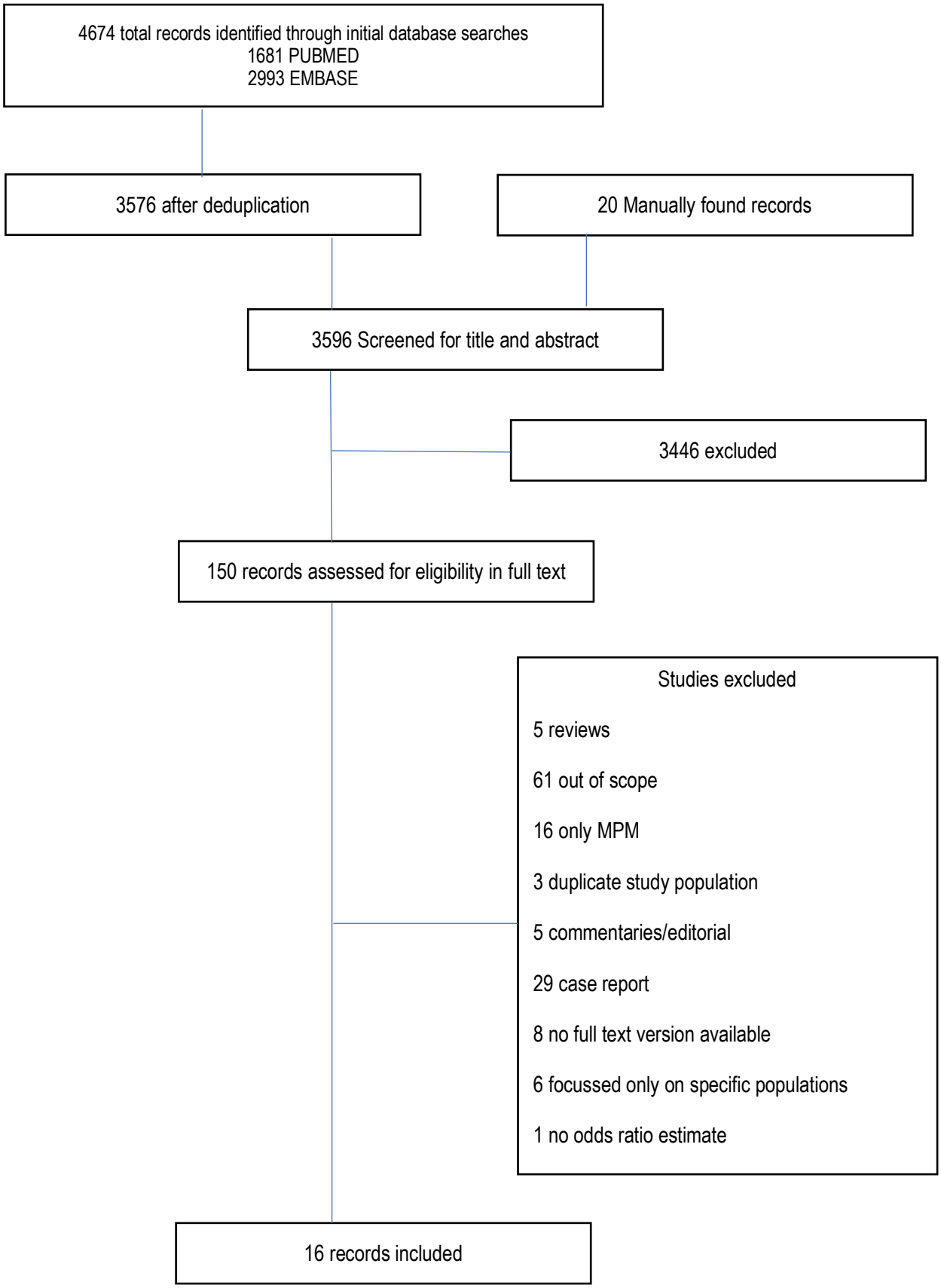


Table 1. This table depicts characteristics of the studies used for the meta-analysis for a certain risk factor.

STUDY	YEAR	COUNTRY	MPM	SPM	FH	DN	BN	HC	SB	FR	EC	PT
Scheibner et al. [342]	1982	Australia	90	3128	x	x						
Slingluff et al. [343]	1993	USA	283	7816	x							
Burden et al. [344]	1999	U.K.	48	108	x	x	x					
Ferrone et al. [335]	2005	USA	385	4484	x	x						
de Giorgi et al. [345]	2010	Italy	40	672	x	x	x					x
Titus Ernsthoff et al. [346]	1988	USA	8	24		x	x	x	x			x
Titus Ernsthoff et al. [347]	2006	USA	27	327	x	x	x	x	x	x	x	x
Sigg et al.[348]	1989	Switzerland	23	257	x							
Begg et al. [336]	2006	Int'l GEM	1210	2470	x		x	x		x	x	x
Kricker et al. [63]	2007	Int'l GEM	1125	2023					x			
Mandelcorn et al. [349]	2011	Int'l GEM	1138	2151								x
Hwa et al. [350]	2012	USA	61	727	x							
Manganoni et al. [351]	2012	Italy	76	2079	x							
Johnson et al. [352]	1998	USA	60	1482				x			x	x
Pastorino et al. [353]	2008	Italy	95	398	x			x		x	x	
Nagore et al. [354]	2008	Spain	36	923	x							

*Legend: MPM=number of participating patients with multiple primary melanomas; SPM= number of participating patients with single primary melanomas; FH= family history, DN= dysplastic naevi, BN= benign naevi, HC=hair color, SB=sunburn, FR=freckles, EC=eye color, PT= phototype

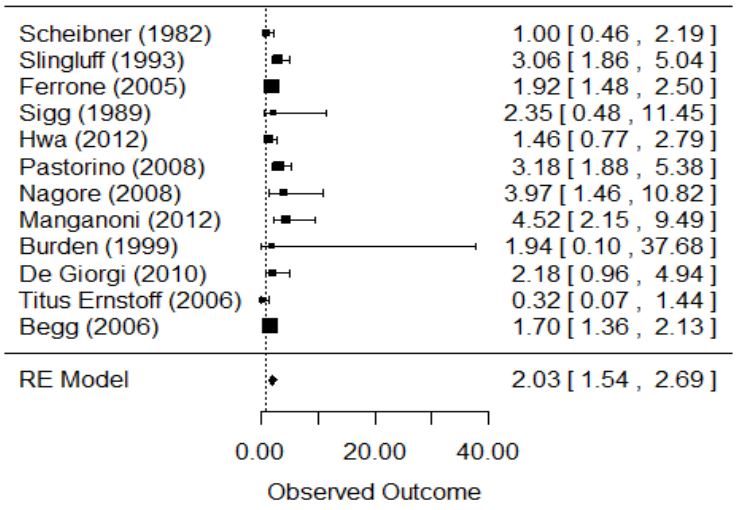
Family history

Twelve studies were included in the analysis (Table 1). Of these, five studies did not give a precise definition of a positive family history, but were specific enough to remain usable. [336,342,345,350,353] Five studies defined family history (FH) to be positive when at least one first-degree family member has a history of melanoma [335,344,348,351,354] whilst two studies [343,347] determined FH to be at least one first or second-degree family member with a melanoma. Four studies provided ORs. [336,344,345,347] One of these studies utilized logistic regression analysis, which produces log ORs as results, which the authors identified by the term relative risk. [344] The OR was calculated from crude data in eight studies. [335,342,343,348,350,351,353,354]

All estimates were transformed into log OR and sampling variance. An inverse-variance weighted meta-analysis was performed to obtain an estimate of the overall log OR, which was back-transformed to the OR scale to ease the interpretation. The overall OR was significant OR 2.0 (95% HPD: 1.5-2.7), which means that the odds for a positive family history is twice as large in the MPM group compared to the SPM group. Substantial heterogeneity between studies was not found to be present (estimated tau^2 = 0.107, 95% HPD: 0.0-0.5). However, this is likely due to the small sample size and our selection of a very small value for the prior for the heterogeneity parameter. Figure 2 shows a forest plot of these results.

A sub-analysis of the 5 studies which defined a family history as positive only when melanoma occurred in first-degree family members, produced an OR = 2.50 (95% HPD: 1.6-5).

Figure 2. Forest plot of the ORs and confidence intervals of studies on family history of melanoma.

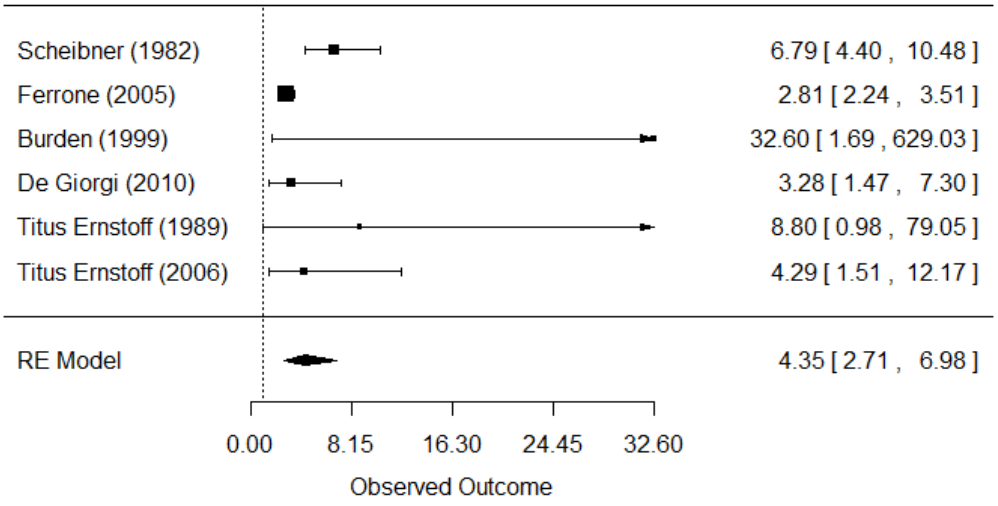


Naevi

In total five studies on common naevi were deemed eligible. Three studies defined common naevi count dichotomously with varying cut-off points. [344,346,347] One study had five ordinal groups whereby the estimate of the highest level was included in the analysis [336] whilst one study analysed common naevi count as a continuous variable. [345] Benign nevi was counted for the whole body in three studies [336,346,347] but was not defined in two. [344,345] All estimates were transformed into log OR and a Bayesian random effects meta-analysis was applied, obtaining an OR of 1.8 (95% HPD: 0.8-4.5) which is not significant.

Odds ratios could be retrieved from six studies on dysplastic naevi. Concerning the definition of DN (clinical versus histological), three studies observed DN clinically [342,345,347] whilst two included both clinical DN and histologically confirmed DN. [335,346] One study presented separate results for clinical and histological DN. [344] Concerning the quantity of DN: three studies compared the presence of ≥1 DN vs none. [335,344,345] One study had two groups for clinical DN: group 1 (>10 vs 2-9 vs 1 vs 0) and group 2, (>2 vs <2) whilst histological DN was categorized as 0 vs rest. [346] Another study used 3 categories (≥ 3 vs 1-2 vs 0). [347] Scheibner et al. compared > 20 DN versus <20.[342] Using log ORs from the analyses most accounting for covariates adjusted analyses performed by each of the papers, we obtained an aggregated OR of 4.4 (95% HPD: 2.7-7), showing that there is a significant risk of multiple melanoma if at least one DN is observed (Figure 3). A sub-analysis without the Scheibner et al. paper produced an OR of 3.2 (95% HPD: 2.2-6.1), which further bolsters our findings.

Figure 3. Forest plot of the ORs of studies on dysplastic naevi.

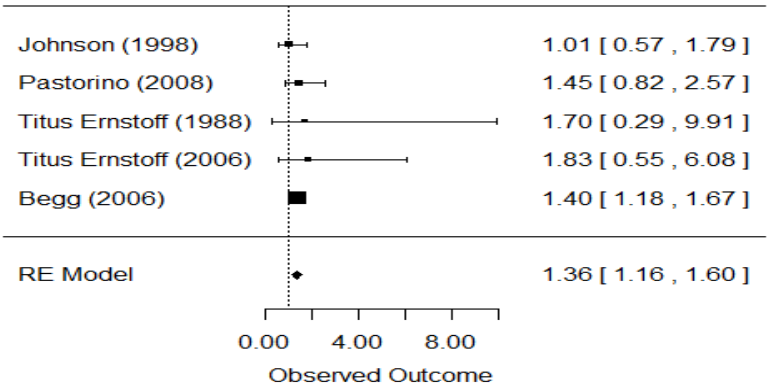


Phenotypical features

Six studies on phototype were examined of which three compared phototype higher than 2 (darker skin) to phototype 1-2 (fair skin). [345,349,352] Begg et al. [336] measured tannability but was in the same collaboration group as Mandelcorn et al. [349] which mentioned phototype; a more precise definition. The latter article was therefore included for this risk factor. Both papers by Titus-Ernstoff et al. [346,347] with two different populations mentioned tannability. The first paper had only one estimate (tanning vs no tanning) whereas the latter article published two results: tannability after short term sun exposure vs long term. For the latter study, tannability after long term exposure was chosen because of its higher dependability.

Using the category fair vs dark, gives an OR 1.36 (95% HPD: 0.93-2.01) which is not significant. There were five studies on hair color included. Titus-Ernstoff et al. compared red/light hair vs dark in their first publication [346] but mentioned three categories in their second [347] (blonde/red vs brown/redbrown vs black/brown). Begg et al. [336] had estimates for a dichotomous categorization (light vs dark) and for 4 groups (red vs blonde vs light brown vs black/dark- brown). Johnson et al. [352] and Pastorino et al. [353] each had 4 and 3 groups respectively whereby ORs were calculated from crude data (non-dark hair vs dark hair). For this analysis, individuals with the lightest hair color classification were compared to all individuals with darker hair classifications resulting in a dichotomization of the groups (light vs dark hair). The mean effect estimate was significant: OR 1.4 (95% HPD: 1.2-1.6) (Figure 4). Three studies mentioning freckles were found, with all three defining freckles as present vs not present. The pooled REM effects were insignificant OR 1.1 (95% HPD: 0.5-1.7), but the sample size is small. Therefore, we performed a sensitivity analysis with fixed effects model, OR 1.2 (95% CI 1.02-1.45), which is significant. We also examined four studies mentioning eye color. Begg et al. [336] had two estimates of which the one for light vs dark was used. For the 3 studies without an estimate for a dichotomous variable (light vs dark) [353 347 352], ORs were calculated from the available crude data (non-brown color vs brown). For eye color, the pooled REM effects were insignificant: OR 1.0 (95% HPD: 0.7-1.6). Due to the small sample size, a FEM was estimated, also finding insignificance: OR 1.1 (95% CI: 0.7-1.7). Only two studies [353 347] mentioned skin color with two different ascertainment methods and were therefore not included in the meta-analysis.

Figure 4. Forest plot of the ORs of studies hair color.



Sun exposure

Three studies on sun burn were included. Two studies [346,347] had dichotomous categories (no sun burn versus one or more episodes). An additional study [63] had multiple ordinal categories of which the highest level (versus no sunburn) was included in the analysis. Using a random effects model, the mean effect estimate was OR 1.1 (95% HPD: 0.1-7.2). However, sensitivity analysis using a fixed effects model resulted in an OR of 1.4 (95% CI 1.1-1.8). In addition to sun burn, three studies on intermittent sun exposure were found but two were from the same collaboration study (GEM). We finally decided not to include the papers in this meta-analysis because of the insufficient number. Articles on actinic damage were sought but only two articles [63,342] were deemed eligible with two completely different sets of variables analytic methods; therefore, a meta-analysis was not conducted.

3.5 DISCUSSION

In this meta-analysis, we examined whether certain melanoma risk factors are positively associated with the development of a second melanoma. We investigated the following major melanoma risk factors: family history, benign naevi count, dysplastic naevi, phenotypic features (hair color, freckles, eye color) and sun exposure. We only included papers which compared major melanoma risk factors in a multiple melanoma population against a population with a single melanoma only. There are papers which either investigated risk factors within a multiple melanoma population whilst other papers compared multiple melanoma groups with healthy controls. However, because some melanoma risk factors have relatively low prevalence in healthy, population-based controls, such as family history of melanoma and dysplastic naevi, Begg et al. [336] proposed the utilization of single melanoma

populations as controls to increase the power of the study. The major findings of this study were that family history and dysplastic nevi are significantly positively associated with multiple melanoma when compared to a single melanoma population; with dysplastic naevi displaying a much stronger association. These findings concur with the results of meta-analyses on risk factors for melanoma development in the healthy population. [12,14] Most studies focused on family history, which was rather unsurprising because genetic susceptibility is known to play a major risk for melanoma development. About 10% of melanoma cases are familial with high penetrant genes such as *CDKN2A* (on chromosome 9p21.3) and *CDK4* (on chromosome 12q14) accounting for melanoma risk in 20-57% of familial melanoma, but a very low frequency in sporadic cases. [139,355,356] This implies that other susceptibility genes exist for melanoma susceptibility like low penetrant genes.

The presence of dysplastic naevi is a risk factor associated with the highest risk of acquiring multiple melanoma. The presence of even one dysplastic naevi is found to indicate risk. However, most studies that were included categorized dysplastic naevi dichotomously and only one study had ordinal categories for this risk factor which makes a sub-analysis at the level of ordinal groups impossible. Conversely, *CDK6* and *XRCC1* variants were shown to be associated with DN risk but not with melanoma development in melanoma families implying that other genetic or extrinsic factors (e.g. sun protection usage in risk individuals, solar exposure) might influence the transformation of DN into melanoma. [357] Our findings on light hair color versus dark indicate a significant positive association with multiple melanoma, albeit to a lesser extent. However, the number of papers on phenotypic features is generally low. The estimate for freckles in REM was insignificant in contrast to that of FEM but given the small sample size and the insignificant estimate in the conservative REM, we consider this sensitivity analysis an indication that further studies are needed, rather than conclusive. Findings on phototype and eye color were not significant.

It is evident that studies on sun exposure and indicators of actinic damage in relation to multiple melanoma versus single melanoma populations are lacking. Only sunburn had enough studies to carry out a meta-analysis albeit consisting of only three studies. Nevertheless, summary estimates for sunburn were non-significant in the Bayesian analysis but significant in the FEM model. The interpretation of these contradictory results is delicate because the Bayesian approach used for the REM is inherently sceptical, and the sample size is small. The estimates for intermittent sun exposure suggests a positive influence but this is based only on two papers [63,342] (results not shown). This result indicates that further research on this risk factor is advisable. In meta-analyses, heterogeneity

between papers can be a limitation when interpreting the results. To conduct our analyses, we estimated random effects models, to account for heterogeneity between papers, using a Bayesian procedure. Due to the small sample sizes for many of the risk factors, Bayesian estimation methods were preferred because they do not require large samples to obtain useful intervals on the estimated log ORs, unlike maximum likelihood. Furthermore, we selected sceptical priors such that the Bayesian models will only give weight to the information in the data if it is very convincing, so that risk factors would only be found significant if the evidence was very strong. This approach seemed prudent given how few papers address some risk factors. Sensitivity analyses were also performed using fixed effects models. Another limitation is that results could be affected by subgroup differences. Due to small number of papers, we could not always conduct analyses on differences between subgroups for each risk factor. Also, this study focused only on the clinical risk factors for melanoma as shown by the meta-analyses conducted by Gandini et al. [12–14]Histopathological risk factors (e.g. site, subtype, etc.) falls outside the scope of this review. Papers without a risk factor besides age and gender were not included in our analysis, so a comprehensive meta-analysis of these patient characteristics was not conducted. However, we performed analyses on these two factors with the studies that were included and which contained sufficient data. Results on age of first diagnosis based on 3 studies were inconsistent and non-interpretable (results not shown). Concerning gender, 7 studies contained enough data in the articles. A random effects model reports an OR of 0.6 (95% HPD 0.1-3.8), indicating that gender is not significant. In addition to this result, we note that one study, Sigg et al., [348] reported that gender was not significant in their sample, which is consistent with our more general findings, but sufficient details were not provided and their study was therefore not utilized in the analysis.

Overall, this meta-analysis underlines the need for surveillance for the development of more primary melanomas in already diagnosed melanoma patients with at least one dysplastic nevus and positive family history. Light hair color seems to indicate that fair features might also be associated with multiple melanoma development but other fair phenotype markers failed to show significance, which might be due to the limited amount of papers and presence of heterogeneity. The same problem applies to sun exposure and actinic damage indicators where no significant outcomes were found in the Bayesian approach. Clearly, there is a need for additional studies regarding this subject.

CHAPTER 4: INVERSE ASSOCIATION BETWEEN ATOPY AND MELANOMA: A CASE-CONTROL STUDY

Derived from: Vivien Marasigan, Marie-Anne Morren, Julien Lambert, Karen Medaer, Steffen Fieuws, Tamar Nijsten, Marjan Garmyn. Acta Derm Venereol. 2016 Jun 8. Doi: 10.2340/00015555-2476.

4.1 ABSTRACT

Heightened cutaneous immune surveillance in atopic patients may inhibit development of melanoma. The aim of this study was to analyse the association between atopy and melanoma (development and outcome). A total of 188 cases of melanoma and 596 healthy controls were interviewed by telephone with a standardized questionnaire on atopic, demographic and melanoma characteristics. Cases were matched with controls on important confounders (age, sex, sunburn sensitivity, hair colour, number of moles, sunburn as juvenile, ever sunbed use, familial melanoma). Melanoma outcome data (disease relapse and death) within cases were retrieved. Analysis showed a general inverse association between atopy and melanoma development, but this was statistically significant only for a history of personal atopy (odds ratio 0.53, 95% confidence interval: 0.30–0.96, p-value=0.04). Among melanoma patients, atopy did not affect survival or progression. In conclusion, this study suggests an inverse association between a history of atopy and melanoma development, but not with disease progression.

