Phenotypes and Genotypes of Melanoma Patients

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For my Parents

Content

Abstract

Introduction

Melanoma is most common in populations with European ancestry. The highest incidence rates are reported from Australia and New Zealand with more than 50/100,000 new cases and 6/100 000 deaths per year compared to around 20/100,000 new cases and 3/100 000 deaths in Western Europe. Established host risk factors are the total body naevus number, fair skin and a family history of melanoma. In the last decade several new genetic loci identified through genome-wide association analyses (GWAS) were shown to influence pigmentation traits and melanoma risk. Recently an intronic SNP in PPARGC1B has been shown to modify tanning ability and melanoma survival. The aims of this thesis were to conduct phenotypic and genetic comparisons of early and late stage melanoma patients, and to compare melanoma patients from Brisbane, Australia and Tübingen, Germany. This included associations of PPARGC1B and PPARGC1A coding polymorphisms with pigmentation traits, melanoma risk and progression in both these populations.

Methods

Two cohorts of melanoma patients and controls from Tübingen (n=614) and Brisbane (n=893) have been collected and analysed to investigate phenotypes and genotypes associated with melanoma risk and progression. Data and material included questionnaires, clinical assessments, melanoma specific parameters and germline DNA. Genotyping was performed by Sanger Sequencing, TaqMan SNP assays and with a high throughput Illumina CoreExome Chip assay. Phenotype and genotype associations were analysed using cross tabulation and logistic regression models. Survival probabilities were assessed genome-wide and for single variables using Kaplan Meier estimates, and comparisons were done with the log rank test.

Results

Patients with high naevus counts had no favourable outcome in the analysed cohort from Tübingen, however being a carrier of a MC1R r allele was associated with an improved survival probability ($p=0.049$). Two SNPs, the intronic variants rs7551288 in DHCR24, and rs12146110 in USH2A, were found in a genome-wide association analyses to be associated with overall survival and furthermore with progression in stage IV ($p<0.001$). Melanoma patients from Brisbane had favourable prognostic tumour characteristics and a higher rate of familial and multiple melanomas compared to patients from Tübingen. Furthermore, the influence of the

PPARGC1 transcription factors (PGC-1) on pigmentation was investigated. The exon SNP rs3736265 (T612M) in PPARGC1A was found for the first time to reduce naevus count. The intronic SNPs rs251468 and rs32579 in PPARGC1B were both confirmed to increase tanning ability and decrease naevus count, the SNP rs32579 additionally to improve survival in a meta-analysis.

Conclusion

Genetic variants have been identified that influence pigmentation traits, melanoma risk and progression. New loci of potential relevance for naevus count, melanoma risk and progression have reached statistical significance. UV radiation has an overriding influence on the impact of genetic predisposition, especially in countries with high UV exposure and is leading to an increase of preventable, initially less aggressive melanomas.

UV protection, early recognition and early treatment remain the most important strategies to overcome the rise of preventable melanomas and to reduce melanoma deaths.

German Abstract

Einleitung

Das Melanom ist am häufigsten in Bevölkerungen mit europäischer Abstammung. Australien und Neuseeland haben die höchsten Inzidenzraten mit mehr als 50/100 000 Neuerkrankungen und 6/100 000 Todesfällen pro Jahr, verglichen mit ungefähr 20/100 000 Neuerkrankungen und 3/100 000 Todesfällen in Westeuropa. Etablierte Risikofaktoren sind die Gesamtzahl der melanozytären Nävi, helle Haut und eine positive Familienanamnese für Melanom. Im letzten Jahrzehnt wurden einige neue genetische Marker durch genomweite Assoziationsstudien entdeckt, für die ein Einfluss auf die Pigmentierung und das Melanom Risiko gezeigt wurden. Kürzlich konnte gezeigt werden, dass ein intronischer SNP in PPARGC1B das Bräunungsverhalten und das Melanom Überleben modifiziert. Die Ziele dieser Thesis waren, phänotypische und genetische Vergleiche zwischen Melanomen im frühen und im späten Stadium durchzuführen und Melanom Patienten von Brisbane, Australien und Tübingen, Deutschland zu vergleichen. Dies beinhaltete Assoziationen von PPARGC1B und PPARGC1A codierenden Polymorphismen mit Pigmentierung, Melanom Risiko und Progression in beiden Populationen.

Methoden

Zwei Kohorten aus Melanom Patienten und Kontrollen von Tübingen (n=614) und Brisbane (n=893) wurden erfasst und ausgewertet, um Phänotypen und Genotypen zu untersuchen, die mit Melanom Risiko und Progression assoziiert sind. Daten und Material beinhalteten Fragebögen, klinische Untersuchungen, Melanom spezifische Parameter und Keimbahn DNA. Genotypisierungen erfolgten mit Sanger Sequenzierung, TaqMan SNP Assays und mit dem Hochdurchsatz Illumina CoreExome Chip Assay. Phänotyp- und Genotyp-Assoziationen wurden mittels Kreuztabellen und logistischen Regressionsmodellen analysiert. Überlebenswahrscheinlichkeiten wurden genomweit und für einzelne Variablen unter Verwendung von Kaplan-Meier-Schätzungen berechnet, und Vergleiche wurden mit dem Log-Rank-Test durchgeführt.

Ergebnisse

Patienten mit hoher Zahl melanozytärer Nävi hatten in der untersuchten Tübinger Kohorte keine günstigere Prognose, jedoch war das Vorhandensein eines MC1R r - Allels mit einer verbesserten Überlebenswahrscheinlichkeit assoziiert (p = 0,049). Zwei SNPs, die intronischen

Varianten rs7551288 in DHCR24 und rs12146110 in USH2A, wurden in einer genomweiten Assoziationsanalyse gefunden, die sowohl mit dem Gesamtüberleben als auch mit der Progression im Stadium IV assoziiert waren ($p < 0{,}001$). Melanom Patienten aus Brisbane hatten günstige prognostische Tumor Merkmale und eine höhere Rate an familiären und multiplen Melanomen im Vergleich zu Patienten aus Tübingen. Weiterhin wurde der Einfluss der PPARGC1 Transkriptionsfaktoren (PGC-1) auf die Pigmentierung untersucht. Der Exon SNP rs3736265 (T612M) in PPARGC1A wurde erstmals mit einer reduzierten Nävuszahl in Verbindung gebracht. Die Assoziationen der beiden intronischen SNPs rs251468 und rs32579 in PPARGC1B mit erhöhtem Bräunungsvermögen und reduzierter Anzahl an Nävi wurde bestätigt, der SNP rs32579 war zusätzlich mit einem verbesserten Überleben in einer Metaanalyse assoziiert.

Schlussfolgerung

Es wurden genetische Varianten identifiziert, die die Pigmentierung, das Melanom Risiko und die Progression beeinflussen. Neue Loci von potentieller Relevanz für Nävuszahl, Melanom Risiko und Progression haben statistische Signifikanz erreicht. Vor allem in Ländern mit hoher UV-Exposition hat die UV-Strahlung einen dominierenden Einfluss auf die genetische Prädisposition und führt zu einer Zunahme von vermeidbaren, zunächst weniger aggressiven Melanomen.

UV-Schutz, Früherkennung und frühzeitige Behandlung bleiben die wichtigsten Strategien, um den Anstieg von vermeidbaren Melanomen zu überwinden und Melanom-Todesfälle zu reduzieren.

1. Introduction

The work of this thesis is focussing on melanoma patients from two countries with different daily UV exposure intensities, Germany and Australia. The main research interests included the phenotypic and genotypic differences of early and late stage melanoma patients, the different characteristics of melanoma patients from Australia and Germany and the effect of genetic germline variants.

This thesis document commences with in a general introduction chapter and a method chapter giving an overview of how patient data and material was collected and processed in the laboratory and which information technologies were used for this work.

The general introduction will give an overview on the disease melanoma, including the different melanoma subtypes, historical aspects, the incidence, risk and prognostic factors of the disease. Biological aspects of the melanocytes, which are the cells of origin of naevi and melanoma will be described as well. The introduction will furthermore cover the process of naevogenesis, the development of naevi on the skin to understand the background of the phenotype characteristic “total body naevus count” and the meaning of naevi as potential precursor of melanoma. The next topic will be melanoma susceptibility genes, those genes which are thought to influence the breakout and the course of the disease. The last chapter of the introduction is focussing on the transcription factors PGC-1α and PGC-1β (peroxisome proliferator-activated receptor gamma coactivators alpha and beta) and its biologic interactions. This will establish the potential meaning of genetic variants within the coding regions for these proteins in relation to the pigment system and melanoma outcome.

The following four chapters are covering the four different research areas: 1) the pilot study, which was initiated in 2013 in Tübingen, 2) the comparison of early stage and late stage melanoma patients from a large cohort study conducted in Tübingen combining phenotypes and genotypes with disease specific survival data, 3) the comparison of melanoma patients from Australia and from Germany with a focus on phenotype and genotype characteristics as well as melanoma specific data and 4) the association analyses of genetic variants coding for PGC-1 family members. Each of these four chapters are structured in a short introduction and methods chapter, with specific aspects not covered by the general introduction and method chapter followed by a result, discussion and conclusion chapter.

The thesis finishes with a summary of findings and a final conclusion chapter.

1.1. Melanoma

Melanoma is a high mortality risk cancer originating from pigment producing cells, the melanocytes. Given the predominant localisation on the outer skin this type of cancer is exceptionally vulnerable to the main carcinogen UV radiation but also suitable for early detection.[1] Melanomas either develop from single melanoma cells or evolve from existing naevi, original benign conglomerations of melanocytes in the skin. Tumour thickness is the main prognostic factor, which is raising the risk of tumour spread and the hazard of distant organ metastases even years after primary excision. Affecting adults of all races and also at a younger age compared to other solid tumours, melanoma is among those cancers with the most substantial impact on years of life lost.[2] The incidence rates are still rising globally and are the highest among fair skinned populations in environments with intense UV radiation.[3]

Despite the high mortality risk of metastatic melanoma, it is important to note that today most melanomas are cured by excision after early detection. The individual prognosis of a patient however drops dramatically if the primary tumour has reached a tumour thickness of 2mm or greater, and if other adverse factors like an increased mitoses rate or ulceration of the primary are present. Metastases are rarely found at primary diagnoses but develop frequently in high risk patients after a latency of commonly 1-3 years, yet can also occur more than 10 years after primary excision in single cases. Patients with solitary metastases can still be cured with surgery, some patients with disseminated metastases do experience long term remissions with the recently developed systemic treatments, but in general survival chances diminish if the disease has spread more widely.

1.1.1. Subtypes

According to the distribution of melanocytes throughout the body, melanoma develops predominantly in the skin but can also primarily arise in nail beds, mucosa and in the uveal tract of the eye. In respect to these locations melanomas are categorized in cutaneous melanoma, mucosal melanoma and uveal melanoma. The most common cutaneous melanomas have been classified as different melanoma subtypes in regard to their clinical and histopathological appearance: Lentigo maligna melanoma, superficial spreading melanoma, nodular melanoma and acral lentiginous melanoma.[4] The clinical presentation of the different subtypes is shown in Figure 1. Lentigo maligna melanoma is linked to the amount of cumulative sun exposure,

superficial spreading melanoma to intermittent sun exposure, while the subtypes nodular melanoma and acral lentiginous melanoma are thought to develop independently of UV as carcinogen.[1]

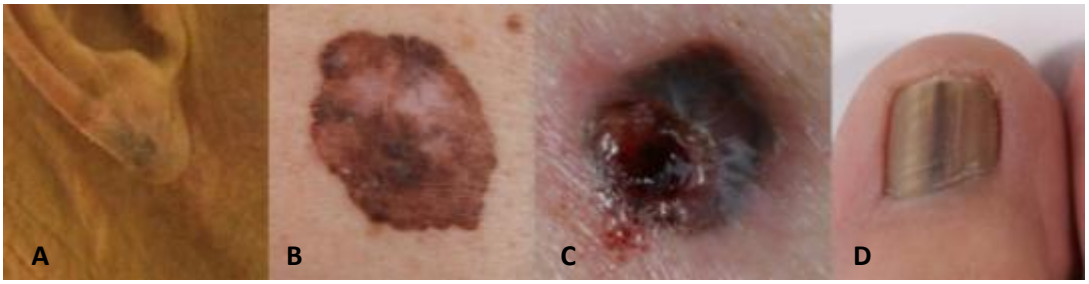


Figure 1: Clinically images of the diverse subtypes, underpinning the variety of clinical melanoma presentations. 1A Lentigo Maligna Melanoma, 1B Superficial spreading melanoma, 1C Nodular melanoma (with ulceration), 1D Acral lentiginous melanoma. (Photographs: Department of Dermatology, Tübingen, Germany)

1.1.2. Historical Background

The first report on melanoma retrieved in PubMed was from the year 1905. It was a case on a melanoma of the choroid written by DeSchweinitz and Shumway.[5] In this report the authors stated that the pathologist Virchow was the first who described malignant tumour growth on pre-existing pigmented naevi on the skin. The pathologists at that time called those tumours sarcomas. Other common terms were naevocarcinoma, melanosarcoma and melanocarcinoma.[6,7] Interestingly to note is that initially the term melanoma was used synonymously for the terms “moles” or “pigmented naevus”.[8] Only with using the term “malignant melanoma” the malignant counterpart was correctly described, whereas today it is more and more common to use solely the term melanoma. The pathologists in the early 20th century proposed that the pigmented hairy mole was a sensory organ of the mammal, as they described the nerve fibres in the lesion and compared it with tactile organs of reptiles.[9] The pigmentation of melanoma metastases was considered to be extruded chromatin from the nuclei.[6] The first case reports are predominantly covering the topic uveal melanoma or metastatic findings.[10-12] In the 1940s, the first clinical cohort studies were published with a detailed description of 35, 117 and 862 melanoma cases respectively and calculated overall survival probabilities below 20%.[13-15] The authors considered mechanical trauma or irritation to be the main cause of disease at that time and pointed out the necessity of deep and wide surgical excision. The

association of sunlight and melanoma was described in the 1950s.[16,17] In the 1970s families with multiple and atypical moles were reported and their increased risk for developing melanoma as well as contributing hereditary factors were discussed.[18,19] These reports marked the beginning of an ongoing exploration of the genetic and environmental risk factors for naevus and melanoma development.

1.1.3. Incidence

The incidence and mortality rates for melanoma are steadily increasing over the last decades and are projected to further increase in most regions of the world.[20] In Germany 6-8 melanomas per 100 000 inhabitants were registered in the eighties of the last century, while 18 cases per 100 000 were reported in 2011.[21 22] Australia and New Zealand have the highest incidence rates of melanoma in the world with a lifetime risk of 3.6% compared to 1.9% in the white US population and up to 1.6% in European countries.[23] In Australia the age-standardised mortality rate in 2007 was 5.8 per 100,000 for men and 2.5 per 100,000 for women in Australia and even higher in New Zealand with 6.4 and 3.4 per 100,000, respectively.[24] Recently it was shown that the incidence of thin melanomas was declining in Queensland for the first time, the incidence of melanomas >1mm tumour thickness however was still remaining stable. The decline of thin melanomas was linked to the successful prevention program “Slip Slap Slop”, which was introduced in Queensland 30 years ago. The stable incidence of thick melanomas was thought to be explained by melanomas with a different biology, e.g. fast growing melanomas or not sun-induced melanomas.[25] This for the first time reported decline in diagnosed melanoma cases was criticised by the argument that the incidence rates were diluted with a high number of immigrants from “low risk” countries, e.g. from Asia, who were not susceptible for melanoma as were the inhabitants with European ancestry.[26] However, even under the assumption that all population increases were due to immigrants from “low risk” countries, the decline in incidence rates was confirmed.[27,28] Thus, for the first time, a decline in incidence rates was observed which is most probably linked to an effective primary prevention via UV protection.

1.1.4. Risk Factors

Environmental, phenotypic and genetic risk factors for the development of primary melanoma have been explored in the past. Genetically determined host risk factors for the development of melanoma are fair skin, low tanning capacity and a family history of melanoma. There is evolving knowledge of genes that influence melanoma development and prognosis. The only known environmental risk factor to date is sun exposure. Sun exposure and genetic predisposition both influence the total number of acquired melanocytic naevi, which is the strongest known risk factor for primary melanoma.[29]

The typical phenotype of a melanoma patient is fair skinned with blue eyes and blond or red hair or with multiple naevi. The first studies investigating the association with pigment traits and constitutional factors with melanoma risk were published in the 1980's. One of the first case-control study reported naevus count as the strongest risk factor. Further traits were inability to tan, susceptibility to sunburn and hair colour, whereas skin and eye colour had no independent effect.[30] Having red or blond hair was also reported as risk factor as well as naevus count in another study.[31] The strong relation between increasing numbers of benign naevi and melanoma risk was repeatedly reported.[32-34] With the combined assessment of 16 case-control studies, based on more than 3000 cases, the lower impact traits like hair colour, eye colour, freckling, skin colour were also confirmed as independent risk factors besides naevus count.[35] In subsequent studies the presence of atypical naevi were included in the analyses and were added as independent risk factors.[29] The largest study so far included 12,387 individuals from 15 case cohort studies into a pooled dataset to calculate a risk prediction model based on seven different risk factors. The strongest individual risk factor based on this dataset was the total naevus body count, followed by the existence of large naevi (5mm) on the body, hair colour, family history of melanoma, freckling, hair colour, and the history of sunburns.[36] Apart from this well-established phenotype risk factors, which are mainly based on pigment traits, research has been done in the last years to find additional factors, such as obesity. Obesity is a long established risk factor for several types of cancer, e.g. breast, endometrium, colon and kidney.[37] A meta-analyses published in 2013 found an increased melanoma risk for obese male but not for obese female individuals.[38] No association was found in a large prospective cohort study from Denmark.[39] However, detailed data to adjust for UV exposure were not available and the authors stated that further studies are needed to address this question.

UV Exposure is the main carcinogen for melanoma and for skin cancer in general. The sun's impact on the development of melanoma is revealed by clear geographical differences, with rising incidence rates for Caucasians living closer to the equator.[23] Furthermore, sun seeking behaviour, especially in early childhood, as well as occupational sun exposure is a frequently reported melanoma risk factor in epidemiological studies.[40-42] The dramatically increased melanoma risk of patients with xeroderma pigmentosum, a hereditary syndrome with a deficiency in nucleotide excision repair and UV hypersensitivity, strongly supports the role of UV associated DNA damage in melanoma development as well.[43] Despite those findings, the role of UV exposure was still a controversial topic in recent years. In contrast to keratinocyte cancers (KCs), which occur predominantly on chronically sun-exposed body areas, only the lentigo maligna subtype is clearly associated with chronical sun damage, whereas some melanomas develop in intermittent or not sun exposed areas.[44] The absence of UV signature mutations in BRAF and other melanoma related genes, as well as the absence of p53, a driver mutation in KCs with UV signature mutations[45] seemed to lower the role of UV in melanoma. Moreover an UV independent pathway, based on oxidative damage, was described recently in a mouse model with and without pheomelanin production.[46] However, the presence of multiple UV signature mutations in melanoma was finally revealed with new sequencing approaches, including the discovery of a new set of driver mutations in melanoma, highlighting the role of UV light in melanoma pathogenesis.[47-49] Beside possible alternative pathways to melanoma, UV exposure remains the most important acquired melanoma risk factor.

1.1.5. Prognosis, Staging and Prognostic Factors

The term prognosis derived from the Greek word πρόγνωσις which means forecast. It is describing the attempt to declare what will occur within a specific area of interest in future. In melanoma these outcomes of interest are the prediction of progression, in particular the event of new metastases, as well as the prediction of survival for an individual patient. These predictions are generally made based on experiences with other melanoma patients in the past. This information can be obtained by personal observations, by adapting observations made by others or obtained from the literature, ideally based on systematic scientific analyses of a large group of patients. The analyses of differences in melanoma patients and their outcomes permit the establishment of prognostic factors.

The currently used melanoma staging system was updated in 2009 and uses the factors tumour thickness, mitoses in the primary tumour, number of lymph node metastases, site of distant metastases and the blood marker LDH. Tumour thickness and the presence of ulceration or mitoses in the primary tumour are defining the T Stage, the N Stage is summarizing the number and size of lymph node metastases and the presence of satellite or in-transit metastases and the M Stage is defined by the presence and site of distant metastases and the LDH blood level.[50] In January 2017 the 8th Edition staging system was presented.[51] Relevant revisions included the definition of the primary tumour with a 0.8mm threshold for T1 and the remove of the mitotic rate as staging criteria. The updated TNM definitions are summarized in Table 1. The melanoma stage is based on the TNM definitions. The new staging system will be implemented in 2018. The pathological staging of the lymph nodes is requiring a sentinel lymph node biopsy which is generally performed at primary diagnoses. The recommended threshold for this procedure in Germany is currently a tumour thickness of 1mm.[52]

The 20 year survival rates for patients with a clinical stage IB, comprising tumours with a thickness below 2mm and the absence of lymph node involvement are above 70%. The survival curves in stage IV based on the different sites of metastases are shown in Figure 2.

T Category	Thickness	Ulceration status
TX: primary tumor thickness cannot be assessed (e.g., diagnosis by curettage)	Not applicable	Not applicable
T0: no evidence of primary tumor (e.g., unknown primary or completely regressed melanoma)	Not applicable	Not applicable
Tis (melanoma *in situ*)	Not applicable	Not applicable
T1	≤1.0 mm	Unknown or unspecified
T1a	<0.8 mm	Without ulceration
T1b	<0.8 mm 0.8–1.0 mm	With ulceration With or without ulceration
T2	>1.0–2.0 mm	Unknown or unspecified
T2a	>1.0–2.0 mm	Without ulceration
T2b	>1.0–2.0 mm	With ulceration
T3	>2.0-4.0 mm	Unknown or unspecified
T3a	>2.0–4.0 mm	Without ulceration
T3b	>2.0–4.0 mm	With ulceration
T4	>4.0 mm	Unknown or unspecified
T4a	>4.0 mm	Without ulceration
T4b	>4.0 mm	With ulceration

	M Criteria	
M Category	Anatomic site	LDH level
M0	No evidence of distant metastasis	Not applicable
M1	Evidence of distant metastasis	See below
M1a	Distant metastasis to skin, soft tissue including muscle, and/or nonregional lymph node	Not recorded or unspecified
M1a(0)		Not elevated
M1a(1)		Elevated
M1b	Distant metastasis to lung with or without M1a sites of disease	Not recorded or unspecified
M1b(0)		Not elevated
M1b(1)		Elevated
M1c	Distant metastasis to non-CNS visceral sites with or without M1a or M1b sites of disease	Not recorded or unspecified
M1c(0)		Not elevated
M1c(1)		Elevated
M1d	Distant metastasis to CNS with or without M1a, M1b, or M1c sites of disease	Not recorded or unspecified
M1d(0)		Normal
M1d(1)		Elevated

Suffixes for M category: (0) LDH not elevated, (1) LDH elevated.
No suffix is used if LDH is not recorded or is unspecified.

Extent of regional lymph node and/or lymphatic metastasis		
N Category	Number of tumor-involved regional lymph node	Presence of in-transit, satellite, and/or microsatellite metastases
NX	Regional nodes not assessed (e.g., SLN biopsy not performed, regional nodes previously removed for another reason)	No
	Exception: pathological N category is not required for T1 melanomas, use cN.	
N0	No regional metastases detected	No
N1	One tumor-involved node or in-transit, satellite, and/or microsatellite metastases with no tumor-involved nodes	
N1a	One clinically occult (i.e., detected by SLN biopsy)	No
N1b	One clinically detected	No
N1c	No regional lymph node disease	Yes
N2	Two or three tumor-involved nodes or in-transit, satellite, and/or microsatellite metastases with one tumor-involved node	
N2a	Two or three clinically occult (i.e., detected by SLN biopsy)	No
N2b	Two or three, at least one of which was clinically detected	No
N2c	One clinically occult or clinically detected	Yes
N3	Four or more tumor-involved nodes or in-transit, satellite, and/or microsatellite metastases with two or more tumor-involved nodes, or any number of matted nodes without or with in-transit, satellite, and/or microsatellite metastases	
N3a	Four or more clinically occult (i.e., detected by SLN biopsy)	No
N3b	Four or more, at least one of which was clinically detected, or presence of any number of matted nodes	No
N3c	Two or more clinically occult or clinically detected and/or presence of any number of matted nodes	Yes

Table 1: TNM disease classification used by the latest AJCC staging system from 2017. Figure from Gershenwald et al. 2017[51]

Table 2: Updated melanoma staging system published by the American Joint Committee on Cancer in 2017. Adapted from Gershenwald et al. 2017[51]

Clinical stage group*	Clinical Staging			Pathologi-cal stage group**	Pathologic Staging		
	T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Any T	≥N1	M0	IIIA	T1a/b–T2a	N1a or N2a	M0
				IIIB	T0	N1b, N1c	M0
					T1a/b–T2a	N1b/c or N2b	M0
					T2b/T3a	N1a–N2b	M0
				IIIC	T0	N2b, N2c, N3b or N3c	M0
					T1a–T3a	N2c or N3a/b/c	M0
					T3b/T4a	≥N1	M0
					T4b	N1a–N2c	M0
				IIID	T4b	N3a/b/c	M0
IV	Any T, Tis	Any N	M1	IV	Any T, Tis	Any N	M1

*Clinical staging includes the microstaging of the primary melanoma and evaluation for metastases
**Pathological staging includes the microstaging of the primary melanoma and pathological information about the regional lymph nodes after sentinel lymph node biopsy.

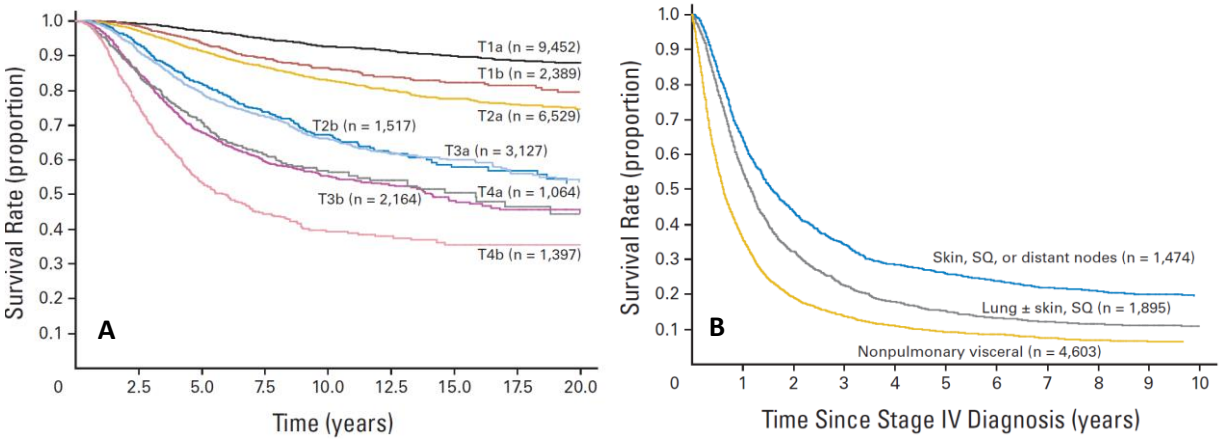


Figure 2: Survival rates based on the different T categories from time of diagnoses (Fig 2A) and survival rates calculated from time of Stage IV disease comparing different site of distant metastases (Fig 2B). Image from Balch et al. 2009[50]

Apart from the factors which the American Joint Committee on Cancer used to build the currently used staging system, several other prognostic factors were assessed and discussed in the literature, some having a clear prognostic impact and some of them with inconsistent results. Those investigated factors include tumour specific factors, phenotype factors, blood based biomarkers and hereditary genetic factors.

Regression is a regularly observed phenomenon in primary melanomas and is thought to be an immunologic event in which malignant cells are diminishing and are being replaced by fibrosis. These regression zones can already be observed visually, notably with the use of dermoscopy, and more accurately diagnosed in the histologic specimen. A systematic review and meta-analysis including more than 8 000 patients found a significantly decreased risk of death for patients with histologic regression in their primary melanoma.[53]

The majority of melanoma patients develop a single melanoma, while a subset will have further melanomas in their lifetime. Those patients having multiple melanomas were investigated in regard to their outcome and several studies found a better survival rate for those patients compared to patients with a single melanoma.[54-56] This favourable association was confirmed in a large multicentre melanoma cohort study for a subset of patients, those with thick melanomas above 4mm.[57]

Antigen expression on primary melanomas is widely studied and some expression patterns were found to be associated with prognosis, albeit none of those markers have transferred into clinical routine so far.[58]

The phenotypic characteristic total body naevus number is the most important risk factor for melanoma. A favourable outcome for patients with high total body naevus count was reported by Ribero et al. in 2015 based on a cohort of more than 2 000 patients. The results remained statistically significant even after adjustment for known prognostic and other factors, such as age, tumour thickness, sex, site of primary, presence of ulceration, the mitotic rate and the country of the patients.[59] However, there are concerns about the validity of this association due to the used statistical model, assuming a constant linear association of tumour thickness and prognosis, and due to the suspected over-diagnosis of indolent screen-detected melanomas, notably in this group of high risk patients.[60]

Several studies found a better outcome for woman with melanoma across all stages.[61-63] The favourable prognosis was discussed to be associated with thinner melanomas in women due to a better participation in screening programs but remained statistically significant after adjustment for other prognostic factors. A pooled analysis of three large studies showed an enhanced survival of women with metastatic melanoma which supports the biologic influence rather than a behavioural difference of females on disease outcome.[64]

Obesity was assessed as risk factor for melanoma in many studies and is thought to be associated with an aggressive course of disease as well, as reviewed by Clement et al. based on molecular mechanisms and animal models.[65] One study showed an increased rate of thick melanomas in obese patients.[66] Clear evidence of a prognostic survival impact of obesity is still lacking.

Elevated levels of the serum biomarker LDH are associated with impaired survival. This biomarker is used by the current staging classification. Other biomarkers with a prognostic impact have been studied as well, such as MIA, CRP and S100b. Serum S100b protein is routinely used as a diagnostic biomarker.[58]

Germline variants in the genome are increasingly investigated given the modern and fast evolving sequencing techniques. Variants were found to contribute to melanoma susceptibility as discussed later in this introduction chapter, as well as having a prognostic impact on disease outcome.[67]

Somatic gene alterations in the primary melanoma and in metastases have been discovered in an increasing number with clinical significance and some of them with evidence of prognostic impact. The first candidate that resulted in a novel effective treatment strategy was the BRAF mutation which was published in 2002.[68] A prognostic impact was not found for patients with BRAF positive melanomas.[69-71] The Cancer Genome Atlas (TCGA), a project conducted by the National Cancer Institute and the National Human Genome Research Institute of the United States, has published a genomic classification of cutaneous melanoma in 2015. The extensive analyses of primary melanomas and melanoma metastases from 333 patients resulted in 4 distinct subtypes, the BRAF, RAS, NF1 and triple WT subtype. An improved survival was found for melanomas with an overexpression of immune related genes compared to those who were classified as “keratin” subclass.[72]

There are several aspects connected with the search for prognostic factors. Understanding of those specific characteristics that are associated with poor prognoses not only allows for the potential prediction of an individual course of the disease but can also serve as a foundation for clinical interventions. These potential interventions could be the implementation of adapted surveillance strategies to enhance secondary prevention as well as the discovery of new therapeutic targets.

1.2. Melanocytes

Malignant melanomas and melanocytic naevi have their cell of origin in common. This is the melanocyte, a pigment producing, dendritic cell derived from the neural crest during embryonal development with a subsequent migration into skin, eye and other tissues.[73] The main localisation of melanocytes is the lower epidermis and the precursor cells, the melanoblasts are located in the hair follicles.[74] However, melanocytes are rare in the skin compared to other cell populations with 1,500 melanocytes per square millimetre epidermis.[75] One important function of melanocytes is the production of melanin, the so called melanogenesis.[76] The melanin is packed in melanosomes which are membrane-bound organelles. The dendrites of the melanocytes enable the distribution of the pigment packed melanosomes towards the surrounding keratinocytes, where they built a layer over the nuclei of keratinocytes to protect the DNA from UVR damage.[77,78] The neighbourhood and organisation between keratinocytes and the corresponding melanocyte is called “the epidermal melanin unit” and includes an estimated sum of 40 keratinocytes per melanocyte.[79] The production of melanin is initiated by DNA damage of the keratinocytes typically caused by UV light.[80] As a result keratinocytes secrete α-melanocyte-stimulating hormone (αMSH)[81] which binds to the melanocortin-1 receptor (MC1R) on the surface of melanocytes and activates the tanning response through MITF. The initiation of this pathway requires the induction of the transcription factor p53.[82] The enzymatic cascade to produce melanin pigments involves tyrosinase, tyrosinase-related protein-1 (TYRP1), and tyrosinase-related protein 2/dopachrome tautomerase (DCT).[83] The high penetrance for TYR wildtypes correlating with darker pigmentation was demonstrated in cultured melanocytes.[84] There are two types of melanin, the orange-yellow pigment pheomelanin and the brown-black eumelanin which has better UV protection properties. Both were detected in hair and epidermis in the same relative proportions with lower concentrations of eumelanin

in individuals with skin type I.[85] The lighter pigment pheomelanin is more frequent among individuals with MC1R germline variants.[86] Those individuals have at higher risk for melanoma.[87] Apart from the impaired UV protection properties of pheomelanin with a higher probability of UV induced mutations, pheomelanin also induces reactive oxygen species (ROS). ROS in turn contributes to mutations such as $BRAF^{V600E}$ which are relevant for both melanoma and naevus development (naevogenesis).[88]

1.3. Naevogenesis

Melanocytic naevi (from now on referred as naevi) are common benign conglomerates of melanocytes and result from oncogenic mutations.[89] They develop in utero as congenital naevi or as acquired naevi in the first decades of life. Some naevi remain completely stable over the lifetime, while others change in size and pattern or involute completely, resulting in lower naevus numbers in elder individuals.[90] Naevi can occur throughout the body wherever melanocytes are found as well. This includes the most common location of sun exposed skin, such as trunk, extremities and face, but naevi can also arise in sun protected areas like the scalp, soles and the genitals. Naevi locations outside the skin involve nailbeds, iris, conjunctivae, mucosae and menginges.[91,92] Common acquired naevi of the skin can be characterised according to typical clinical and histological features. Junctional naevi are flat lesions and have nests of clustered melanocytes at the dermoepidermal zone. Compound naevi are usually light brown papules with additional nests in the dermis containing melanocytes with smaller cell nuclei and with lower pigment production. Dermal naevi are skin-coloured nodules having only dermal nests of non-pigmented melanocytes.[93] Nesting is one histopathological feature of naevi. Maturation, the decrease in size and pigment density in the lower areas is the other typical feature and is used as separation criteria towards melanoma.[94] Special entities of common naevi are Halo naevus (Sutton's naevus), with a depigmented surrounding, Blue naevus, with dermal nests of spindled melanocytes and a characteristic blue-grey colour, Naevus spilus, typically a large light brown macula including sprinkled dots and Spitz naevus, a common melanoma simulator in children with atypical cells.[93]

Different from common naevi are "dysplastic" (histopathological description) or "atypical" (clinical description) naevi. This entity was first described in melanoma families as irregular coloured and shaped lesions of larger size (frequently 5-10mm) than common naevi as part of

the so-called B-K mole syndrome.[18] Several epidemiological studies evaluated the presence of dysplastic naevi as an independent melanoma risk factor.[29] However, the individual risk for one dysplastic naevi to develop into a melanoma is low and prophylactic excisions are not reasonable.[95,96] Melanoma arise in most cases from single, isolated melanocytes and only in about one third from nested melanocytes of common or dysplastic naevi.[97] Models of a linear progression from melanocytes through dysplastic towards malignant lesions as well as the location and type of precursor cell are still under debate.[98,99]

1.3.1. Development Models and Cell of Origin

For a long time, initiating naevus cells were thought to be located in the epidermis, based on Unnas concept “Abtropfung” (drip down) from 1893. He postulated a downwards movement of naevus cells from the epidermis towards the dermis.[100] This concept was questioned in the last decades and the concept “Hochsteigerung” (rise up) was opposed, arguing the cells move upwards instead.[101] Both directions of naevus cell migration have been demonstrated in follow up images using dermoscopy which supported either of the two concepts.[102] Again based on dermoscopic follow up images, different naevogenesis pathways were introduced. A constitutional pathway, with persisting dermal proliferations and an acquired pathway with epidermal proliferations that grow and disappear later in life were postulated.[103] Evidence for both location of origin models, the epidermal as well as dermal precursor models are on hand as reviewed by Grichnik et al, a view replacing the one-direction models of the past.[98]

The initiation of naevus development is requiring oncogenic mutations, in most cases one that activates the MAPK pathway.[104] It has been demonstrated that most naevi show clonality[105,106], assuming that one melanocyte initially gains a mutation, consequently enters a proliferating state and forms the lesion. In the absence of additional mutations, the lesions remain benign and enter senescence. The concept of oncogene induced senescence was established on the observation that the induction of an oncogenic HRAS mutation resulted paradoxically in a growth arrest in vitro.[107] Most naevi seem to remain in a senescence state due to a $BRAF^{V600E}$ mutation.[108] Somatic alteration which for example lead to an activation of the Wnt signalling pathway[109], TERT promoter mutations or alterations of CDKN2A[99] can overcome this senescence and turn the faith towards a malignant lesion.

The kind of precursor cell of melanocytic naevi is still not defined. Beside mature or immature melanocytes of the skin, other cells like multipotent stem cells[110] or Schwann cells[111] have been proposed to serve as cells of origin as well. Some clinical observations support the immature progenitor cell model in contrast to the concept that already differentiated melanocytes develop initiating mutations. Immature mutated cells would remain silent until activation. This behaviour explains the association of childhood sun exposure and the delayed development of naevi and melanoma, as well as the occurrence of eruptive naevi under certain medications or other modulating circumstances such as pregnancy, as reviewed by Ross et al.[112]

1.3.2. Naevus Susceptibility Genes

Several germline variants have been identified in the last years which were demonstrated to be associated with naevus count and characteristics. One of the first reports of a genetic background of dysplastic naevi and melanoma was published in 1983 and postulated an autosomal dominant trait with a localisation of the respective gene on chromosome 1.[113] A few years later this suspected single responsible gene was located on the chromosome band 1p36.[114] Heterogeneity of the genetic basis however was suggested based on results of a subsequent study from Australia[115], and the immense complexity of genetic causes has been confirmed in the following years. The inherited background of naevus count and characteristics was proven in multiple twin studies, all demonstrating a higher correlation between monozygous compared to dizygous twins.[116-121] Specific genetic loci with an influence on naevi were retrieved by candidate approaches as well as genome-wide analyses with an ongoing increase of detected markers. A case-control analysis from Queensland found three variants, *MTAP* rs10757257, *PLA2G6* rs132985 and *IRF4* rs12203592 which had a significant association with naevus number.[122] A meta-analysis of genetic studies exploring the association of loci with naevi and melanoma confirmed two of those loci, *MTAP* and *PLA2G6*.[123] Not only coding variants but also variants outside the coding region of a gene were identified, such as the marker *D9S942* which is adjacent to the *CDKN2A* gene.[124] A genome-wide search for naevus count found two loci at chromosomes 9p21 and 5q31-32 in subjects from the UK Adult twin registry.[118] The *NID1* locus was identified in a GWAS from the USA conducted in a large cohort of individuals with European ancestry.[125] A candidate approach was used to identify *PAX3* as a candidate nevus gene.[126] Variants in *IRF4* were found to be associated with melanoma risk

and with naevus number. One allele, the rs12203592*T, was associated with flat naevi on the trunk in young adults and with melanoma.[127] The most recent meta-analysis of 11 naevus GWAS including more than 50 000 phenotyped individuals from Australis, Netherlands, United Kingdom and the United States confirmed known loci such as *MTAP*, with the strongest association, followed by *PLA2G6* and *IRF4*, 9q31.2, *CYP1B1*, *PPARGC1B*, and others and found additional SNPS with strong associations in *DOCK8*, *KITLG*, *HDAC4* and other genes. Most of these genes with the exception of *KITLG* were also associated with melanoma risk. (Duffy et al., in submission)

1.3.3. Somatic Mutations and Naevus Subtypes

Mutations in protein coding genes of the MAPK pathway are in most cases the underlying cause of naevogenesis.[104] The mutations seem to occur mutual exclusive, indicating a functional redundancy with no additional advantage of secondary mutations in this pathway, as reviewed by Damsky et al.[128] The activating BRAF mutation is the most common mutation in naevi and was found with about 80%[129,130] in a higher frequency than in primary melanomas.[68] Yazdi et al. found the BRAF mutation most frequently in papillomatous naevi (78%) but not in Spitz and in blue naevi.[130] Spitz naevi were found to have frequently a HRAS activation through copy number gains.[131] HRAS mutations in Spitz naevi were additionally found in several studies in a frequency between 11% and 26%.[132] NRAS mutations are less frequent than BRAF mutations in acquired naevi. However, all acquired naevi without a BRAF mutation seem to harbor a NRAS mutation.[133] NRAS mutations are in contrast common in congenital naevi.[134] Mutations in GNAQ, again resulting in an activation of the MAPK pathway were found in 83% of investigated blue naevi.[135] The use of BRAF inhibitors in the treatment of metastatic melanoma enabled the study of naevus behaviour in vivo. A report described the appearance of new lesions, nevi increase and nevi involution in one patient under BRAF inhibitor treatment. The involuted lesions were predominantly elevated papillomatous nevi with a globular dermoscopic pattern.[136] The speculation that these nevi must have been BRAF positive was directly demonstrated in a similar case using microbiopsy sampling and genotyping techniques.[137]

Life Cycle of Naevi

Naevi are considered to be benign but neoplastic lesions. While some naevi are stable throughout the life, others undergo a currently accepted model of a naevus life cycle. This cycle starts with an initiating mutation. An activation step is required to enter the promotion state which involves the proliferation of the lesion. The naevus growth stops in the senescence state, which is different in melanoma lesions. The last step is the involution of the naevus, which results in an incomplete or complete regression.[112]

1.4. Melanoma Susceptibility Genes

Several genes were discovered in the last years that influence melanoma susceptibility and prognosis. These genes are generally classified as high-, moderate- and low risk alleles. Variants in low risk genes are common in the population but their penetrance is low in contrast to high risk genes which are rare but have a high penetrance of development of cutaneous melanoma.[67,138]

1.4.1. High Penetrance Genes

The cell cycle regulators *CDKN2A* and *CDK4* are considered as high-penetrance melanoma susceptibility alleles. Both are discovered among melanoma families.[139] *CDKN2A* is located on chromosome 9p21 and can be translated via alternative splicing into 2 different proteins, p16/Ink4a and p14/Arf. Inactivating mutations of the *CDKN2A* gene are found in 20-40% of all familial melanoma cases and constitute the most frequent cause for melanoma susceptibility.[140] *CDK4* is located on chromosome 12q13 and represents a target of p16/Ink4a. All three proteins are part of the retinoblastoma (Rb) pathway and important players in tumour suppression.[141]

In the last years several new high risk genes were discovered, such as new loss-of-function variants in the protection of telomeres 1 gene (*POT1*), which were identified in familial melanoma patients.[142,143] Other mutations were found in the genes *BAP1*, *TERT*, *ACD* and *TERF2IP*.[144] An overview on the prevalence of high-risk melanoma susceptibility genes identified in melanoma-prone families so far is given in Figure 3.

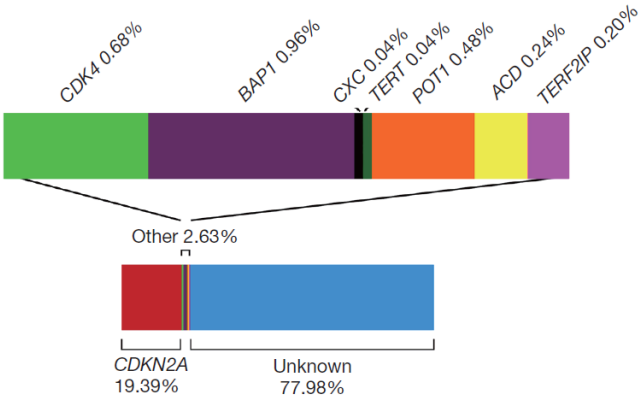


Figure 3: Prevalence of high-risk genes which were identified in melanoma families. Figure from Potrony et al. [138]

1.4.2. Moderate Penetrance genes

MC1R (melanocortin-1 receptor) is a key regulator of skin pigmentation and a moderate penetrance melanoma risk gene.[145] The gene encodes for a 7-transmembrane G-protein coupled receptor on the surface of melanocytes and is activated by α-melanocyte-stimulating hormone (MSH), which is secreted by keratinocytes as a response to UV exposure. The receptor protein is highly polymorphic. A large number of single nucleotide exchanges were described which lead to amino acid substitutions within the coding region and alter receptor function.[146] A decrease of MC1R function is shifting the balance from the black, photo protective pigment eumelanin towards the reddish pheomelanin, resulting in the red hair and fair skin phenotype.[147] The different *MC1R* variants were classified in big R types with a strong influence on pigmentation traits and small r types, those with a weak influence.[148] Carriers of *MC1R* variants have a significant higher risk of melanoma development and were also shown to increase penetrance of *CDKN2A* mutations.[149,150] Activation of MC1R induces the expression of MITF (microphthalmia transcription factor), a regulator of melanocyte proliferation and survival.[151] A novel *MITF* variant allele leading to an aminoacid change E318K was found in familial and sporadic melanoma and was associated with multiple primary melanomas. This allele increased melanoma risk by approximately 5-fold compared to the general population.[152,153] The encoded protein is deficient in SUMOylation and allows melanocytic cells to more efficiently overcome BRAF induced senescence, potentially favouring melanoma progression.[154]

1.4.3. Low Penetrance genes

As fair skin and decreased tanning ability is a risk factor for developing melanoma, not only *MC1R* but also other genes involved in pigmentation pathways were found to influence melanoma risk. The association of several candidate genes, like *ASIP* (Agouti signalling protein, nonagouti homolog (mouse) gene), *TYR* (tyrosinase), *TYRP1* (tyrosinase-related protein 1), *OCA2* (oculocutaneous albinism type II), *SLC24A4* (solute carrier family 24, member 4), *SLC45A2* (solute carrier family 45, member 2) and *IRF4* (interferon regulatory factor 4) have been discovered in the recent years.[155] Conflicting risk associations of the *IRF4* rs12203592*C/T alleles have been reported, with either increased melanoma risk attributed to the T allele[127,156], or to the C allele[122,157], while the latter association was found to be age dependant.[122] A potential prediction of melanoma subtypes based on divergent pathways was reported later, showing an association of melanoma with solar elastosis and the T allele and of melanoma with naevus residues and the C allele.[158,159] Further evidence of the significance of *IRF4* in melanoma is the association of the T allele with increased tumour thickness[160] and an impaired survival[161]. The influence of the C/C and T/T genotype on IRF4 protein levels and its response to UV radiation have been recently demonstrated in melanoblasts, highlighting the role of IRF4 in melanoma development.[162]

1.4.4. Genome Wide Association Studies

Genome-wide association studies (GWAS) allow the exploration of multiple genetic markers across the genome to search for uneven distributions of the markers frequencies in regard to a specific trait or disease among the study population. This method however does not allow a prediction of a causal relationship between the genetic variant and the trait or the impact of its influence. The first GWAS in melanoma were performed in 2008. A study from Iceland found variants near *ASIP*, in the genes *TYR* and *TYRP1* to be associated with melanoma risk.[163] Brown and MacGregor et al. and detected variants on the 20q11.22 locus associated with melanoma.[164] One year later a GWAS from the GenoMEl consortium was published including 1650 cases and 4336 controls. Loci within the genes *MC1R*, *TYR*, *MTAP*, *CDKN2A* were identified.[165] In 2011 Amos et al detected two loci in 2011 on chromosome 15 (locus 15q13.1, *HERC2/OCA2* region) and on chromosome 16 (locus 16q24.3, *MC1R* region) associated with melanoma risk.[166] Another study from the GenoMeL consortium replicated previous findings and

reported three novel loci (*ATM*, *MX2* and *CASP8*) which seemed to be independent to naevus or pigmentation associations.[167] A meta-analyses of genetic association studies including genome-wide association studies found four loci with genome wide significance (16q24.3 (*MC1R*), 20q11.22 (*MYH7B/PIGU/ASIP*), 11q14.3 (*TYR*), and 5p13.2 (*SLC45A2*).[168] An Australian GWAS confirmed previous loci and detected two novel loci at chromosome 1, 1q21.3 in proximity of the genes *ARNT* and *SETDB1* and at 1q42.12 in the DNA repair gene *PARP1*.[169] The GWAS performed by Nan et al. identified *NID1* as susceptibility locus.[125] A pathway based approach was used by Schoof et al. to focus on regions with previously known association to immunosuppression pathways as potential melanoma susceptibility loci. However, initial significant associations were not replicated in a second phase.[170] Investigating 156 melanoma cases from high-risk pedigrees three SNPs with genome-wide significance were detected on chromosome 10.[171] The latest GWAS with the largest study population to date was published by Ransohoff et al. using the 23andMe research cohort with self-reported melanoma cases and controls of European ancestry and a smaller stage 2 cohort for replication. The study confirmed 20 previously known loci and found a novel SNP (rs187843643) downstream of *BASP1* at chromosome 5.[172] The clinical significance of this region is supported by a prior report of the prognostic impact of *BASP1* methylation in human melanoma samples.[173]

1.5. PGC-1 Family Coactivators

The first member of the PGC-1 family (Peroxisome proliferator-activated receptor gamma co-activator encoded by the *PPARGC1A* gene) was identified in 1998 by Puigserver et al. They found a strong expression of PCG-1α upon cold exposure in mice. Expression of PCG-1α was associated with an increased function of PPARγ and an increased amount of mitochondrial DNA.[174] Two other members of the PGC-1 family, PCG-1ß (*PPARGC1B*) and a more distant relative, PGC-1 related coactivator (PRC) were found through their similar amino acid sequence. PCG-1ß was first cloned in 2002 revealing a conserved amino acid motif between PCG-1α and PCG-1ß.[175] This motif serves as a binding site for nuclear receptors to enhance transcriptional activity. PCG-1α and PCG-1ß both work as co-regulatory proteins together with estrogen-related receptors (ERRs) to regulate the expression of metabolic and mitochondrial genes and to maintain energy homeostasis.[176] High levels of PGC-1 are found in energy demanding tissues like brown adipose tissue, heart, brain and kidney which have also high numbers of mitochondria. PGC-1 coactivators directly influence mitochondrial biogenesis through up-regulation and coactivation of nuclear respiratory factor 1 (NRF1).[177] Other roles of PGC-1 are the control of hepatic gluconeogenesis [178] and Glut4 expression in muscle cells.[179]

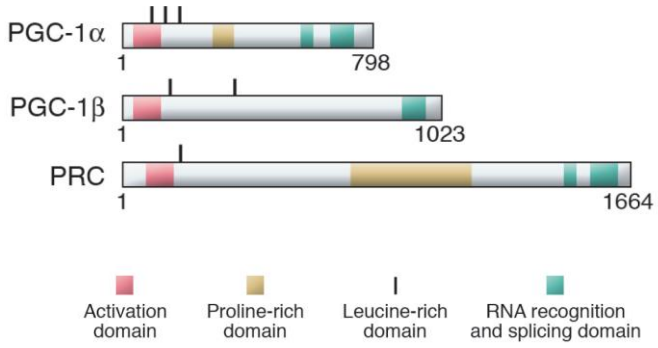


Figure 4: PGC-1 family members: PGC-1alpha, PGC-1beta and PRC share important primary sequence characteristics. Image from Finck et al. 2006 [180]

1.5.1. Role in Melanocytes

Recently Shoag et al. described for the first time a role in the skin for two members of the PGC-1 family.[181] They could show that both, PCG-1α and PCG-1ß, are involved in melanogenesis and are induced by secretion of a-MSH as a response of UV radiation. In turn PCG-1α and

PCG-1ß lead to the induction of MITF and other melanogenic genes. Furthermore, there is evidence for an association between a PGC-1ß variant (rs32579, located in intron 4) and increased tanning ability.[182] A recent analysis done by David Duffy et al. found evidence for a decreased naevus count and decreased melanoma risk for the minor allele at rs251464, located in intron 1 (Duffy et al., in submission).