4.2 INTRODUCTION

Melanoma is the most frequent lethal form of cutaneous malignancy among Caucasians. An increase in mortality rates and incidence rates of melanoma has been detected, [11] whereby a further increase in incidence rates of melanoma in the coming years is forecasted. [334] Melanoma accounts for approximately 75% of all skin cancer deaths, which translates into the loss of thousands of lives. In 2008, 45,000 deaths due to melanoma were reported worldwide. [9] Although there are several novel treatment options for advanced melanoma currently under investigation, mortality rates remain high. It has been hypothesized that a possible negative link between atopy and melanoma might exist due to the hyperactive immune system in atopic patients, which might prevent the development of malignancy through increased immune surveillance. [70,358] Moreover, the fact that melanoma is an immunogenic tumour might render this disease susceptible to the heightened immune surveillance in atopic patients. [72] In addition, it has been shown that fewer naevi are present in patients with atopic dermatitis (AD), which implies that AD might protect against melanoma, since a high naevi count is a well-known risk factor for melanoma.

[76] However, atopy is an inflammatory disease, and inflammation has been linked with tumour development and has been postulated as one of the hallmarks of cancer. It has been hypothesized that inflammation can contribute to genetic instability and might even sustain a pro-oncogenic environment. [70,71] To date, studies that have been conducted on the association between atopy and melanoma are scarce and contradictory. Three studies in large cohorts of subjects with AD have reported lower incidence ratios of melanoma cases in AD vs. non-AD populations, [70,77,78] while a recent study found a significant positive association between AD and melanoma. [79] These studies examined the incidence of melanoma in AD vs. non-AD subjects, but did not take other melanoma risk factors into account. Three other studies comparing the incidence of AD in a melanoma case vs. a healthy control population, demonstrated a significant negative [80] and non-significant positive association [81] between melanoma and AD, but these studies also did not correct for melanoma risk factors, except for a recent Dutch study that corrected for phototype. [82] To the best of our knowledge, none of the studies conducted on atopy and melanoma investigated the association between atopy and melanoma survival or progression. Therefore, we sought to examine the association of atopy-related variables and melanoma development and progression in a case-control population, using a validated questionnaire for AD diagnosis, controlled for the major confounders of melanoma.

4.3 METHODS

Data collection

Cases and controls were recruited from March 2004 to January 2008. Local ethics committees gave approval for the study (S number: 24960). A cohort of consecutive cutaneous patients with melanoma treated at the Department of Dermatology in University Hospitals Leuven and Antwerp University Hospital in Belgium were included after obtaining informed consent. All participants were informed about the aim (further insight in the cause of skin cancer and prevention of skin cancer) and content of the study (answering a questionnaire). They were also informed about the possible risks, the advantages of the study, the protection of their privacy, the right to information, and their right to withdraw from the study at any time. Two hundred and one consecutive melanoma cases were approached for the study. For each case, at least 2 controls were selected from the same street (or residence area) using the telephone book. Both groups were interviewed via a telephone call with a standardized questionnaire for the demographic data (age, sex and year of birth) and melanoma risk factors. The following major melanoma risk factors were registered: sunburn sensitivity (ability to burn after sun exposure in the beginning of the summer and after 1 h without sun protection), ability to tan (the ability to develop a tanned skin at the end of the summer after

chronic sun exposure), natural hair colour, eye colour, number of moles, sunburn as juvenile, sunburn in the last 5 years, ever sunbed use, and presence of melanoma in first-degree family members were noted. Potential participants were given a schematic diagram along with the introduction letter for the study to instruct them how to assess their number of moles. For the ascertainment of AD, a questionnaire, namely the UK Working Party's diagnostic criteria for AD was used [359] (Table I), which has been validated in several studies with a sensitivity of 80% and a specificity of 97% .[360]

Table 1. Questions used for the definition of the atopy related variables and their definitions.

UK Working Party's diagnostic criteria [a]

1. Did you ever had an itchy skin condition?
2. If yes, was this present before the age of two years?
3. Did you ever have eczema on the elbows or knees, ankles, neck, around the eyes or ears?
4. Did you ever have asthma (wheezing) or hay fever (sneezing, runny nose, itchy eyes in the summer)?
5. Did you have dry skin in the last twelve months?

Definitions

AD [b] : question number 1 = yes plus at least 3 other questions were answered with yes

Personal atopy: if AD is present or question no. 4 was answered with yes

Respiratory atopy: if question no. 4 was answered with yes

Extra Questions on

Active AD: Do you have eczema (red, irritated, itchy rash) on the knee or elbow folds currently?

Familial atopy: Did any of your siblings, children and/or parents ever had hay fever, eczema and/or asthma?

[a] adapted from Williams et al 1994 [361]

[b] AD = Atopic Dermatitis

Atopic dermatitis was defined as having an itchy skin plus at least 3 of the following criteria: itchy condition present before the age of 2 years, eczema on the elbows or knees, ankles, neck, around the eyes or ears, a history of asthma or hay fever and/or dry skin in the last year. The same questionnaire and 2 additional questions were used to define other atopy-related variables, namely personal atopy, respiratory atopy, active atopy and familial atopy (Table I). Personal atopy was defined as either having had AD or respiratory atopy. Respiratory atopy was defined as having had asthma or hay fever. Active atopy was defined as eczema (red, irritated, itchy rash) on the knees or

elbow folds at the time of the interview. Familial atopy was positive when any of the siblings, children and/or parents ever had hay fever, eczema and/or asthma. For the survival analyses, histopathological data and data on relapse date, date of last follow-up and date of death were collected from hospital records.

Statistical Methods

The analyses consisted of 2 parts. First, we analysed the possible correlation between atopy and presence of melanoma through comparison of the melanoma cases with the controls. Secondly, which concerns only the melanoma cases, the correlation between atopy and outcome (progression-free survival (PFS) and overall survival (OS)) was evaluated. Descriptive statistics, such as Fisher's exact and Mann-Whitney U tests, were used to evaluate distribution differences in characteristics between cases and controls with regard to demographic (age, sex) and major melanoma risk factors. A 1:1 matching was performed between cases and controls, matching for age, sex, and on all confounders found to be statistically significantly different between cases and controls in the descriptive analyses, more specifically sunburn sensitivity, tanning ability, natural hair colour, eye colour, number of moles, sunburn as juvenile, sunburn in the last 5 years, ever sunbed use, and presence of melanoma in first-degree family. Exact matches on the statistically significant confounders were made, except for age, where a maximum difference of 5 years has been allowed. Cases and controls within pairs were compared on atopy-related variables using exact McNemar's tests. Conditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CI). Given that fewer naevi were seen in patients with AD, and that naevi count was the strongest predictor for melanoma risk amongst the potential confounders (i.e. highest area under the curve (AUC) in univariable analyses; results not shown), a sub-analysis was performed within the group with no/few naevi count. For the outcome analysis within the melanoma cases, histopathological data on relapse date, date of last follow-up and date of death were collected. Patients were followed from the time of diagnosis. The cut-off date for follow-up was 31 December 2014. Survival period was calculated from the time of diagnosis until the last follow-up visit if the patient was still alive, or until date of death. Any cause of death is considered an event for the OS and death due to melanoma for melanoma-related death (MRD). Progression-free period was calculated from time of diagnosis until date of progression or any cause of death or last follow-up. Cox proportional hazards models were used to analyse the association of atopy with OS, MRD and PFS. Due to the low number of events, corrections for the confounder's age and staging were performed separately (referred to as bivariable models). Significance level was set at $p=0.05$. All tests were 2-sided. Analyses were performed in SAS (version 9.2 of the SAS System for

Windows). Copyright 2002 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks of SAS Institute Inc., Cary, NC, USA).

4.4 RESULTS

Out of the 1,058 healthy controls approached, there were 237 refusals and 225 individuals could not be reached for the actual interview. A total of 596 controls were therefore included. Of the 201 cases contacted, there was 1 refusal, and 5 could not be contacted. Seven cases were excluded because the diagnosis of melanoma could not be confirmed on revision, leading to a final total of 188 cases. Baseline characteristics found to be significantly associated with presence of melanoma in the univariate analyses were: age, sunburn sensitivity, hair colour, number of moles, sunburn as juvenile, sunbed use, and familial melanoma (Table II). Matching cases with controls for age, sex, and the aforementioned 7 confounders resulted in a final total of 116 pairs. For all atopy-related variables, a negative association was found with the melanoma risk, i.e. the prevalence was lower for the cases compared with the controls, resulting in ORs lower than 1 (Table III). However, only for personal atopy was this negative relation also significant (OR 0.53; 95% CI 0.30–0.96; p-value 0.04). In the univariate analysis of the major confounders for melanoma risk, high naevi count was found to be the most important. Therefore, a sub-analysis in the group with no/few moles was conducted. This subgroup analysis on 79 pairs confirmed a relevant difference between cases and controls in personal atopy (29% vs. 13%, p=0.03) and respiratory atopy (27% vs. 13%, p=0.06) (detailed data not shown). Cox analysis within the group of melanoma cases did not reveal a significant association between all atopy-related variables and PFS. The bivariable analysis, whereby a correction for staging or for age was conducted, did not alter the conclusions (Table IV). Also, for OS and melanoma-related death, no evidence was found for an association between atopy variables and outcome (data not shown).

Table II. Baseline characteristics of the study population.[a]

	CONTROLS	CASES	P-VALUE
N	596	188	
Age	57.4	52.2	**<0.001**
Gender			
Male	224	69	0.863
Female	372	119	
Sunburn sensitivity			
Yes	440	163	**<0.001**
No	156	25	
Tanning ability			
Brown/very brown	548	170	0.547
Never/very mild	48	18	
Hair color			
Light	262	111	**<0.001**
Dark	332	77	
Eye color			
Light	438	143	0.700
Dark	151	45	
Moles			
No/few	498	107	**<0.001**
Multiple/many	81	78	
Sunburn as juvenile			
No	354	83	**<0.001**
Yes	224	99	

Table II continued

Sunburn last five years			
No	507	158	0.812
Yes	85	28	
Ever sunbed use			
No	315	69	**<0.001**
Yes	280	117	
Familial melanoma (first degree)			
No	564	163	**<0.001**
Yes	24	23	

[a] Results of the univariate analysis of the differences in baseline characteristics between cases and controls.

Matching cases with controls for age, gender and the aforementioned seven confounders resulted in 116 pairs. For all atopy related variables, a negative association was found with the melanoma risk, i.e. the prevalence was lower for the cases compared to the controls, resulting in odds ratios smaller than one (Table III). However, only for personal atopy was this negative relation also significant (OR=0.53, CI: 0.30;0.96; p-value: 0.04).

In the univariate analysis of the major confounders for melanoma risk, high nevus count was found to be the most important. Therefore, a sub-analysis in the group with no/few moles was conducted. This subgroup analysis on 79 pairs confirmed a relevant difference between cases and controls in personal atopy (29% versus 13%, p=0.03) and respiratory atopy (27% versus 13%, p=0.06) (detailed data not shown).

Table III. The association of atopy related variables and melanoma development.

	CONTROLS	CASES	P-Value [a]	Odds Ratio[b] (95%CI)	P-value[b]
Atopic Dermatitis	11 (9.5%)	5 (4.3%)	0.21		
Yes				0.46 (0.16;1.31)	0.14
No				#	
Personal Atopy	35 (30.2%)	20 (17.2%)	0.04		
Yes				0.53 (0.30;0.96)	**0.04**
No				#	
Respiratory Atopy	32 (27.6%)	19 (16.4%)	0.07		
Yes				0.55 (0.30;1.02)	0.06
No				#	
Active Atopy	5 (4.3%)	2 (1.7%)	0.45		
Yes				0.40 (0.08;2.06)	0.27
No				#	
Familial Atopy	58 (50.0%)	52 (44.8%)	0.52		
Yes				0.82 (0.49;1.36)	0.44
No				#	

[a] Mc Nemar's test results after 1:1 matching on important confounders and gender.

[b] Results of the conditional logistic regression models.

reference category

Table IV. Results of Cox regression analyses for the association between atopy related variables and progression-free survival [a]

	Univariable Results Hazard ratio (95%CI)	P-value	Bivariable Results corrected for staging Hazard ratio (95%CI)	P-value corrected for staging	Bivariable Results corrected for age Hazard ratio (95%CI)	P-value corrected for Age
Atopic dermatitis	1.49 (0.35;6.30)	0.61	3.02 (0.69;13.34)	0.20	2.01(0.46;8.80)	0.39
Personal atopy	1.02 (0.41;2.50)	0.97	1.05 (0.41;2.69)	0.91	1.28(0.51;3.23)	0.61
Respiratory atopy	1.09 (0.45;2.68)	0.84	1.10 (0.43;2.80)	0.84	1.37(0.55;3.41)	0.52
Active atopy	0.00 (0.00;ND[b])	0.11	0.00 (0.00;ND[b])	0.21	0.00 (0.00;ND[b])	0.17
Familial atopy	0.00 (0.00;ND[b])	0.11	0.00 (0.00;ND[b])	0.21	0.61(0.28;1.37)	0.22

[a] Results of univariable and bivariable Cox regressions analyses. In the bivariable model, staging or age was added as a confounder. Since Wald tests are not valid in case there are no events for a specific level of the predictor, all reported p-values are obtained with likelihood-ratio tests (with 1 and 3 degrees of freedom for the effects of atopy and staging, respectively).

[b] ND=not defined.

4.5 DISCUSSION

In this clinically well characterized case-control population, we found a significant negative association between personal atopy and melanoma development, while no significant associations were found between atopy and melanoma outcome. A strength of this paper is the investigation of the relationship between atopy and melanoma development in a clinically well-characterized case-control population. A huge effort has been made to minimize selection bias of controls by choosing healthy, unrelated controls. Another strength of this paper is the use of an extensive questionnaire with which all major melanoma risk factors were meticulously registered, both in the melanoma population and control population. The study by Hajdarbegovic et al. [82] was the only previous study on melanoma and atopy that took account of phototype as confounder. Although phototype affects melanoma risk, it

is not the only confounder, as demonstrated in this study. When matched for important melanoma confounders (sunburn sensitivity, hair colour, number of moles, sunburn as juvenile, ever sunbed use, familial melanoma), age and sex, negative associations with melanoma development were observed for all 5 variables referring to atopy. This observed inverse association between atopy and melanoma is in line with previous literature which also found a lower risk of melanoma in atopics. [70,77,78,80,362] In our study, the correlation was only significant for personal atopy and we have to acknowledge that a strong claim about this correlation is not possible in the absence of a correction for multiple testing. Note that the power to detect differences between cases and controls depends on the prevalence, which differs strongly between the atopy-related variables. For example, in the matched analysis less than 5% of the controls have active atopy. To detect a significant reduction among the cases for this variable, a much larger study is required. Larger studies would help to further determine the nature of the link between melanoma and atopy. Another possible limitation is response bias. Although the response rate in the case group is high, that of the controls is not. It is probable that those who were contacted and had AD are more prone to cooperate if they think that there could be some personal benefit to be gained. It is also possible that recall bias might play a role in both groups. Recall bias might be present, since atopy tends to be more prominent at a young age. However, the case-control setting and random selection of controls should attenuate this bias. Also, the use of a validated questionnaire for AD diagnosis, whereby a combination of factors was considered before a positive diagnosis is made, might also reduce recall bias. The association between AD and melanoma OS, melanoma-related death, and recurrence-free survival were investigated, but this yielded no significant associations. It could be that the higher immunosurveillance associated with atopy may protect against development of melanoma, but is not enough to protect against progression. In addition, lack of any association should be regarded with caution, since there were relatively few events in our study population. In summary, this study suggests an inverse correlation between personal atopy and melanoma development when matched for important confounders. In addition, no evidence has been found for an association between atopy and melanoma survival or progression.

CHAPTER 5: SOLAR LENTIGINES ARE ASSOCIATED WITH BETTER OUTCOME IN CUTANEOUS MELANOMA

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5.1 ABSTRACT

The rising incidence of cutaneous melanoma and its stable high mortality rates despite innovative cancer care, require better prediction of its clinical outcome. In a large cutaneous melanoma population, we explored whether known clinical risk factors for melanoma susceptibility (nevus phenotype, phototype, family and personal history of melanoma and sun damage) affect melanoma outcome. A total of 1530 melanoma patients were included. Multivariable analysis adjusting for age, gender, melanoma stage, localization and subtype showed that familial melanoma, solar lentigines on head and neck, the back of hands, arms and shoulders were associated with better relapse free survival. The presence of atypical naevi was associated with increased risk of relapse. After Bonferroni correction, the correlation between presence of solar lentigines on the back of hands and arms remained the most robust and significant prognosticator for relapse free survival in cutaneous melanoma patients.

5.2 BACKGROUND

Cutaneous melanoma (CM) is the most common lethal skin disease worldwide, affecting mainly Caucasians[1].[16] The rising incidence and stable high mortality rates of CM, despite continuously improving therapies, calls for a better prediction of its clinical outcome.

The American Joint Committee on Cancer (AJCC) for melanoma staging identified clinico-histopathological risk factors that influence melanoma outcome, including Breslow thickness, mitosis, ulceration, sentinel node status, regional macro metastatic disease and distant metastatic disease. [170] Age, sex, localization and subtype have also been associated with melanoma outcome, although these are not implemented yet in the staging criteria. [363] In addition, several risk factors have been linked to melanoma susceptibility, including a high count of common nevi, presence of atypical nevi, sun exposure, low phototype, freckles, actinic damage, personal and family history of melanoma. [12–14,55]

Previous studies have investigated associations of individual melanoma susceptibility risk factors, including sun exposure, [241,245,364,365] family [366] or personal history of melanoma [61], and nevus phenotype [44,367] with melanoma outcome. Most of these studies are based on questionnaires and in-person interviews on sun exposure in relatively small populations. Two large studies with respect to the impact of nevus burden on melanoma outcome, [44,367] have reported conflicting results, while other susceptibility risk factors including skin lesions representative of chronic sun exposure, lentigines and actinic keratosis, and phototype were poorly studied, if analyzed at all. Therefore, we set out to perform a comprehensive exploratory analysis of a large panel of known clinical risk factors for melanoma susceptibility in a large and well-phenotyped contemporary Belgian melanoma population and examined their effect on melanoma outcome.

5.3 METHODS

Data Collection

The study was designed and approved by the local ethics committee of the University of Leuven in 2004. Patients with a histologically proven diagnosis of cutaneous melanoma who attended the outpatient clinic of the Dermatology Department at University Hospitals Leuven, Belgium, were phenotyped at the time of inclusion and prospectively followed for relapse till 2014. Patients with in situ, mucosal melanomas and unknown primary melanomas were excluded.

Trained dermatology residents performed a full skin examination for melanoma susceptibility risk factors. Sun damage signs (solar lentigines on head and neck, the back of hands, arms and shoulders, actinic keratosis, ephelides, idiopathic guttate hypomelanosis) were carefully annotated. Solar lentigines were classified as sharply defined, brown, round/polycyclic macular lesions. Ephelides were determined from a self-reported history of freckles during in-person interviews and through physical examination. Idiopathic guttate hypomelanosis, preferentially occurring on chronic sun-exposed sites was clinically assessed as small, asymptomatic round to oval hypopigmented macules. Childhood sunburn (sunburn before 18 years of age) was assessed through questioning. Sunburn was defined as a painful erythema, with or without blistering, peaking 24 h after sun exposure, was self-reported and verified by the medical doctor during history taking. Melanocytic nevi, defined as light/dark brown macular or elevated lesions ≥2 mm, were counted over the entire body and categorized into 2 groups: < 50 and ≥50. The presence and number of nevi on the dorsum of the feet, the scalp and the gluteal region were quantified and atypical nevi were defined according to the presence of at least ≥3 of the following parameters: ill-defined

borders, diameter ≥5 mm, variable pigmentation, irregular border and erythema. [47] Histologically proven atypical nevi were also included. Every patient's phototype was ascertained according to Fitzpatrick's classification. [53] Patients were categorized as having a positive family history if they reported at least one affected first- or second-degree relative with melanoma. Histopathological and relevant medical data were retrieved from the electronic hospital records. Melanomas were categorized into subtypes i.e. superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo malignant melanoma (LMM), and a group consisting of other rare subtypes (acrolentiginous melanoma (ALM), desmoplastic melanoma, spitzoid melanoma and nevoid melanoma).

The following clinical and histopathological established prognostic factors for CM were recorded for further analysis: age at diagnosis, sex, Breslow thickness, mitosis, ulceration, stage at diagnosis (according to AJCC 7th edition), localization, subtype and the above mentioned known clinical risk factors for melanoma susceptibility. In case of multiple melanomas, the most aggressive case was included for further analysis. Data on relapse date, last follow-up and date of death were collected. End of study date was set at December 31, 2014. Relapse free survival (RFS) was calculated from the time of melanoma diagnosis until date of relapse or end of the study date. Relapse was defined as any recurrence/relapse of the disease, (most often locoregional recurrence of melanoma such as lymph node metastasis or in transit metastasis). Relapses were confirmed by histological analysis and /or imaging.

Statistics

Data analysis was performed using SPSS statistical software (IBM SPSS Statistics 24; Chicago, IL). Missing data were excluded from the database, but in view of the extremely low number, no imputation was performed for missing data. Descriptive statistics were calculated for the clinicopathological variables. Differences in length of follow-up were taken into account using survival analysis technique, the Cox regression model. [368] The median follow-up time was 6.2 years (IQR 3.1-9.5 yrs).