1.5.2. Role in Cell Survival

The survival of cells is regulated in a complex way with opposing pathways promoting either death or life. Cell death can occur through different routes e.g. via apoptosis, the well sorted deconstruction of a cell, or via necrosis which is a more chaotic process involving acute rupture of cell organelles. One important strategy of cancer cells is to resist cell death.[183] Dysregulation of mitochondrial cell death pathways can result in either neurodegenerative and autoimmune diseases or malignant transformation, suggesting an important role of PGC-1 as a master regulator of mitochondria in these diseases.[184,185] The mitochondrial death pathway can be activated by mitochondrial Ca2+ accumulation and overexpression of PCG-1α has been shown to reduce mitochondrial Ca2+ uptake and thus enhance survival in a cervical cancer cell line.[186] PGC-1 family members are physiologically expressed in tissues with a high energy need. Stress conditions like fasting, cold exposure and exercise increase the expression of PGC-1 and subsequently lead to an elevated mitochondria density.[180] Overexpression of PGC-1 was demonstrated to support the cells coping with oxidative stress, e.g. through increased detoxification of mitochondrial and cytosolic reactive oxygen species (ROS).[187] Cancer cells are dependent on an increased cell metabolism. A study published by Vasquez et al. could show that overexpression of PGC-1α in melanoma cells resulted in increased survival under oxidative stress conditions based on enhanced ROS detoxification capacities. PGC-1α negative melanoma cells were more sensitive to ROS-inducing drugs.[188] Furthermore, activation of the PGC-1α – MITF axis may support the increased energy demand in melanoma cells as reviewed by Ze'ev Ronai.[189]

1.6. Hypothesis and Aims

Hypotheses

Melanoma risk is based on genotype, phenotype and environmental factors. Each of these factors also influence naevus development, which is the most important melanoma risk factor. Understanding the genetic determinants of naevus pattern might foster the understanding of melanoma development.

Melanoma risk is higher for individuals with high total body nevus count, but these patients are more likely to have low risk melanomas and an improved prognosis. The typical patient at high risk of melanoma is not the typical patient at high risk of progression and death.

UV exposure is the only predominant environmental risk factor.[190] Comparing melanoma patients from two countries with different daily UV exposure might reveal the impact of this factor on melanoma risk and prognosis.

Variants of the genes *PPARGC1B* and *PPARGC1A* might influence pigment traits and melanoma risk and prognoses.

Research Questions

Chapter 3:

- Is the dominant dermoscopic naevus pattern different between early and late stage melanoma patients?
- Is the dominant dermoscopic naevus pattern of an individual patient genetically determined?

Chapter 4:

- Are there differences between early and late stage melanoma patients?
- Do melanoma patients with multiple melanocytic naevi have a more favourable prognosis as compared to melanoma patients with few naevi?

Chapter 5:

- Are there differences between German and Australian melanoma patients?

Chapter 6:

- Is there an influence of *PPARGC1B* and *PPARGC1A* germline coding variants on naevus count, skin reaction, melanoma risk and melanoma progression?

Aims

Aim 1

To initiate a Melanoma cohort study in Tübingen with phenotypic, genotypic and dermoscopic data in order to test initial hypotheses / generate further hypothesis

- Chapter 3: Case Reports of a Pilot Study from Tübingen

Aim 2

To establish a German dataset similar to the dataset of the Brisbane Naevus Morphology Study in order to perform comparative analyses with two independent cohorts

- Chapter 4: Comparisons of early stage and late stage melanoma patients from Tübingen
- Chapter 5: Comparisons of German and Australian melanoma patients

Aim 3

To generate a library of SNP data derived from germline DNA of two independent cohorts, the Australian and the German cohort of melanoma patients and controls.

- Chapter 4: Genome-wide association with survival
- Chapter 6: PGC-1 Associations

Outlook: First genome wide association study (GWAS) on melanoma risk as future project with the use of the generated SNP library and healthy controls from the KORA dataset

2. Methods

2.1. Ethical Clearance

The work of this PhD thesis was based on data, images and biomaterial from melanoma patients and controls of two different countries, which were sampled during different time periods by the candidate and by colleagues. A total of 4 different ethical approvals were obtained to gather and to work with the data and material of the individuals.

A positive ethic vote was obtained in 2013 by the candidate for the pilot study with the title "Genetics and naevus pattern in melanoma patients". After submission of the protocol description, the planned assessments and a copy of the patient information and consent sheet, the positive ethic vote was assigned by the ethic committee of the Eberhard Karls University on the 8th July 2013 with the ethic number 326/2013BO2.

The Brisbane Naevus Morphology Study was initiated in 2009 by Prof. H. Peter Soyer to investigate melanoma patients and controls from Brisbane with phenotype and genetic data. The study was approved from the Metro South human ethics committee with the reference number HREC-09-QPAH-162. The UQ human ethics number was 2009001590.

The second cohort from Tübingen was drawn from a study with the title "Hereditary defects and cutaneous malignant melanoma", which was approved by the ethic committee of the Eberhard Karls University on the 12th December 2007, the ethics number was 376/2007B01. The study was designed to examine genetic and environmental risk factors for the development of melanoma. One analysis with collaborators at the Copenhagen University Hospital in Denmark was performed with the data in the past and published in 2011.[191] Future analyses of genetic associations were approved by the ethics committee in 2007.

In 2014 the candidate applied for an ethic approval to use data and material from the study 'Hereditary defects and cutaneous malignant melanoma', in order to perform comparative analyses with the data from the Brisbane Naevus Morphology Study. An institutional human research ethics approval was obtained from the University of Queensland (Approval Number 2014001223).

2.2. Study Populations

The present work and the analyses of this PhD thesis are based on three study populations. The first study was the pilot study, which was initiated and conducted by the candidate in Tübingen and consisted of 16 melanoma patients. The patients were recruited in July and August 2013. The second study was the Brisbane Naevus Morphology Study, initiated by Prof. H. Peter Soyer. This study has been commenced in 2009 and still has an ongoing recruitment. The candidate was involved in patient recruitment in 2014. The third study was the study with the title 'Hereditary defects and cutaneous malignant melanoma' from Tübingen and was initiated by Dr. Thomas Eigentler and Prof. Claus Garbe in 2007. The candidate was involved in patient recruitment as a resident in the department of Dermatology. The last study participant was included in 2011.

Recruitment and patient selection for the analyses as the basis of this PhD thesis will be described for each of the three studies in the following.

2.2.1. Pilot Study Tübingen

The patients were recruited at the University Hospital Tübingen in the Department of Dermatology. They were asked during their regular visit in the melanoma outpatient clinic if they would be interested to take part in a clinical study. The patients gave written informed consent before any study specific procedures were performed. A total of 16 melanoma patients were recruited in July and August 2013.

None of the patients had withdrawn their consent and all patients were included in the analyses.

2.2.2. Australian Cohort Study

The patients and controls were recruited at the Dermatology Department and the Melanoma Unit of the Princess Alexandra Hospital in Brisbane, Australia as well as in community private dermatology clinics. Participants who had no personal history of melanoma were recruited by public advertisement and by contacting participants from a previous study, the Brisbane Twin Naevus Study.[127] All participants had given written informed consent prior to study entry.

For the purpose of the analyses for this PhD thesis all consecutively recruited individuals with the first entry at the 19th Oct 2009 until a defined cut-off date (12th Oct 2015) were considered. The definition of this cut-off date was based on the last patient whose germline DNA was submitted for CoreExome genotyping by 13th Dec 2016, which was the time point of dataset closure. One patient (Patient ID 1374MB) was excluded, as no data at all were obtained after enrolment. Participants with missing genetic data were excluded as well. Those were 12 participants (Patient IDs: 141JB, 384KN, 550BB, 704JG, 709SJ, 747LH, 761PO, 957TJ, 1009MF, 1016RC, 1028PM and 1049PC) who did not give saliva at study entry and two participants (Patient IDs: 745SS, 903JA) whose samples did fail for genotyping on the Illumina CoreExome Chip.

A total of 893 participants of the study were thereby considered for the Australian cohort, including 454 melanoma patients and 439 controls.

2.2.3. German Cohort Study

The patients and controls were recruited at the Department of Dermatology of the University Hospital Tübingen. Melanoma patients were asked to participate at the study during their follow up visits in the melanoma outpatient clinic, participants without a history of melanoma were recruited during other dermatologic consultations, mainly at the general dermatologic outpatient clinic, or from clinic staff. All participants had given written informed consent prior to study entry. The first participant was included in 2007, the last one in 2011. A first report using the data was published in 2012.[191]

For the purpose of the analyses for this PhD thesis all participants with available frozen blood samples were considered. These were 558 samples from melanoma patients and 204 samples from controls, which were shipped to Australia. Two EDTA blood tubes were stored for one participant without a history of melanoma, reducing the number of control cases to 203 cases. All melanoma samples and 58 control samples were genotyped with the Illumina CoreExome Chip. Two cases of the melanoma group showed a mismatch of their recorded sex and the gender based on the genotype result (M316 and M372). One was recorded as female and showed a male genotype, one was recorded as male and showed a female genotype. Both cases were melanoma patients. Most likely those two blood samples were swapped by

accident. Both cases were excluded from the analyses for safety reasons. All participants with available genetic data were included.

A total of 614 participants of the study were considered for the German cohort, including 556 melanoma patients and 58 controls.

2.3. Study Measures

The study specific procedures consisted in all three studies of i. the performance of physical assessments, ii. the completion of questionnaires by the physicians and by the participants and iii. the sampling of blood or saliva of the participants to obtain germline DNA.

Naevus images and overview images were taken from the participants of the pilot study Tübingen and of the Australian cohort study, but not of the German cohort study.

Measurements of the skin reflectance as a marker for the skin colour were only done for participants of the Australian cohort study.

2.3.1. Pilot Study Tübingen

The detailed description of the study measures is given in chapter 3.2.3. The procedures were similar to those of the Australian participants as explained in the next paragraph.

2.3.2. Australian Cohort Study

The study participants of Brisbane were investigated by research assistants in the study centre of the Princess Alexandra Hospital. Prior to the study examinations they were asked to fill out a questionnaire which covered information of their ethnical background, hair- and eye colour, response to sun exposure, sun behaviour, personal and family history of melanoma and medication. Skin reflectance measurements were done at the inner and outer forearm to quantify constitutive and facultative skin pigmentation. The total naevus count of naevi from 5mm was recorded and each naevus was classified as flat or raised. Additionally, a total body photography as well as dermoscopic imaging of each individual naevus was performed using a digital computer dermoscope (FotoFinder Dermoscope, Bad Birnbach, Germany). Each participant

donated 2ml saliva using an Oragene-DNA self-collection kit (DNA Genotec, Ottawa, ON, Canada) in order to obtain germline DNA.

Melanoma specific data, such as localisation of the primary, histology and specific details of the histopathological report like ulceration, naevus association and signs of regression as well as subsequent metastases during the follow up were retrospectively recorded by research assistants. The first reports with data from study participants of the Australian cohort study were published in 2014 and 2016.[152,192]

2.3.3. German Cohort Study

The study participants of Tübingen were investigated by physicians of the melanoma outpatient clinic during their regular appointment. The patients were asked to complete a questionnaire which covered information on the ethnical background, pigmentation traits, like hair and eye colour, response to sun exposure, personal sun behaviour, personal and family history of melanoma or other cancers and medication. Participants were also asked to estimate the total number of their moles. Part of the study was a full body examination by a dermatologist. In order to quantify the presence of melanocytic naevi, the total number of naevi was grouped into 6 categories, which were 0-10, 11-30, 31-50, 51-100, 101-150 and above 150 naevi. All naevi with a diameter from 3mm were included. The number of dysplastic naevi was additionally recorded within the categories 0, 1-5, 6-10, 11-20 and above 20 dysplastic naevi. Furthermore, the Fitzpatrick skin type, signs of sun damage and the presence of actinic keratoses were documented. Blood was drawn to extract the germline DNA of each participant.

Data from all melanoma patients from Tübingen are generally included into the Central German Melanoma Registry after written informed consent. Therefore, detailed melanoma specific data were additionally available for the patients of the German cohort study. These included data related to the primary melanoma, such as the date of excision, localisation, histology and initial stage of disease. Furthermore, date and localisation of subsequent metastases, subsequent clinical stages, date and cause of death were recorded. These data were based on prospectively obtained follow up reports provided by the responsible physicians and entered by research assistants of the Central German Melanoma Registry.

2.4. Genotyping

Germline DNA from all selected study participants were processed for genetic analyses. These were 16 and 556 melanoma patients and 58 control cases from Tübingen (N=630) as well as 454 melanoma patients and 439 control cases from Brisbane (N=893). The first set of 16 patients from Tübingen and the individuals from Brisbane gave saliva samples with an Oragene-DNA self-collection kit (DNA Genotec, Ottawa, ON, Canada) for DNA extraction. The second set of samples from Tübingen consisted of frozen EDTA blood samples.

2.4.1. DNA Extraction

2.4.1.1. Saliva Samples

The germline DNA extraction of the saliva samples from Brisbane and the first 16 samples from Germany were done using the prepIT^{TM} L2P kit from DNA Genotek®. The saliva samples from Germany were extracted at the Institute for Molecular Bioscience in Brisbane. The saliva samples from Brisbane were collected between 2009 and 2015. The extractions were done at the Institute for Molecular Bioscience in Brisbane and from 2014 at the Dermatology Research Centre (DRC), Translational Research Institute (TRI) in Brisbane.

The DNA purification was done according to the manufacturer's protocol as follows. Each sample was mixed by inversion and was incubated in a water bath at 50°C for one hour to release the DNA and to inactivate nucleases in the probe. For one extraction step, 500ul of the sample were transferred to a 1.5ml microcentrifuge tube and 20ul of PT-L2P was added to precipitate impurities and inhibitors. After incubation on ice for 10 minutes the sample was centrifuged for 5 min at 13,000rpm. The supernatant was transferred to a microcentrifuge tube and 600ul ethanol was added to precipitate the DNA. The sample was left for 10min at room temperature and then centrifuged for 2min at 13,000rpm. The supernatant was removed with a pipette tip and discarded. After a washing step with ethanol the DNA pellet was dissolved with 100ul of TE solution. The DNA was incubated for an hour at room temperature followed by vortexing. The rehydrated DNA was stored in TE solution at 4°C.

The DNA quality and concentration were measured using a Spectrophotometer (NanoDrop™). For each sample the volume, the concentration (mg/ul), the absorbance at A260, the A260/A280 ratio and the total yield (uq) was recorded in an excel sheet.

2.4.1.2. Blood Samples

The germline DNA extraction of the blood samples from Germany were done using the column based QIAamp DNA Blood Midi Kit. The blood samples were collected during the years 2007 and 2011 and were stored in EDTA tubes at -80°C in Germany. In 2014 they were shipped on dry ice to Australia and stored again at -80°C. The extractions were done between December 2014 and March 2015 at the DRC, TRI in Brisbane.

The extractions were performed with each sample following the instructions of the Qiagen protocol as follows. Two ml of the thawed blood sample were mixed with 200ul Protease in a 15ml centrifuge tube. As a next step 2.4ml Buffer AL was added and after inverting 15 times and shaking for 1min the probe was placed in a 70°C water bath for 10min. After adding 2ml ethanol, half of the sample was transferred into a QIAamp Midi column and centrifuged at 3 000 rpm for 3min. The filtrate was discarded and the rest of the sample was transferred, centrifuged and the filtrate discarded. Subsequently two washing steps by adding Buffer AW1 and Buffer AW2, 2ml each, and centrifugation at 5 000rpm for 1min and 15min respectively were performed. The column was then transferred into a clean 15 ml centrifuge tube. An amount of 300ul Buffer AE was placed on the column membrane and the DNA was solved during a 5min incubation followed by a centrifugation at 5000rpm for 2min. This last step was repeated in order to increase the DNA yield.

The DNA quality and concentration were measured using a Spectrophotometer (NanoDrop™). For each sample the volume, the concentration (mg/ul), the absorbance at A260, the A260/A280 ratio and the total yield (uq) was recorded in an excel sheet.

2.4.2. Sanger Sequencing

Sanger Sequencing was performed with 175 germline DNA samples of the Brisbane cohort and with 16 samples of the German pilot study samples. Two different loci were sequenced. The first one was the melanocortin 1 receptor *(MC1R)* coding sequence at chromosome 16. The

second locus was on chromosome 5 within the gene *PPARGC1B*, coding for the protein Peroxisome proliferator-activated receptor gamma coactivator 1-beta.

The steps are described below in detail. At a glance they included the amplification of the germline DNA template via Polymerase chain reaction (PCR) with the use of oligonucleotide primers. A nested PCR with suitable primers was already established for the *MC1R* locus.[149] Customized primers were designed for the PGC-1b locus and a single PCR was performed. The subsequent steps for both loci were the separation on a gel via Electrophoresis, gel extraction, quantification and quality check with a spectrophotometer and the performance of the sequencing reaction. The samples were then submitted to the Australian Equine Genetics Research Centre (AEGRC, Brisbane, Australia) for sequencing. The retrieved data was analysed using the Sequencher® software (Company: Gene Codes Corporation).

2.4.2.1. PCR

MC1R

A nested PCR was performed to amplify the long *MC1R* locus. Two sets of primer pairs were used.

The oligonucleotide sequence of the primers for the first step, the outer PCR were:
N-outer (5’ -AGATGGAAGGAGGCAGGCAT-3’)
C-outer (5’ -CCGCGCTTCAACACTTTCAGAGATCA-3’)

The oligonucleotide sequence of the primers for the second step, the inner PCR were:
N-inner (5’-CCCCTGGCAGCACCATGAACT-3’)
C-inner (5’ -TGCCCAGGGTCACACAGGAAC-3’)

A 25ul system was used with an amount of 50-100ng germline DNA, dNTPs, primers, DMSO, $MgCl_2$, Promega buffer and Taq enzyme in defined amounts as listed below.

1. 10X Promega buffer 2.5ul
2. DMSO 2.5ul
3. dNTPs 2.0
4. N primer 1.0
5. C primer 1.0
6. $MgCl_2$ 0.75
7. Taq 0.25
8. DNA 50-100ng
9. H_2O bring total up to 25ul

The components No 1 – No 7 of the system were multiplied with the number of the processed samples +1 to set up a master mix. Taq polymerase was added as the last component and the master mix was kept on ice. It was instantly distributed on the prepared PCR tubes containing the germline DNA of each sample and the calculated amount of Millipore water (water purified with a Milli-Q® Water System).

The probes were then transferred into a standard PCR thermal cycler. The cycling steps were programmed with the following specifications:

First cycle (initialization step)	94°C for 3 min
34 cycles (denaturation, annealing, extension), each:	94°C for 1 min
	55°C for 1 min
	72°C for 2 min
Last step (final elongation)	72°C for 7 min

The resulting PCR product of the outer PCR was used as a template for the inner PCR. The master mix with the inner primers was set up as above with 1ul of the DNA template. The PCR program was repeated as described above.

PGC-1b

A single PCR was performed to amplify a selected region of PGC-1b at exon 5. Primers were designed using Primer3, an online tool, available at http://biotools.umassmed.edu/bio-apps/primer3_www.cgi.

The reference sequence of the PGC-1b region of interest was given in the 5´→ 3´direction and a forward and backward primer were calculated according to the predefined standard settings

The oligonucleotide sequences of the primers were:

Forward primer: 5'-TGAGACACATGGGAGGAGTG-3'

Reverse primer: 5'-AGGTAAGGATCGCAGCTTCAC-3'

The master mix components were set up using the following ingredients per sample.

1. 10X Promega buffer 5ul
2. DMSO 2.5ul
3. dNTPs 1.0
4. N primer 1.0
5. C primer 1.0
6. $MgCl_2$ 0.5
7. Taq 0.5
8. DNA 50-100ng
9. H_2O bring total up to 50ul

The PCR program was performed in an analogous fashion to the above described PCR of *MC1R.*

2.4.2.2. Gel Electrophoresis

A 1% agarose gel was prepared to run a gel electrophoresis with the PCR products. Sybr® Safe was used to visualise the DNA. The PCR products were mixed with loading dye on the chambers of the gel, which was placed on the gel tray within Tris Acetat EDTA Buffer. The Electrophoresis was run at 100V. Images of the bands were documented on a Gel Doc Imaging

system. Gels with clear bands around 1000bp for the *MC1R* PCR and around 700bp for the PGC-1 PCR were transferred to an ultraviolet light station and the bands were excised with a scalpel and stored in an Eppendorf tube.

2.4.2.3. DNA Gel Extraction and Quantification

The single stranded DNA products of the PCR reaction were extracted from the gel using a Gel Extraction Kit from QIAGEN. The extractions were performed according to the manufacturer´s protocol as described below. The excised DNA fragments from the agarose gel were added with 3 volumes Buffer QG per volume gel and incubated at 50°C for 10min and the sample was mixed every two minutes until the gel slice has completely dissolved. Isopropanol was added. The sample was transferred to the spin column and centrifuged for one minute. In the next steps 500ul Buffer QG and 750ul Buffer PE were added consecutively, each time centrifuged and the flow-through was discarded. Finally, the DNA was eluted by adding 50ul Buffer EB to the centre of the membrane, followed by a centrifugation for one minute.

The purified DNA was measured using a Spectrophotometer (Nanodrop®). After calibrating the system with Millipore water, an amount of 0.7ul sample was pipetted onto the pedestal. With closing the arm, a sample column was formed. Measurements were performed at the wavelengths of 260nm and 280nm. The concentration (mg/ul), the absorbance at A260, the A260/A280 ratio were displayed by the spectrophotometer and recorded.

2.4.2.4. Sequencing Reaction und Submission for Sequencing

For the sequencing reaction a master mix was set up using the following components in a system of 20ul per sample.