The low number of melanoma-related deaths precluded a sufficiently powered analysis of the effect of melanoma susceptibility risk factors on melanoma cancer deaths. Therefore, only RFS was analysed. The curves of probability of recurrence were conducted with the Kaplan–Meier method and intercategorical differences were assessed with the log rank test. Univariable Cox proportional hazards models (Cox PH) were conducted to assess the associations between each of the clinicopathological variables and RFS. The multivariable analyses consisted of two steps. In the first step, results were reported from a multivariable Cox proportional hazards model for RFS, with age, sex, stage, localization and subtype as predictors. In the second step, this model is extended with each of the variables

(n=16) referring to nevus phenotype (n=5), phototype, family history, multiple primaries and sun damage (n=8) separately. A Bonferroni correction was applied, controlling the familywise type-I error for the results of these variables (derived from the 16 different models, p=0.003). Hazard ratios (HR) and associated 95% confidence intervals (CI) were reported. All values are two-sided, and a p-value less than 0.05 was considered statistically significant. To simplify the interpretation of the Cox proportional hazards results, we reorganized the variables stage, atypical nevi, phototype and multiple primaries into a new subgroup including stage IA, IB, II, III and IV, atypical nevi (yes versus no), phototype (3,4 versus 1, 2) and multiple primaries (yes versus no), respectively.

5.4 RESULTS

In total, 1530 patients were included in the study (931 females and 599 males). Demographic and clinicopathological characteristics of the study population are depicted in Table I. During the follow-up period of the study, 230 melanoma patients had a relapse and 169 patients died, but only 89 patients died from their melanoma. Univariable analysis of the clinicopathological associations with RFS is shown in Table II.

Multivariable analysis of prognostic factors for melanoma outcome

In the multivariable analysis, we adjusted for age, sex, stage, localization and subtype of melanoma for each predictor variable (Table II). We observed that family history of melanoma was associated with a better RFS (HR=0.61, p=0.048), while the presence of atypical naevi was associated with increased risk of relapse (HR=1.34, p=0.04). The presence of solar lentigines on the back of hands (HR=0.64, p=0.002), on arms (HR=0.66, p=0.002) and on shoulders (HR=0.70, p=0.005), was associated with improved RFS (Table II). After Bonferroni correction, the correlation between presence of solar lentigines on the back of hands and arms remained the most robust and significant prognosticator for RFS in CM patients. Figure 1 illustrates the effect of lentigines by showing the Kaplan Meyer estimates in some strata defined by age and/or stage. Note that this figure suggests that the effect of solar lentigines was stronger at a younger age. A further exploration within the multivariable Cox regression model indeed confirmed a significant interaction between solar lentigines on the back of the hands and age (p=0.036), but not with stage (p=0.35) (results not shown).

5.5 DISCUSSION

This explorative study in a large contemporary and carefully phenotyped Belgian cutaneous melanoma population investigated the association between known clinical risk factors for melanoma susceptibility and melanoma outcome. In a multivariable analysis, with correction for age, sex, melanoma stage, location and subtype, we

observed better relapse free survival in patients with solar lentigines. These findings indicate that within a group of patients with the same age, same sex and similar melanoma characteristics, those with solar lentigines have a better melanoma outcome compared to patients without solar lentigines. The absence of such correlation in univariable analysis is not surprising, because of the tight positive relation between age and presence of solar lentigines, confounding the correlation with clinical melanoma outcome. This confounding effect of age on protection associated with solar lentigines is also revealed in the Kaplan-Meier survival stratified by age and stage. Solar lentigines are signs of chronic photodamage and typical for photoaging. [369] [370]

Our observations of a salutary effect on relapse-free survival in a large melanoma population are consistent with other studies reporting a possible association between melanoma survival and elastosis, another sign of chronic photodamage . [371,372]

Berwick et al. also reported an inverse association between solar elastosis and melanoma-related death (MRD), independent of the strong association of MRD with melanoma site, lesion thickness and mitosis, [245] which, however, could not be confirmed in a later study. [246]

A possible association between sun exposure and melanoma outcome is further supported by epidemiological studies demonstrating a beneficial effect of sun exposure or sunny holidays on melanoma outcome [365,373] and an inverse association between average annual sunlight in different countries and melanoma mortality. [364]

Our observations of solar lentigines associated protection against relapse raise the intriguing question on the biological significance and potential mechanisms whereby chronic sun damage affects melanoma outcome. Several molecular pathways may contribute to better melanoma prognosis following chronic sun exposure. First, the sun is an important source of Vitamin D (VD) and active VD has pleiotropic anticancer effects, also on melanoma. [374]

Previous observational studies have shown that increased levels of VD at diagnosis predict a better outcome, and that metastatic melanoma and its poor prognosis are associated with decreased VD levels. [375] [376] Second, human skin possesses different photo adaptive mechanisms, which may counteract melanoma-associated mutations resulting in less aggressive melanomas. [377]

Finally, sun exposure may be associated with a healthier lifestyle and higher socioeconomic status. Another potential mechanism for the observed relationship between solar lentigines and survival are studies conducted on a melanoma variant, the melanocortin 1 receptor (*MC1R*). *MC1R* variants have been associated not only with

increased melanoma risk and melanoma risk factors such as freckling and solar lentigines but also with a better melanoma survival. It has been postulated that although *MC1R* variants are at increased melanoma risk through decreased (UV) DNA damage repair, *MC1R* variants also confer increased susceptibility to apoptosis and decreased cell proliferation, leading to a better survival. [157,293,378]

In addition to solar lentigines, we also investigated the association of family history and nevus phenotype, two other well-known melanoma susceptibility risk factors, with melanoma outcome.

First, family history is a risk factor for the development of primary melanoma and associated with inherited mutations in melanoma susceptibility genes, such as *CDKN2A*. [379] Previous studies have demonstrated that germline *CDKN2A* status is not only associated with melanoma susceptibility, but seems also to be important for prognosis. [249]

Consistent with these earlier findings a positive family history appears also to have a favorable impact on melanoma outcome in our population. Lead time bias whereby family members of melanoma patients may seek help earlier, and may therefore be diagnosed at an earlier stage, might be an explanation but needs to be confirmed. Although in our study a favourable outcome of familial melanoma remained significant in multivariable analysis, the association was weaker than in the univariable setting [380,381], emphasizing the need for follow-up studies.

Second, previous studies have investigated the impact of nevus phenotype (more specifically nevus count) on melanoma outcome. In our study, not only did we investigate nevus count, but also other characteristics of nevus phenotype (i.e. presence of atypical nevi and localisation of the nevi) and their relation with melanoma outcome. We observed that some characteristics of atypical mole syndrome [47], have a better outcome in the univariable model, while in the multivariable model, patients with atypical naevi have a higher risk for relapse. A recent study by Taylor et al. showed similar results [44] while Berwick et al. did not find a significant association between nevus count and melanoma-related death. [245] In contrast, Ribero et al. reported a beneficial association between high nevus count and survival in melanoma patients. [367] The discrepant results may be attributable to different methodologies and study populations.

We recognize the limitations of our study. First, we used a broad definition of family history and only a limited number of events were present for survival analysis in this population. Familial history was self-reported and was not histopathologically or genealogically confirmed. Second, the lack of sentinel node data in the majority of the patients might have led to a potential underreporting of stage III cases. However, only 10% (n=162) of our study

population had undergone a SLN biopsy which had been implemented only since the end of 2012 in our institution and a positive sentinel was observed in 15 patients (9.3%) only. Third, systemic melanoma treatments, administered predominantly for stage IV disease, were not taken into account. Finally, no adjustments were made for residual confounders including healthy lifestyle factors, socio-economic status and other causes of death such as cardiovascular disease. Despite all the above, the large number of participants followed in a single centre, the uniform study procedures and detailed clinical annotation of melanoma susceptibility risk factors validate the major findings of this comprehensive analysis.

In conclusion, in this exploratory study we report that clinical signs of cumulative photodamage are inversely associated with melanoma outcome after adjustment for clinical and histopathological prognosticators. This observation, however, does not prove a causal relationship between sun exposure and melanoma outcome, but may offer clinicians an additional tool to better risk stratify their melanoma patients. Further analyses are necessary to understand the biological, molecular and genetic mechanisms that account for these correlations.

Table 1: Clinical and histopathological parameters of all patients

Demographic characteristics	
Patients, n(M/F)	1530 (599/931)
Age mean (SD)	52(15)
Primary tumor characteristics	
Stage at diagnosis, n (%)	
IA	492 (32)
IB	607 (40)
II	355 (23)
III + IV	39 (3)
ND	37(2)
Localisation, n(%)	
Extremity	792(52)
Trunk	532(35)
Head and Neck	204 (13)
Subtype,n(%)	
SSM	1106 (72)
NM	146 (10)
LLM	49 (3)
Others	227 (15)
Clinical risk factors for melanoma susceptibility	
Nevus phenotype	
Number of common nevi, N (<50, ≥50)	1054/378
Presence of atypical nevi, N (no, yes)	938/478
Nevus on the dorsum of the feet, N (no, yes)	1110/420
Nevus on the scalp, N (no, yes)	1433/97
Nevus on the gluteal region, N (no, yes)	873/657
Phototype, N (1 and 2/3 and 4)	1074/417
Family history, N (no, yes)	1243/139
Multiple primaries, N (no, yes)	1441/89
Sun damage, N (no, yes)	
Solar lentigines: head and neck	614/916
Solar lentigines: dorsum of hands	633/897
Solar lentigines: arms	548/982
Solar lentigines: shoulder	562/968
Actinic keratoses	1264/135
Ephelides	1135/395
Idiopathic guttate hypomelanosis	1405/125
Sunburn before 18 years	907/621

Abbreviations: SD: Standard Deviation; ND: not defined; SSM: Superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo malignant melanoma .[a] Age is expressed in years . Missing values are not depicted.

Table 2: Survival association result from univariate and multivariable Cox proportional hazards models

	Univariable Cox proportional hazard model			Mulitvariable Cox proportional hazard model		
	Relapse free survival			Relapse Free Survival		
Variable	HR	95% C.I.for HR	p value	HR	95% C.I.for HR	p value
Gender : male vs female	1.595	1.272-2.001	**<0.001**	1.250	0.969-1.613	0.085
Age[a] continuous	1.040	1.032-1.049	**<0.001**	1.032	1.022-1.041	**<0.001**
Stage at diagnosis :			**<0.001**			**<0.001**
IB	4.043	2.475-6.604	**<0.001**	3.780	2.303-6.206	**<0.001**
II	10.753	6.666-17.348	**<0.001**	8.358	5.077-13.759	**<0.001**
III+IV	10.979	5.690-21.184	**<0.001**	11.396	5.877-22.096	**<0.001**
IA(ref)						
Localisation :			**0.014**			0.105
Trunk	1.173	0.908-1.515	0.223	1.356	1.019-1.805	**0.037**
head and neck	1.606	1.165-2.213	**0.004**	1.065	0.718-1.580	0.755
Extremity(ref)						
Subtype :			**<0.001**			0.377
NM	3.059	2.228-4.201	**<0.001**	1.221	0.865-1.724	0.255
LMM	1.730	0.963-3.109	0.067	1.526	0.777-2.995	0.220
others	1.862	1.411-2.459	**<0.001**	1.202	0.891-1.621	0.228
SSM (ref)						
Nevus phenotype (yes vs no)						
Number of common nevi: ≥50 vs <50	0.883	0.667-1.168	0.382	1.321	0.974-1.791	0.073
Presence of atypical nevi	0.879	0.684-1.129	0.312	1.336	1.017-1.755	**0.038**
Nevus on the dorsum on the feet	0.655	0.499-0.858	**0.002**	0.924	0.697-1.226	0.585
Nevus on the scalp	0.693	0.412-1.166	0.167	0.804	0.456-1.416	0.450
Nevus on the gluteal region	0.735	0.582-0.927	**0.009**	1.034	0.801-1.336	0.797
Phototype (3,4 versus 1,2)	1.003	0.774-1.301	0.980	0.852	0.650-1.118	0.249
Family history (yes vs no)	0.552	0.342-0.892	**0.015**	0.609	0.372-0.996	**0.048**
Multiple primaries (yes vs no)	1.086	0.696-1.694	0.716	0.993	0.628-1.567	0.974
Sun damage (yes vs no)						
Solar lentigines: head and neck	1.266	0.994-1.611	0.056	0.790	0.609-1.024	0.075
Solar lentigines: backs of hands	1.298	1.014-1.662	**0.039**	0.643	0.488-0.846	**0.002**
Solar lentigines: arms	1.129	0.879-1.451	0.341	0.659	0.505-0.859	**0.002**
Solar lentigines: shoulder	0.993	0.780-1.263	0.951	0.701	0.547-0.899	**0.005**
Actinic keratosis	1.299	0.865-1.949	0.207	0.647	0.418-1.002	0.051
Ephelides	0.722	0.553-0.941	**0.016**	1.035	0.784-1.366	0.808
Idiopathic guttate hypomelanosis	1.024	0.680-1.544	0.908	1.021	0.664-1.572	0.923
Sunburn before 18 years	0.790	0.625-0.999	**0.049**	1.117	0.866-1.440	0.394

Abbreviations: ref: reference; C.I.: confidence interval; HR: Hazard Ratio; SSM: Superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo malignant melanoma. [a] Age is expressed in years. The multivariable analyses consisted of two steps. In a first step (n=291/1489), results were reported from a multivariable Cox proportional hazards model for RFS, with age, gender, stage, localization and subtype as predictors. In a second step, this model is extended with each of the variables (n=16) referring to nevus phenotype (n=5), phototype, family history, multiple primaries and sun damage (n=8) separately. A Bonferroni correction was applied controlling the familywise type-I error for the results of these variables (derived from the 16 different models, p=0.003).

CHAPTER 6: MELANOMA SUSCEPTIBILITY VARIANT rs869330 IN THE *MTAP* GENE IS ASSOCIATED WITH MELANOMA OUTCOME

Derived from: Marasigan V, Güvenç C, van den Oord JJ, Stas M, Boecxstaens V, Bechter O, Wolter P, Lambrechts D, Garmyn M. Melanoma Research. 2019 Dec; 29(6):590-595. doi: 10.1097/CMR.0000000000000578

6.1 ABSTRACT

Background: The rising incidence of cutaneous melanoma (CM), an aggressive skin cancer, emphasizes the need for novel biomarkers to guide personalized care and better predict outcome. Genetic factors including germline risk variants are promising candidates for this aim.

Objective: We explored the association between germline risk variants and melanoma outcome in a large genetically homogenous Belgian melanoma population, focusing on single nucleotide (SNP) which generated the highest association with melanoma susceptibility.

Methods: Between 2004 and 2014, blood samples of 1088 patients with histologically confirmed cutaneous melanoma patients were collected and genotyped for 9 variants. Cox proportional hazard models were used to assess the association between each SNP and relapse-free survival (RFS) and overall-survival (OS), adjusted by age, gender, melanoma stage, site, and subtype.

Results: We identified significant associations for rs869330 (in the methylthioadenosine phosphorylase - *MTAP* gene) with OS (HR=0.760, p-value= 0.048, 95% CI: 0.580-0.998) and RFS (HR=0.800, p-value=0.020, 95% CI: 0.650-0.970).

Conclusion: This exploratory study is the first to show a significant association between the rs869330 variant (in the *MTAP* gene) and outcome in a large cutaneous melanoma population.

6.2 INTRODUCTION

Cutaneous melanoma (CM) is a deadly type of skin cancer, the incidence of which is rising significantly. Although considerable efforts have been made to mitigate the disease through prevention, screening programs, and novel therapies, recent calculations from cancer registries in six susceptible populations show a rising incidence with at

least 3% per year between 1982 and 2011, except for Australia. Even more alarmingly, the incidence is estimated to further climb in all six populations until 2031. [382]

When diagnosed and treated in a late stage, CM markedly diminishes overall survival, because of its high propensity to metastasize and resistance to systemic therapy. Although many relapses in CM arise from tumors with high Breslow thickness, the best known prognostic factor for CM relapse [170], a subset of melanomas with low Breslow thickness ultimately relapse with metastatic disease. Hence, there is a need for additional biomarkers, including genetic factors, to more accurately predict the prognosis of melanoma patients at the time of diagnosis.

Genetic factors such as genes identified by linkage analysis of family cases and single nucleotide polymorphisms (SNPs), as identified by genome-wide association studies (GWAS), are implicated in the risk to develop melanoma. [383] Previous studies have also shown significant associations between SNPs and melanoma outcome, like SNPs in DNA repair genes, metastasis-driven genes and immunomodulatory genes, as reviewed by Vogelsang et al. [383] Moreover, melanoma susceptibility SNPs have been associated with prognosis, as reported in an exploratory study by Rendleman et al. [383], investigating the association of 108 melanoma risk variants with melanoma outcome, in 891 patients with melanoma. Other studies started from a single gene and showed associations between genetic melanoma-susceptibility variants, *MC1R, PARP, ASIP and IRF1* [157,293,299,384] and melanoma outcome.

In this exploratory study, rather than limiting our scientific strategy to a hypothesis-driven analysis of a single polymorphism, we took advantage of a recently reported fine mapping study by Barrett et al. [151] This study refined association signals for genomic regions harboring melanoma-susceptibility variants, to a set of SNPs most significantly associated with melanoma susceptibility. We have carefully analyzed these variants in our study and investigated their association with melanoma overall survival and relapse-free survival in a Belgian melanoma population.

6.3 PATIENTS AND METHODS

Data collection

Patients who attended the Dermatology Department of the University Hospitals Leuven in Belgium between March 2004 and December 2014 were eligible for the study after obtaining histological proof of melanoma diagnosis and a written informed consent which includes donating blood samples for genetic analysis. Most patients were White individuals of European ancestry (94%), that i.e., all four grandparents were Europeans.

Clinical details, histopathological data and date of progression, death or last follow-up were sourced from electronic hospital records. Exclusion criteria were in situ and mucosal melanomas. For patients with multiple melanomas, the study entry was triggered by the lesion with the highest Breslow. This study was carried out in accordance with the Helsinki guidelines for clinical trials and received approval from the institutional Ethics Committee of the University Hospital Leuven.

Selection and genotyping of single nucleotide polymorphisms

SNPs that were most significantly associated with melanoma susceptibility, as fine mapped by Barrett et al. [151], were selected. These were 18 SNPs in total. Two additional SNPs, reported to be associated with melanoma outcome by Rendleman et al, rs7538876 and rs9960018, were also included. [309] From these 20 SNPs, six SNPs could not be designed using SEQUENOM MassArray technology (see below) leaving 14 SNPs for the study. We extracted DNA from peripheral blood samples of 1088 participants and genotyped germline DNA using SEQUENOM MassArray technology (VIB Center for Cancer Biology, KU Leuven, Belgium) which provides flexible, effective, rapid, and accurate SNP genotyping. Of the 14 SNPs that were genotyped, five SNPs did not meet the post genotyping criteria, which included exclusion of SNPs with minor allele frequency (MAF) less than 1%, exclusion of SNPs with a call rate less than 95% as well as exclusion of SNPs with a significant deviation from Hardy Weinberg equilibrium (HWE) ($p<0.001$). Finally, genotyping data of nine SNPs were available for downstream analysis (Table 2).

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was assessed with $\chi 2$ test. Cox proportional hazards models were used to assess the associations between each SNP and recurrence-free survival (RFS) and overall survival (OS). Survival curves for RFS were generated using the survival plots. For each SNP separately, a multivariable Cox proportional hazards model was used adjusting for age, sex, stage, localization and subtype. Results for sex, age, stage, localization and subtype were obtained from the multivariable model with only these variables as predictors. SNP associations were analyzed under both a co-dominant model and an additive genetic model.

End of study date was 31 December 2014. OS was defined as the time from diagnosis of the primary melanoma until death owing to any cause, date of last follow-up or end of the study, whichever was first. RFS was calculated from the time of diagnosis of the primary melanoma until date of relapse or date of last follow-up or the cutoff date, whichever was first. A p-value less than 0.05 was considered statistically significant. Correction for multiple testing

was conducted by implementing the Bonferroni method. Statistical analyses were conducted with SPSS version 22 (IBM SPSS Statistics 22; Chicago, Illinois, USA). Because of the low number of events (only 59 patients died from their melanoma), no analyses were performed for melanoma-related death.

6.4 RESULTS

We included 1088 patients that consented for this study of whom 665 (61,1%) were women and 423 (38.9%) men. Mean age at diagnosis was 50 years. The diagnosed stages (according to AJCC 7th edition [170]) and respective distribution were as follows: IA (33.5%), IB (38.4%), II (23.0%), III and IV (2.5%); 2.7% of patients had an unknown stage. The primary tumor was located in 598 (55.1%) patients on the extremities, in 356 (32.8%) on the trunk and in 131(12.1%) on the head and neck. Superficial spreading melanoma (SSM) was the predominant histological subtype of CM in 804 (73.9%) of patients, followed by nodular melanoma (NM) [92 (8.5%)] and lentigo malignant melanoma (LMM) [34(3.1%)]. Other histological subtypes of melanoma consisted of 158 (14.5%) cases (summarized in Table 1).

In this exploratory study, we tested the associations of nine melanoma risk variants with RFS and OS (Tables 2 and 3). In general, no significant associations were seen between the susceptibility variants and melanoma outcome (Supplementary Table 1, Supplemental digital content 1, http://links.lww.com/MR/A114)), except for one variant. The additive model indeed showed that rs869330 (GG) in methylthioadenosine phosphorylase (*MTAP*) was significantly associated with OS [hazard ratio (HR)=0.759, P=0.048] in the univariate analysis. In the codominant model, rs869330 GG-carriers were associated with a favorable OS prognosis (HR=0.546, P=0.044; Table 2). In the multivariable analysis, adjustments were made for age, sex, melanoma stage, localization and subtype for each SNP. In the additive model, the association between rs869330 and OS remained significant after correction for these prognostic covariates (HR=0.751 and P=0.048). Interestingly, after correction, the association with RFS also became significant (HR=0.793 and P=0.022), highlighting the biological relevance of genomic risk factor. In multivariable analysis of the codominant model, rs869330 GG-carriers again exhibited a favorable prognosis, both for OS (HR=0.513, P=0.037) and RFS (HR=0.61, P=0.019; Table 3). Figure 1 shows the effect of the *MTAP* variant on RFS for patient in stage IB (a) and stage II (b) based on the multivariable Cox regression after correction for age, gender, stage, location and subtype. Patients with rs869330 GG exhibited a favorable prognosis compared with the patients without rs869330 GG for RFS. This protective effect was observed in stage IB (HR:4.588, P<0.001) and stage II (HR: 9.902, P<0.001).

6.5 DISCUSSION

In this large contemporary CM population, we demonstrated that the rs869330*GG variant in the *MTAP* gene is associated with a significantly better overall survival and relapse free survival after correction for prognostic covariates including age, sex, melanoma stage, localization and subtype. The variant rs869330 is located in chromosome 9p21 in the *MTAP* gene, which encodes an enzyme involved in polyamine metabolism and restoration of adenine and methionine. Earlier mapping studies of this region reported a strong link for the SNP rs869330 with melanoma susceptibility [385]. Other SNPs in the *MTAP* region such as, rs7023329, rs10811629 and rs10757257 have also been found to be associated with melanoma susceptibility [367,386,387]. However, none of the earlier studies have investigated the effect of *MTAP* variants on clinical outcome in melanoma patients. SNPs in *MTAP* were also found to be associated with underlying clinical risk factors for melanoma development, including low phototype (rs10811629) [367] and high nevus count (rs4636294, rs7023329, rs10757257 and rs 10811629). [387] Interestingly, Ribero et al. recently reported better melanoma outcome in patients with a high nevus count .[247] We could not replicate the findings of Ribero in our melanoma population (unpublished data). Moreover, the rs869330 in *MTAP*, was not associated with nevus phenotype characteristics in our melanoma population (V. Marasigan, C. Güvenc, J.J. van den Oord, M. Stas, V. Boecxstaens, O. Bechter, P. Wolter, D. Lambrechts, M. Garmyn, unpublished data).

In our study, the rs869330*GG *MTAP* gene variant was associated with significantly improved melanoma outcome. The *MTAP* gene and the *CDKN2A* gene, a well-known tumor suppressor located near *MTAP*, are co-deleted in many cancers and this is correlated with poor survival as reported for peritoneal mesotheliomas [387] and non-small cell lung cancer. [388] However, there is growing evidence that the *MTAP* gene might act as an independent tumor suppressor and that *MTAP* protein expression is inversely correlated with melanoma progression in tissue samples ranging from benign nevi to metastatic melanomas. [389] *MTAP* loss has also been demonstrated to be part of a signature of seven biomarkers, independent of *CDKN2A*, that can predict melanoma outcome. [390] *MTAP* deletion leads to loss of the MTA-catabolizing enzyme MTAP, which in turn results in higher MTA levels. Loss of MTAP and subsequent accumulation of MTA characterize several tumor entities. Henrich et al. demonstrated that MTA contributes to immune evasion, by inhibiting proliferation, activation, differentiation and effector function of antigen-presenting T cells. [391] We speculate that a similar immune modulatory effect on antigen-specific T-cells may modulate melanoma growth and this needs targeted follow-up studies.

In our melanoma population, the better survival in rs869330*GG positive patients might be attributable to better sustained MTAP levels or activity and hence better-preserved tumor suppressor function of *MTAP*. The mechanism of this altered tumor suppression function is unclear and beyond the scope of the present large genotype-phenotype long-term follow up study. One possibility is that altered (i.e. less) susceptibility to epigenetic silencing might play a role. The rs869330 variant is located in a non-coding region and has not been functionally investigated before but might have a regulatory role that affects transcription [392]. Further molecular investigations are therefore needed to determine whether or not the rs869330*GG allelic variant for *MTAP* affects protein expression levels during tumor progression, and maintains *MTAP* expression in the deepest invasive parts of melanoma in comparison to wild-type allele carriers.

We could not replicate the findings of Rendleman and colleagues in our melanoma population as the rs7538876 variant was not associated with survival. One of the explanations could be the difference in genetic background, as the melanoma population in the study of Rendleman et al. consisted of a genetically different and more diverse population. [309]

One of the strengths of our study is the homogenous White population. Almost 95% of the study population had all four grandparents of European ancestry. In addition, because of the fact that this is a single center study, data collection, treatments and follow-up regimens were very consistent in our patient population.

We acknowledge the limitations of our study. First, in the longitudinal analysis, systemic therapies were not taken into account. The therapeutic landscape in melanoma has substantially changed over the past years and overall survival has significantly improved. It is therefore possible that patients, who entered later into the study, could have influenced the survival analysis. However, as only very few patients were treated in an adjuvant setting, the effect of systemic therapy on RSF is negligible. Second, our study population only harbors a few higher stage melanoma cases and the lack of sentinel node data in the majority of the patients might cause potential underreporting of stage III cases. Consequently, only few patients died from melanoma in the follow-up period of the study, making the power too low for melanoma-related death analysis. Third, our selected cohort of European ancestry up to the third generation might limit generalizability to other populations. Finally, the association between rs869330*GG and melanoma outcome did not pass the Bonferonni correction in this explorative study. Therefore, we need to replicate our findings in additional melanoma populations with more advanced stages of the disease

at higher risk, for relapse and melanoma related death.

Conclusion

This study is the first to show an association between *MTAP* rs869330*GG genotype and better prognosis in a large contemporary CM population. Follow-up studies are now required to further elucidate the biological significance of the *MTAP* rs869330 variant in patients with melanoma.

Table 1: Patient and primary tumor characteristics of the study population

Demographic characteristics	
Patients, n(M/F)	1088 (665/423)
Age mean (SD)	51(15)
Primary tumor characteristics	
Stage at diagnosis, n (%)	
IA	364 (33.5)
IB	418 (38.04)
II	250 (23.0)
III + IV	27 (2.5)
ND	29 (2.7)
Localisation, n(%)	
Extremity	598(55.1)
Trunk	356(32.8)
Head and Neck	131(12.1)
Subtype,n(%)	
SSM	804(73.9)
NM	92 (8.5)
LLM	34 (3.1)
Others	158 (14.5)

Abbreviations: SSM: Superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo maligna melanoma. ND: Not defined

*Age is expressed in years and Breslow thickness in mm. Missing values are not depicted.

Table 2: Survival association results from genetic univariate Cox proportional hazards model

				Overall survival				Relapse free survival		
SNP	**Gene**	**Frequency (%)**	**N(#events/total)**	HR	95% C.I.for HR	p value	**N(#events/total)**	HR	95% C.I.for HR	p value
rs12596638	*FTO*		108/1080				220/1083			
GG		64.5				0.971				0.938
AG		31.8		1.051	0.700-1.578	0.811		1.046	0.787-1.392	0.755
AA		3.7		1.001	0.365-2.743	0.999		1.078	0.549-2.118	0.827
additive				1.031	0.738-1.44	0.858		1.043	0.827-1.316	0.722
rs16891982	*SLC45A2*		108/1078				220/1081			
GG		95.8								
GC		4.2		1.23	0.540-2.802	0.623		0.925	0.498-1.719	0.805
rs1858550	*PARP1*		108/1065				219/1068			
CC		47.5				0.935				0.840
CA		42.3		1.07	0.720-1.592	0.737		0.942	0.711-1.248	0.677
AA		10.2		0.983	0.499-1.934	0.960		0.879	0.534-1.447	0.612
additive				1.021	0.767-1.358	0.889		0.939	0.764-1.156	0.555
rs443099	*MX2*		108/1078				220/1081			
TT		40.3				0.194				0.370
GT		46.9		1.249	0.839-1.859	0.274		1.175	0.884-1.562	0.266
GG		12.8		0.686	0.334-1.407	0.304		0.911	0.587-1.413	0.676
additive				0.96	0.727-1.267	0.774		1.012	0.836-1.225	0.906
rs6059655	*ASIP*		108/1072				220/1075			
GG		80.6				0.964				0.982
GA		18.1		0.951	0.579-1.561	0.842		0.976	0.690-1.381	0.890
AA		1.3		0.825	0.115-5.926	0.848		1.094	0.271-4.418	0.899
additive				0.942	0.601-1.476	0.795		0.988	0.719-1.358	0.941
rs73283859	*MC1R*		108/1080				220/1083			
GG		80				0.97				0.914
GC		18.8		0.94	0.573-1.543	0.807		0.928	0.654-1.318	0.676
CC		1.2		0	0.000-5.481E+192	0.960		0.000	0.000-6.073E+146	0.947
additive				0.842	0.527-1.347	0.474		0.843	0.604-1.177	0.316
rs7538876	*PADI6*		108/1076				220/1079			
GG		42.1				0.551				0.857
GA		47.3		0.890	0.594-1.333	0.571		1.065	0.803-1.412	0.664
AA		10.6		1.231	0.677-2.24	0.496		1.118	0.704-1.776	0.635
additive				1.036	0.775-1.385	0.813		1.060	0.863-1.302	0.578
rs869330	*MTAP*		107/1078				219/1081			
AA		28.6				0.132				0.187
GA		49.9		0.846	0.556-1.288	0.437		0.900	0.663-1.223	0.502
GG		21.5		0.546	0.303-0.985	**0.044**		0.699	0.475-1.029	0.069
additive				0.759	0.578-0.998	**0.048**		0.843	0.699-1.017	0.074
rs9405705	*IRF4*		107/1074				218/1077			
GG		76.1				0.543				0.326
GC		21.3		1.272	0.830-1.950	0.270		0.992	0.721-1.364	0.958
CC		2.6		0.000	0-2.165E+200	0.959		0.418	0.133-1.311	0.135
additive				0.973	0.667-1.418	0.885		0.875	0.666-1.148	0.334

Abbreviations: ref: reference;

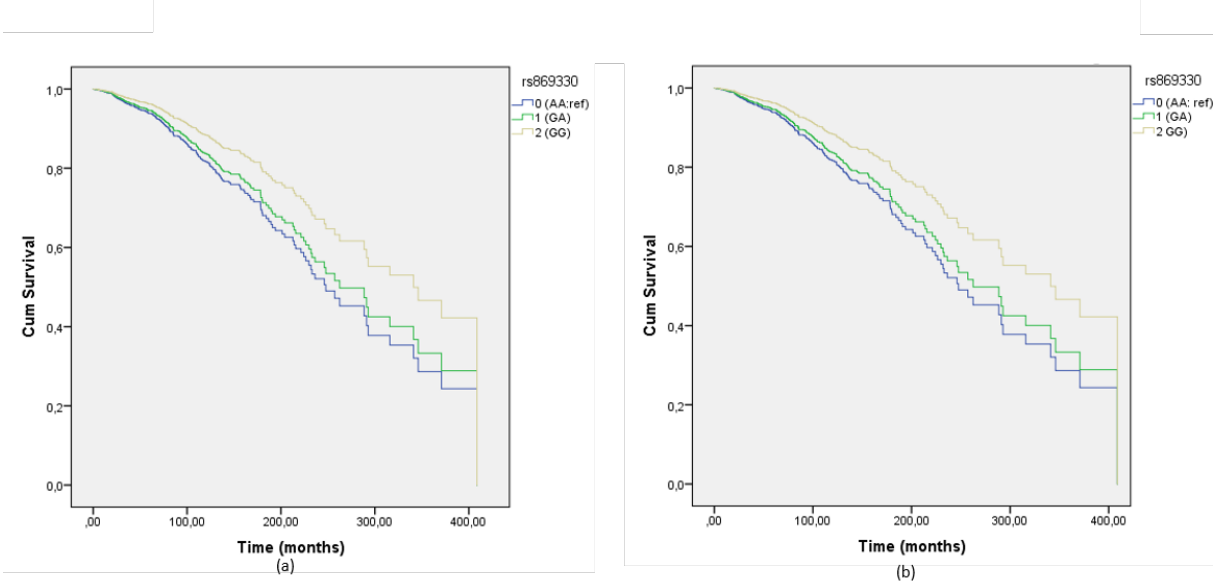
C.I.: confidence interval ; HR: Hazard Ratio; SSM: Superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo maligna melanoma

Table 3: Survival association result from multivariable Cox proportional hazards models

			Overall Survival				Relapse free survival		
Prognostic variables for melanoma outcome	**Frequency (%)**	**N(#events/total)**	HR	95% C.I.for HR	p value	**N(#events/total)**	HR	95% C.I.for HR	p value
Gender : male vs female		102/1053	1.409	0.950-2.088	0.088	209/1053	1.158-0.878	0.878-1.528	0.300
Age[a] continuous			1.038	1.023-1.054	**<0.001**		1.025	1.014-1.036	**<0.001**
Stage at diagnosis					**<0.001**				**<0.001**
IB			4.073	1.694-9.792	**0.002**		4.483	2.425-8.289	**<0.001**
II			10.527	4.402-25.176	**<0.001**		9.820	5.286-18.242	**<0.001**
III+IV			26.143	9.352-73.077	**<0.001**		10.498	4.554-24.198	**<0.001**
IA(ref)									
Localisation					0.062				**0.026**
Trunk			1.621	1.033-2.542	**0.035**		1.425	1.033-1.966	**0.031**
Head and neck			1.625	0.896-2.947	0.110		1.612	1.029-2.528	**0.037**
Extremity (ref)									
Subtype					0.928				0.862
NM			1.079	0.604-1.925	0.798		1.042	0.683-1.590	0.848
LMM			1.320	0.466-3.744	0.601		1.413	0.627-3.184	0.405
others			1.140	0.671-1.936	0.629		1.059	0.736-1.522	0.759
SSM(ref)									
SNP (gene)									
rs869330 (MTAP)		101/1049				208/1049			
AA	28.6				0.101				0.060
GA	49.9		0.906	0.583-1.408	0.660		0.880	0.639-1.21	0.431
GG	21.5		0.513	0.273-0.962	**0.037**		0.61	0.404-0.923	**0.019**
additive			0.751	0.566-0.998	**0.048**		0.793	0.650-0.966	**0.022**

Abbreviations: ref: reference; C.I.: confidence interval ; HR: Hazard Ratio; SSM: Superficial spreading melanoma ; NM: nodular melanoma; LMM: lentigo maligna melanoma. *Age is expressed in years. For each clinical parameter separately, a multivariable Cox proportional hazards model was used adjusting for age, gender, stage, location and subtype. Results for gender, age, stage, localization and subtype were obtained from the multivariable model with only these variables as predictors.

Figure 1: Illustration of effect of rs869330 for patient in stage Ib (a) and stage II (b) based on the multivariable Cox regression after correction for age, gender, stage, location and subtype.



CHAPTER 7: GENERAL DISCUSSION

7.1 MPM: GENETIC AND/OR ENVIRONMENTAL?

Our first aim was to investigate risk factors for multiple primary melanomas (MPM) versus single primary melanomas (SPM). In the third chapter, we demonstrated that compared to SPM, MPM patients were more likely to have a positive familial history for cutaneous melanoma, dysplastic nevi and light hair color. There is evidence that these three factors are intertwined. For example, multiple primary melanomas have been linked to a higher prevalence of *CDKN2A* mutations in population based studies. [393] *CDKN2A* mutation carriers in turn are positively associated with multiple melanomas in a familial melanoma vs sporadic population. [394] Furthermore, atypical nevi are more common in familial melanoma (versus sporadic melanoma) and *CDKN2A* carriers (versus non *CDKN2A* carriers). Non-*CDKN2A* mutation carriers on the other hand are correlated with light-coloured hair and atypical naevi in a familial melanoma vs sporadic population. [394] Of note, the study conducted by Marghoob et al. was excluded as their cases consisted only of patients with classic atypical mole syndrome (CAMS) which is a combination of nevi phenotypes. This syndrome was associated with familial melanoma and certain melanoma risk phenotypes.[341] The inclusion of CAMS only, a highly select group, as cases in Marghoob et al.'s study can be viewed as a form of selection bias and given that the other studies that were included in our analyses had unselected cases of the two groups that we were interested in, we have excluded the aforementioned study. As for the study by Siskind et al., which reported hazard ratios, their raw data would have secured inclusion in our study. However, this data was not available in their article. [62]

MPM and genetics

Genetic variants have been demonstrated to be related to pigmentation and melanoma. In addition, individuals that harbour CM susceptibility genes are more likely to have familial melanoma and MPM. [395] Major CM susceptibility genes are *CDKN2A, CDK4* and *MC1R* (see Introduction). Interestingly, polymorphisms in rare oculocutaneous albinism (*OCA*) genes such as *TYR, OCA2, TYRP1* and *SLC45A2* are prevalent in familial cutaneous melanoma and might confer a moderate risk for melanoma. [396] Of note, *OCA* genes regulate pigmentation of the eyes, hair and skin. Moreover, other 9p21 SNPs were found to be associated with CM risk

(three SNPs in the *ACO1* gene) and tanning ability (*IFNW1* and *TUSC1* variants) in melanoma families. [397] In a MPM cohort, a combination of familial melanoma and germline *CDKN2A* (versus controls) is not only linked to a lower age of diagnosis of a second melanoma but also has an increased risk for more than two melanomas. In the same MPM population, those who harbour *CDKN2A* germline alteration had a threefold higher risk of a dismal outcome versus familial melanoma or sporadic wild-type melanomas. [296]

MC1R gives signals to adenylate cyclase through a G protein coupled receptor. This stimulates micropthalmia-associated transcription factor (*MITF*) gene that binds the alpha melanocyte stimulating hormone (α-MSH), which is involved in melanin production. [398] *MITF* has been marked as a medium-penetrance melanoma susceptibility loci. [399] [400] Conversely, *MITF* variations are present in 4-21% of all somatically mutated melanomas, [108,401] whereby its frequency is positively related to disease stage. [110] The combination of low phototype (i.e. red hair and fair skin), genetic susceptibility, and environmental risk factors such as solar exposure contributes to a higher melanoma risk. [402] Moreover, melanin synthesis by melanocytes in the suntan response has been linked with DNA damage of keratinocytes via the p53-MC1R pathway. [403] Another pigmentation locus is the *E318K* variant in microphthalmia associated transcription factor (*MITF*) gene which dysregulates normal MITF, a transcription factor involved in regulation of melanocytes. [404] Carriers of this *MITF* variant tend to not only have a high nevi count, number of freckles and non-blue eye color but are also prone to having multiple melanomas and/or family history of melanomas. The *MITF* locus confers a twofold melanoma risk making it a moderate risk variant. [400] [399]

Atypical naevi, genetics and MPM

With regard to atypical nevi, it was previously mentioned that the risk of melanoma development is increased in those with atypical nevi vs none in melanoma prone families [65,66]. It has been reported that individuals who harbour at least one *CDKN2A* variant versus none had been reported to more likely have atypical nevi but not benign nevi. [405] An Italian cohort study demonstrated the correlation of dysplastic nevus associated melanoma (NAM) and harboring atypical naevi versus common naevi NAM. [406] In addition, dysplastic NAM was also found to be associated with *MC1R* non-synonymous variants and thinner tumor lesions. We hypothesize that these associations might explain the link between dysplastic nevi and MPM since thinner lesions might lead to a better survival, therefore giving more time to accumulate subsequent melanomas. Moreover, it can be posited that since

individuals with *MC1R* variants tend to be diagnosed with melanomas at a younger age, this may also confer more time to develop additional primary melanomas. For example, *CDK6* and *XRCC* polymorphisms have been revealed in melanoma families to be associated with the presence of dysplastic nevi but not with CM after adjusting for age, sex, CMM, and *CDKN2A* status which suggests that other factors, either genetics or environmental, might be needed for DN development into melanoma. [407] Notably, *CDK6* variants have a stronger link with DN in families harboring *CDKN2A* mutations implying synergy and the requirement of additional genetic mutations for melanoma development. Furthermore, there is still an ongoing debate on whether dysplastic nevi are predestined to develop into a melanoma or whether it is induced by solar exposure over the years or a combination of both. A model for melanoma risk in melanoma prone families implementing *CDKN2A* gene mutation status and clinical risk factors such as DN, total nevi and solar injury revealed a positive interaction between DN (and total nevi) and *CDKN2A*. [136] However, solar damage did not significantly make the model better. Of noteworthy, a gene analysis through exome sequencing reported a much decreased mutational load for dysplastic naevi and a lower frequency for UV mutational signatures compared to melanomas suggesting that melanoma progression necessitates more genetic alterations that affect major tumor suppressors. [408]

Environmental Risk Factors and MPM

As for the interplay between genetic risk factors and solar risk factors, a study of 53 melanoma families also reported an increased melanoma risk in those that harbour *CDKN2A* mutations, dysplastic nevi, high total nevi count and history of sunburn with sunburn conferring the highest risk of approximately five fold. [66] It should be noted however that melanoma families might already have the genetic predisposition to develop melanoma and exposure to an environmental melanoma risk factor such as sunburn might confer additional risk, which are likely to explain these findings. The role of immunosuppression and carcinogenesis had been reviewed in the Introduction chapter and will be discussed further in this chapter.

Our findings suggest a possible strong role of genetic influences for multiple melanoma development. However, since few studies had been conducted comparing sun behaviours and sun damage between MPM and SPM, environmental influence cannot be excluded. We hypothesize that a cumulative risk for melanoma develops when genetic susceptibility and UVR exposure are both present.

7.2 ATOPY AND MELANOMA RISK: ADAPTIVE VERSUS INNATE IMMUNITY OR BOTH?

In our second study, a general opposite association was observed between atopy and melanoma risk. However, the only statistically significant association was seen between a personal history of atopy which remained significant even after stratifying for nevi count. No association was seen between atopy and melanoma outcome among melanoma cases. But first, the implications of the new AJCC staging criteria for melanoma outcome are discussed below since disease stage has been used as a variable in three of our studies.