1. Big dye terminator v3.1	1ul
2. Seq buffer (5XB)	3.5ul
3. 3.2pmol Primer	1ul
4. DNA	20ng
5. sH_20	bring total up to 20ul

For the *MC1R* sequencing reaction two separate reactions for N and C-inner were prepared to obtain a bi-directional sequencing.[149] The PGC-1b sequencing was performed with one sequencing reaction per sample, using the forward primer of the PCR reaction.

The PCR tubes were placed in an Eppendorf PCR machine with the following sequence reaction program:

Step 1:	94°C, 3 min
Step 2 was repeated 30 times:	94°C, 12 sec
	50°C, 8 sec
	60°C, 4 min
Step 3	hold at 4°C

The samples were submitted to the Australian Equine Genetics Research Centre (AEGRC, Brisbane, Australia) were the reading out of the sequences was performed.

2.4.2.5. Analyses of Chromatograms

The chromatograms with the analysed sequences were provided as “ab1” and “seq” files. The data was assessed with the program Sequencer 5.1 (Gene Codes Corporation). The reference sequences of the analysed DNA region of *PPARGC1B* was obtained from the online SNP database (https://www.ncbi.nlm.nih.gov/snp) of the National Center for Biotechnology Information. The reference of the analysed region within *MC1R* was already available in the working group and was provided for the analyses.

In average a set of four chromatograms from different samples were imported together with the respective reference sequence in one project file at one time. The sequences were automatically assembled to the reference sequence. The overview function of the program displayed the sequenced fragments as lines and provided an assessment if the full expected length of each sample was obtained. Going into detail of the base sequences, the bases were displayed as characters – A, T, G and C - as well as chromatograms in the colours green, red, black and blue, respectively. The program highlighted variants in comparison to the reference sequence automatically with a certain amount of false positive and false negative findings.

Thus, all chromatograms were throughout visually investigated and compared with the reference sequence and each other. Homozygous variants were detected as one peak in a different colour on the chromatogram, heterozygous variants with two equally high peaks in different colours and insertions or deletions through a shift of the expected sequence alignments. The analyses were repeated for each set of samples. The results were recorded first manually and then transferred into excel sheets.

2.4.3. TaqMan Assay

TaqMan SNP genotyping assays were performed on a 384-well-plate format using a ViiA™ 7 Real-Time PCR system and analysis was carried out using the QuantStudio™ Real Time PCR software v1.1 (Applied Biosystems).[193]

The TagMan assay was not performed by the candidate, but by Kasturee Jagirdar and Hilary Yong.

The four additional SNPs that were genotyped using the TaqMan assay consisted of three *MC1R* polymorphisms; rs885479 R163Q, rs1805005 V60L, rs1805009 D294H, and a PGC-1β non-coding variant rs251468.

2.4.4. CoreExome Chip Array

In respect to the fast-evolving next generation high through put SNP assay, the CoreExome Chip array from Illumina was used from 2014 to genotype the samples of the Brisbane cohort study and the German cohort study. For the purpose of the present studies a total of 630 samples from Tübingen and 893 samples from Brisbane were genotyped. This resulted in data for more than 500 000 exon and tagging SNP locations per sample.

The samples were prepared in the lab as described below and submitted to the UQ Centre for Clinical Genomics (UQCCG) at the Translational Research Institute (TRI) where the genotyping was performed. The data which were provided by UQCCG consisted of binary files in the ".bed", ".bim", ".fam" format for Plink analysis.

2.4.4.1. DNA Preparation and Submission

Genomic DNA samples which were prepared for genotyping using the Illumina HumanCoreExome-24 platform, were required to contain a minimum of 2.5ug of DNA with concentrations ranging from 100 to 300ng/ul, resulting in a volume between 20 and 25ul dependant on the DNA concentration of the sample.

After DNA extraction from the EDTA blood samples, some of the samples were available in the right concentrations, some had higher and a large number of samples had lower concentrations than required.

Samples with higher concentrations were re-diluted and measured with Nanodrop® until the values were in the desired range.

A total of 435 samples had concentrations below 75ng/ul. Those samples were processed with a vacuum concentrator (SpeedVac) as follows.

A set of 20 selected samples were processed at one time. The samples were placed in 1.5ml tubes in a heated centrifuge chamber which was connected to a vacuum pump. The low pressure resulted in a decline of the boiling point and facilitated an evaporation of the DNA solvents. The target volume was calculated by using the equation:

C_1 (ng/ul) x V_1 (ul) = C_2 (ng/ul) x V_2 (ul)

C_1 was the measured concentration, V_1 the volume processed on SpeedVac(ul), which was in general 150ul, C_2 (ng/ul) was the concentration aimed at (120 ng/ul) and V_2 (ul), was the estimated target volume.

The time duration of processing the samples in the SpeedVac ranged from 10 to 40min until the volume was equal or less the calculated value.

The samples were subsequently re-suspended with Buffer AE Elution buffer if necessary to reach the exact target volume. The target concentration was measured with a spectrophotometer and re-concentrations or re-dilutions were performed if required.

For quality assessments all samples were measured with NanoDrop™ prior submission and the absorbance at A260 as well as the A260/A280 ratio were recorded in excel sheets.

For submission the samples were aliquoted in 96-well semi-skirted PCR plates (Axygen Scientific) and sealed with Clear Self-Adhesive Topseal (PerkinElmer).

An excel sheet which assigned the plate coordinates (Plate No, A01, A02,…) to the sample ID, gender information, DNA concentration (ng/ul), Volume (ul) and Date, was submitted along with the PCR plates.

2.4.4.2. Interpretation of the CoreExome Chip Results

The genotype approach using the Illumina HumanCoreExome BeadChip resulted in raw data files and additionally files in a binary format. The raw data were charts with coloured dots within the x-axis and y-axis. Genotype information was displayed in one chart per SNP. The location of the dots in the chart was determined by fluorescent intensity measurements. They indicated the result of each analysed individual at the representative SNP, having either two major alleles, one minor and one major allele, or two minor alleles at the respective locus.

The files in the binary format (“.bed”, “.bim”, “.fam”) included the genotype for each of the 550,601 markers. The genotype was assigned to the sample IDs and the gender data. The genotype for each marker was given in the format “homozygous genotype” or “no homozygous genotype”, coded with 1 or 0, as well as in the format “number of minor alleles”, coded with 0, 1 and 2. The data was extracted from the files using the program Plink as described below in 2.6.1. The data was retrieved as csv file (comma separated values) and was imported in other programs such as SPSS and Excel for further analyses.

2.5. Data Management

The data management included the sampling, storage and processing of patient data and material, the documentation of laboratory work and the storage of electronic files related to this work.

2.5.1. Patient Data Management

Patient data used in this work were sampled through three different study protocols.

The recruitment of the pilot study patients resulted in the following documents and material per patient: the patient information and consent, a clinical assessment documentation and a patient questionnaire, images and saliva samples. The patients were pseudonymised using patient IDs (TU-01, TU-02, TU-03, ….). TU stood for Tübingen and the number represented the sequence of patient recruitment. The documents were kept exclusively as paper copies at the Eberhard Karls University of Tübingen. Informed consent sheets and the patient identification list were kept at the office rooms of the Central German Melanoma Registry, University of Tübingen. The information on the assessment and questionnaire files were transferred by the PhD candidate as variables into SPSS files using exclusively the patients ID. The pseudonymised SPSS files were stored at the University of Queensland and the Eberhard Karls University of Tübingen. The patient images were labelled with the patient IDs and stored at the University of Queensland and the Eberhard Karls University of Tübingen. The overview images did not allow a personal identification of the patients. The patient saliva samples were labelled with the Patient ID and transferred to Brisbane. The pseudonymised genotyping results from the saliva samples were recorded manually on paper and were then transferred into excel sheets and SPSS files.

The recruitment of the cohort study from Brisbane resulted in the following documents and material per patient: the patient information and consent, clinical assessment sheets, a patient questionnaire, images and saliva samples. The patients were pseudonymised using consecutively allocated numbers and the patient's initials (1AR, 3MM, 4PF, …). The data was transferred to excel sheets by research assistants. Images, saliva samples as well as the genotyping results were stored at the University of Queensland. Copies of the excel sheets with pseudonymised data were stored additionally at the Eberhard Karls University of Tübingen.

The recruitment of the cohort study from Tübingen resulted in the following documents and material per patient: the patient information and consent, clinical assessment sheets, patient questionnaire and blood samples. The patients were pseudonymised using either the letter K for study participants without a history of melanoma or M for melanoma patients in the combination with consecutively allocated numbers (K1, K2, … M1, M2, …). The paper sheets were

stored at the office rooms of the Central German Melanoma Registry, University of Tübingen. In 2014 the pseudonymised questionnaires and assessment sheets were scanned and send on a web based data cloud of the University Hospital Tübingen to share the data with the PhD candidate. The patient identification list and the informed consent sheets remained in Tübingen at all time. Blood samples were labelled with the IDs and were stored initially in Tübingen until they were transferred to Brisbane in 2014. The patients were additionally assigned to the Central German Melanoma Registry. A registry extract with melanoma specific data of the selected melanoma patients was send electronically to Brisbane in 2015. The different data sources were merged using the Patient IDs as key identifier and stored pseudonymised in a SPSS file.

2.5.2. Documentation of Laboratory Work

The laboratory work of the PhD candidate was done at two different laboratory institutes in Brisbane. In the years 2013 and 2014 the candidate was located at the Institute for Molecular Bioscience (IMB) and from 2014 to 2015 at the DRC, TRI in Brisbane. Each work step was documented by the candidate in a paper lab book at the bench. The lab books were provided by the institutes and were archived on site after the departure of the candidate.

2.5.3. Storage of Electronic Files

Electronic files related to this work included pseudonymised patient data, result data, documentation and literature. These electronic files were initially stored at the local hard drive allocated to the working group at the Institute for Molecular Bioscience (IMB). They were then transferred to the folder of the DRC, TRI. Both locations had regular safety backups performed by the institutional information technology services. After the relocation to Germany a VPN (virtual private network) connection was established to enable a continuous data exchange. In addition, the files were transferred to the hard drive of the Center of Dermatooncology with regular backups performed by the information technology service of the University Tübingen.

2.6. Statistical Analyses

The gathered phenotypic, genotypic and disease specific data of the patients, which were used for the present work, were processed and analysed using different statistical programs and packages. The involved programs included the open source projects Plink[194], R[195] and the Sib-pair package, which was programmed by the co-supervisor David Duffy as well as the commercially available programs SPSS and Stata.

2.6.1. Plink

Plink is an open-source C/C++ tool set which was developed to perform genome association studies.[194] The software was downloaded from the URL http://pngu.mgh.harvard.edu/purcell/plink/ and installed at the notebook of the candidate. The command-line program Plink was used as a first step to extract the SNPs of interest. Therefore, the single batches of binary files, which contained the genotype results from the Illumina CoreExome Chip were loaded step by step into the software. Providing a separate txt file of the required SNPs identificators a cvs file was generated containing the patient IDs together with gender data and the genotype of the requested SNPs.

2.6.2. Sib-pair

The software Sib-pair was developed by David Duffy to work with genetic datasets. Along with general genetic analyses capabilities the program has a special advantage to deal with related study participants for genetic association studies. The software is available from the URL https://genepi.qimr.edu.au/staff/davidD/#sib-pair. The Version 1.0 beta, 6.10.2012 was downloaded by the candidate and used for association analyses and to perform the genome-wide association analyses on survival.

2.6.3. R

The R package is an open source program language and at the same time the name of a free software package. A free download is available from the internet. The software was used for the graphs in Chapter 5.3. and for computing summary statistics and the corresponding forest plot in Chapter 5.4.5.

2.6.4. SPSS

SPSS (Statistical Package for the Social Sciences) is a Java based software program for statistical analysis. A software licence was available at the University of Queensland and the Center of Dermatooncology in Tübingen. The University of Queensland provided additionally a student licence for a time limited use of the software on the candidate´s notebook. The program has a graphical user interface, and the data is displayed in analogue to data in excel sheets. These features facilitated the necessary steps of merging the data from different sources and clearing the data. Different stages of patient recruitment within the Brisbane cohort study and manually recorded patient IDs resulted in differing patient IDs in the various data sources. The data view as tables was therefore helpful in identifying data mismatches and confirming congruencies. The multiple steps of merging and dividing cohorts for the present work were supported by the functionality of the program as well. Statistical analyses, such as analyses of frequencies, association analyses with fisher's exact test and Chi square tests, regression models and survival analyses were carried out as described in the respective chapters. SPSS was also used to generate figures such as bar charts and Kaplan Meier survival plots. Due to an extended display function for the plots, the presented Kaplan Meier survival plots were built using Stata.

2.6.5. Stata

The software Stata (**Sta**tistics and da**ta**) is a software package developed 1985 by the company StataCorp.[196] A Stata licence was available at the Center of Dermatooncology in Tübingen. The software was used to compose Kaplan Meier survival plots with the automated inclusion of a number at risk table. The presented p-values of the statistical test were derived from the analyses performed with SPSS.

3. Pilot Study with 16 Melanoma Patients from Tübingen

3.1. Introduction

A pilot study was initiated as a first step of the PhD project in Tübingen in 2013. The aim was to sample clinical data, general information and biomaterial of melanoma patients from Germany in order to compare these data with Australian melanoma patients. The special focus was the dermoscopic pattern of naevi in melanoma patients and the comparison of those patterns between metastasized and non-metastasized patients. The study was designed in accordance with the Brisbane Naevus Morphology Study (NHMRC Project Grant Application: APP1004999, Scientific Title: Effects of naevogenesis susceptibility genes and phenotypic correlation with dermoscopic characteristics of naevi – Soyer HP, Sturm RA, Duffy D, Smithers M) to enable comparative analyses. The material was then transferred to Australia in order to process the samples and to analyse the data.

3.2. Methods

3.2.1. Ethics

The pilot study with the title "Genetik und Nävusmuster bei Melanompatienten" (English translation: "Genetics and naevus pattern in melanoma patients") was planned in 2013 in Tübingen. A detailed description of the protocol, the planned assessments, a copy of the patient information and consent sheet was submitted to the ethic committee of the Eberhard Karls University Tübingen. The ethic committee asked for a remission regarding in- and exclusion criteria and patient ID. After resolution of the requested issues, the positive ethic vote was obtained on the 8th July 2013 with the ethic number 326/2013BO2.

3.2.2. Study Population and Study Setting

A total of 16 melanoma patients were included into the study. All patients were recruited during their clinical visit in the melanoma outpatient clinic of the University Hospital in Tübingen. The melanoma outpatient clinic belongs to the Department of Dermatology and is part of the general outpatient clinic. About 5 000 contacts with melanoma patients were

recorded per in the year 2016. Most of the patients are referred after primary excision for the secondary excision with the final safety margin and a concomitant sentinel node biopsy. They are subsequently included in a structured follow up program which is recommended in Germany and paid by the public and private health insurances. In single cases, such as the first case in the pilot study, patients are seen at their first visit with the suspicion of a primary melanoma. During the follow up visits the patients get a full body examination to exclude secondary melanomas and in-transit or lymph node metastases. Blood examinations and imaging procedures are completing the follow up checks. Some of the high-risk patients receive adjuvant treatments which are prescribed and monitored by the melanoma clinic. Patients who develop metastases and ultimately die of their disease are also seen and treated at the clinic either as outpatient or inpatient at the department of dermatology.

Patients were asked during their visit at the melanoma outpatient clinic by the candidate if they would be interested in taking part of a research study. The study procedures and the voluntariness of their participation were explained to the patients and a copy of the patient information with the patient consent sheet was handed out to the patient. The study specific assessments were performed after the patients had given written informed consent. A copy of the consent sheet as well as the patient information was handed over to the patient. None of the patients had withdrawn their consent.

3.2.3. Data Collection

The data collection of the Pilot Study was planned to obtain comprehensive information about each patient including the general pigment traits, the number of moles, their sun seeking behaviour as well as the personal response to sun exposure, information about their ancestry, the family history of melanoma along with their medical history and the collection of germline DNA. All these data and materials were gathered within different data collection procedures, which are explained in detail below.

The data collection procedures were organized in four parts after the patient had given written informed consent.

First part was the completion of the physician sheet, which required firstly to collect melanoma specific data from the medical records of the patient and secondly a full body examination of the patient.

Details to be recorded by the physician after the full body examination were:

i. Constitutive skin colour, assessed at the ventral forearm within the categories

1. Fair, 2. Medium, 3. Olive;

ii Facultative skin colour, assessed at the dorsal forearm within the categories

1. Fair, 2. Medium, 3. Olive;

iii Eye colour, assessed within the categories

1. Blue/grey, 2. Green/hazel, 3. Brown;

iv Hair colour, assessed with the categories

1. Red/red-brown, 2. Blonde, 3. Light brown, 4. Dark brown, 5. Black;

v Freckling scores, assessed at three body regions, face, dorsal hand and shoulders within the categories 1. None, 2. Mild, 3. Moderate, 4. Severe;

vi Naevus count, recorded within 18 regions at the front and 16 regions at the back and summed up to the total naevus body count.

The 18 regions at the front of the body were: face, ear right, ear left, neck front, palmar right, forearm right, upper arm right, chest, abdomen, palmar left, forearm left, upper arm left, thigh ventral right, lower leg ventral right, foot right, thigh ventral left, lower leg ventral left, foot left. The 16 regions at the back of the body were: head, neck back, dorsal hand left, forearm left, upper arm left, back, buttocks, dorsal hand right, forearm right, upper arm right, thigh dorsal left, lower leg dorsal left, plantar left, thigh dorsal right, lower leg dorsal right, plantar right. Naevi were recorded from a size of 3mm. The last item to be recorded was vii the presence of a cutis rhomboidalis nuchae as a sign of chronic sun damage, measured in three categories, 0, + and ++.

The second part of the data collection procedures was the imaging of the melanocytic naevi. After completion of the body examination and filling out of the physicians sheet the naevi of the patient were recorded using a digital computer based dermoscopic imaging system (FotoFinder Dermoscope®, Bad Birnbach, Germany). The FotoFinder Dermoscope® is a dermoscope system including a hand-held epiluminescence microscopy camera which is connected to a computer with the Fotofinder software. The system enables video documentation with follow up images of naevi and other skin lesions. The first step was to capture four overview images – upper body front, lower body front, upper body back and lower body back – which were loaded into the Fotofinder Software. The images were then presented on the computer screen and all melanocytic naevi which were chosen to be imaged via dermoscopy were marked and

consecutively numbered. In a next step all numbered lesions were imaged with the FotoFinder hand-held camera. The moles were imaged with direct skin contact using a disinfectant spray to reduce skin reflections.

Part three of the data collection procedures was a questionnaire which had to be filled out by the patients. The questionnaire contained four sets of questions which covered the topics

i General informations,

ii Sun exposure,

iii Skin cancer and moles and

iv Medications.

The questions for the patients are listed per area below:

i General informations – 6 items

1) Sex: male, female; 2) Date of birth; 3) Place of birth; 4) Ancestry: Western European, Southern European, Eastern European, other; 5) Height; 6) Weight.

ii Sun exposure – 7 items

1) Where have you been employed: 1. Mainly outdoors, outdoors and indoors, mainly indoors.

2) Where have your leisure activities been: 1. Mainly outdoors, outdoors and indoors, mainly indoors.

3) During the last year, how much time did you spend outdoors during sunrise and sunset, a. on a typical week day, b. on a typical weekend day: 1. Hardly ever (up to one hour), 2. Less than 50% of the time (2-4 hours), 3. More than 50% of the time (5 hours and more)

4) When you have been outside in the sun, on average, how often have you, a. worn sunglasses, b. worn a hat, c. applied sunscreen: 1. Never, 2. Less than 50% of the time, 3. More than 50% of the time.

5) If you were exposed to strong sun for the first time in summer for an hour with no protection would: 1. Always burn, never tan, 2. Burn, then tan, 3. Only tan.

6) How many times in your life have you had a painful sunburn: 1. Never, 2. Once, 3. 2-3 times, 4) 4-5 times, 5. More than 10 times.

7) What SPF of sunscreen are you currently using

iii Skin cancer and moles – 5 items

1) What were the location of your melanoma/melanomas and the date of diagnosis

2) Have you had any other skin cancer in the last two years, a. body region, b. date of diagnoses

3) How many moles have you had excised in your lifetime, 1. None, 2. 1-5, 3. 6-10, 4. more than 10

4) Have any of your family members (first or second-degree relatives) ever been diagnosed with melanoma

5) Do any of your immediate family members (parents, siblings, children) have a history of more than 50 moles

iv medications – 2 items

1) Have you taken the following medications during the past 5 years, a. ASPIRIN (e.g. ASS 100), b. Anti-inflammatory medications (e.g. Celebrex, Ibuprofen), c. Steroids (Kortison) (e.g. Decortin H, Prednison, Urbason), 1. Never, 2. Occasionally, 3. Less than once a month, 4. 2-3 times a month, 5. Once a week, 6. 2-3 times a week, 7. 4-7 times a week, 8. Twice or more a day.

2) Other medication: please indicate medications which you take on a regular base and indicate frequency and indication.

The questionnaires were stored as paper sheets.

The last part of the data collection procedures was the sampling of saliva with the Oragene collection tubes. The patients were requested to provide a 2ml volume of saliva in a collection tube if possible. By bending and closing the cap, the storage solution contained within the cap was automatically instilled into the tube. Shaking the tube a few times was necessary to ensure an optimal mixture and preservation of the genomic DNA. The tubes were labelled with the patient ID and stored until they were processed in Australia.

3.3. Results

A total of 16 melanoma patients were collected. The characteristics of the patients are summarized in Table 3. The individual case reports are presented in Appendix 1.

Most of the patients had stage I or stage II disease at the time of diagnoses and 5 patients had stage III or stage IV disease. Two patients had progressive disease and died during follow up. The follow up time of the patients was between 2 and 21 years (median = 3.5 years). Nine patients were male and 7 patients were female. The age ranged between 18 and 79 years. Most patients had a superficial spreading melanoma, followed by the subtype nodular melanoma. Other presented melanoma subtypes were lentigo maligna melanoma, acral lentiginous melanoma, dermal melanoma and desmoplastic melanoma, each with one case. The reported tumour thickness ranged from 1.0 mm to 5.0 mm. The patients with progressive disease had a tumour thickness above 2mm. Only two of the non-metastasized patients, TU-03 and TU-11 had a thick primary melanoma. Most patients had blue eyes (n=11, 69%), 3 patients had green eyes (19%), one brown eyes (6%) and one did not indicate the eye colour. The hair colour was brown in 9 patients (56%), blonde in 6 patients (38%) and one patient had black hair (6%). Of the five metastasized patients all but one had brown or black hair. The BMI ranged from 17.3 to 38.1 (median = 24.3). The total body naevus count ranged from 0 to 364 naevi (median = 28). The patients with multiple moles, those who had more than 100 melanocytic naevi on their body, were TU-01, TU-3, TU-05 and TU-08. All these four patients were non-metastasized patients. The dominant naevus pattern was reticular in 8 patients and unspecific in 4 patients. Three patients had no predominant naevus type and one patient had no naevus at all. Most of the patients (n=8, 50%) indicated they would burn and then tan after sun exposure, three patients would only burn (19%), three only tan (19%) and two patients skipped the question. The genotyping results were summarized for the gene loci of *MC1R* and exon 5 of *PPARGC1B*. *PPARGC1B* is the gene coding for the protein PGC-1b. Variants in *MC1R* were very frequent in this small cohort of melanoma patients. Only 4 patients had no variant allele (25%). Three of those four wild type patients were metastasized patients. Detected variants were R160W, R163Q, I155T, R151C, T95M and V60L. The variants R160W and R151C are considered as high penetrance alleles in regard to hair and skin colour and marked as “R” variants, the variants R163Q, I155T and V60L as low penetrance allele and marked as “r” variants. [197] The variant T95M is a very rare variant. A frequency of 0.002 was reported in a

Mediterranean population.[198] Of the 12 patients (75%) with *MC1R* variants, 9 had blue eyes, 6 blonde hair and 8 indicated they were sun sensitive, with either "burn then tan" or "only burn" as a skin reaction after sun exposure.

Variants at exon 5 of *PPARGC1B* were rare. Only three patients had one or two variant alleles. The detected variants were R226Q and V240I. Two non-metastasized patients and one metastasized patient had a variant.