Implications of AJCC Staging Criteria Changes

Recently, the 7th edition of the AJCC staging criteria had been replaced by the 8th edition. As for the implications for our conclusions, we believe that our results would still be robust with the new AJCC staging criteria. At the time of data collection, the AJCC 7th version was valid and therefore utilised. Furthermore, disease stage was only used to correct for confounding in our study and is not part of the explanatory variables of interest as disease stage is already a well-established prognostic factor as evinced by published literature. In addition, clinical staging remained the same in both versions. Moreover, restaging to the 8th edition has no impact to our stage III patients as we did not use subgroups but had taken stage III patients as a whole group.

Also in support of our statement, Verver et al. showed that restaging from the 7th to the 8th AJCC edition only increased pT1b patients by 7%. [409] The difference in SLNB positivity rate for pT1b and pT1a patients was 3% and was non-significant and was supported by relatively identical survival curves between the two AJCC versions. Another study population showed a transfer of 8% from T1a to T1b after implementation of AJCC 8th edition. [410] In terms of DFS, there is a significant difference of 5% between T1a and T1b for AJCC 8 versus a non-significant 2% for AJCC 7. Both studies demonstrated that the low percentages of population shift when AJCC 8 is utilised instead of the 7th version implies that the staging criteria change does not affect our results significantly.

Atopy and Cancer

Findings on the association between atopy and cancer in general have been discordant. [411,412] Some studies have reported a role for immunology in some cancer types. Two studies showed a statistically significant decreased incidence for atopic dermatitis in oncology patients versus controls. [413,414] A recent publication showed a

significantly reduced overall incidence of allergies in breast cancer patients compared to controls, in particular 83% lower in those with AD. [415] Different studies support these findings whereby AD was associated with reduced cancer risk in general and of various types such as gastric cancer [416], gliomas [417], CNS cancers, pancreatic, lung cancers and NMSCs. [418] A meta-analysis found lower risks between AD and certain cancer types (i.e. CNS, pancreatic cancer, lung and respiratory cancers) among case-control studies. [419] However, the three studies on AD and melanoma that were analysed in the same meta-analysis did not show any significant correlation between AD and melanoma.[82,414,420] Interestingly, Vojtechova et al. found an association between atopy with allergen specific IgE or positive skin prick test and prostate cancer in their meta-analysis but found no association between asthma, hay fever or any allergy and certain cancers such as colorectal, breast and prostate cancer. [421] Moreover, a meta-analysis [413] and a prospective study [422] reported inverse weak associations between IgE and cancer.

In contrast, Arana et al. conducted a population study which demonstrated a higher overall risk for cancers in those with AD and for specific cancer subtypes e.g. melanoma, NMSC and lymphoma after correcting for age and gender. [79] D'Arcy et al. also showed AD to be linked with lymphoma. [423] Hagstromer et al. found an elevated cancer risk in those diagnosed with AD versus the general population in Sweden including brain, lung, lymphoma and NMSC.[77] Melanoma incidence was more or less similar in the two groups. A meta-analysis found an elevated statistically significant association between AD and keratinocyte carcinoma, central nervous system, renal and pancreas cancers in cohort studies. In contrast, lower risks were found between AD and certain cancer types (i.e. CNS, pancreatic cancer, lung and respiratory cancers) among case-control studies. Notably, there was a lack of association between AD and melanoma. [419] Olesen et al. reported an increased risk of cancer consisting predominantly of keratinocyte carcinomas in a cohort diagnosed with AD. Their group however noted an absence of melanoma cases in the same population demonstrating a negative association between AD and melanoma. [78]

Hagstromer et al. conducted a retrospective cohort study on cancer incidence in a Swedish AD population.[77] They reported an incidence ratio of 0.6 (observed vs expected cases: confidence interval 0.3-1.2) for melanoma among this AD cohort consisting of 15 666 AD patients. The authors warned that the results of the study should be interpreted with caution because of chance findings due to few observed patients. Olesen et al. reported an absence of melanoma cases in a cohort study of 6275 AD patients who were hospitalized in Denmark.[78] The number of expected cases of melanoma was 2.4 demonstrating a negative association between AD and

melanoma. Furthermore, a non-significant negative association was found by the group Synnerstad et al. in a retrospective Swedish cohort study which examined the risk of melanoma in an AD population consisting of 6280 patients.[70] In contrast, Arana et al. found a significant positive association (incidence rate ratio= 1.71; CI:1.67-1.76) between AD and melanoma risk.[79] This study looked into the incidence of melanoma, as the first occurrence of cancer, in AD vs non-AD subjects. The authors did comment that they lack data concerning the treatments AD patients had undergone, which might have contributed to the positive association, and the possibility of detection or surveillance bias because AD patients tend to seek medical help more often than non-AD patients. [79]

Unlike our study, all four studies used hospital diagnosis codes for AD and melanoma whereas ours used a validated questionnaire and histological verification of the melanoma diagnosis by expert melanoma dermatopathologists. Also, we corrected for more prognostic factors for melanoma. In contrast to three aforementioned studies, their studies were conducted with only AD patient cohorts. [70,78,424] As for Arana et al., although their study included non-AD patients like our study, it did not take any other melanoma risk factors into account similar to the other three aforementioned studies. [70,78,79,424]

Two studies compared the incidence of atopy in a melanoma versus non-melanoma subjects. The investigation conducted by Kolmel et al. demonstrated a significant negative association. [80] The diagnosis of atopic dermatitis was made through an interview. This German study reported three subjects out of 331 in the melanoma group who had eczema as opposed to 11 in the 380 controls. However, no OR was mentioned, only the p-value ($p<0.05$) unlike our study. The calculated OR though was 0.31(95%CI:0.08-1.11, p-value: 0.07). Furthermore, atopy was analyzed as one group whereas our study made distinctions in the different presentations required for the diagnosis of atopy. The other study done by Beral et al., which analysed the association between melanoma and cutaneous factors (among which was eczema), rendered a non-significant positive association (relative risk 1.1).[81] A history of eczema was obtained through a questionnaire although no further details on which questions were asked specifically were mentioned by the authors.

Atopic disposition other than AD and having allergies were also found to be linked with lower cancer risk overall [422,425] and certain subtypes such as lung cancers, [426,427] GI cancers, [428] breast cancer [429] and NMSC. [425] Vena et al. found lower cancer risk in lungs, GI and urinary tract in men with a history of allergy whilst the same is true for females regarding digestive and reproductive systems. [430] Allergic rhinitis but not asthma or AD was demonstrated to be less likely in individuals affected with breast[413], skin [413], pulmonary [413], GI cancers[423,431] and gynecological

cancers.[423] Other studies reported allergic rhinitis to be more likely in lung cancer patients but a lack of association with hematological cancers. [432–434] However, Hwang et al. reported atopic individuals to have an elevated risk with brain cancer, renal and urinary bladder tumours. Moreover, overall cancer risk was not increased in atopic patients and seems to be influenced by gender, age, type of atopic symptoms and cancer site. [425] Interestingly, the more atopic symptoms one has, the lower the general cancer risk.

Possible Explanations of Inconsistent Findings

Several other reasons might explain the inconsistent findings regarding the association between atopy and melanoma. One of them is the nature of atopy assessment whereby different methods were utilised such as questionnaires and interviews predominantly but also IgE levels and skin prick testing. [421,435] Questionnaires and interviews are prone to recall bias and misclassification of exposure. [436] Also, measurement of IgE levels do not always correlate with clinical allergy signs. One of the main stumbling blocks for atopy-cancer association studies is the lack of unity in the definition of atopy. [73] However, although four cohort studies that looked specifically into the melanoma incidence in AD vs non-AD populations had used hospital diagnosis codes for AD and melanoma, their results were still inconsistent. [77 78 70 79]

In addition, other atopic immunological reactions may vary on each site. [422,437,438] Neoplasma located on body surfaces with direct interaction to the outside environment (skin, GI, respiratory) may show a more obvious opposite association between allergy and tumor development whereas it might be less obvious in body locations which lack this exposure. [411] It has been proposed that the endodermal origin of cancers in the lungs and GI tract can be heavily exposed to carcinogens which might explain some of the disparity. [413] Moreover, the time between atopic symptoms or reaction and cancer development should be considered since there had been study findings that imply that the shorter the time period, the better the protective effect. [439] Also, case-control designs are dependent on the method of selection controls for its validity which might affect outcome. [412,418]

Inconsistent findings might also be due to the dual capacity of Th2 pathway to stimulate and mitigate immunosurveillance. Interestingly, it has been shown that adult AD patients have less nevi compared to non-AD subjects. [76] In vitro studies demonstrated that certain pro-inflammatory cytokines (eg T-helper 2 (Th2) type), interleukins (IL1,4,5) and TNF-alpha inhibit human melanocyte expression and proliferation. [440,441] It can be inferred

from these data that AD might protect against melanoma since having a large number of nevi is a risk factor for melanoma development.

In addition, IgE produced indirectly by Th2 cells via B cells has been suggested to play a role in carcinogenesis. IgE production is stimulated by epithelial cells in distress. Increased levels of IgE may therefore be part of immune-surveillance and can be potentially triggered by neoplasms. IgE can counteract tumor development by identifying cancer antigens and by inducing cytotoxic cells e.g. mast cells, eosinophils, basophiles and natural killer cells. [442–444] This supports the utilisation of cancer subtype-specific IgE as immunotherapy. However, there had been studies suggesting that immune cells from those who are atopic are less effective in counteracting tumor compared to non-atopics due to the fact that relevant receptors are already attached to allergen-specific IgE. [445] Also, concentration levels of IgE might not correlate with immunological activity and instead might be a signal of inadequate immunological response utilising the Th2 pathway which mitigates cytolytic activity. However, it can also be construed that Th1 process attenuation might obstruct carcinogenesis since Th1 is an inflammatory response and inflammation has been proposed to be one of the hallmarks of cancer. [437,438] Another hypothesis that has been put forward is that IgE levels might be correlated with another anti-tumor determinant that might act site or subtype specific. [446] In addition, cancer therapy such as chemotherapy or radiation therapy has been suggested to influence IgE levels usually by increasing it. [447–449] Interestingly, AD has allergic (high IgE levels) as well as non-allergic factors (e.g. autoimmune diseases and infections) and has therefore more diverse determinants compared to allergic rhinitis. [413]

AD and immune response

Innate immunity reacts to non-specific antigens with rapid response within hours. It consists of dendritic cells, natural killer (NK) cells, complement, phagocytes, macrophage, neutrophil, eosinophil and basophils, although this list is not exhaustive. Eosinophils are present in the skin lesions of AD patients. Furthermore, acute AD involves the participation of innate immune cells such as dendritic cells (CD1+ and CD 11+) which intensifies in chronic disease. [450]

Adaptive immune response consists of humoral immune response (B cells) and cell mediated immune response (T cells). The discussion hereafter will concentrate on the latter group. Of note, IgE production by B cells in AD

patients has been discussed above.

Cell mediated immune response

Cell mediated immune response consists of CD8 and CD4 cells. Adaptive immunity reacts to specific pathogens and responds usually within days. Pro-inflammatory cytokines, interleukins, TNF-alpha, and T-lymphocytes are present in the skin lesions of AD patients. T-lymphocytes, a major player in cancer immunity, are divided into two groups namely CD8+ and CD4+ cells. The former has direct anticancer function whilst the latter reject tumors indirectly. [451] Furthermore, acute AD not only involves the participation of T cells (CD3+) but also innate immune cells such as dendritic cells (CD1+ and CD 11+) which intensifies in chronic disease. [450]

Autoreactive CD8+ T cells, which produce IL4 and IFN-gamma, had been demonstrated in AD cases. [452] CD8 T cells recognize MHC class I molecules on cells which include antigen presenting cells (APC) and exerts direct cell mediated cytotoxicity upon activation. [453] However, to restrain immune response and avoid excessive tissue damage, most T cells undergo apoptosis. CD8 T cells' role in cancer is well established. CD8+ T cells are regarded as more vital to inducing cancer cell death than CD4+ T cells. [454] Nevertheless, CD8 T cells can lose their function and become exhausted during chronic infections and cancer. CD4 T cells not only play a vital role in supporting CD8 T cells but also exhibit direct anti-tumoral effects through cytokines, stimulate B cells and dendritic cells and coordinate innate immunity. CD8+ T cells need to be activated and primed to become cytotoxic T lymphocytes (CTLs). [455] [456] In this process, innate immunity (dendritic cells (DCs) and natural killer cells (NK)) cooperate with adaptive immunity (CD4+). CTLs are effector CD8+ T cells with cytotoxic capacity. [457] CTLs can initiate tumor invasion and stop cancer cells. CTLs are capable of killing tumor cells through interferon gamma and tumor necrosis factor alpha. [458] IFN gamma is a proinflammatory cytokine produced by CTLs, M1 and Th1 cells. [459] They enhance adaptive immunity [460] and inhibit angiogenesis in tumor cells. [461] On the other hand, IFN gamma also have pro-cancer capabilities.[462] [463] For example, IFN gamma can promote T cell exhaustion and tumor progression. IFN gamma can also stimulate tumor cells evasion of the immune system by producing PD-L1, STAT3 and IDO1. In turn, they stimulate IFN gamma production by T cells leading to T cell exhaustion.

Interaction between innate and adaptive immunity in countering carcinogenesis

Interestingly, CD4 T cells have been demonstrated to be capable of countering tumors and metastases through

Th2 cells.[464–467] Some studies postulate that CD4 T cells' anticarcinogenic effect is facilitated by eosinophils and that eosinophils can induce tumor regression. [464,467] Eosinophils are part of the innate immunity. They are not only frequently seen in AD lesions but are also Th2 mediated in the adaptive immune response. Eosinophils have the capability to induce the release of proinflammatory cytokines, initiate adaptive immunity and cause tissue damage. [468] They can also secrete cytotoxic mediators such as cationic protein, basic protein and peroxidase. [469] However, the role of eosinophils in oncology still needs to be investigated further.

CD4 T cells have been demonstrated to collaborate with NK cells in order to optimize antitumorigenic capacity. Interestingly, it has been found that natural killer T cells are expressed in skin lesions of AD. [470] NK cells are a type of lymphocytes and are also part of the innate immunity. NK cells have dual cancer activities. Undifferentiated NK cells are anti-cancerous whilst differentiated NK cells are pro-cancerous through PD receptor expression. [459] Innate immunity with NK cells and DCs as effectors is faster than adaptive immunity. [471] NK cells can recognize cancer cells which lack or have low MHC expression. [469] They are capable of producing IFN-ɣ and IL-4 and mediates cytotoxicity via perforin, granzyme B and FasL. Cancer cells however can develop escape mechanisms to avert NK cell attack or mechanisms that can stimulate defective NK cell production. [469]

Innate immune cells such as dendritic cells (DCs) also facilitate the development of adaptive immune response.[469] For example, DCs produce various cytokines and chemokines such as TNF-alpha, IFNs, IL-1/4/6/10/12/23, which also influence T cell responses. [472] DCs can exert CD4+ T cell to differentiate to antigen specific effector T cells. [473] Furthermore, basophils can produce atopy related molecules and IL-4 and also has anti-cancer potential but the process require investigation. [474]

TNF alpha, which can be found in AD skin lesions, can be both pro and anti-cancerous. TNF alpha produced by CTLs exerts anticancer activity through angiogenesis inhibition and through direct or indirect (TGF beta) stimulation of apoptosis. [461] TGF beta has an immunosuppressive function within the tumor. On the other hand, TNF alpha can obstruct CD8 T cell tumor infiltration and cancer cell immune response through indirect influences to tumor micro environment. For example, TGF beta produced by cancer cells can counteract NK cells and CTLs. [475]

Naïve CD4 T cells (Th0) can evolve into various subtypes with sometimes contradicting functions. [476] CD4+ cells can produce T-helper (Th) cells: Th1 and Th2. Th2 cells are mostly present in acute lesions of atopic dermatitis whereas Th1 predominate in chronic AD lesions. Th1 cells, which mainly produces IL-2 and IFN gamma plays a

role in cellular immunity. Th1 cytokines reactivates cellular senescence, which dampens tumor development. [477] IL-12 is an important Th1 cytokine. It has the capability to activate both innate and adaptive immune systems. IL-12 enhances CTL priming and differentiation, CD4 T cells priming and production of NK. [478] Together with interferon gamma, IL-12 can counteract metastasis.[460] Moreover, IL-12 promotes polarization of macrophages into antitumor M1 phenotype.[479]

As for Th2, it predominantly express IL-4, IL-5 and IL-10 which are essential for humoral immunity.[480] In the acute phase, Th2 is the main process which switches to Th1 in the chronic phase.[450] Th2 cytokines (including IL-4, IL-5 and IL-13) levels are elevated in acute AD lesions especially in extrinsic type.[481] In chronic lesions, Th1 cell production of IFN-gamma is higher whilst IL4 and IL13 levels are decreased which is mostly present in intrinsic type. [482] [481] Especially IL4/IL13 related molecules downstream are implicated in AD severity. [483]

It is assumed that CD4+ cells, specifically Th1 cells, play only a supportive role in CD8+ activation.[484] However, there are findings suggesting that CD4+, Th2 in particular, have more potent anti-carcinogenic characteristics than CD8+ T cells and Th1 cells. [464–466] Murine experiments showed that CD4+ T cells were able to eliminate CD8+ resistant tumor cells by recognizing tumor specific antigens, expression of Th2 cytokines such as IL-4 and partnering with natural killer cells. In addition, CD4 cells can induce CD8+ T cell priming through cytokine release after interacting with MHC class II antigens. [485] CD4 T cells can influence cellular and humoral immune response by providing vital support to CD8 T cells and B cells. [486] Moreover, CD4 T cells, NK and DCs can collaborate to induce CD8+ T cell priming. CD4+ cells can also induce DCs which produce IL12 and 15 stimulating proliferation and differentiation of CTLs. [487,488]

In contrast, inflammation does not only activate immunosurveillance but can also be pro-oncogenic due to its ability to induce genetic changes through impairment of DNA repair mechanisms and pivotal cell-cycle checkpoints. [71] Inflammation can also sustain a pro-tumor environment through stimulation of cell proliferation, angiogenesis and impediment of apoptosis. [489] In addition, the role of the different components of the immune system on carcinogenesis is still not fully elucidated. For example, cytokines such as TNF alpha, which has been originally shown to suppress cancer development, have been implicated in skin carcinogenesis.[490] Other cytokines such as IL-10, which is upregulated in AD [491], also possess a dual role in cancer development.[492,493]

The exact mechanisms of what makes elements of the immune system to be pro-oncogenic in one situation and

anti-cancerous in another is currently undergoing investigation.

Our atopy study has limitations of which some have been discussed in Chapter 4. Other limitations include the following. First, the number of melanoma related death (N=10) and progression events (N=21) observed in our study were few and as such may potentially increase the risk of a random effect. Second, residual confounding that can affect overall survival such as lifestyle factors (smoking, lack of physical activity and BMI)[494] or other diseases e.g. infectious, cardiovascular, autoimmune etc.[495] may exist as these can vary between the comparison groups. Other confounding factors for cancer have not been taken into account in our study such as low socioeconomic factors because the data was not available.[496] Third, other risk factors associated with AD might have influenced our findings such as (higher) education levels and (male) gender. [413,429] Although our study has accounted for gender, there was no data on the education level in our population. It is possible that AD patients who are well educated might also belong to the higher economic class and might tend to seek medical help for their disease. However, health resources are easily accessible for the majority of the Belgian population. Furthermore, a potential confounding by CM molecular subtype is possible, but this data was not available at the time of analysis. Last but not least, different allergy types may reflect different processing of toxins and subsequent levels.[73]

To our knowledge, our study is the first case-control study to assess the incidence of AD in a melanoma case-control group with a valid questionnaire. The UK working party diagnostic criteria for AD has been validated in several studies with a sensitivity of 80 percent and a specificity of 97 percent. [360] Melanoma PFS was not related with AD in our study. Of note, the control group consisted of unmatched melanoma patients without AD. One study reported no significant differences in Breslow-thickness and metastases between atopic and non-atopic melanoma patients. [83] However, a direct comparison regarding melanoma outcome using survival statistical analysis between AD and non-AD patients in melanoma patients was not conducted unlike our study.

Some recommendations for future studies consist of gathering other information such as bio-banked samples and data on other immunological discriminators. [422] In addition, other variables should be considered such as IgE levels, allergy type, cancer type and site and risk factors related to either atopy or melanoma.

7.3 MELANOMA CLINICAL AND HISTOPATHOLOGICAL RISK FACTORS AND OUTCOME: GENETICS AND UV EXPOSURE

Our (aim 3) study further confirmed the prognostic influences of gender, age, disease stage, site and subtype on relapse free melanoma survival. However, only age and stage remained statistically significant in the multivariable analysis.

We also observed statistically significant associations between family history and atypical nevi on one hand and melanoma outcome on the other hand, underlining a possible role of genetics in melanoma outcome. Moreover, non-UV melanomas may occur and are usually located in the eyes, on mucosal and acral sites implying a more prominent role for genetics in their etiology. [497] Although the exact etiology of atypical nevi is still under investigation, correlations with family history and *CDKN2A* gene mutations have been reported.