Table 3: Characteristics of the 16 patients of the pilot study

Patient	Sex	Age at D	Histology	Td	Stage at D	Stage FU	Follow up	died	Eyes	Hair	BMI	Naevus count	Naevus pattern*	Sun reaction	*MC1R*	*PPARGC1B*
TU-01	m	43	SSM	1.65	IIA	IIA	3y	no	blue	brown	-	364	retic	b/t	R160W-/-	wt
TU-02	f	34	na	-	na	IV	21y	no	blue	blonde	-	51	unsp	-	R160W-/-	wt
TU-03	m	59	SSM	2.5	IIA	IIA	4y	no	blue	brown	35.8	128	retic	b/t	wt	wt
TU-04	m	17	derm	-	IIA	IIA	6y	no	green	brown	20.5	77	unsp	b/t	R163Q+/-	wt
TU-05	m	76	SSM	1.0	IA	IA	6y	no	blue	brown	25.0	123	retic	b/t	I155T+/-	R226Q +/-
TU-06	f	49	SSM	1.1	IB	IB	6y	no	blue	blonde	24.8	19	unsp	b	R151C+/-	wt
TU-07	m	75	LMM	1.0	IB	IB	3y	no	blue	blonde	31.6	0	na	b/t	R151C+/- T95M+/-	wt
TU-08	m	28	NM	1.2	IB	IB	3y	no	blue	blonde	25.1	170	no	b/t	V60L+/-	wt
TU-09	f	75	ALM	1.4	IIA	IIA	6y	no	blue	blonde	20,8	5	unsp	t	V60L+/- R160W+/-	wt
TU-10	f	75	SSM	1.0	IA	IA	4y	no	blue	brown	29.1	22	retic	b	R160W+/-	wt
TU-11	f	63	desm	2.7	IIA	IIA	2y	no	-	brown	28.0	62	no	t	R160W+/-	V240I +/-
TU-12	m	48	SSM	1.2	IB	IB	3y	no	blue	blonde	23.8	34	retic	b/t	R160W+/-	wt
TU-13	f	29	na	3.3	IIIB	IV	2.5y	yes	green	brown	22.9	22	no	b	wt	wt
TU-14	m	77	SSM	2.4	IIIA	IIIA	3y	no	green	brown	38.1	1	retic	-	wt	R226Q +/- V240I +/-
TU-15	m	61	NM	5.0	IIIA	IV	4y	yes	blue	brown	-	9	retic	b/t	wt	wt
TU-16	f	18	NM	4.5	IIIA	IIIA	3y	no	brown	black	17.3	9	retic	t	V60L+/- R151C+/-	wt

*dominant naevus type (>40% naevi belong to one pattern)

Metastasized patients (stage III/IV) are highlighted in grey, those patients who progressed and died in dark grey.

at D = at diagnosis

3.4. Discussion and Conclusion

These prospectively included melanoma patients from Tübingen, Germany represent an in depth described cohort with comprehensive medical history, the report of behavioural data in addition with the phenotype and genotype of the patients.

The sample size of 16 patients did not allow statistical comparisons. However, the profound and systematic view on individual patients allowed a deeper understanding on the different personal courses of the disease. Furthermore, the summary of the characteristics resulted in a renunciation of the initially hypothesis put forward and formed the base for new research questions as discussed below.

The obtained digital dermoscopic images were classified and a dominant naevus pattern was seen in 12 of the 16 patients (75%). This was in accordance to a previously reported rate of a pre-dominant global pattern in individuals without a personal history of melanoma.[199] However, this dominant type was based on a threshold of 40% as suggested in the literature. The overall variance of different naevus types in the present cohort was high and not distinctive. A difference in late and early stage patients was not detectable. In the literature a change in naevus pattern was demonstrated with a dermoscopic follow-up imaging study in children and young adults. More than half of the observed naevi evolved into different patterns, even from atypical naevi into common naevi.[200] Another study found an age dependent association of naevus pattern and no differences between melanoma patients and controls.[201] The high intra-individual variability of naevi as observed in the presented patients as well as the reported variation over time in terms of pattern did not encourage to pursue investigations on dermoscopic naevus pattern in metastasized and non-metastasized melanoma patients in this work.

The exploration of the present cohort of melanoma patients did not allow reflection on melanoma risk due to the lack of controls, but did allow a comparison of non-metastasized patients with patients who had progressive disease and the search for contributing factors.

High impact factors can be detected even in small numbers, whereas low impact factors need to be confirmed in a larger group. In respect to the general frequency of this contributing factors one has to consider true positive and false positive findings in small cohorts.

The main prognostic factor tumour thickness for melanoma progression was clearly revealed in this small cohort. All 5 patients with lymph node or distant metastases (stage III and stage

IV) had a primary tumour thickness above 2mm, whereas only two of the eleven early stage patients had a thick primary. The second clear association of an established factor was the histological subtype, with a high rate of nodular melanomas among the late stage patients.

Host specific characteristics included eye and hair colour, BMI, sun reaction, naevus count and the genotype. Brown or black hair seemed to be more common among patients with metastatic disease, while there was no difference for hair colour, BMI or the individual reaction of the skin after sun exposure. The total body naevus count was higher among patients in early stages. This finding was in accordance with a personal observation of frequently seeing metastatic patients in the melanoma outpatient clinic without or with only few melanocytic naevi. Variants in *PPARGC1B* were found in 3 patients without a distinct distribution. Variants in *MC1R* were found in 11 out of the 16 patients, which represented a higher number than expected compared to the general population. Only one patient among the early stage patients had a *MC1R* wild type compared to three among the five late stage patients. Both patients who died had no germline variant at the *MC1R* loci. These findings were in accordance to a previously reported survival benefit of patients with *MC1R* variants by Davies et al.[202] and lead to the exploration of the prognostic impact of genetic and phenotypic markers in a larger cohort.

In conclusion the completion of the pilot study resulted in a set of extensively well described melanoma patients, including early stage and late stage patients. The initial research goal to investigate the dermoscopic pattern of melanocytic naevi in metastasized and non-metastasized patients was dropped based on these initial findings. Succeeding research interests included the exploration of identified phenotypic and genotypic characteristics of interest, such as naevus count, hair colour and *MC1R* germline variants in a larger German patient cohort.

3.5. Appendix 1 Case Reports

Patient TU-01

The first study participant was a 43-year-old male patient from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IIA.

Medical history: The patient was seen with the suspicion of a superficial spreading melanoma on the lower back in August 2013. The histopathology report confirmed the diagnoses and described the melanoma as an ulcerated secondary nodular superficial spreading melanoma with a tumour thickness of 1.65mm, Clark level IV without an association to a pre-existing naevus. Regression areas were described in the specimen and a number of four mitoses per mm^3 were counted. The patient was referred to a sentinel node biopsy based on the tumour thickness of his primary tumour. However, the sentinel was not detectable and the procedure was not performed. The patient received an adjuvant treatment with subcutaneous Interferon-alpha 3 MIU 3 times per week from September 2013 to September 2014. The treatment duration was shortened due to adverse reactions, such as flue like symptoms, concentration disorder, and diminished performance. None of his moles had been excised before. He had no other skin cancers and no other medical condition. He was taking occasionally acetylsalicylic acid and anti-inflammatory medications. There are no other family members diagnosed with melanoma so far, but his father and his brother did have multiple naevi. The patient had been participating in clinical follow up visits on a regular basis and he was without recurrence or secondary melanoma at the time of last follow up (8th Dec 2016).

Phenotype: The patient had blue/grey eyes and light brown hair. His constitutive skin colour was fair, his facultative skin colour medium. He had mild freckles at the face and the dorsal hand, severe freckles at the shoulders and a cutis rhomboidalis at his neck. His total body naevus count was 364 (>3 mm) and the area with the largest number of naevi was his back with 82 naevi.

Dermoscopy: A total of 81 naevi were imaged with dermoscopy. The predominant naevus type was of reticular pattern with 50 reticular, 8 reticular/globular, 2 globular and 21 unspecific pattern.

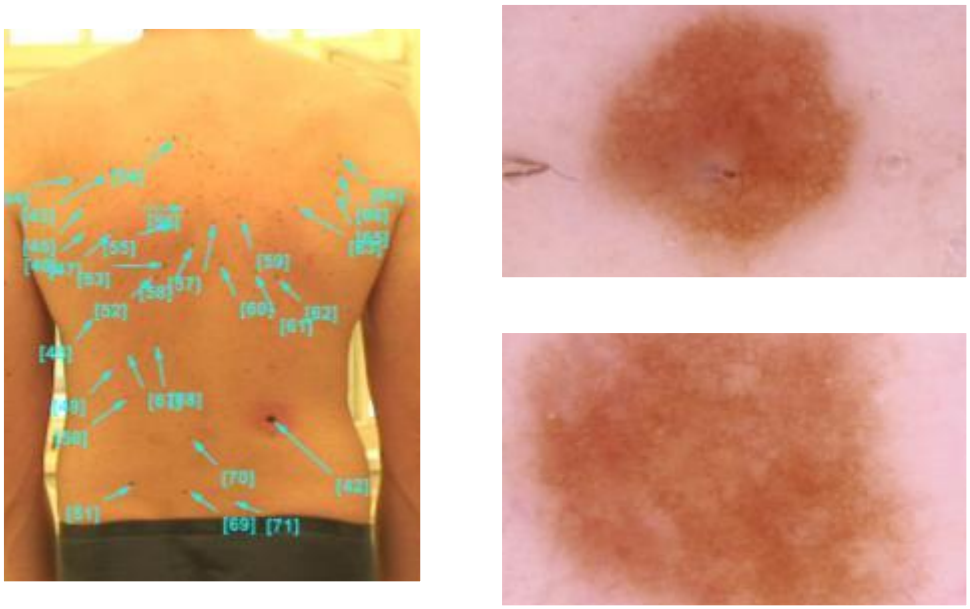


Figure 5: Overview Image upper back with the primary melanoma (No 42) and two naevi captured with dermoscopy, both with reticular pattern

Sun behaviour: The patient had been mainly working indoors and his leisure activities were located indoors and outdoors. On a typical weekday he would stay less than 50% of the time outside while he would be outside in the sun more than 5 hours a day on the weekend. He would have protected himself from the sun in less than 50% of the time with sunglasses and sunscreen with a protection factor of 25 and never with a hat. If he would have exposed himself to the sun unprotected he would have burned and tan. He indicated that he had between two and five painful sunburns in his life.

Genotype: *MC1R*: one homozygous variant at R160W was detected which is considered a R variant. *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-02

The second patient was a 52-year-old woman from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IV.

Medical history: The patient was diagnosed with lymph node metastases in the right groin and iliac lymph node metastases in May 1996 at the age of 34. The patient said that three years before a mole had been excised at her upper leg without a histopathologic examination. The pathology review of 1996 found that 5 of 13 inguinal lymph nodes and 2 of 9 iliac lymph nodes were affected with metastases from malignant melanoma. The patient received an adjuvant combination treatment with subcutaneous Interferon alpha 60 MIE 3 times a week and 40mg Isotretinoin a day. The treatment with Isotretinoin was stopped in August 2001. Interferon alpha was continued with 6 MIE until September 2006, the dose was subsequently reduced to 3MIE 3 times a week and the treatment was terminated in October 2008 after a total period of 12 years of adjuvant therapy. More than 10 of her moles had been excised. She had no other skin cancer. Her other medical conditions were a Hashimoto Thyreoditis and hay fever. Her regular medication was levothyroxine sodium 50mg a day and desloratadine 5mg a day. She was taking acetylsalicylic acid and anti-inflammatory drugs on an occasionally base. There were no other family members diagnosed with melanoma. She is indicating that her children have more than 50 moles. The patient had been participating in clinical follow up visits on a regular basis and she was without recurrence or secondary melanoma at the time of last follow up (7th Feb 2017).

Phenotype: The patient had blue/grey eyes and blonde hair. Her constitutive and her facultative skin colour were fair. She had mild freckles at the face and the dorsal hand, moderate freckles at the shoulders and a cutis rhomboidalis at her neck. Her total body naevus count was 51 (>3 mm) and the area with the largest number of naevi was her back with 9 naevi. The head, hands and feet were free of naevi.

Dermoscopy: A total of 11 naevi were imaged with dermoscopy. There was no predominant naevus type with 5 reticular and 6 naevi of unspecific pattern.

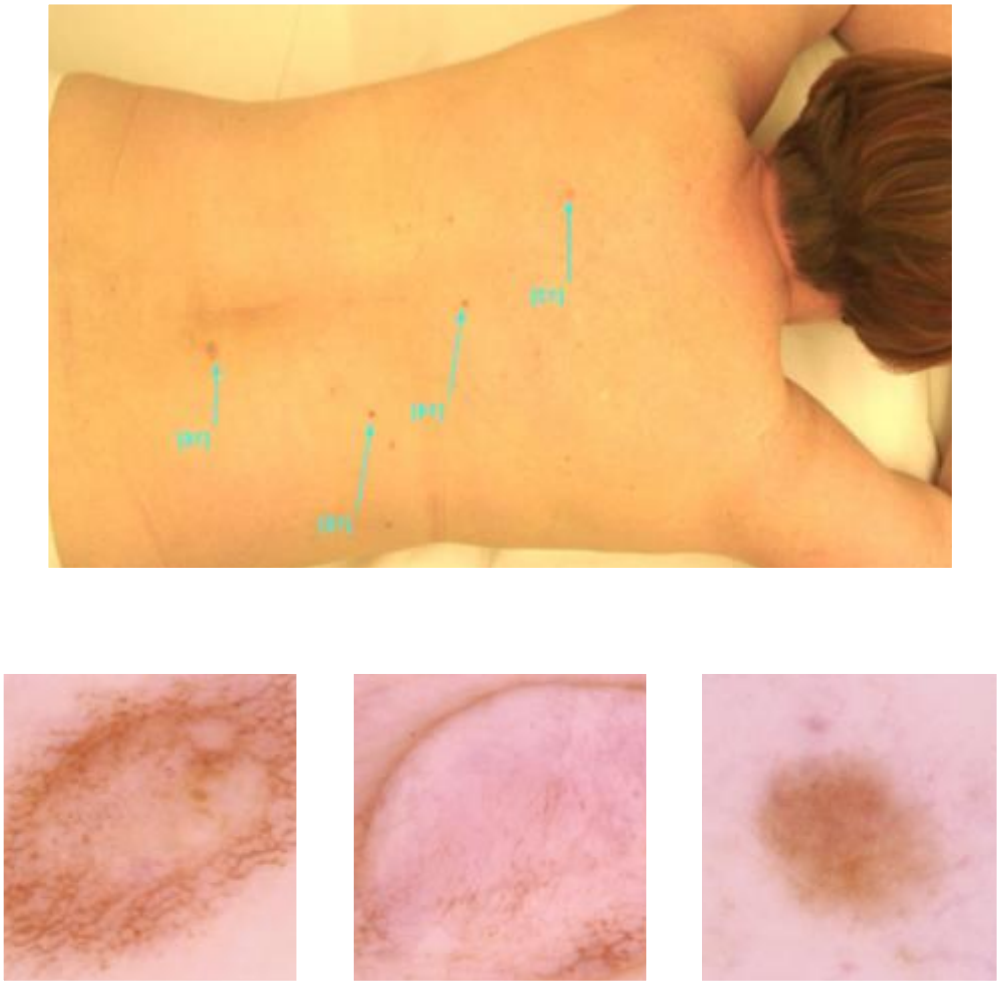


Figure 6: Overview Image upper back and three naevi captured with dermoscopy, one with reticular and two with unspecific pattern

Sun behaviour: The patient had been mainly indoors for work as well as for her leisure activities. On a typical weekday she would have been outside less than one hour and on the weekend less than 50% of her time. For her sun protection she used sunglasses and sunscreen with a protection factor of 50, both of it more than 50% of the time and sometimes she used a hat. She remembered more than 10 painful sunburns in her life.

Genotype: *MC1R*: one homozygous variant at R160W was detected which is considered a R variant. *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-03

Patient TU-03 was a 61-year-old man from Kazakhstan with Eastern Europe ancestry and a Malignant Melanoma Stage IIA.

Medical history: The patient was diagnosed with a superficial spreading melanoma on the left shoulder in May 2011. The tumour thickness was 2.5mm without ulceration. A sentinel lymph node biopsy was performed and no metastases were found. An adjuvant treatment was not given to the patient. Between 1 and 5 moles had been excised so far. He had no other skin cancers. His other medical condition was a peripheral vascular disease. His regular medication was Marcumar. His son was diagnosed with a melanoma in 1999 and both, son and daughter have multiple moles. The patient had been participating in clinical follow up visits on a regular basis and he was without recurrence or secondary melanoma at the time of last follow up (7th Sep 2015).

Phenotype: The patient had blue/grey eyes and light brown hair. His body height was 172cm and his weight was 106kg. His constitutive skin colour was fair, his facultative skin colour medium. He had mild freckles at the face, the dorsal hand and the shoulders and a cutis rhomboidalis at his neck. His total body naevus count was 128 (>3 mm) and the area with the largest number of naevi was his back with 28 naevi.

Dermoscopy: A total of 31 naevi were imaged with dermoscopy. The predominant naevus type was of reticular pattern with 18 reticular and 13 naevi of unspecific pattern.

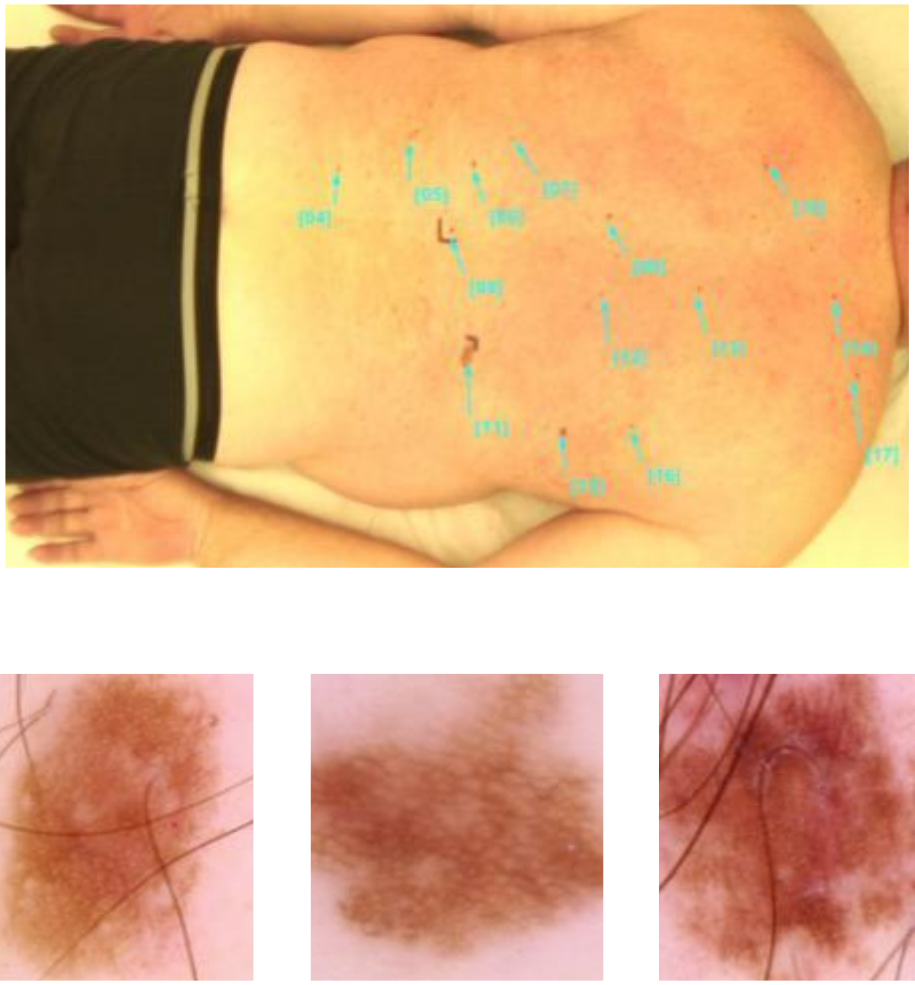


Figure 7: Overview Image upper back and three naevi captured with dermoscopy, all three with reticular pattern

Sun behaviour: The patient had been working as a welder mainly indoors and his leisure activities were indoors as well. He would have been less than 50% of his time outside in the sun on a weekday as well as on weekends. He never used sun glasses, a hat or sunscreen for sun protection. His skin would react with sunburn and tan after sun exposure. He cannot remember any painful sunburn in his life.

Genotype: *MC1R*: wildtype; *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-04

Patient TU-04 was a 20-year-old man from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IIA.

Medical history: The patient was diagnosed with a dermal melanoma on the scalp in December 2010. The histopathology report described a suspicious cell clone with nuclear polymorphisms and a high proliferation index within a congenital naevus. A comparative genomic hybridisation (CGH) was performed and revealed multiple quantitative chromosomal aberrations which supported the classification of the lesion as malignant melanoma. A sentinel node biopsy was performed and three lymph nodes were found without evidence of metastases. An adjuvant therapy was not given to the patient. Between 1 and 5 moles had been excised in the past. He had no other skin cancers and no other medical condition. No family member was affected with melanoma and he indicated that his mother had more than 50 moles on her skin. He was taking no regular drugs but occasionally acetylsalicylic acid and anti-inflammatory medications. The patient had been participating in clinical follow up visits on a regular basis and he was without recurrence or secondary melanoma at the time of last follow up (27th Feb 2017).

Phenotype: The patient had green/hazel eyes and light brown hair. His body height was 185cm and his weight was 70kg. His constitutive and facultative skin colour was fair. He had no freckles at the face, the dorsal hand and the shoulders and no cutis rhomboidalis at his neck. His total body naevus count was 77 (>3 mm) and the area with the largest number of naevi was his back with 15 naevi, followed by his face with 10 naevi.

Dermoscopy: A total of 24 naevi were imaged with dermoscopy. There was no predominant naevus type with 4 reticular, 3 globular and 17 naevi of unspecific pattern.

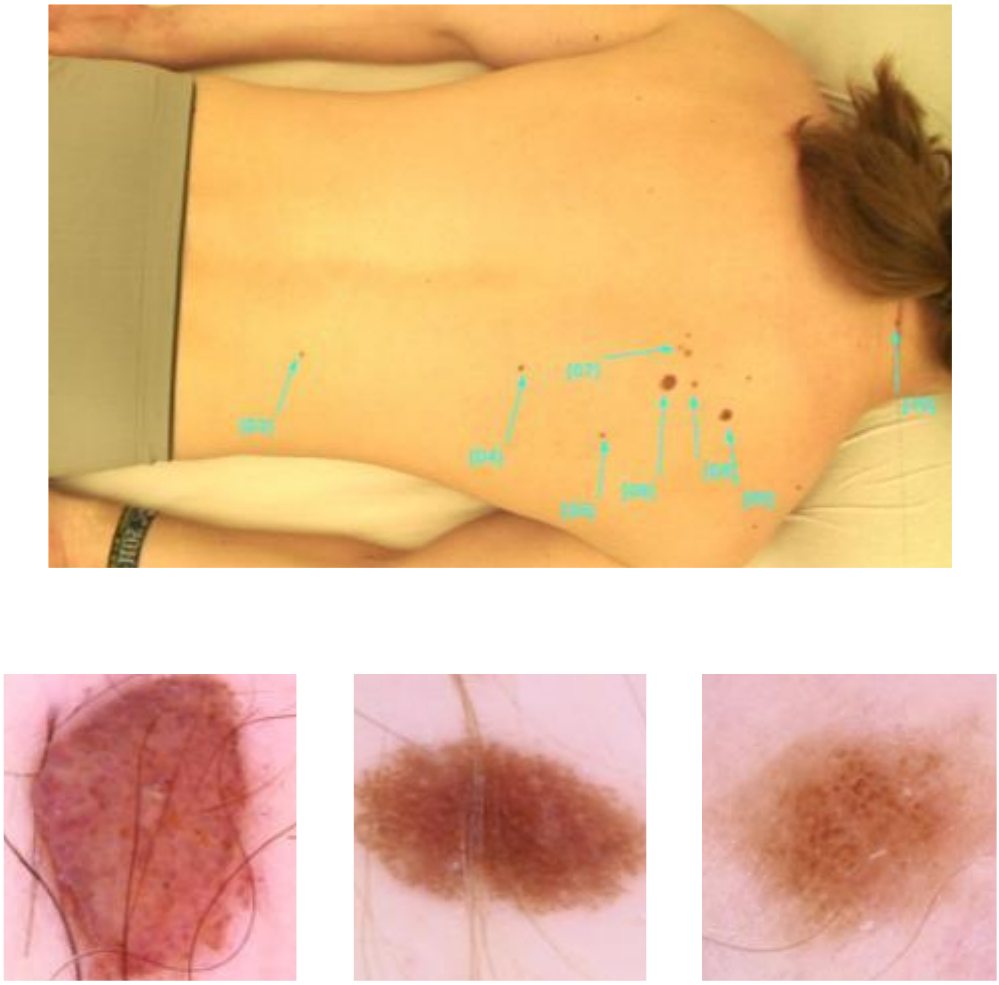


Figure 8: Overview Image upper back and three naevi captured with dermoscopy with globular, reticular and unspecific pattern.

Sun behaviour: The patient had been mainly working indoors and his leisure activities were located indoors and outdoors. He would stay less than 50% of the time outside on a typical weekday as well as on the weekend. He would have protected himself from the sun in less than 50% of the time with sunglasses, a hat and sunscreen with a protection factor of 30. If he would have exposed himself to the sun he would have burned and tan. He indicated that he had between two and five painful sunburns in his life.

Genotype: *MC1R*: R163Q+/-; *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-05

Patient TU-05 was a 79-year-old man from Middle Germany with Western Europe ancestry and a Malignant Melanoma Stage IA.

Medical history: The patient was diagnosed with two superficial spreading melanomas in the year 2010. The first one was located on the left shoulder with a tumour thickness of 0.3mm and the second melanoma was located on the right upper leg and had a tumour thickness of 1.0mm. Both melanomas were not ulcerated. A sentinel lymph node biopsy was not performed and no adjuvant treatment was given to the patient. He had no excision of naevi in the past and no other skin cancer. His other medical conditions were Parkinson´s disease and coronary heart disease. His regular medications consisted of Ropinirole 12mg, Asagiline 1mg, Furosemide 40mg, Mirtazapine 30mg, Simvastatin 20mg and Fenofibrate 160mg, all on a daily base. He was taking occasionally acetylsalicylic acid and anti-inflammatory medications. His family members are not affected with melanoma. His children had more than 50 moles on their skin. The patient had been participating in clinical follow up visits on a regular basis and was without recurrence or secondary melanoma at the time of last follow up (1st Aug 2016).

Phenotype: The patient had blue/grey eyes and light brown hair. His body height was 171cm and his weight was 73kg. He had moderate freckles at the face and the dorsal hand and severe freckles at the shoulders. His total body naevus count was 123 (>3 mm) and the area with the largest number of naevi was his left ventral thigh with 22 naevi.

Dermoscopy: A total of 27 naevi were imaged with dermoscopy. The predominant naevus type was of reticular pattern with 22 reticular and 5 naevi of unspecific pattern.

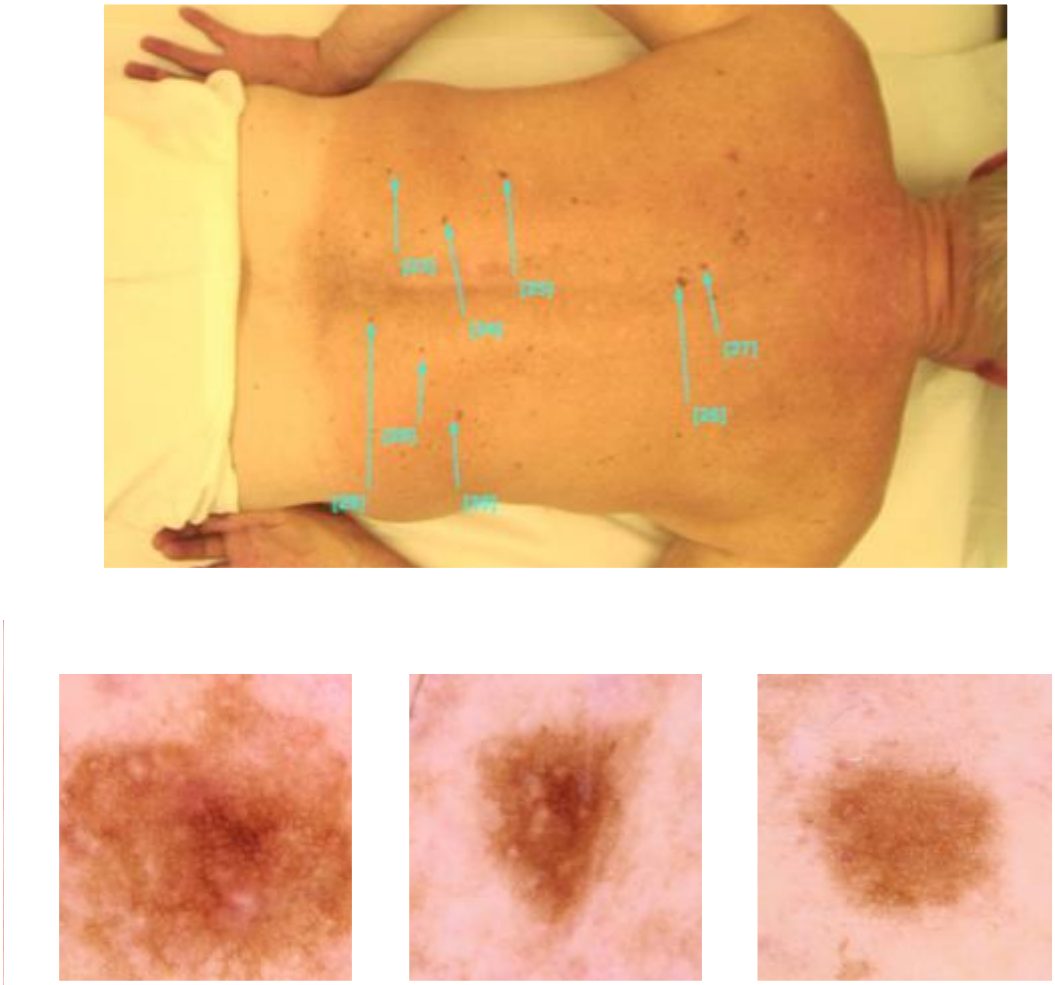


Figure 9: Overview Image upper back and three naevi captured with dermoscopy, all three with reticular pattern

Sun behaviour: The patient had been working indoors and outdoors and his leisure activities were located mainly outdoors. He would have stayed less than 50% of the time outside on a typical weekday and on the weekend. He never used sunscreen but protected himself from the sun in less than 50% of the time with sunglasses and a hat. If he would have exposed himself to the sun unprotected he would have burned and tan. He remembered that he had more than 10 painful sunburns in his life.

Genotype: *MC1R*: I155T+/-; *PGC-1b*: Arg226Gln A/G

Patient TU-06

Patient TU-06 was a 52-year-old woman from Austria with Western Europe ancestry and a Malignant Melanoma Stage IB.

Medical history: The patient was diagnosed with a superficial spreading melanoma on the right upper arm in May 2010. The melanoma was 1.1mm thick and not ulcerated. A sentinel lymph node biopsy was performed with no pathologic findings. An adjuvant treatment was not given to the patient. Between 1 and 5 naevi had been excised in the past. No other skin cancer was diagnosed. Her other medical conditions were breast cancer and thyroid disease. She had been taking tamoxifen and levothyroxine sodium on a daily base. There were no family members with melanoma or multiple moles. The patient had been participating in clinical follow up visits on a regular basis and was without recurrence or secondary melanoma at the time of last follow up (2st Aug 2016).

Phenotype: The patient had blue/grey eyes and blonde hair. Her body height was 175cm and her weight was 76kg. Her constitutive and her facultative skin colour were fair. She had moderate freckles at the face, the dorsal hand, the shoulders and a cutis rhomboidalis at her neck. Her total body naevus count was 19 (>3 mm) and the area with the largest number of naevi was her back with 4 naevi. The head, hands and feet were free of naevi.

Dermoscopy: A total of 12 naevi were imaged with dermoscopy. There was no predominant naevus type with 3 reticular, 2 reticular/globular and 7 naevi with unspecific pattern.

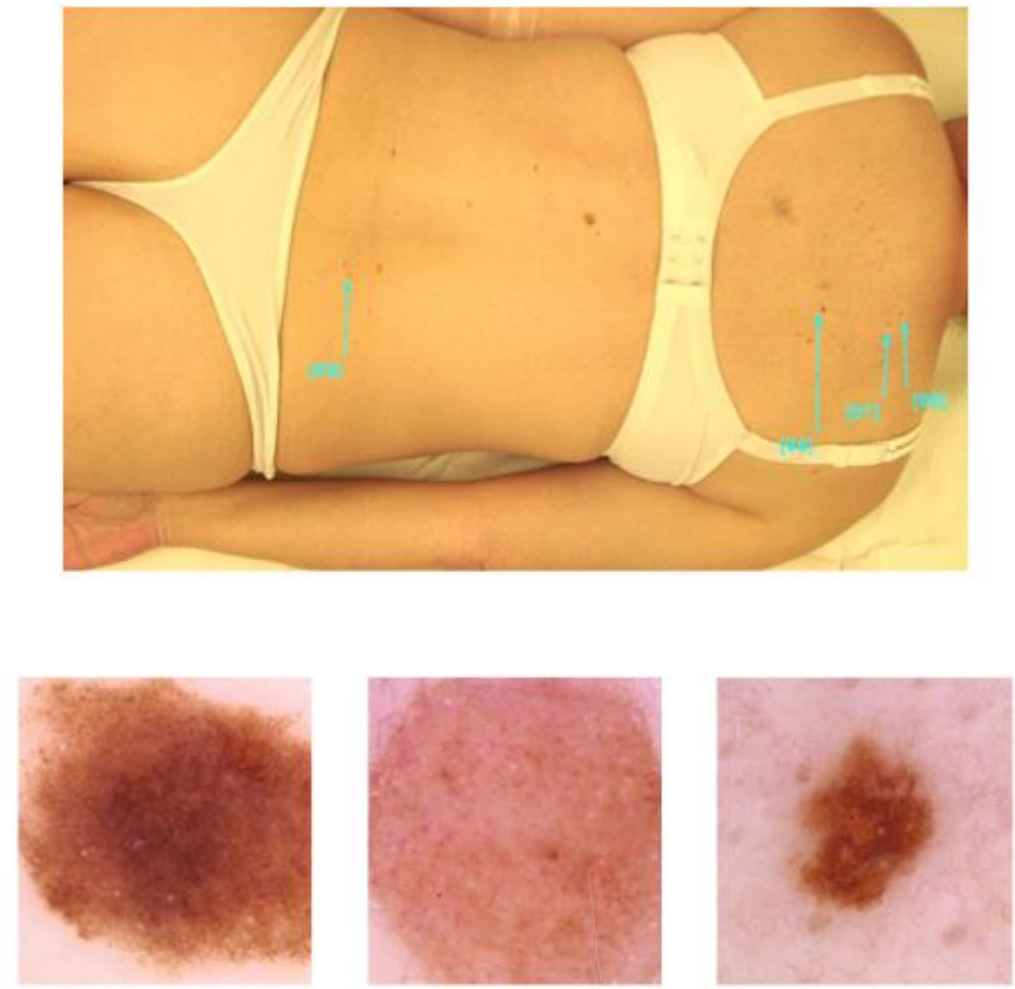


Figure 10: Overview Image upper back and three naevi captured with dermoscopy, one with reticular/globular, one with unspecific and one with reticular pattern.

Sun behaviour: The patient had been mainly indoors for work and her leisure activities were located indoors and outdoors. On a typical weekday she would have been less than 2-4 hours outside and on the weekend more than 50% of her time. For her sun protection she used sunglasses and sunscreen with a protection factor of 50 more than 50% of her time. She never used a hat. If she would have exposed herself to the sun unprotected she would have always burned. She remembered between 2 and 5 painful sunburns in her life.

Genotype: *MC1R* R151C+/-; *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-07

Patient TU-07 was a 76-year-old man from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IB.

Medical history: The patient was diagnosed with lentigo maligna melanoma with a tumour thickness of 1.0mm on his right forehead in October 2012. A sentinel node biopsy was not performed based on the wish of the patient. No adjuvant treatment was given. No moles had been excised before. He had no other skin cancers. He had been taking Marcumar due to a thrombosis in his medical history. His family members had no history of melanoma and no multiple moles. The patient had been participating in clinical follow up visits on a regular basis and was without recurrence or secondary melanoma at the time of last follow up (14th Jul 2015).

Phenotype: The patient had blue/grey eyes and blonde hair. His body height was 164cm and his weight was 85kg. His constitutive and facultative skin colour was fair. He had severe freckles at the face and the dorsal hand, mild freckles at the shoulders and a pronounced cutis rhomboidalis at his neck. His total body naevus count was zero.

Dermoscopy: No melanocytic naevi were detected.

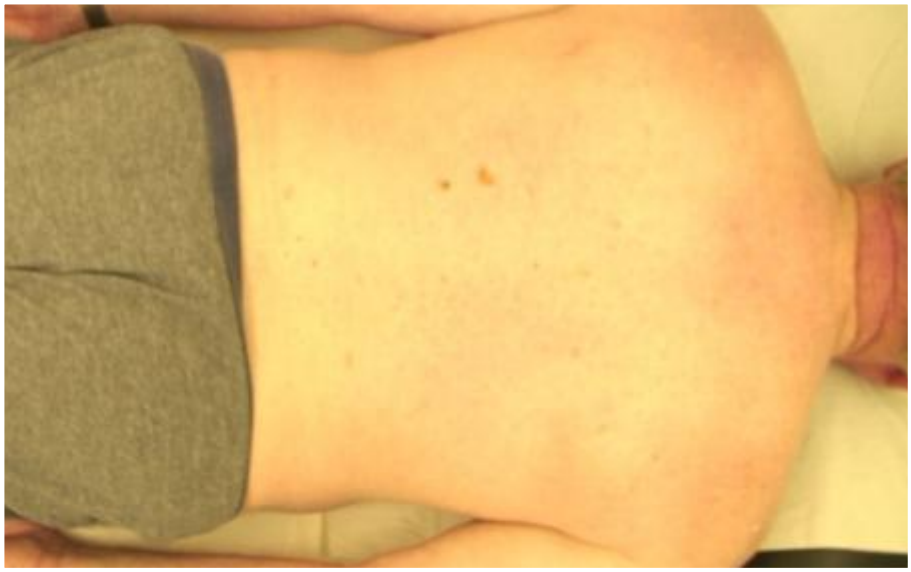


Figure 11: Overview Image upper back

Sun behaviour: The patient had been working indoors and outdoors and had been mainly outdoors for his leisure activities. On a typical weekday he would have been less than 2-4 hours outside in the sun. For his sun protection he used sunglasses and a hat in less than 50% of the time. He never used sunscreen. His skin would react with sunburn and tan after unprotected sun exposure. He remembered between 2 and 5 painful sunburns in his life.

Genotype: *MC1R* R151C+/- T95M+/-; *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-08

Patient TU-08 was a 29-year-old man from Saxony with Western Europe ancestry and a Malignant Melanoma Stage IB.

Medical history: The patient was diagnosed with a nodular malignant melanoma on his left knee in May 2012. The tumour was not ulcerated and 1.2mm thick. A sentinel node biopsy was performed with not pathological results. He did not get an adjuvant treatment. Between 6 and 10 moles had been excised in the past. He had no other skin cancers and no other medical conditions. He was not taking any medications. His mother and his aunt were diagnosed with a melanoma as well in the years 2012 and 2013 respectively. His mother has also multiple moles. The patient had been participating in clinical follow up visits on a regular basis and he was without recurrence or secondary melanoma at the time of last follow up (16th Feb 2015).

Phenotype: The patient had blue/grey eyes and blonde hair. His body height was 184cm and his weight was 85kg. His constitutive skin colour was fair, his facultative skin colour medium. He had mild freckles at the face, no freckles at the dorsal hand and no cutis rhomboidalis at his neck. His total body naevus count was 170 (>3 mm) and the area with the largest number of naevi was his back with 41 naevi, followed by the chest with 25 naevi.

Dermoscopy: A total of 27 naevi were imaged with dermoscopy. The predominant naevus type was of globular pattern with 8 reticular, 2 reticular/globular, 10 globular and 7 naevi of unspecific pattern.

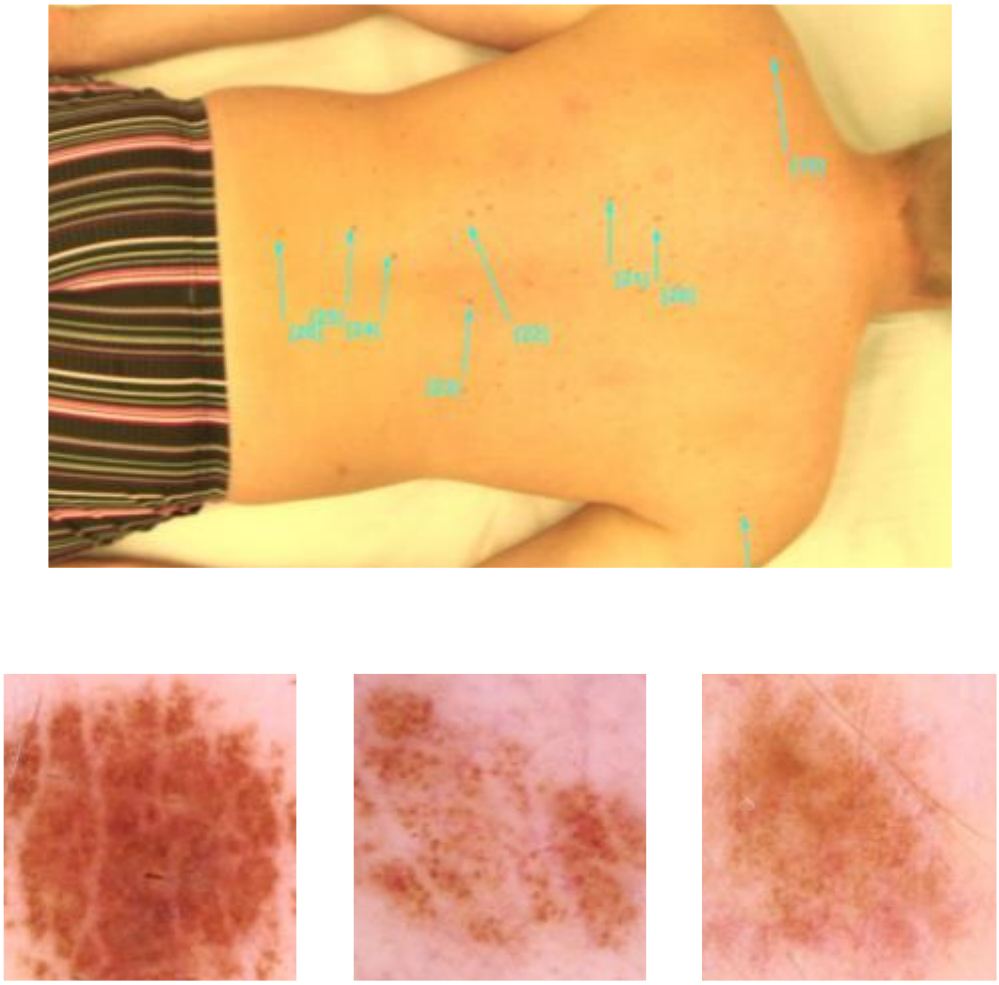


Figure 12: Overview Image upper back and three naevi captured with dermoscopy, two of globular and one of reticular pattern.

Sun behaviour: The patient had been working mainly indoors and his leisure activities were indoors and outdoors. He would have been less than 1 hour outside in the sun on a weekday and between 2 and 4 hours on weekends. He never used sun glasses, and less than 50% of the time sunscreen (SFP 25-30) and a hat for sun protection. His skin would react with sunburn and tan after sun exposure. He remembered 2 to 5 painful sunburns in his life.

Genotype: *MC1R*: V60L+/-; *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-09

Patient TU-09 was a 77-year-old woman from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IIA.

Medical history: The patient was diagnosed with a acral lentiginous melanoma on her left forefinger in January 2011. The tumour was 1.4mm thick and was ulcerated. A sentinel lymph node biopsy was performed and no melanoma metastases were found. The patient received an adjuvant treatment with subcutaneous interferon alpha 3MIU three times a week from June 2011 to January 2012. The treatment was terminated prematurely due to a pronounced weight loss. No moles had been excised in the past and the patient had no other history of skin cancer. Her other medical conditions were hypertension, atrial fibrillation, chronic obstructive pulmonary disease, osteoporosis and a history of an apoplex. She was inhaling tiotropium, fluticasone and salmeterol and taking verapamil, candesartan, digitoxin and phenprocoumon on a daily base. Her family members were not affected with melanoma and none of the family members had multiple moles. The patient had been participating in clinical follow up visits on a regular basis and she was without recurrence or secondary melanoma at the time of last follow up (14th Feb 2017).

Phenotype: The patient had blue/grey eyes and blonde hair. Her body height was 170cm and her weight was 60kg. The constitutive skin colour was fair and her facultative skin colour was medium. She had moderate freckles at the face and the shoulder, severe freckles at the dorsal hand and a cutis rhomboidalis at her neck. Her total body naevus count was 5 (>3 mm) and the area with the largest number of naevi was her back with 3 naevi. The head, hands and feet were free of naevi.

Dermoscopy: A total of 5 naevi were imaged with dermoscopy. There was no predominant naevus type with 2 reticular and 3 naevi of unspecific pattern.

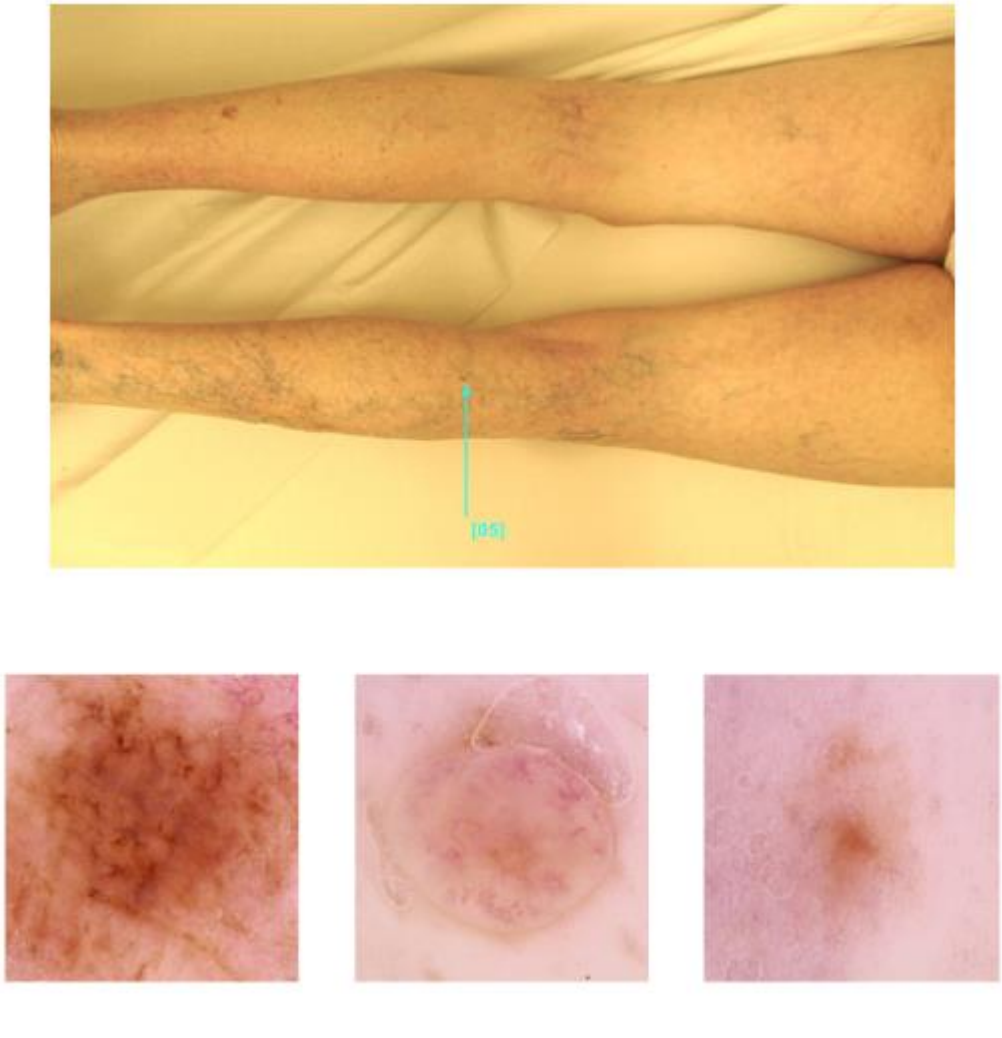


Figure 13: Overview Image of the lower legs and three naevi captured with dermoscopy, all three of unspecific pattern.

Sun behaviour: The patient had been indoors and outdoors for work as well as for her leisure activities. She would have been outside between 2 and 4 hours on a typical weekday and on the weekend. She never used sunglasses, sunscreen or a hat for her sun protection. If she would expose her skin to the sun she indicated that she would only tan. She remembered one painful sunburn in her life.

Genotype: *MC1R*: V60L+/- R160W+/-; *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-10

Patient TU-10 was a 77-year-old woman from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IA.

Medical history: The patient was diagnosed with a superficial spreading melanoma on her right calf in November 2011. The tumour was 1.0mm thick and not ulcerated. She was referred to sentinel node biopsy and no melanoma metastases were found in the lymph node. No adjuvant treatment was given to the patient. She had between one and five moles excised in the past and had no other skin cancer. She had no other medical condition and was taking no medications. No family members were affected with melanoma but her son had multiple moles. The patient had been participating in clinical follow up visits on a regular basis and he was without recurrence or secondary melanoma at the time of last follow up (19th Oct 2015).