As discussed in previous chapters, published studies have conflicting results regarding nevus phenotype and scarce studies have been conducted between atypical nevi (or features of atypical mole syndrome) [47] and melanoma outcome. [44,245,367]

We observed that some characteristics of atypical mole syndrome [47], have a better outcome in the univariable model, while in the multivariable model, patients with atypical naevi have a higher risk for relapse. A recent study by Taylor et al. showed similar results [44] while Berwick et al. did not find a significant association between nevus count and melanoma-related death. [245] In addition, Ribero et al. reported a beneficial association between high nevus count and survival in melanoma patients. [367] Most dysplastic nevi are biologically stable and are generally seen as a cutaneous melanoma precursor. However, dysplastic nevi have been correlated with all of familial melanomas and 60% of sporadic melanomas. [498]

Furthermore, NM and other subtypes were observed to have a worse relapse free survival compared to SSM in the univariate analysis consistent with previous studies. LMM was not statistically significantly (p=0.067, 95% CI 0.96-3.11, HR=1.730) different from SSM regarding relapse free survival. In the multivariate analysis, subtype did not remain significant. These findings suggest that other potentially stronger risk factors may influence the association between subtype and melanoma outcome.

With regard to site, published literature demonstrated favourable melanoma outcome in melanomas situated on the hand and extremities. In our univariable analysis, melanomas located on the extremities have better survival

compared to head and neck melanomas in the univariate analysis and compared to trunk melanomas in the multivariable analysis. Interestingly, a recent publication of an Australian prospective cohort study reported a better melanoma specific survival of lesions located on forearms/hands and anterior upper arms compared to upper back after correction for other prognostic factors. [499] The same study revealed increased MSS for intermittent and chronically sun-exposed locations compared with rarely exposed body sites after adjustment for other variables. Also, the Australian cohort showed a decreased prognosis for posterior scalp albeit compared to trunk melanomas in a multivariate analysis which was almost similar to our study whereby head and neck melanomas were seen to confer a worse relapse free survival compared to extremities in the univariate analysis but not in the multivariate analysis.

There are several explanations for why site might be correlated with melanoma outcome. As suggested by some authors, the visibility of these sites might play a role making self-examination more challenging since lesions on difficult to see locations such as the back might be easily missed. [499] [500] [501] [502] Another possibility is that sun exposure of these sites leads to beneficial effects of vitamin D. UV exposure is the only carcinogen listed by the WHO that also can be beneficial through vitamin D production which has been shown to have anticarcinogenic effects. However, although vitamin D can counter tumorigenesis and progression, other factors such as irreparable DNA damage and host susceptibility to melanoma may tip the balance in favour of cancer. Last but not least, host response factors affecting cancer biology might play a role. This has been discussed in the introduction and earlier this chapter. [246,503,504]

In our study, solar lentigines as a sign of benign photodamage on moderately intermittent exposed sites such as the back of hands, arms and shoulder are correlated with better survival in a melanoma population. Chronic UVR exposure sites such as the head and neck as melanoma site were associated with poor prognosis in the univariable analysis whereas head and neck as site of presence of solar lentigines was not statistically significantly correlated with favourable survival. Of note, melanomas on the trunk, a site which has been presumed as either on-chronic or highly intermittent sun exposure site, has a statistically significant worse prognosis compared to melanomas situated on the extremities in our study, unlike melanomas on the head and neck.

Moreover, melanoma subtypes associated with chronic sun exposure generally have a good prognosis, whereas nodular and acral melanoma which are associated with low levels of sun exposure tend to have worse survival. For our analysis of the association between solar lentigines and melanoma outcome, we have taken site and

subtype into account and adjusted for these variables. Although the main aim of our study was not focused on melanoma sites and survival but rather melanoma clinical risk factors and survival, our finding of a chronic photodamage clinical sign and a better relapse free survival aligns with Gordon et al.'s study results after correction for prognostic variables including melanoma sites. [500] In addition, although our study also lacked information on individual UVR exposure which can be obtained through questionnaires but can be prone to recall bias, our metric of a more objective clinical sign of photodamage is less susceptible to biases.

Our third study also demonstrated other solar damage indicators such as ephelides and sunburn before 18 years of age to be protective against relapse in the univariate analysis but not in the multivariable analysis. Actinic keratoses was not found to be statistically significant with relapse free survival in both types of analyses. One might surmise that it was borderline significant at best and that it is leaning towards a protective trait in the multivariable analysis.

The inverse association between clinical photodamage signs and melanoma outcome might be counterintuitive at first. Various measurements of sun exposure including questionnaire on outdoor activities, satellite measures, latitude as proxy for solar exposure had been used to study its relation with melanoma mortality and reported conflicting results . [505] These methods include using variables such as season of diagnosis, sun hours and geography (latitudes). Contradicting results have been reported and Berwick et al. concluded that there is not enough evidence for a role of season in melanoma diagnosis and melanoma mortality. Ecological studies according to Berwick et al. also have conflicting findings and suggested that "the weight of evidence no longer supports a strong positive association between latitude or UV exposure and mortality from melanoma". [505] It has been suggested that ecological data might be an indicator of current rather than cumulative lifetime sun exposure when the UV exposure is inferred from the place of diagnosis or death due to migration of study patients. [506] However, this does not apply in our case since ours is not an ecological study.

It has been argued that melanoma patients might avoid the sun at the time of diagnosis or after diagnosis and therefore might miss the advantages of solar exposure on mortality. [505] Gandini et al. reported that sunny holidays prior and post a diagnosis of melanoma are correlated with decreased Breslow and lower relapse rates respectively in an Italian melanoma population. [365] The authors offered vitamin D serum levels, socio-economic factors and factors related with sun seeking individuals as an explanation of their findings rather than a causal effect of solar exposure on survival. [365] Stable low vitamin D levels might mask association with vitamin D levels. The dual effect

of UV exposure which not only induces vitamin D and its anticarcinogenic effects but UV also exerts immunosuppressive effects. In the case of carcinogenesis, the balance might tip over towards the harmful UV effects leading to melanoma, which is an immunogenic tumor.

Berwick et al. demonstrated an opposite correlation between self-reported history of severe sunburns, high levels of intermittent sun exposure and presence of solar elastosis in the proximity of the melanoma lesion. [505] The study also gathered information on skin examination management by the patients, their doctors and partners which suggested that skin awareness might have contributed to better survival. In addition, the authors posited that the increased survival might have been due to vitamin D or due to development of less aggressive melanomas [507] incited by previous solar exposure via DNA repair [246] and stimulated pigmentation which provides protection. [403] High levels of vitamin D was shown to confer improved survival through inhibition of proliferation and induction of apoptosis. [508,509]

More individual measurements such as sun exposure based on occupation had been also conducted. Meta-analyses on outdoor occupation suggest a decreased risk of melanoma death unlike indoor workers. [13,87,510] Regarding melanoma risk and clinical solar damage factors, one study which only included women, reported sunburn to be correlated with all major melanoma body sites and solar keratoses to be associated with a higher risk of head and neck melanoma. [511] As stated earlier, one may surmise that solar damage signs are more specific and reflect individual exposure better compared to ambient solar measurements and are more objective than questionnaires which are prone to recall bias.

On the other hand, solar lentigines are benign signs of chronic sun damage. [369] [370] Lentigines are mostly present on chronic sun exposed skin such as the face, forearms and back of the hands and unaffected by seasonal changes. [512] Solar lentigines have been related to older age and older age has been reported to confer a worse melanoma survival. [512] In our study, we have corrected for age along with other melanoma prognostic factors which makes our results robust. [229] Of note, solar radiation has been purported to play a major pathogenic role in the increasing incidence of skin cancers including melanoma. [513]

Interestingly, other signs of chronic sun damage such as solar elastosis had been associated with better melanoma outcome [243] which some authors hypothesized might be due to longer telomere lengths. [514] In addition, we have mentioned in the discussion of our aim 3 article that other studies had reported better melanoma prognosis with

regard to solar exposure (measured as chronic photodamage signs such as elastosis, ambient sunlight or sunny holidays).[245,364,365,371–373]

Solar lentigines are associated with better outcome after multivariate analysis in our study and the effect is even larger in younger age groups. A possible role of the anticarcinogenic effects of *MC1R* (photoadaptation) and vitamin D variants may exist. UV induced melanomagenesis involves several processes which can be interrupted at each point for example by vitamin D levels. A vitamin D receptor variant involved in VDR expression had been reported to be associated with worse melanoma specific survival [303] whilst another vitamin D variant (a vitamin D binding protein) was found to be correlated with better MSS.[290] The latter was even demonstrated to have an effect on gene methylation status. [290] However, there is a lack of literature on the relationship between solar lentigines in younger age and vitamin D levels.

It is plausible that solar lentigines, sometimes also called as pigmented spots, could be related to increased vitamin D levels as solar lentigines are a sign of sun damage resulting from sun exposure. However, vitamin D levels can fluctuate and are subject to a multitude of factors. A meta-analysis of two cohorts found a positive association between vitamin D levels and higher perceived age and wrinkles but not with pigmented spots. [515] Of noteworthy, the authors defined pigmented spots as 'light, patchy, mottled hyperpigmentation, actinic lentigines, seborrheic keratosis, and solar freckling'. The same group also did not observe a link between genetic variants related to a higher number of pigmented spots and vitamin D level. Another study conducted by the Leiden Longevity Study demonstrated a correlation between elevated 25-hydroxyvitamin D serum concentrations and a higher count of pigmented spots on the face. [516] Beneficial effects of 1,25-hydroxyvitamin D had been reported by in vitro studies that showed protection against skin aging and cell damage from UVB radiation through stimulation of keratinocyte proliferation and differentiation. [517,518] Nevertheless, other factors that could lead to high vitamin D levels should be considered in in vivo studies such as outdoor activities (more sun exposure or exercise), better diet and associated decreased fat mass .[515,519] The authors of the study that showed no association between facial skin aging and higher genetically determined vitamin D levels postulated that this might be attributable to lower average vitamin D levels in the study participants compared to the in vitro experiments. [515] One of the limitations of their study was that cumulative sun exposure might not have been measured accurately with the utilised UV variables. Lack of power might also be an issue as well as the time lag between when the facial photographs were taken and

the time that vitamin D sample was collected. They do recommend however that vitamin D levels on the skin be measured in future studies to clarify the relevance of in vitro findings.

Low intensity chronic sun exposure -as opposed to high intensity intermittent exposure- was reported to be beneficial by several authors. [520] It can be hypothesized from these findings that perhaps a moderate dosage of UV exposure should be sought to be able to reap its benefits. The number of weeks of sunny holidays was shown to be inversely associated with Breslow depth in a dose-related manner. [365] Because of this, advocating complete avoidance of the sun might be potentially harmful rather than beneficial. Currently, a phase 3 randomised, double blind clinical trial of vitamin D supplementation is being conducted at the Department of Dermatology, University Hospitals Leuven.

Although solar lentigines are predominantly environmentally determined in particular through cumulative skin photodamage, it has been shown that their development is also dependent on pigmentation genes such as *MC1R* [378,521] and *SLC45A2* variants as well as mutations in *FGFR3* and *PIK3CA* genes. [522–524] In addition, melanoma risk is approximately fourfold higher in those with pre-malignant and skin cancer lesions compared to those who lack these lesions. [14] Nevertheless, it has been posited that a lower intentional UV exposure post melanoma diagnosis significantly diminishes the development of subsequent melanomas. [63]

Interestingly, skin pigmentation variants have been linked with 25(OH)D serum concentration. [525] Datta et al. asserted that although skin pigmentation was statistically significantly linked with UVB-induced vitamin D levels, this link disappeared after adjustment for sex, height, age and pigmentation SNPs located in *ASIP*, *MIR196A29,* Solute Carrier Family genes and *MTAP* gene (rs6475555). [526] Notably, our study also showed another *MTAP* variant to be associated with progression free survival among melanoma patients. This will be discussed in the next section.

UV as carcinogen, UV protection and Vitamin D supplementation

UVR exposure has been declared by Europe as the main cause of skin cancer. The 4th edition of the European Code Against Cancer recommended to “Avoid too much sun, especially for children. Use sun protection. Do not use sunbeds.” They also advocate the following measures such as seeking shade when solar radiation is at its maximum, through physical protection with appropriate clothing, sunscreen use and avoiding sunbeds. [527]

Advantageous UVR exposure effects e.g. vitamin D production can be achieved via alternative methods such as increased dietary vitamin D intake, supplementation and vitamin D fortification. Recommended target vitamin D

levels vary between ≥25 to ≥50 nmol/L (≥10-≥20 ng/mL) and a daily vitamin D intake between 400 to 800 IU. Vitamin D levels of <50 nmol/L is prevalent in 53% of the European population implying that vitamin D fortification at the general population level would be beneficial. [528] Concentrations of vitamin D lower than 50 nmol/L or 20 ng/ml can lead to bone loss and fractures whilst levels below 30 nmol/L or 12 ng/ml confers elevated risk for mortality, infections and other ailments. [529]

The World Health Organization (WHO) stated that a UV Index (UVI) of at least three requires UV protection. [530] However, UVA doses can vary with this UVI threshold, which can also induce melanoma. High dose sunscreen use with UVI between 3 and 5 may in theory hamper vitamin D production. A review on the effects of sunscreen on vitamin D showed field trials and observational studies reporting a lack of evidence that sunscreen use has a negative effect on vitamin D levels. [531] However, studies were conducted on moderate sun protection of SPF 16 on average. There was a paucity on literature regarding very high sun protection use which is currently recommended. Sun exposure campaigns should also include the relevance of duration exposure and that sun protection should be expanded accordingly depending on the UV dose. [530]

The IARC acknowledged sun exposure as the major environmental determinant of CM and keratinocytic cancers. [532] Advocating longer sun exposure with the aim of increasing vitamin D level is unsupported by literature. Vitamin D production reaches its height after approximately 5 to 10 mins of UVB exposure depending on a range of variables. Beyond this point the cutaneous cancer risk increases without further vitamin D increase. IARC stated that it has been proposed that vitamin D status be increased either through more UV exposure or higher vitamin D supplementation intake but is wary of the fact that effects of prolonged high levels of vitamin D of more than one year is still undetermined. [533] Nevertheless, the beneficial effects of vitamin D supplementation are supported by laboratory and observational studies. [533,534] Interestingly, a recent meta-analysis showed that cancer death risk is reduced by 16% with vitamin D supplementation. [535]

Challenging this is the fact that population supplementation which has been thought initially as advantageous has proven to be harmful instead as was the case with antioxidants. [536] Moreover, there had been studies reporting elevated CVD and mortality risk correlating dose-dependently with serum vitamin D levels. [537–539] Prior to implementing a population wide supplementation programme, further investigations are needed to clarify the association between vitamin D status and mortality or morbidity including cancer. The IARC proposed conducting RCTs that measure the serum 25-hydroxyvitamin D levels before and during the study. [533]

Since sun exposure is an established risk factor for CM, sun avoidance or solar exposure reduction is often advised which can potentially worsen existing insufficient vitamin D levels and subsequently affect disease and overall survival. Hence, it is recommended that vitamin D levels of melanoma patients be ascertained at time of diagnosis. [540]

Results from observational studies suggest causality between vitamin D levels and melanoma although the evidence is weak. [540] Limitations of these studies include type 1 errors caused by few study participants and potential attrition bias due to dropouts of participants. [541] Currently randomised controlled trials on vitamin D supplementation in melanoma patients are ongoing.

Opponents of vitamin D food fortification stated the following arguments. First, there can be uncertainty if the right individuals are getting the right dose with inadequate dosing still leading to vitamin D insufficiency without the beneficial direct immunosuppressive effects of UVR exposure. In addition, vitamin D overdose can be toxic especially to those who are not only receiving vitamin D food fortification but also are on vitamin D supplementation as treatment. Nevertheless, a Danish study and the ODIN project concluded that low dose vitamin D food fortification is a safe and effective approach in the prevention of hypovitaminosis D. [542,543] Furthermore, chronic vitamin D supplementation might lead to increased serum lipids. [544]

Proponents of UVR exposure argue its safety when done in moderation. [544] In addition, sun exposure cannot induce vitamin D overdose. [545] Of noteworthy, vitamin D3 production via UV radiation reaches its height a couple of minutes after exposure and at suberythemogenic UV doses. [546–548] Moreover, prolonged sun exposure does not increase vitamin D levels but it is linearly associated with elevated DNA damage. [546]

Last but not least, UVR is available naturally and is less expensive compared to oral vitamin D supplementation.

Conversely, total avoidance of UVR exposure might inflict more harm than benefit in those who are susceptible to low vitamin D levels such as dark-skinned individuals in low ambient solar environment and those who predominantly stay indoors. On the other hand, personalised advice regarding the ideal UVR exposure can pose a challenge as this is dependent on several factors e.g. skin type, diet and season to name a few. [547] However, loosening the sun exposure propaganda might lead to over exposure in some individuals. Also, vitamin D levels or solar exposure might be critical in certain development phases. Nevertheless, vitamin D supplementation may not be sufficient because not all the advantageous effects of UVR can be achieved through UVR induced vitamin D production. [544] Therefore, some sun exposure might be necessary. Although our conclusion suggests that sun

exposure might protect against melanoma progression, we do not advice melanoma patients to refrain from sun protection but rather to employ a combination of sun protection and vitamin D supplementation.

Further limitations of our third study consist of recall bias regarding sun exposure. In addition, past sunburns are frequently measured in sun exposure studies which is more indicative of intermittent versus total sun exposure. [533] Future studies are advised to not only include clinical signs of photodamage but perhaps also temporal changes such as (altered) sun habits after diagnosis i.e. exposure and sunscreen use. Also, combining questionnaires on sun habits and clinical signs at diagnosis might also clarify the issue. Quantifying the severity of solar lentigines or solar risk factors are also recommended. Another limitation is the possibility that molecular subtype could have played a role. For example, *BRAFV600E* can be found in melanoma and nevi on sites exposed to sun. [549] Viros et al. reported in a murine experiment that UVR stimulates *BRAF* dependent melanoma development via *TP53* mutations. [114] However, our study did not have data on *BRAF* and other molecular subtypes. Nevertheless, future studies should consider including all these aforementioned variables when investigating vitamin D levels.

7.4 GENETICS AND MELANOMA OUTCOME

In our fourth study, we showed that a MTAP gene variant, rs869330*G, located in chromosome 9p21 was significantly correlated with a favorable OS and RFS after adjusting for other prognostic factors of melanoma. Although the SNP is located in an intron region, it is possible that it might exert influence on transcription. However, the function of rs869330 has not been studied before. Interestingly, *MTAP* rs10757257 has been reported to be statistically significantly associated with nevi count and overall higher melanoma risk. Also, the same variant was correlated with SSM subtype vs LMM underlining a role for genetics since an association with chronic sun exposure has been shown with the latter subtype. [550]

Previously, it has been mentioned that two hydrogen bonds connect adenine and thymine base pairs whereas three hydrogen bonds ties guanine with cytosine (see Appendix Fig. 3). It can therefore be hypothesized that because there are more hydrogen bonds connecting guanine (with cytosine) compared to that of the variant adenine (with thymine), that perhaps the bond is stronger leading to better resilience of this particular variant against DNA breaks or damage. Nevertheless, this requires further investigation.

We had intended to analyze 20 susceptibility SNPs at first: 18 melanoma susceptibility SNPs as fine mapped by

Barrett et al. including 2 SNPs from *CDKN2A* region and an additional 2 SNPs from Rendleman et al. [159,309] The SNPs from the latter study were shown to be related with melanoma survival. Rs7538876 was correlated with BCC and rs9960018 was associated with tanning. Both SNPs however have not been studied in relation to melanoma risk.

Six SNPs could not be designed using SEQUENOM MassArray technology and 5 SNPs did not meet the post genotyping criteria. It would have been interesting if all these SNPs were analyzed. Especially the two *CDKN2A* variants since the *CDKN2A* gene has high penetrance within melanoma families. The *TERT* variant would have been interesting as well because it has been previously associated with lower melanoma risk. [160] Surprisingly, the tanning and fair phenotype variants (eg *MC1R, ASIP, SCL45A2* and *IRF4*) were not statistically significantly associated with melanoma survival. One explanation could be that these SNPs might contribute to susceptibility for melanoma development but not to invasion and progression.

The relatively low p-value for the *MTAP* variant implies that this variant may not have a major impact on melanoma survival. This can probably be expected from a low penetrance risk variant. As previously discussed in Chapter 6, there is a possibility that our findings on the *MTAP* SNP and melanoma outcome might be attributable to a random effect due to multiple testing. The association is only marginally significant and disappears after correcting for multiple testing. Replication in other populations is therefore recommended. It should be noted though that there are plausible biological reasons which could explain the observed association as discussed below.