Phenotype: The patient had blue/grey eyes and light brown hair. Her body height was 154cm and her weight was 69kg. Her constitutive skin colour was fair and her facultative skin colour was medium. She had moderate freckles at the face, the dorsal hand, the shoulders and a pronounced cutis rhomboidalis at her neck. Her total body naevus count was 22 (>3 mm) and the areas with the largest number of naevi were her back and her left dorsal lower leg with 4 naevi each.

Dermoscopy: A total of 18 naevi were imaged with dermoscopy. The predominant naevus type was of reticular pattern with 10 reticular, 1 reticular/globular and 7 naevi of unspecific pattern.

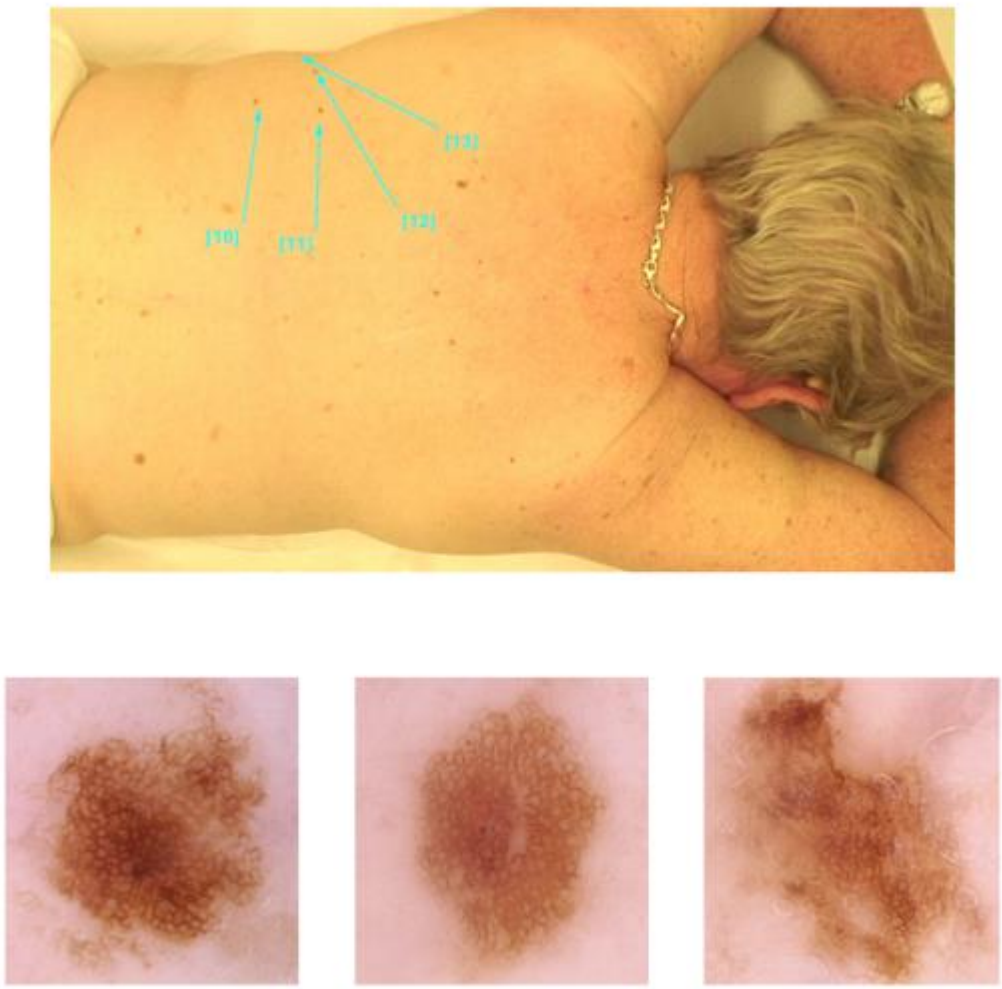


Figure 14: Overview Image upper back and three naevi captured with dermoscopy, all three of reticular pattern.

Sun behaviour: The patient had been mainly outdoors for her leisure activities. On a typical weekday she would have been 2-4 hours outside and on the weekend less than one hour of her time. For her sun protection she never used sunglasses. After sun exposure her skin would always have burned but not tanned. She remembered between 2 and 5 painful sunburns in her life.

Genotype: *MC1R*: R160W+/-; *PGC-1b:* none of the assessed 7 SNPs showed a variant

Patient TU-11

Patient TU-11 was a 63-year-old woman from Croatia with Southern Europe ancestry and a Malignant Melanoma Stage IIA.

Medical history: The patient was diagnosed with a desmoplastic malignant melanoma on her left cheek in April 2013. The tumour was 2.7mm thick and not ulcerated. In July 2013 a sentinel lymph biopsy was performed and none of the two extirpated lymph nodes was affected with melanoma metastases. The patient did not receive adjuvant treatment. She had between one and five moles excised in the past and no other skin cancer. Her other medical conditions were hypertension and depression. She was taking citalopram and candesartane daily and acetyl-salicylic acid less than once a month. None of her family members had a history of melanoma. Her children and siblings had multiple moles. The patient had been participating in clinical follow up visits on a regular basis and she was without recurrence or secondary melanoma at the time of last follow up (19th Oct 2015).

Phenotype: The patient had light brown hair. Her body height was 158cm and her weight was 70kg. Her constitutive and her facultative skin colour were fair. She had no freckles at the face, the dorsal hand and the shoulders and no cutis rhomboidalis at her neck. Her total body naevus count was 62 (>3 mm) and the area with the largest number of naevi was her back with 18 naevi.

Dermoscopy: A total of 11 naevi were imaged with dermoscopy. There was no predominant naevus type with 5 reticular, 1 reticular/globular and 5 naevi of unspecific pattern.

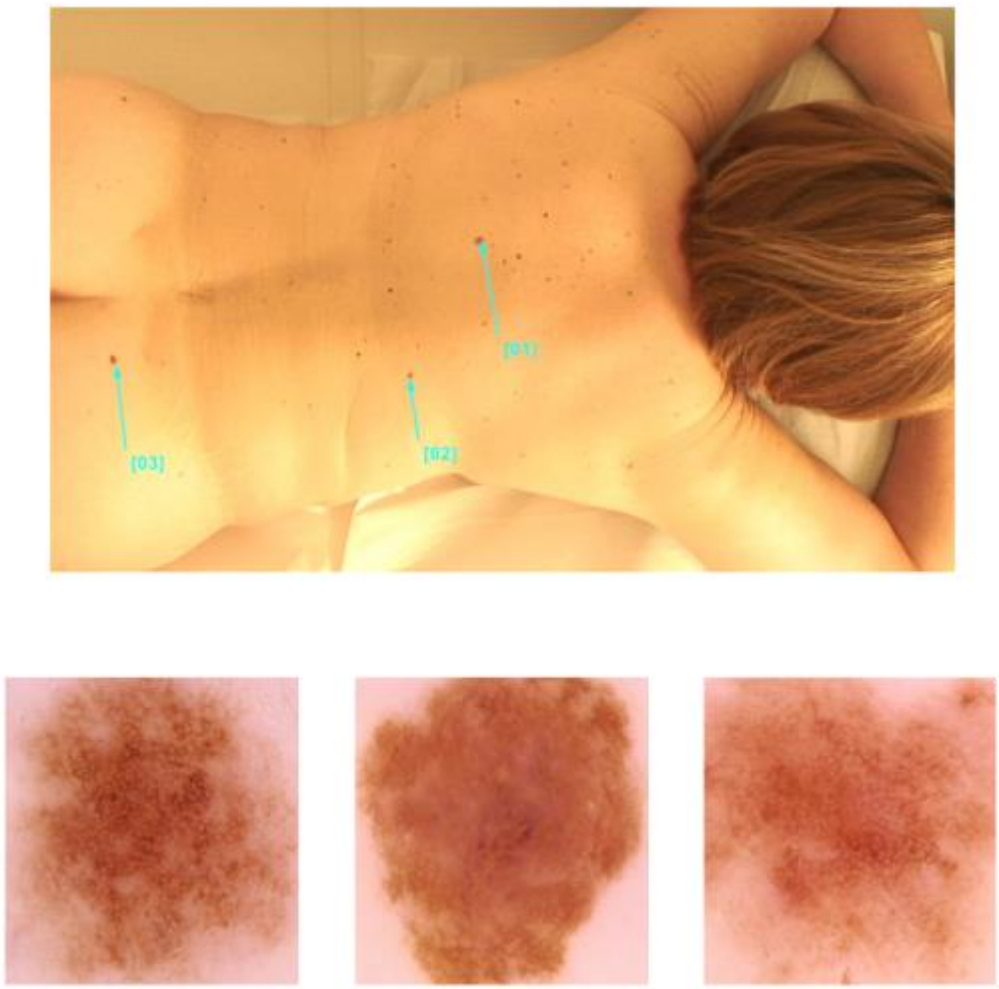


Figure 15: Overview Image upper back and three naevi captured with dermoscopy, two of reticular and one of reticular/globular pattern.

Sun behaviour: The patient had been mainly indoors for work and had been indoors and outdoors for her leisure activities. She would have been less than 50% of her time outside during the week and on the weekend. For her sun protection she never used sunglasses and a hat in less than 50% of the time. After exposure to the sun she would always tan. She remembered between 5 and 10 painful sunburns in her life.

Genotype: *MC1R*: R160W+/-; *PGC-1b*: Val240Ile A/G heterozygous

Patient TU-12

Patient TU-12 was a 48-year-old man from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IB.

Medical history: The patient was diagnosed with a superficial spreading melanoma on his left abdomen in June 2013. The tumour was initially reported to be 1,2mm thick. In a second histopathology assessment the tumour thickness was reported to be 0.9mm, with a Clark level of III, focal regression, no ulceration, no mitoses and an association to a pre-existing naevus was observed. A sentinel lymph node biopsy revealed no pathologic findings. The patient received no adjuvant treatment. The patient had no other skin cancer and no moles had been excised in the past. He had no other medical conditions and no medications. His family members were not affected by a melanoma and had not multiple moles. The patient had been participating in clinical follow up visits on a regular basis and he was without recurrence or secondary melanoma at the time of last follow up (4th Oct 2016).

Phenotype: The patient had blue/grey eyes and blonde hair. His body height was 182cm and his weight was 79kg. His constitutive skin colour was fair and his facultative skin colour was medium. He had moderate freckles at the face, the dorsal hand and the shoulders and a cutis rhomboidalis at his neck. His total body naevus count was 34 (>3 mm) and the area with the largest number of naevi was his back with 18 naevi. The head, hands and feet were free of naevi.

Dermoscopy: A total of 12 naevi were imaged with dermoscopy. The predominant naevus type was of reticular pattern with 8 reticular, 1 reticular/globular and 3 naevi of unspecific pattern.

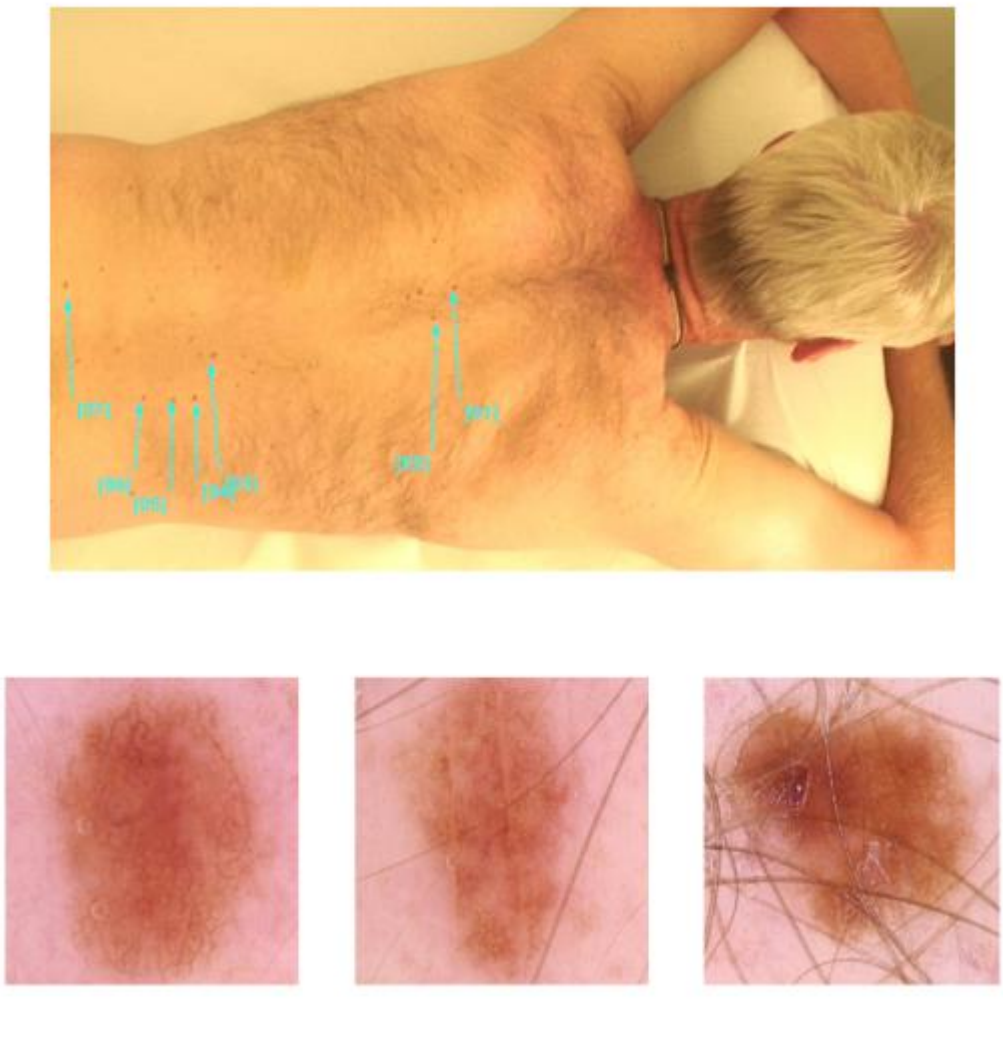


Figure 16: Overview Image upper back and three naevi captured with dermoscopy, all three of reticular pattern.

Sun behaviour: The patient had been working indoors and outdoors and his leisure activities were located mainly outdoors. He would stay between 2 and 4 hours outside on a typical weekday as well as on the weekend. He would have protected himself from the sun in less than 50% of the time with sunglasses, a hat and sunscreen with a protection factor of 20. If he would have exposed himself to the sun he would have burned and tan. He indicated that he had between two and five painful sunburns in his life.

Genotype: *MC1R*: R160W+/-, *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-13

Patient TU-13 was a 31-year-old woman from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IV.

Medical history: The patient was diagnosed with a 3.3mm thick melanoma on the left upper arm in September 2011. The patient said that it had been a fast-growing black nodule on a small naevus. She had shown it to several physicians in a time period of two and a half years before it got excised. In October 2011 a sentinel lymph node biopsy was performed and melanoma metastases were found in two lymph nodes. An adjuvant treatment with subcutaneous interferon alpha 3 MIE three times a week was given to the patient over a period of 1.5 years until April 2013. In August 2013 multiple new distant metastases were detected in the lungs, liver, bones and she had a solitary brain metastasis. Her tumour was BRAF V600E positive. As a single mother she decided for a standard treatment outside a clinical trial in proximity to her home city. She received vemurafenib 960mg and ibandronic acid 50mg daily. Her brain metastasis was treated with stereotactic radiotherapy. In November 2011 she had a mixed response with a reduced size of the lung and liver lesions but with multiple new brain metastases. A whole brain radiation therapy was performed and a combination treatment with vemurafenib and dabrafenib was started in December 2013. In January 2014 she had a deterioration of her general condition and a progressive disease. A polychemotherapy with carboplatin AUC 6, 900mg and paclitaxel 225mg/m², 360mg was started on the 10th January 2014 and scheduled every 4 weeks. The patient died on the 4th March 2014.

She had no other skin cancers. Her regular medication was sumatriptan once a week for migraine. She had no family members with melanoma. Her sister and her brother had multiple moles.

Phenotype: The patient had green/hazel eyes and dark brown hair. Her body height was 155cm and her weight was 55kg. Her constitutive and her facultative skin colour were fair. She had no freckles at the face, the dorsal hand and the shoulders and no cutis rhomboidalis at her neck. Her total body naevus count was 22 (>3 mm) and the area with the largest number of naevi was her chest with 5 naevi.

Dermoscopy: A total of 10 naevi were imaged with dermoscopy. The predominant naevus type was of unspecific pattern with 4 reticular, 1 globular and 5 naevi of unspecific pattern.

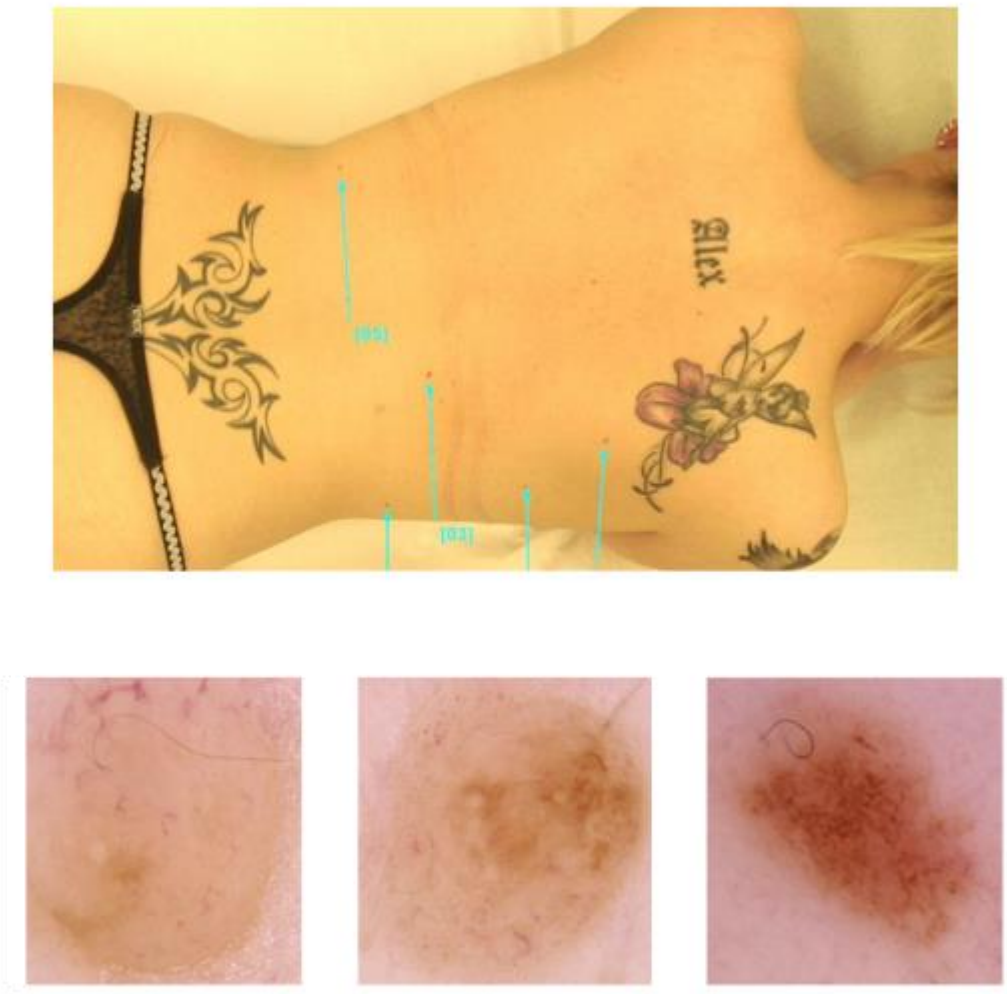


Figure 17: Overview Image upper back and three naevi captured with dermoscopy, two of unspecific and one of reticular pattern.

Sun behaviour: The patient had been mainly indoors for work as well as for her leisure activities. On a typical weekday and on the weekend, she would have been less than one hour outside. For her sun protection she used sunglasses and sunscreen with a protection factor of 50, both of it less than 50% of the time and she never used a hat. She would always burn and never tan after sun exposure. She remembered no painful sunburns in her life.

Genotype: *MC1R*: wildtype; *PGC-1b*: none of the assessed 7 SNPs showed a variant

Patient TU-14

Patient TU-14 was a 78-year-old man from South Germany with Western Europe ancestry and a Malignant Melanoma Stage IIIA.

Medical history: The patient was diagnosed with a secondary nodular, superficial spreading melanoma on this right flank in January 2013. The tumour was 2.4mm thick and not ulcerated. A Sentinel lymph node biopsy revealed one affected lymph node. In March 2013 a completing lymph node dissection of the right axilla was performed. An adjuvant treatment was not given to the patient. He had no excisions of moles before and no other skin cancer. His other medical conditions were hypertension, atrial fibrillation, chronic obstructive pulmonary disease, coronary heart disease and thyroid hypofunction. His medication was valsartan 80mg, levothyroxine sodium 100ug, magnesium, tiotropium, torasemide, verapamil, metamizol and phenprocoumon. He had no family members with melanoma or multiple moles. The patient had been participating in clinical follow up visits on a regular basis and he was without recurrence or secondary melanoma at the time of last follow up (29th Sep 2016).

Phenotype: The patient had green/hazel eyes and dark brown hair. His body height was 170cm and his weight was 110kg. His constitutive and facultative skin colour was medium. He had mild freckles at the face, the dorsal hand and the shoulders and a cutis rhomboidalis at his neck. His total body naevus count was 1 (>3 mm). The naevus was located on his abdomen.

Dermoscopy: One naevus was imaged with dermoscopy. It was of reticular pattern.

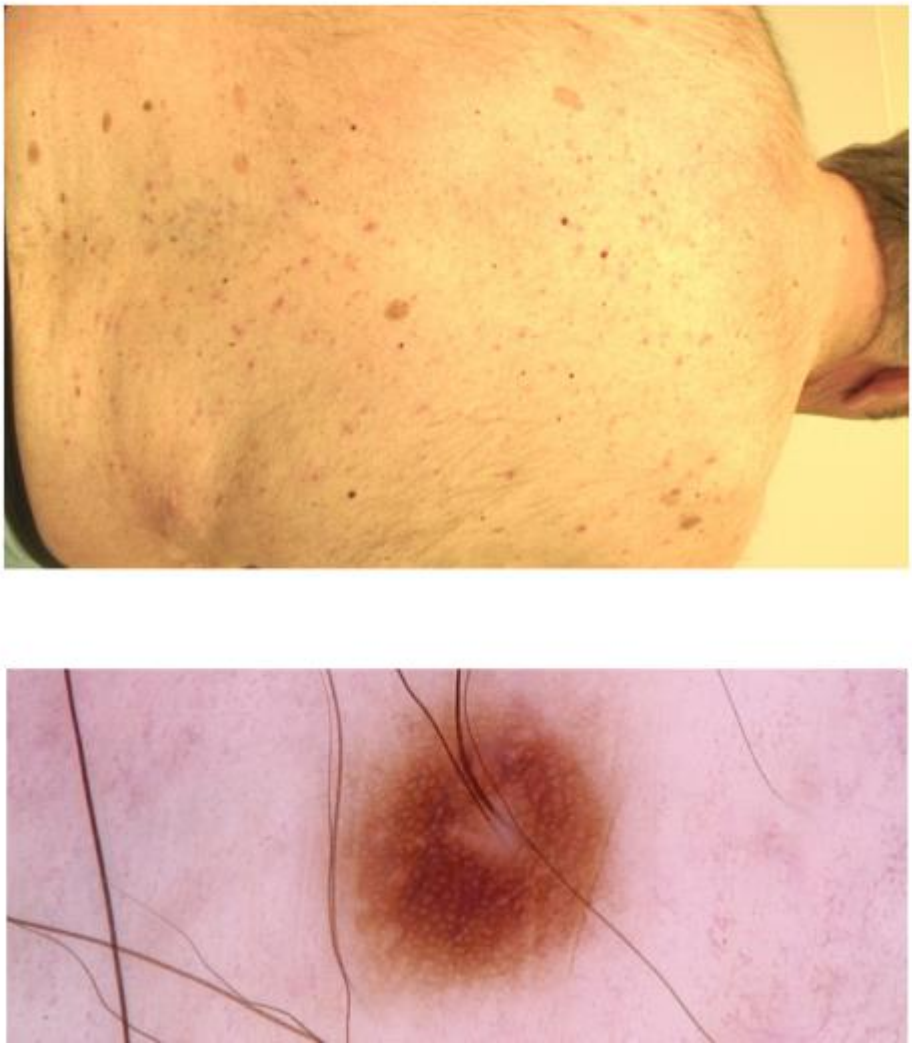


Figure 18: Overview Image upper back and one naevus captured with dermoscopy with reticular pattern.