The *MTAP* gene regulates the polyamine metabolism and is involved in restoration of adenine and methionine. Methylthioadenosine (MTA) is a nucleoside that contains sulfur and is found in different species such as yeast, plants, animals and humans. [551] MTAP is an enzyme that breaks down MTA into 5-methylthioribose-1-phosphate (MTR-1P) and adenine, therefore controlling MTA levels in cells. [552] Cancer cells have been shown to have low MTAP levels or reduced activity leading to MTA accumulation in tumor tissues. For example, MTA levels had been measured to be higher in melanoma patients' skin (around 140 nM) compared to healthy controls (around 10-20 nM). [553]

MTA regulates several cellular processes including gene expression, cell death and proliferation, and lymphocyte activation. [554] Furthermore, MTA has a role in oncogenesis and evasion of the immune system by cancer cells (see

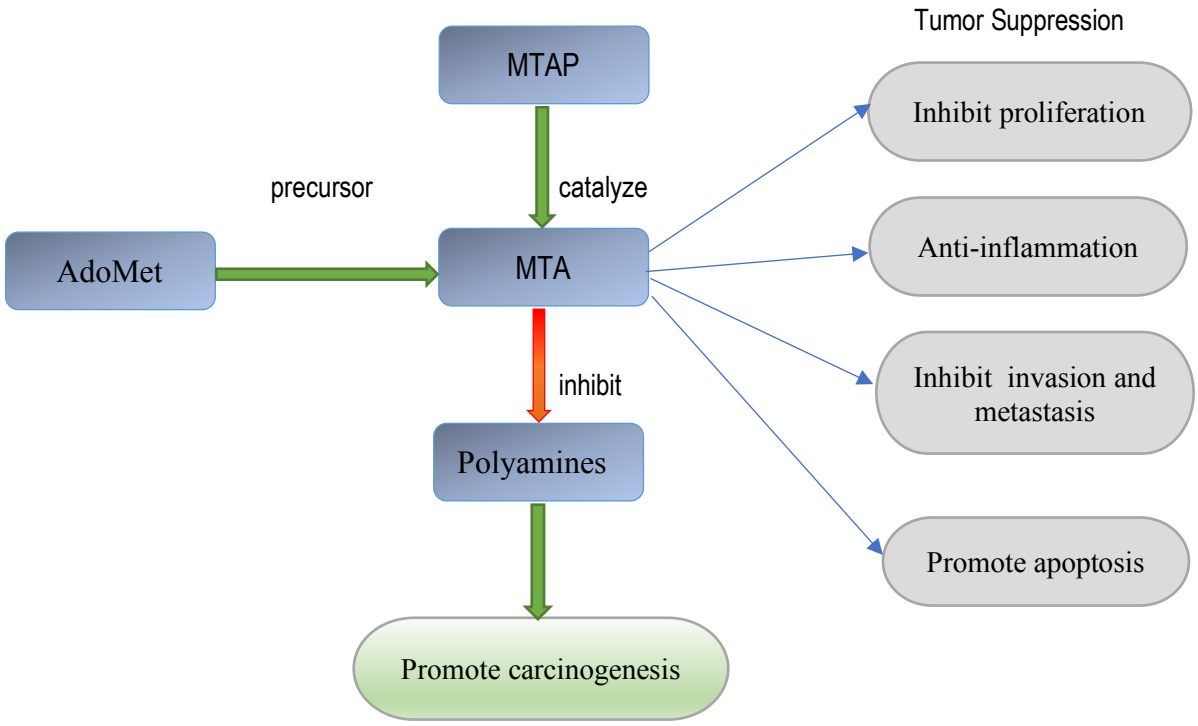
figure 1). A positive correlation was shown between increased MTA amounts in cultured melanoma cells and tumor metastasis indicating that MTA supports cancer progression. [553] As for apoptosis, MTA has pro-apoptotic effects on cancer cells but might inhibit apoptosis in normal cells which can be explained by ratios of genes which stimulate or suppress apoptosis. [555] Another explanation for the apoptotic effect of MTA is threshold dosage. Anti-proliferative effects were observed at lower dosages in a human cancer cell line. [556]

MTA not only has direct effects on tumor cells but also can exert influence in the tumor environment. Upregulation of angiogenesis related growth factors (vascular endothelial growth factor (VEGF) and basis fibroblast growth factor (bFGF) and metastasis associated enzymes (matrix metalloproteinase (MMP) 9 and 14) was demonstrated in melanoma cells post MTA treatment. [553] In contrast, MTA treatment mitigated cancer in a metastatic liver murine model. [556] This discrepancy has been attributed to the different MTA dosages applied in the two studies and the different cell types used. Furthermore, MTA has been reported to have a major inhibitory influence on inflammation in murine models and cell lines, counteracting pro-inflammatory cytokines and enhancing anti-inflammatory cytokines. [557,558] Interestingly, inflammation has been postulated to be a hallmark of cancer and since MTA seems to counter inflammation, this might explain its anti-cancer capabilities. [71]

MTAP is the main enzyme that phosphorylates MTA, thus maintaining MTA cellular levels low. MTAP is present in normal human cells and is predominantly expressed in the liver. [388] Similar to MTA, MTAP activity loss has been demonstrated in several malignancies including melanoma. [389] The *MTAP* gene is located in the 9p21 chromosome and is in near proximity to *p16* and *Interferon alpha and beta* genes which are frequently deleted in cancers especially *CDKN2A (p16INK4)*. [559] Hypermethylation of *MTAP* promoter silences its activity and is related with a reduction of MTAP mRNA expression in melanoma cell lines. [389]

Worse survival rates had been demonstrated in tissues of lung cancer patients with decreased MTAP levels and is an independent prognostic factor for this tumor type. [388] Furthermore, a statistically significant negative association has been observed in melanoma samples between MTAP expression levels and degree of invasive potential ranging from benign nevi to metastatic melanoma. [389] Additionally, heterozygote *MTAP* deletion with associated decreased MTAP levels leads to lymphoma development, indicating that *MTAP* is a tumor suppressor.[560]

Figure 1: Effects of MTAP pathway on tumors.



Polyamines are necessary for cell proliferation and oncogenesis. [561] High polyamine levels and enhanced polyamine transport processes have been reported in various tumor cell types including melanoma. [562] Hence, inhibition of polyamine production through MTA treatments is a potential anti-cancer therapy. [563,564]

Furthermore, interaction between polyamine metabolism and various other oncogenic pathways has been reported including MYC [565], P13/AKT/WNT [566], RAS/RAF/MEK/ERK [562] and PTEN-P13k-mTOR complex 1 [567] probably due to the fact that polyamine is downstream from these pathways. Therapies that target the polyamine metabolism or transport are interesting novel anti-cancer drugs with a lot of potential. Of particular interest is that *RAS* activation enhances polyamine transport in cancer cells. [568] Approximately half of melanoma cases harbour *BRAF* gene mutations, a member of the RAS pathway. [111] Polyamine transport activity has been shown to be elevated in *BRAF* mutant melanoma cells. [562] Results from in vitro studies suggest that polyamine analogue treatment not only kills BRAF mutant melanoma cells specifically unlike wildtype cells but also mitigates the resistance to BRAF inhibitors. [562]

As stated previously, MTAP catalyzes MTA into 5-methylthioribose 1-phosphate and adenine. In turn, 5-methylthioribose 1- phosphate is salvaged into methionine whilst adenine is further converted into AMP and

eventually ATP by adenine phosphoribosyltransferase (APRT) with the help of phosphoribosyl pyrophosphate (PRPP). However, APRT can produce toxic nucleotides by also acting on purine analogues. To counter this, PRPP is diverted and utilised by MTAP instead to produce adenine. [552] Therefore, in *MTAP* loss, PRPP is not being utilised for adenine conversion whereby toxic nucleotide production by APRT is enhanced.

In addition, methionine plays a role in tumor growth in various cancers. *MTAP* deletion leading to lack of methionine salvage increases methionine levels and enhances tumorigenesis. [569] Several therapeutic approaches are being explored that tackle MTAP deficiency. [552] One of the suggested therapies is supplementation with MTA and purine analogues. Interestingly, cancers with *MTAP* deletions leads to dependence on protein arginine N-methyltransferase 5 (PRMT5). Apoptosis of cancer cells is induced by depleting PRMT5. Hence, *PRMT5* inhibitors such as MTA might be another promising novel therapy in cancers that thrive in *MTAP* loss. [570] Polyamine analogues have been reported to induce apoptosis in a human melanoma cell line. [571] However, clinical trials so far had demonstrated only stabilization of the tumor as its greatest result which can be partially attributed to the dosages used. [572] It is noteworthy to mention that some tumors retain the *MTAP* locus such as prostate cancer and can utilize the polyamine metabolism for their growth. [573] For these tumors that express MTAP, MTAP inhibitors or polyamine blocking therapy may be used but this falls outside the scope of this thesis.

Melanoma SNPs ethnicity

Variations in polymorphisms exist in different races or ethnicities. It has been postulated that SNPs that are specific to a certain population or race may be responsible for the racial disparities in cancer risk and related phenotypes. For example, 8q24 variants confer increased prostate cancer risk in African Americans [574] and a *CREBRF* SNP is correlated with obesity among Samoans. [575] Risk alleles vary in prevalence by ancestral population. [576] A meta-analysis found no significant association between a *TERT* SNP and melanoma. [577] Interestingly, a subgroup-analysis of lung cancer by ethnic groups showed a significant increase in Caucasians but not in Asians suggesting that cancer risk SNPs may vary between races. Furthermore, *MC1R, SLC45A2* and *TYR* SNPs were found to be associated with melanoma risk in South Europeans. [578] Also, *MC1R* variants have been seen to increase CM risk in Israeli Jews and is even higher in Ashkenazi Jews. [579] Smit et al. also found high risk *MC1R* alleles to have similar proportions across different races (European, African and Native American) in a Puerto Rican population.

[580] In contrast, *MATP/SLC45A2* SNPs mitigates CM risk among the French. [581] Nan et al. also reported different SNPs and skin cancer development risk in a Caucasian group. [582]

GWAS may be used to elucidate underlying biological mechanisms, aid in identification of unknown environmental risk factors and to develop genetic risk prediction models. Strikingly, a review of the GWAS discovery populations reported that all GWAS for CM had been conducted in European ancestry populations. Notably, GWAS of cancer in general identified more than 700 risk loci which has been discovered first predominantly in European ancestry populations and less than 1 % in African and Latin populations reflecting inequalities in genetic research. [576] This might influence the translational impact of GWAS findings in Caucasians on other races. A recent study on the prediction accuracy of polygenetic risk scores of variants discovered through GWAS revealed that it is largely dictated by the genetic similarity to the population within which the included SNPs were discovered. [583] In sum, polymorphisms associated with melanoma risk may vary per population or ethnicity.

As for admixed populations, Lona-Durazo et al. conducted a meta-analysis of GWAS which provided new insights on the genetic architecture of skin pigmentation in these populations and came to the following conclusions. First, independent functional mutations within the same genes can be found in different populations which was termed as convergent evolution (e.g. *OCA2/HERC2* and fair phenotype in Europeans and East Asians). Second, various independent loci within the same genomic areas may be found in one population group (e.g. *GRM5/TYR* and *OCA2/HERC2*). Third, variants which were reported to have high significant associations appear to have pleiotropic effects (e.g. *HERC2* variants with eye, skin and hair color pigmentation and *EMX2* with skin pigmentation SNPs and tanning). Lastly, the paper highlighted the mechanisms of action of SNPs on pigmentation (e.g. effects on protein structure and epigenetics). [584]

One of the limitations of our study is that our results may not be transferable to other non-Belgian populations or non-Caucasian groups. Nevertheless, Carlson et al. [585] studied the generalization and dilution of GWAS findings discovered in European ancestry populations and found that most of the identified variants, especially if functional, can be usually generalized to other ethnic populations unlike genetic models derived from European ancestry discovery groups which can produce non-accurate results in other races due to discrepancy in effect sizes.

7.5 CONCLUSION

Cutaneous melanoma is the most frequent deadly form of skin cancer. CM incidence is rising and is being diagnosed in an early stage whereby the absolute number of patients in early stage that progress is increasing. [197] Metastatic melanoma has a 5-year survival rate of less than 15%. [148] However, the prognosis of a percentage of melanoma patients cannot be accurately predicted by the AJCC staging criteria reflected by relatively wide ranges of survival. [198] Nevertheless, the earlier the stage CM is diagnosed, the better the disease outcome. Furthermore, novel targeted and immunotherapies have been shown to improve survival in a group of melanoma patients but are challenged by high expenses, therapy resistance and toxic adverse effects. However, few studies have been conducted between CM risk factors such as a history of CM and potential novel determinants or prognosticators such as atopy and genetic susceptibility and their association with CM risk and outcome. Understanding the etiology of CM and potential risk factors contributing to CM development and survival is therefore a pressing matter.

In our first analysis, we demonstrated a difference in risk factors between patients with multiple melanoma primaries compared to single CM diagnosis. MPM patients are more likely to have a positive family history, dysplastic nevi and light hair color versus SPM patients. There is evidence that these three factors are intertwined. Patients with these characteristics should be carefully monitored. Our findings also suggest a possible strong role of genetic influences for multiple melanoma development. However, since few studies had been conducted comparing sun behaviours and sun damage between MPM and SPM, environmental influence cannot be excluded. We hypothesize that a cumulative risk for melanoma develops when genetic susceptibility and UVR exposure are both present.

Our second study showed a general inverse association between atopy and melanoma risk but only a history of atopy was statistically significant after correction for histopathological prognostic factors for the first time. However, atopy was not correlated with melanoma outcome. A strength of this paper is the investigation of the relationship between atopy and melanoma development in a clinically well-characterized case-control population and correction for important clinical melanoma confounders negative associations with melanoma development were observed for all variables referring to atopy. This inverse association between atopy and melanoma has been reported by other studies. [70,77,78,80,362] In our study, the correlation was only significant for personal atopy and that

a strong claim about this correlation is not possible in the absence of a correction for multiple testing. Further investigations between atopy and melanoma outcome are warranted, as the events were few in our study population. Findings on the association between atopy and cancer in general have been discordant. [411,412] Many explanations have been offered including immune system processes. There is evidence from published literature that both innate and adaptive immunity that have been shown to be activated in AD patients can also counteract carcinogenesis. An effective anti-cancer immune response necessitates collaboration and coordination of both innate and adaptive immune systems. However, certain immune system components can possess a dual role in tumor development [492,493] whereby they can either be pro-oncogenic in one situation and anti-cancerous in another of which the exact mechanisms are currently undergoing investigation.

In our subsequent analysis, established clinical CM risk factors, specifically sun damage, family history, presence of atypical nevi, have been shown to affect melanoma survival. In the multivariable analysis with Bonferroni correction, solar lentigines on the dorsum of hands is the only determinant which persisted to have a statistically significant inverse correlation with melanoma relapse free survival. It is plausible that solar lentigines, could be related to increased vitamin D levels as solar lentigines are a sign of sun damage resulting from sun exposure. Elevated 25-hydroxyvitamin D serum concentrations had been correlated with a higher count of pigmented spots on the face. [516] Beneficial effects of 1,25-hydroxyvitamin D had been reported by in vitro studies that showed protection against skin aging and cell damage from UVB radiation through stimulation of keratinocyte proliferation and differentiation. [517,518] Vitamin D variants had been reported to be associated with both worse [303] and better melanoma specific survival.[290] Although solar lentigines are predominantly environmentally determined in particular through cumulative skin photodamage, it has been shown that their development is also dependent on pigmentation .[378,521,524] Skin pigmentation variants including an *MTAP* variant have been linked with 25(OH)D serum concentration. [378,525,526] The ideal UVR exposure can pose a challenge, as this is dependent on several factors. Moreover, vitamin D levels or solar exposure might be critical in certain development phases. A combination of a lower intentional UV exposure post melanoma diagnosis and vitamin D supplementation might be beneficial with regard to melanoma risk and survival. [63,290,535] However, vitamin D and melanoma correlation needs to be clarified further and research is still ongoing.

Lastly, we investigated the relation between melanoma susceptibility SNPs and disease outcome whereby *MTAP*

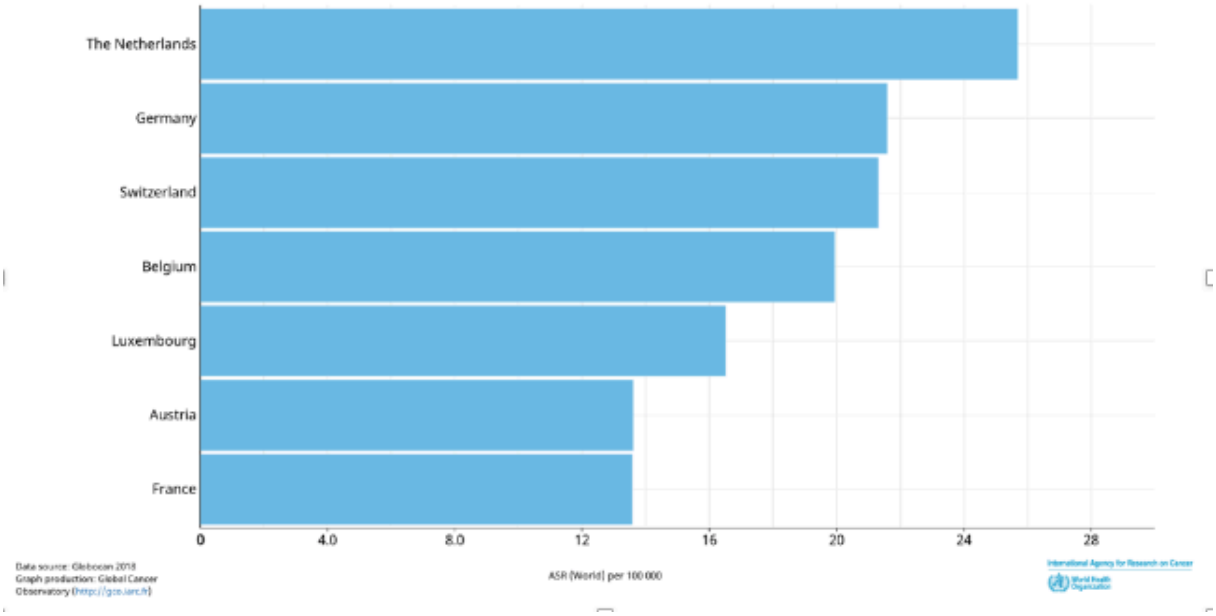
variant rs869330*GG was demonstrated to have a beneficial effect on melanoma relapse free survival in a large contemporary CM population. The *MTAP* gene regulates the polyamine metabolism and is involved in regulation of several cellular processes including gene expression, cell death/proliferation and lymphocyte activation. [554] Cancer cells have been shown to have low MTAP levels or reduced activity leading to MTA accumulation in tumor tissues. [553] *MTAP* activity loss has been demonstrated in several malignancies including melanoma. [389] A major limitation of our study is that our results may not be transferable to other non-Belgian populations or non-Caucasian groups. Hence, validation in other populations is warranted. Furthermore, follow-up studies are recommended to elucidate the biological significance of the *MTAP* rs869330 variant in patients with melanoma.

The strengths and limitations of each study have been thoroughly discussed in each article and in the general discussion. It should be noted however that not only do our analyses include a sizeable representative population of melanoma patients with long follow-up but they also address a number of original research questions that were incompletely answered in published literature. Nevertheless, with regard to our survival studies, the possibility that molecular subtype could have played a role needs to be addressed. Data on melanoma molecular subtype was not available when our investigations were conducted and was therefore not included in the analyses.

In conclusion, we have observed new evidence on the associations between clinical risk factors, genetics and melanoma risk and survival broadening our understanding of the disease and underlining the multiple pathways involved in the disease.

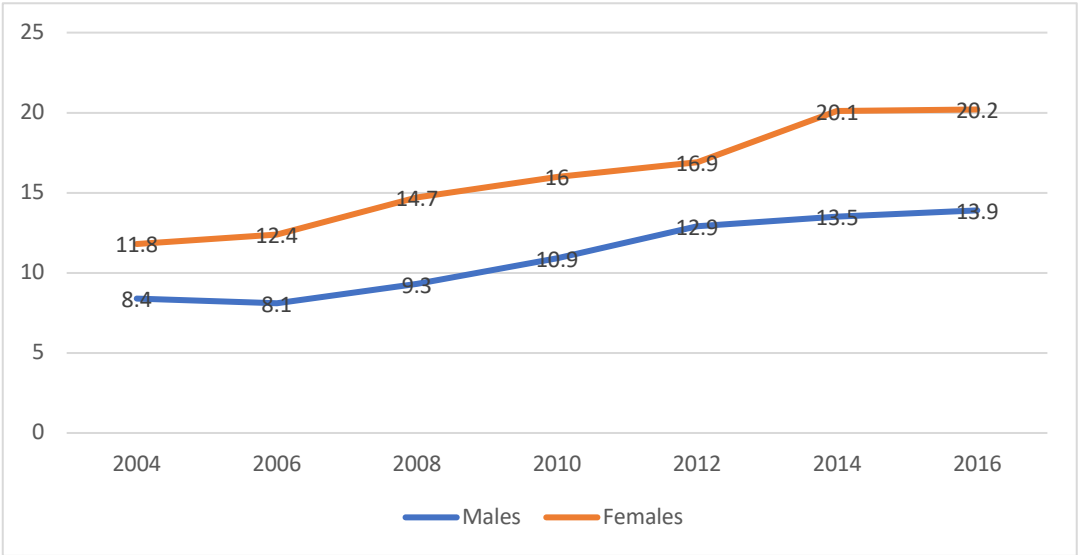
APPENDIX

Figure 1: Estimated cutaneous melanoma incidence rates (age-standardized World) in Western Europe in 2018 (both sexes, all ages)



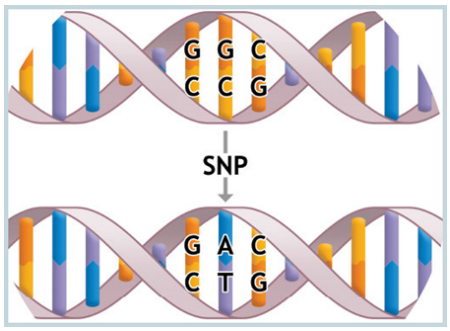
Source: Globocan [2]

Figure 2: Malignant melanoma incidence rates in Belgium (age standardised rate using world standard population, N/100 000 person-years) [5]



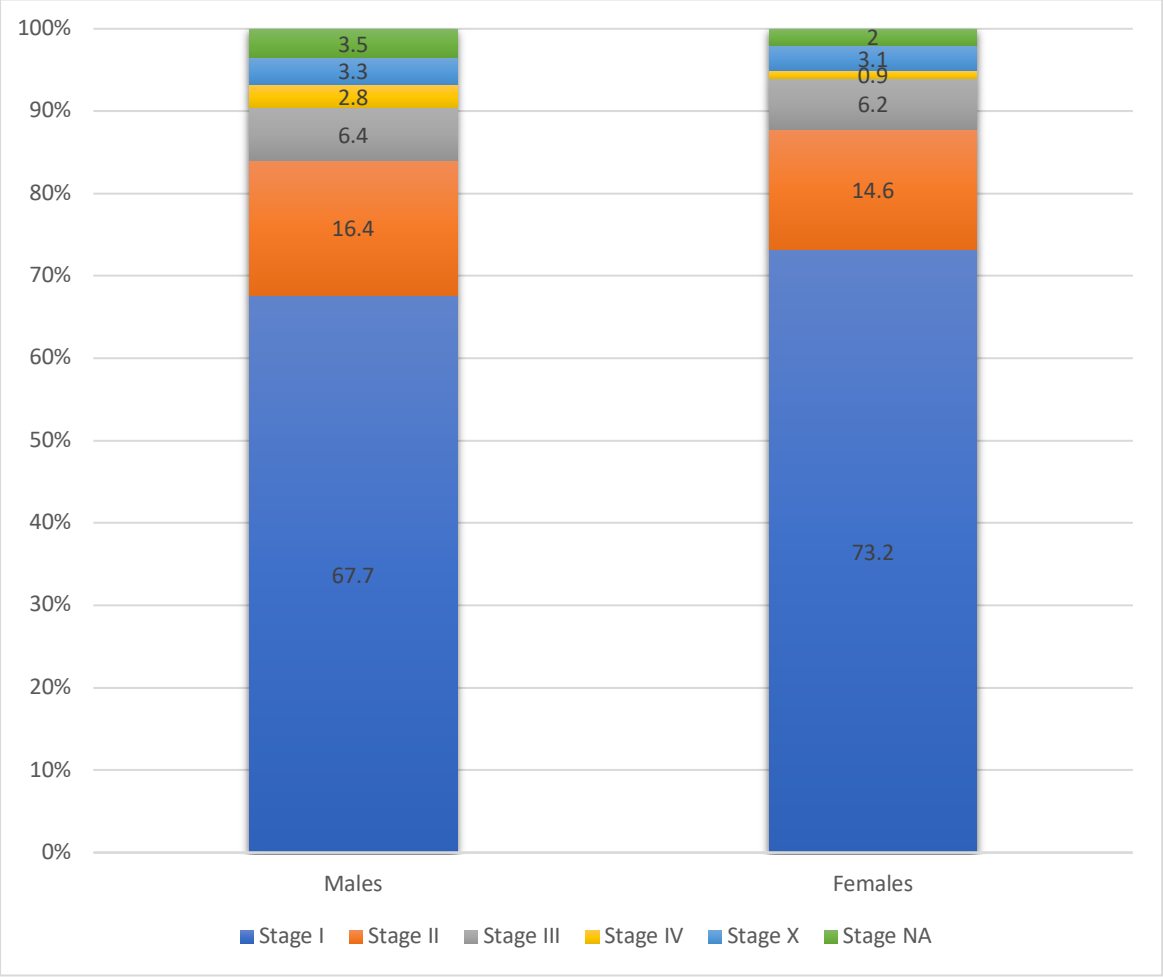
Adapted from Belgian Cancer Registry [5]

Figure 3: A model of single nucleotide polymorphism (SNP).



Source: Wikipedia

Figure 4: Distribution of malignant melanoma stage by gender in Belgium 2016. [5]



Adapted from Belgian Cancer Registry [5]

Table 1. Fitzpatrick Classification of Skin Types.

Type 1	Type II	Type III	Type IV	Type V	Type VI
White skin, always burns, never tans	Fair skin, always burns, rarely tans	Light brown color, sometimes burns, gradual tanning	Medium brown color, rarely burns, fast tanning	Dark brown, rarely burns, tans easily and quickly	Black, almost never burns, fast and dark tanning

Table 2. Melanoma susceptibility SNPs in GWAS studies and after the fine-mapping*

			Original study			Barrett et al. study		
Chromosome	**Gene**	**Reference study**	**SNP name**	**Position**	**R2 for CM risk**	**SNP name**	**Position**	**R2 for CM risk**
1q21.3	ARNT	MacGregor et al. [301]	rs7412746	150860471	0.08	rs3768013	150815411	0.13
1q42.12	PARP1	MacGregor et al. [301]	rs3219090	226564691	0.12	rs1858550	226608104	0.16
2q33-q34	CASP8	Barrett et al. [586]	rs13016963	202162811	0.14	rs2349073	202186986	0.17
5p15.33	TERT	Rafnar et al. [160]	rs401681	1322087	0.25	rs2447853	1333077	0.27
5p13.2	SLC45A2	Guedj et al. [581]	rs16891982	33951693	0.20	as reference		
6p25-p23	IRF4	Duffy et al. [587]	rs12203592	396321	0.04	rs9405705	470384	0.08
9p21	CDKN2A	Bishop et al. [152]	rs7023329	21816528	0.32	rs869330	21804617	0.38
9p23	TYRP1	Duffy et al.[588]	rs2733832	12704725	0.01	rs72706189	11877260	0.11
11q13	CCND1	Barrett et al. [586]	rs1485993	69362414	0.20	rs12422135	69378260	0.22
11q14-q21	TYR	Bishop et al. [152]	rs1393350	89011046	0.30	rs1126809	89017961	0.34
11q22-q23	ATM	Barrett et al.[586]	rs1801516	108175462	0.16	rs4753835	108145249	0.16
15q13.1	OCA2	Amos et al. [589]	rs1129038	28356859	0.02	rs14572014	28468231	0.07
16q12.2	FTO	Iles et al.21	rs12596638	54115829	0.14	as reference		
16q24.3	MC1R	Bishop et al. [152]	rs258322	89755903	1.02	rs73283859	90062520	1.31
20q11.2-q12	ASIP	Bishop et al. [152]	rs2284378	32818707	0.13	rs6059655	32665748	0.26
21q22.3	MX2	Barrett et al. [586]	rs45430	42746081	0.18	rs443099	42743327	0.19
22q13.1	PLA2G6	Bishop et al. [152]	rs6001027	38545619	0.15	rs3891103	38537159	0.20

*adapted from Barrett et al. [151]

Table 3. 5 year relative survival proportion of malignant melanoma in Belgium 2012-2016. [5]

	Males			Females		
	N at risk	**Estimate**	**95% CI**	**N at risk**	**Estimate**	**95% CI**
Overall	5,728	91.2	89.7, 92.7	7,989	95.0	94.0, 96.0
Age group						
15-34 years	335	94.5	90.6, 97.0	790	97.8	96.1, 98.9
35-64 years	2,832	92.2	90.8, 93.6	4,422	96.9	96.2, 97.7
65+ years	2,562	89.5	86.3, 92.7	2,777	90.6	87.8, 93.3

Adapted from Belgian Cancer Registry [5]

Table 4. Characteristics of the most common subtypes of cutaneous melanoma.

Subtype	Prevalence	Dermoscopy	Histology	Location	Other features
Superficial spreading melanoma (SSM)	~70% of melanomas	Atypical pigment network, blue-white veil, multiple brown dots/globules, pseudopods, radial streaming, scar like depigmentation, variegated colors and irregular vascularization. [177]	tumor cells are arranged in a pagetoid manner. Later it grows vertically and become invasive	Typically present on the legs in females and the trunk in males	Usually starts as a flat macule that first spreads horizontally turning into a plaque with variable pigmentation
Nodular melanoma (NM)	15-30%	atypical vascular pattern and/or variegated colors and/or blue grey veil	vertical invasion and lack of radial growth phase	usually presents in men and can be often found on the trunk	not the typical ABCD melanoma lesion. Usually elevated, firm, equally pigmented papule or nodule that evolves rapidly
Lentigo maligna melanoma (LMM)	10-15%	Atypical pseudo network (asymmetric follicular openings, grey annular granular structures, rhomboidal structures)	Atypical melanocytes, solitary units and spindle shape nests along the junction between the dermis and epidermis	typically develops slowly in chronically sun exposed areas e.g. the face, head etc.	asymmetric, brown/black macule with irregular borders
Acral lentiginous melanoma (ALM)	2-3% of melanoma cases worldwide [179] but accounts for 70% of melanomas in darker skin types	parallel ridge pattern	single atypical melanocytes and infiltration of lymphocytes between the dermis and epidermis	typically non-sun exposed sites such as palms, soles, wrists, heels and nail beds. [181]	often diagnosed at a late stage with associated worse prognosis

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CURRICULUM VITAE

Dr. Vivien Marasigan obtained her BSc and MSc diploma in Medicine from the Vrije Universiteit in Amsterdam. She conducted her MD thesis at Antoni van Leeuwenhoek Netherlands Cancer Institute. She then worked for the Royal Dutch Navy as a military physician officer. After her discharge, she filled posts in dermatology clinics in Holland. Subsequently, she was offered a PhD position (Cancer Programme) at the Department of Dermatology in University Hospitals Leuven in Belgium focussing on melanoma, with Prof. Marjan Garmyn as supervisor and Prof. Diether Lambrechts as co-supervisor. As part of the programme, she presented at several departmental, interdepartmental, national and international meetings including the Belgian Association of Dermato-Oncology, European Association of Dermato-Oncology and World Melanoma Congress.

After transferring to Ireland, she worked on clinical trials in Cork University Hospital. In the meanwhile, she was conferred her second Master's degree (Public Health, Advanced Epidemiology pathway) from University College Cork. A collaboration with several renowned professors from University College Cork, University of Liverpool, Institute of Public Health and Royal College of Surgeons Ireland led to the publication of a study aimed at informing the Department of Health in Ireland regarding future national policies. Subsequently, she obtained a post at the Dermatology Department in St. Vincent's University Hospital in Dublin where she was able to sharpen her skills in dermatosurgery, gain experience in psoriasis including the latest biologic treatments, atopic dermatitis, hidradenitis suppurativa and General Dermatology. Thereafter, she worked at the Dermatology Department in University Hospital Limerick where she gained more clinical experience. During this period, she was redeployed to the General Internal Medicine Department to assist in the time of the COVID-19 pandemic. She holds a postgraduate diploma in Clinical Dermatology conferred by University College of Dublin.

Her pastimes are multifarious and include photography and travel, traversing six continents so far. Currently, she is living and working in Ireland.

PUBLICATIONS

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PERSONAL CONTRIBUTIONS

In our first article with meta-analysis and multiple primary melanomas as subject, I collaborated with Dr. Eric Schmitt (PhD Statistics Leuven). I have created the concept, collaborated with librarians regarding keywords for the articles search, conducted the search, filtered the list of articles (with another individual), co-designed the statistical aspects of the meta-analysis, interpreted the results, drafted the article and sent the article for publication. Dr. Eric Schmitt conducted the statistical analysis, helped interpret the results and draft the article.

As for our atopy study, I construed the hypothesis, gathered the clinical and histopathological data, assisted with the statistical analysis, interpreted the outcomes, wrote the article and submitted it to a peer-reviewed journal.

In our third and fourth study, I shared authorship with Mrs. Canan Guvenc. My contributions were the following: I helped create the concept of the study, designed the statistical methods, helped with the statistical analysis, selected potential study participants, included patients, examined and followed-up participants, gathered data, built a database, interpreted the results and wrote the final version of the articles. Mrs. Canan Guvenc helped with gathering data, carrying out the statistical analysis, interpreting the results, writing parts of the statistical methods for the articles and submitting them for publication.

Last but not least, I have disseminated and presented our study results at local, national and international gatherings.

CONFLICT OF INTEREST STATEMENT I have none to report.

LIST OF FIGURES AND TABLES

Chapter 3

Figure 1: Flow diagram of article selection process.....55

Figure 2: Forest plot of the ORs and confidence intervals of studies on family history of melanoma.....57

Figure 3: Forest plot of the ORs of studies on dysplastic naevi.....58

Figure 4: Forest plot of the ORs of studies hair color. 60

Table 1: This table depicts characteristics of the studies used for the meta-analysis for a certain risk factor....56

Chapter 4

Table 1: Questions used for the definition of the atopy related variables and their definitions.....65

Table 2: Baseline characteristics of the study population.....68

Table 3: The association of atopy related variables and melanoma development.....70

Table 4: Results of Cox regression analyses for the association between atopy related variables and progression-free survival.....71

Chapter 5

Table 1: Clinical and histopathological parameters of all patients.....80

Table 2: Survival association result from univariate and multivariable Cox proportional hazards models.....81

Chapter 6

Table 1: Patient and primary tumor characteristics of the study population.....88

Table 2: Survival association results from genetic univariate Cox proportional hazards model.....89

Table 3: Survival association result from multivariable Cox proportional hazards models.....90

Figure 1: Illustration of effect of rs869330 for patient in stage Ib (a) and stage II (b) based on the multivariable Cox regression after correction for age, gender, stage, location and subtype.....90

Chapter 7

Figure 1: Effects of MTAP pathway on tumors. 114

Appendix

Figure 1: Estimated cutaneous melanoma incidence rates (age-standardized World) in Western Europe in 2018 (both sexes, all ages) 120

Figure 2: Malignant melanoma incidence rates in Belgium (age standardised rate using world standard population, N/100 000 person-years) ……..…120

Figure 3: A model of single nucleotide polymorphism (SNP). ……..…121

Figure 4: Distribution of malignant melanoma stage by gender in Belgium 2016…….....121

Table 1: Fitzpatrick Classification of Skin Types……..……..122

Table 2: Melanoma susceptibility SNPs in GWAS studies and after the fine-mapping…….....122

Table 3: 5-year relative survival proportion of malignant melanoma in Belgium 2012-2016……..……...123

Table 4: Characteristics of the most common subtypes of cutaneous melanoma… ……..…...123

LIST OF ABBREVIATIONS

ABCDE	Asymmetry, border, color, diameter, evolution
ACD	Adrenocortical dysplasia
AD	Atopic Dermatitis
AFF3	Lymphoid nuclear protein related to AF4
AFG3L1P	AFG3 like matrix AAA peptidase subunit 1
AJCC	American Joint Committee on Cancer
AMP	Adenosine monophosphate
APC	Antigen presenting cell
API	Asian Pacific Islanders
APRT	Adenine Phosphoribosyltransferase
ARID2	AT-Rich Interaction Domain 2
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASIP	Agouti Signaling Protein
ASR	Age Standardised Rate
ATM	Ataxia Teleangiectasia Mutated
ATP	Adenosine tri-phosphate
AUC	Area Under the Curve
B cells	Humoral Immune Response
BCC	Basal Cell Carcinoma
bFGF	basis Fibroblast Growth Factor
BMI	Body Mass Index
BN	Benign Naevi
BRAF	V-raf murine sarcoma viral oncogene homolog B1
Breg	Regulatory B cell
C(M)M	Cutaneous (Malignant) Melanoma
CASP8	Caspase 8
CAMS	Classic Atypical Mole Syndrome
CCND1	Cyclin D1

CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CDK	Cyclin Dependent Kinase
CDK4 or 6	Cyclin Dependent Kinase 4 Or 6
CDKN2A	Cyclin-dependent Kinase Inhibitor 2A
CGH	Comparative Genomic Hybridization
CI	Confidence Interval
CIITA	Class II Major Histocompatibility Transactivator
CIR	Crude Incidence Rate
CLND	Complete Lymph Node Dissection
CLPTM1L	Cleft Lip and Palate Transmembrane Protein 1 Like
CM	Cutaneous Melanoma
CMM	Cutaneous Malignant Melanoma
CO2	Carbon Dioxide
Cox PH	Cox Proportional Hazards Models
CREBRF	CREB3 Regulatory Factor
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
CTNNB1	Catenin Beta 1
DALY	Disability-Adjusted Life Year
DCs	Dendritic Cells
DCT	Dopachrome Tautomerase
DDX3X	DEAD-Box Helicase 3 X Linked
DLGAP1	Disks large-associated protein 1
DN	Dysplastic Naevi
DNA	Deoxyribonucleic Acid
DSS	Disease Specific Survival
EC	eye color
EGF	Epithelial Growth Factor gene
EGFR	Epidermal Growth Factor Receptor

EMX2	Empty Spiracles Homeobox 2
ERCC	Excision Repair Cross Complementation Group 1
ERK	Extracellular Signal-Regulated Kinases
EU	European Union
EUCAN	European Cancer Estimates
FEM	Fixed effects models
FGFR1	Fibroblast growth factor receptor 1
FH	Family History
FISH	Fluorescence In Situ Hybridization
FR	freckles
FTO	Fat mass and obesity associated
GEM	Genes, Environment and Melanoma
gp100	Glycoprotein100
GRM5	Glutamate Metabotropic Receptor 5
GWAS	Genome-Wide Association Study
HC	Hair Color
HE staining	Haematoxylin and eosin staining
HERC	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase
HERC2	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2
HG	Hypomelanosis guttate
HLA-DQB1	Major histocompatibility complex class II DQ beta 1
HPD	Highest Posterior Density Interval
HR	Hazard Ratio
HRAS	Harvey rat sarcoma viral oncogene homolog
HWE	Hardy Weinberg equilibrium
IC	Immunohistochemical
IDH1	isocitrate dehydrogenase 1
IDO1	Indoleamine 2,3-dioxygenase 1
IFN	Interferon
IFNG	Interferon Gamma

IgE	Immunoglobulin E
IL	Interleukin
IRF	Interferon regulatory factors
KIT	tyrosine protein kinase
KRAS	Kirsten rat sarcoma viral oncogene
L(M)M	Lentigo (Maligna) Melanoma
LAK	Lymphokine-Activated Killer
LDH	Lactate Dehydrogenase
LM	Lentigo Maligna
log OR	natural logarithm of the ORs
M	Macrophage
M1	Classically Activated Macrophage
M2	Alternatively Activated Macrophage
MAF	Minor Allele Frequency
MAP2K1	Mitogen-Activated Protein Kinase 1
Mart1	Melanoma Antigen Recognized by T cells 1
MC1R	Melanocortin 1 Receptor
MDM2	Mouse Double Minute 2
MEK	Mitogen-Activated Protein Kinase Kinase
MFSD12	Major Facilitator Superfamily Domain Containing 12
MHC	Major Histocompatibility Complex
MITF	Microphthalmia associated transcription factor
MLE	Maximum Likelihood Estimation
MMP	Matrix Metalloproteinase
MPM	Multiple Primary Melanomas
MRD	Melanoma Related Death
MSS	Melanoma Specific Mortality
MTAP	Methylthioadenosine Phosphorylase
MTR-1P	5-methylthioribose-1-phosphate
MX2	MX Dynamin Like GTPase 2

MYH7B	Myosin Heavy Chain 7B
MYO7A	Myosin VIIA
NAM	Nevus Associated Melanoma
ND	Not Defined
NF1	Neurofibromatosis 1
NK	Natural Killer (cell)
NM	Nodular Melanoma
NMSC	Non-Melanoma Skin Cancer
NRAS	Neuroblastoma RAS viral oncogene homolog
NTRK1	Neurotrophic Receptor Tyrosine Kinase 1
OBFC1	OB Fold containing Protein 1
OCA2	Oculocutaneous albinism II
OR	Odds Ratios
OS	Overall Survival
OTRs	Organ transplant recipients
p14ARF	ARF tumor suppressor
p16INK4A	also known as CDKN2A
P53	Protein 53
PADI6	Peptidyl Arginine Deiminase 6
PAF	Population Attributable Fraction
PARP	Poly(ADP-Ribose) Polymerase
PD-1	Programmed Death-1
PD-L1	Programmed death ligand 1
PDGFD	Platelet Derived Growth Factor D
PDGFRA	Platelet Derived Growth Factor Receptor Alpha
PH	Proportional Hazards
PLA2G6	Phospholipase A2 Group VI
PMEL	Promelanosome Protein
POT1	Protection of Telomeres 1
PPP6C	Protein Phosphatase 6 Catalytic Subunit

PRPP	Phosphoribosyl Pyrophosphate
PT	Phototype
PTEN	Phosphatase and Tensin Homolog
PUVA	psoralens plus UVA
RAC1	Ras-related C3 botulinum toxin substrate 1
RAS	Rat Sarcoma Gene
RB1	RB Transcriptional Corepressor 1
RCC2	Regulator of Chromosome Condensation 2
PADI6	Peptidyl Arginine Deiminase 6
RCT	Randomised Controlled Trial
Ref	Reference
REM	Random Effects Models
RFS	Relapse Free Survival
RNA	Ribonucleic acid
RR	Relative risk
RTEL1	Regulator of Telomere Elongation Helicase 1
RXRA	Retinoid X receptor Alpha
SB	Sunburn
SCOPE	Skincare in organ transplant patients
SD	Standard Deviation
SETDB1	SET Domain Bifurcated Histone Lysine Methyltransferase 1
SIR	Standardized Incidence Ratio
SLC24A5	Solute Carrier Family 24 Member 5
SLC45A2	Solute Carrier Family 45 Member 2
SLN	Sentinel Lymph Node
SNB	Sentinel Node Biopsy
SNPs	Single Nucleotide Polymorphisms
SPEN	Spen family transcriptional repressor
SPM	Single Primary Melanomas
SPSS	Statistical Package for the Social Sciences

SSM	Superficial spreading melanoma
STAT3	Signal transducer and activator of transcription 3
T cells	T cell mediated immune response
TERC	Telomerase RNA component
TERF2	Telomere repeat binding factor 2
TERF21P	Telomeric repeat binding factor 2 interacting protein 1
TERT	Telomerase reverse transcriptase
Th	T-helper cell
TIL	Tumor Infiltrating Lymphocytes
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TNM	Tumor, Node, Metastasis
TP53	Tumor Protein 53
Treg	Regulatory T Cell
TYR	Tyrosinase
TYRP1	Tyrosinase Related Protein 1
UHL	University Hospitals Leuven
UV(R)	Ultraviolet radiation
VD	Vitamin D
VDBP	Vitamin D Binding Protein
VDR	Vitamin D Receptor
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WSR	Age-Standardised Rate, using the World Standard Population
XRCC1	X-ray repair cross-complementing protein 1
YLLs	Years of Life Lost