Sun behaviour: The patient had been working as a carpenter and caretaker indoors and outdoors. His leisure activities were located mainly outdoors. He would stay between 2 and 4 hours outside on a typical weekday as well as on the weekend. He would have protected himself from the sun in less than 50% of the time with sunglasses, more than 50% of the time with a hat and never used sunscreen.

Genotype: *MC1R*: wt +/+ ; *PGC-1b:* Arg226Gln A/G homozygous, Val240Ile A/G homozygous

Patient TU-15

Patient TU-15 was a 65-year-old man born in Siberia with Western Europe ancestry and a Malignant Melanoma Stage IV.

Medical history: The patient was diagnosed with a 5.0mm thick nodular melanoma on this right upper arm in March 2010. He had a positive lymph node and received completing lymph node dissection in May 2010. An adjuvant treatment with subcutaneous Interferon alpha 3MIE three times a week was initiated in November 2010. In May 2013 a subcutaneous intransit metastasis was resected, followed by resection of lung metastases and lymph node metastases of the hilum. Multiple new distant metastases were diagnosed in August 2013. His mutation status was BRAF negative. He was screened for two clinical trials but failed to meet the inclusion criteria. Chemotherapy with dacarbazine was initiated in October 2013. The brain metastasis was treated with stereotactic radiation in November 2013. Follow up investigation in January 2014 showed a mixed response and a therapy with ipilimumab was recommended and scheduled for February 2014. The patient died on the 25th January 2014.

He had no other skin cancers. His other medical conditions were deep vein thrombosis, pulmonary embolism and diabetes mellitus. He had no family members with melanoma or multiple moles.

Phenotype: The patient had blue/grey eyes and light brown hair. His constitutive skin colour was fair and his facultative skin colour was medium. He had no freckles at the face and the dorsal hand and mild freckles at the shoulders and no cutis rhomboidalis at his neck. His total body naevus count was 9 (>3 mm).

Dermoscopy: A total of 9 naevi were imaged with dermoscopy. The predominant naevus type was of reticular pattern with 8 reticular and one naevus of unspecific pattern.

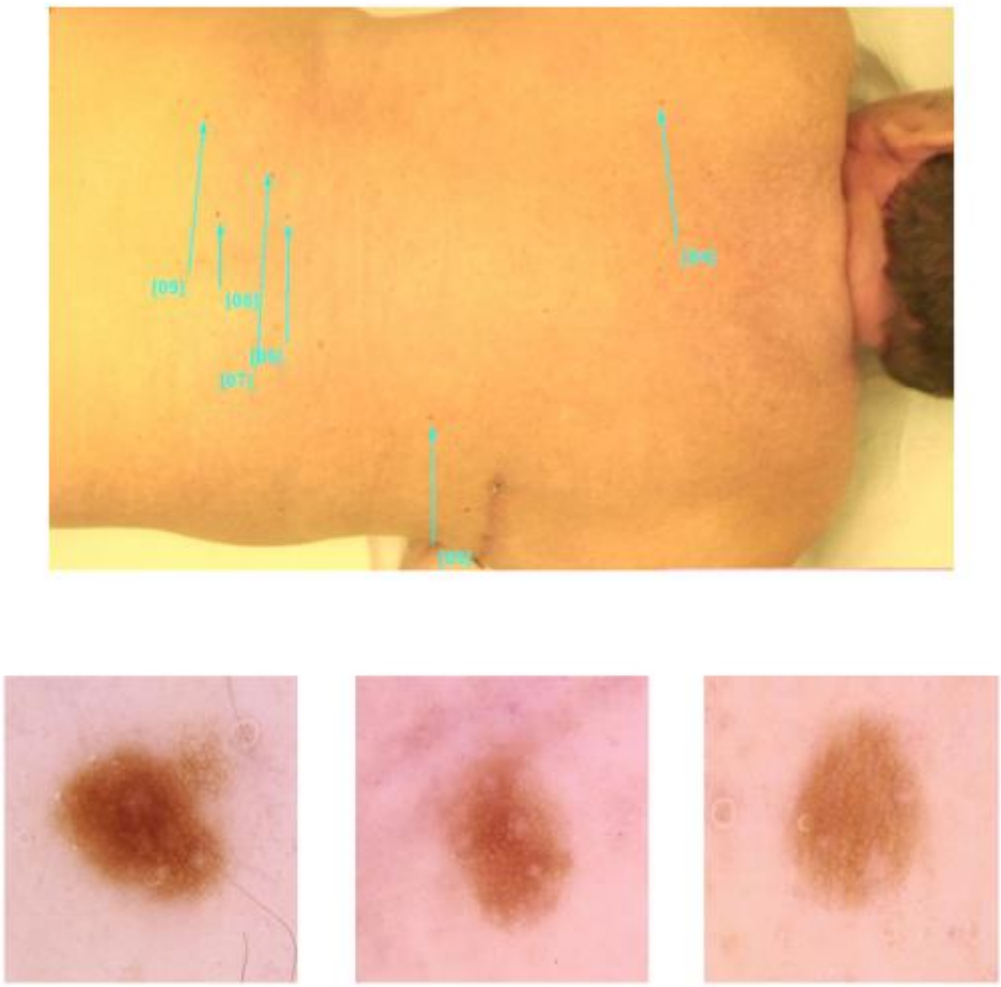


Figure 19: Overview Image upper back and three naevi captured with dermoscopy, all three of reticular pattern.

Sun behaviour: The patient had been working as a cheesemaker mainly indoors and his leisure activities were located mainly indoors as well. He would stay between 2 and 4 hours outside on a typical weekday as well as on the weekend. He never used sun glasses, a hat or sunscreen for sun protection. He would have reacted with burn and tan to unprotected sun exposure. He remembered between 2 and 5 painful sunburns in his life.

Genotype: *MC1R*: wildtype; *PGC-1b*: of the assessed 7 SNPs there was no variant detectable

Patient TU-16

Patient TU-16 was an 18-year-old girl born in Stuttgart with Middle East Ancestry and a Malignant Melanoma Stage IIIA.

Medical history: The patient was diagnosed with a melanoma on her left shoulder in January 2013. She said the lesion had been growing in a time period of 6-8 months. The histopathology report described an abnormal spitzoid melanocytic lesion which was classified as nodular melanoma with a tumour thickness of 4.5mm. The patient was referred to sentinel lymph node biopsy and two HMB 45 positive cells were found in the lymph node. An adjuvant treatment with subcutaneous interferon alpha 3MIE three times a week was given to the patient. She had no excisions of moles before and no other skin cancer. Her other medical condition was a thyroid disease and she took levothyroxine sodium on a daily base. Her family members were not affected with melanoma and had no multiple moles. The patient had been participating in clinical follow up visits on a regular basis and she was without recurrence or secondary melanoma at the time of last follow up (28th Oct 2015).

Phenotype: The patient had brown eyes and black hair. Her body height was 165cm and her weight was 47kg. Her constitutive and her facultative skin colour were olive. She had no freckles at the face, the dorsal hand and the shoulders and no cutis rhomboidalis at her neck. Her total body naevus count was 9 (>3 mm) and the area with the largest number of naevi was her left lower leg with 2 naevi.

Dermoscopy: A total of 7 naevi were imaged with dermoscopy. The predominant naevus type was of reticular pattern with 5 reticular, 1 reticular/globular and 1 naevus of globular pattern.

Figure 20: Overview Image upper back and three naevi captured with dermoscopy, one of globular and two of reticular pattern.

Sun behaviour: The patient had been mainly indoors for work as well as for her leisure activities. She spent 3 years of her life in Melbourne, Australia at the age of 13 to 15. On a typical weekday and on the weekend, she would have been less than 50% of her time outside. For her sun protection she used sunglasses and a hat less than 50% of the time and sunscreen with a protection factor of 50 more than 50% of the time. She would always tan after sun exposure. She remembered no painful sunburn in her life.

Genotype: *MC1R*: V60L+/- R151C+/-; *PGC-1b*: none of the assessed 7 SNPs showed a variant

3.7. Appendix 2 Questionnaires Sample

Clinical assessment, physicians sheet, page 1

Pat ID: ☐☐☐☐☐ Datum:__ /__ /____ 1

MC1R Studie - Klinische Untersuchung

Melanom

Multiple Melanome O ja O nein wenn ja, Anzahl der Melanome:_____

Lokalisation _____ Stadium _____

Histologie, Td _____

Primärexzision _____

Sentinel _____

Dissektion _____

Weitere Therapien _____

Pigmentierung

Angeborene Hautfarbe (ventraler Oberarm): O 1. hell O 2. Medium O 3. Olive

Fakultative Hautfarbe (dorsaler Unterarm): O 1. hell O 2. Medium O 3. Olive

Augenfarbe: O 1. blau/grau O 2. grün/hasel O 3. braun

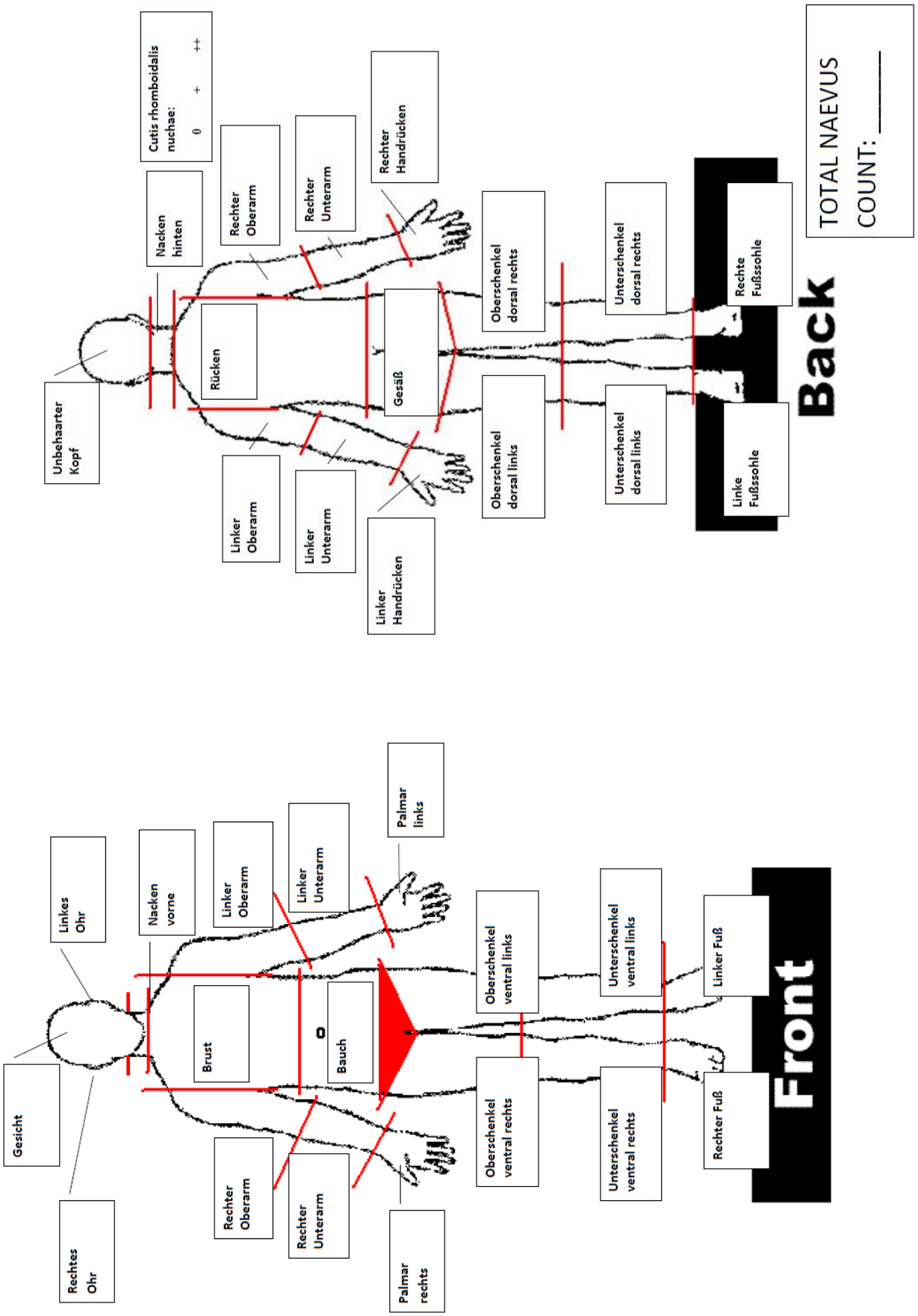
Natürliche Haarfarbe : O 1. Rot/rotbraun O 2. blond O 3. hellbraun O 4. dunkelbraun O 5. schwarz

Freckling Scores

Körperregion	1. None	2. Mild	3. Moderate	4. Severe
Gesicht				
Handrücken rechts				
Schultern				

➔bitte wenden

Clinical assessment, physicians sheet, page 2


Back
Front
TOTAL NAEVUS COUNT: _____
Cutis rhomboidalis nuchae: 0 + ++
Nacken hinten
Rechter Oberarm
Rechter Unterarm
Rechter Handrücken
Unbehaarter Kopf
Rücken
Gesäß
Linker Oberarm
Linker Unterarm
Linker Handrücken
Oberschenkel dorsal rechts
Oberschenkel dorsal links
Unterschenkel dorsal rechts
Unterschenkel dorsal links
Rechte Fußsohle
Linke Fußsohle
Linkes Ohr
Nacken vorne
Linker Oberarm
Linker Unterarm
Palmar links
Gesicht
Brust
Bauch
Rechtes Ohr
Rechter Oberarm
Rechter Unterarm
Palmar rechts
Oberschenkel ventral links
Oberschenkel ventral rechts
Unterschenkel ventral links
Unterschenkel ventral rechts
Linker Fuß
Rechter Fuß

Questionnaire, patients sheet, page 1

Study ID: ☐☐☐☐☐ Datum:__ /__ /____ 1

Fragebogen zum Hautkrebsrisiko und Sonnenverhalten

Bitte nehmen Sie sich einige Minuten Zeit um diesen Fragebogen auszufüllen. Dies hilft uns Ihr Hautkrebsrisiko besser einstufen zu können. Wir werden Ihre Angaben vertraulich behandeln und möchten uns ganz herzlich für Ihre Teilnahme bedanken!

1.Allgemeine Informationen

1. Name: _____

2. Geschlecht: 1. männlich O 2. weiblich O

3. Geburtstag: _____ **4. Geburtsort:** _____

5. Bitte geben Sie uns Ihre Abstammung an:
O Westeuropäer/in O Südeuropäer/in O Osteuropäer/in O _____

6. Größe: _____ **7. Gewicht:** _____

2.Sonnenexposition

1. Wo waren Sie insgesamt beruflich hauptsächlich beschäftigt:
O 1. Hauptsächlich im Freien O 2. Im Freien und in geschlossenen Räumen O 3. Hauptsächlich in geschlossenen Räumen

2. Wo haben Ihre Freizeitaktivitäten hauptsächlich stattgefunden:
O 1. Hauptsächlich im Freien O 2. Im Freien und in geschlossenen Räumen O 3. Hauptsächlich in geschlossenen Räumen

3. Wieviel Zeit haben Sie im letzten Jahr zwischen Sonnenaufgang und –untergang im Freien verbracht:

	a. an einem typischen Wochentag?	b. am Wochenende?
1. Fast nie (bis zu 1 Stunde)	O	O
2. Weniger als 50% der Zeit (2-4 St.)	O	O
3. Mehr als 50% der Zeit (5+)	O	O

4. Wenn Sie sich in der Sonne aufhielten, wie oft haben Sie dann in den letzten 5 Jahren durchschnittlich:

	a. Eine Sonnenbrille getragen?	b. Einen Sonnenhut getragen?	c. Sonnencreme aufgetragen?
1. Nie	O	O	O
2. Weniger als 50% der Zeit	O	O	O
3. Mehr als 50% der Zeit	O	O	O

5. Wenn Sie sich im Sommer erstmals der Sonne für 1 Stunde aussetzen, was trifft für Sie dann zu:
O 1. Immer Sonnenbrand, keine Bräune? O 2. Sonnenbrand und Bräune? O 3. Nur Bräune?

6. Wie oft hatten Sie im Leben einen schmerzhaften Sonnenbrand?
O 1. Nie O 2. Einmal O 3. 2-5 mal O 4. 5-10 mal O5. mehr als 10mal

7. Wie hoch ist der Sonnenschutzfaktor Ihrer Sonnencreme die Sie benutzen? _____

➔bitte wenden

Questionnaire, patients sheet, page 2

Study ID: ☐☐☐☐☐ Datum:__ /__ /____ 2

3.Hautkrebs und Leberflecken

1. Wo befand sich Ihr Melanom / Ihre Melanome?

Körperregion _____ Diagnosedatum _____

_____ _____

2. Hatten Sie in den letzten 2 Jahren einen anderen Hautkrebs (z.B. Basalzellkarzinom, Plattenepithelkarzinom)? *Falls ja, geben Sie es bitte hier an:*

Körperregion/Diagnose _____ Diagnosedatum _____

_____ _____

3. Wieviele Leberflecken wurden in Ihrem Leben bisher entfernt?

O 1. Keine O 2. 1-5 O 3. 6-10 O 4. Mehr als 10

4. Wurde in Ihrer Familie jemals ein Melanom diagnostiziert (Verwandte ersten oder zweiten Grades)?

Verwandte _____ Diagnosedatum _____

_____ _____

5. Hat irgendjemand Ihrer unmittelbaren Verwandten (Eltern, Geschwister, Kinder) mehr als 50 Leberflecken?
Falls ja, geben Sie bitte an welche(r) Verwandte(r): _____

4.Medikamente

1. Haben Sie die folgenden Medikamente innerhalb der letzten 5 Jahre eingenommen?

ASPIRIN (z.B. ASS 100)

O Nie O Gelegentlich O weniger als 1x/Monat O 2-3x/Monat
O 1x/Woche O 2-3x/Woche O 4-7x/Woche O 2x oder öfter täglich

2. Antientzündliche Medikamente (z.B. Celebrex, Ibuprofen)

O Nie O Gelegentlich O weniger als 1x/Monat O 2-3x/Monat
O 1x/Woche O 2-3x/Woche O 4-7x/Woche O 2x oder öfter täglich

3. Steroide (Kortison) (z.B. Decortin H, Prednison, Urbason)

O Nie O Gelegentlich O weniger als 1x/Monat O 2-3x/Monat
O 1x/Woche O 2-3x/Woche O 4-7x/Woche O 2x oder öfter täglich

4. Andere: Bitte geben Sie Ihre bisher nicht angegebenen Medikamente an, die Sie regelmässig einnehmen:

Medikamente	Wie häufig? (Angabe wie oben)	Für welche Erkrankung?

Vielen Dank für Ihre Zeit!

➔bitte wenden

4. Comparison of Early and Late Stage Melanoma Patients from Tübingen

4.1. Introduction

About 80% of all melanoma patients are cured today. More than half of all patients never experience metastatic disease, and a significant proportion of those who do develop metastases are cured with surgical and medical treatments. To date, mainly tumour specific factors which promote progression have been investigated in order to predict and understand the individual course of disease. The thickness of the primary tumour, irrespective of the histology and the general size, is the most important established factor and is included in the AJCC staging classification. Other tumour specific factors are the presence of ulceration and mitoses in the primary tumour and the evidence of skin, lymph node or distant metastases. Serum lactate dehydrogenase (LDH) is a host biomarker with prognostic impact[203] and is included in the AJCC staging system for sub classification in Stage IV.

While it is clear which individuals are at risk to develop melanoma and which types of melanoma – the thick and ulcerated ones- tend to progress, it has been rarely investigated if there is a specific phenotype of patients who are at risk of developing progressive disease or if there is a specific protective phenotype.

The aim of the following analyses was to explore if there are any differences between early and late stage melanoma patients – those patients who are cured and those who develop metastases.

4.2. Methods

Three different approaches were used to investigate phenotype and genotype differences between early and late stage melanoma patients. These were the comparison of non-metastasized and metastasized patients (Stage I/II and Stage III/IV at end of follow up), the comparison of patients who survived and those who died and the calculation of survival probabilities according to the respective variables with single variables and in a genome wide approach.

To compare the phenotype and genotype characteristics between early and late stage melanoma patients a dataset was created. The data was set up with a SPSS sheet and was extracted from the cohort of the study „Hereditary defects and cutaneous malignant melanoma“, which was approved by the ethics committee from Tübingen on the 12th December 2007, the ethics number was 376/2007B01. For the purpose of this analysis all melanoma patients were included and the control patients were excluded. The last update of follow up data including survival dates was performed on the 13th December 2016.

The patient recruitment and data management were described in Methods, Chapter 2.

Variables of interest were patient specific and tumour specific data. These were:

i. general traits of the patients, in detail: sex, age, BMI, ancestry, hair colour, eye colour, skin type Fitzpatrick, skin reaction to UV exposure, naevus count, dysplastic naevus count, UV damage und field cancerosis (the presence of multiple confluent actinic keratoses as a sign of chronic sun damage)

ii medical history and family history of the patients, in detail: history of a secondary cancer, history of psoriasis, family history of melanoma, family history of cancer,

iii sun behaviour of the patients, in detail: sunburns in childhood, usage of sunbeds, frequency of sun holidays, usage of sunscreen, occupational and leisure sun exposure,

iv genetic data, in detail: the presence of a germline variant within *MC1R*, *PGC-1α* and *PGC-1b*, including a deeper analysis with data of the *MC1R* locus and a set of 356 384 chip array derived variants, and

v tumour specific data, in detail: histology of primary tumour, tumour thickness, tumour localisation, Clark level, presence of ulceration, presence of regression and the association to naevi.

The counts for the variables were calculated with SPSS. For ordinal and nominal variables, the numbers and percentages were extracted from the frequency tables. The missing values were indicated and classified with na. The valid percentages, which did not include missing cases, were quoted in the tables. For metric variables, such as age and BMI, the median, standard deviation and the minimum and maximum values (range) was displayed.

Comparisons between metastasized and non-metastasized patients – Stage I/II and Stage III/IV were made with crosstab calculations. For ordinal and nominal variables, the associations were tested with a two-sided Pearson Chi-Square test.

To select the appropriate statistical test for comparisons of the metric variables age and BMI the distribution of the frequencies was tested for both variables using a histogram with a normal distribution curve. The variable “age” showed a bimodal distribution while the variable “BMI” had a normal distribution.

Therefore, the Mann-Whitney U test, a nonparametric test for two independent samples was chosen for the variable “age”. The significance level to reject the null hypothesis was set to 0.05.

For the variable “BMI”, a parametric test, the t-test of equality of means, an independent sample test, was chosen. The equal variance was not assumed and the p-value was presented for a two-tailed test. The significance level to reject the null hypothesis was set to 0.05.

The survival analyses were calculated with the Kaplan Meier method. The survival time was calculated between the date of first diagnoses and the date of last follow up. All cases with death of another reason and patients who were still alive at the time of last follow up were censored. The significance was tested pooled over strata and the log rank (Mantel-Cox) test was used to test if the null hypothesis could be rejected. The significance level to reject the null hypothesis was set to 0.05.

For the genome wide approach, the survival analyses were performed on 356 384 genetic variants distributed over all 23 chromosomes. The rare SNPs with a minor allele frequency

below 0.1 were kept for the analysis in order to not miss rare variants with a high impact. The variants with the highest significance levels were further analysed to filter artefactual results.

4.3. Results

4.3.1. Cohort Description

A total of 556 patients with a history of malignant melanoma were included into the analyses. The majority of patients (n=365) were without metastases at the end of follow up and were classified as Stage I or Stage II disease according to the tumour thickness and properties of the primary tumour. The other 191 patients were either initially metastasized or developed metastases during the time of follow up. They were classified as Stage III or Stage IV with the evidence of regional or distant metastases respectively.

The median time of follow up was 8 years for all 556 patients. Patients with Stage I or Stage II had a follow up time of 9 years and patients who were Stage III or Stage IV had a median follow up time of 8 years at the time of databank closure (Dec 2016). Patients who survived or died of other causes had a median follow up time of 9 years, whereas patients who died of melanoma had a median follow up time of 4 years.

Of the 556 patients, 44.6% were female and 55.4% male. The majority of patients had German parents, only 6.8% of the patients had another ancestry. The median age was 59 years, the youngest patient was 17, the oldest 91 years old. The birthdate was available for all patients. The age distribution showed two peaks, one around 50 years and one around 70 years (Figure 21a). The data for both height and weight was available for 526 patients. The mean BMI was 27 and the curve was normally distributed (Figure 21b).

The age and BMI distribution between early and late stage melanoma patients were similar (Figure 21c+d).

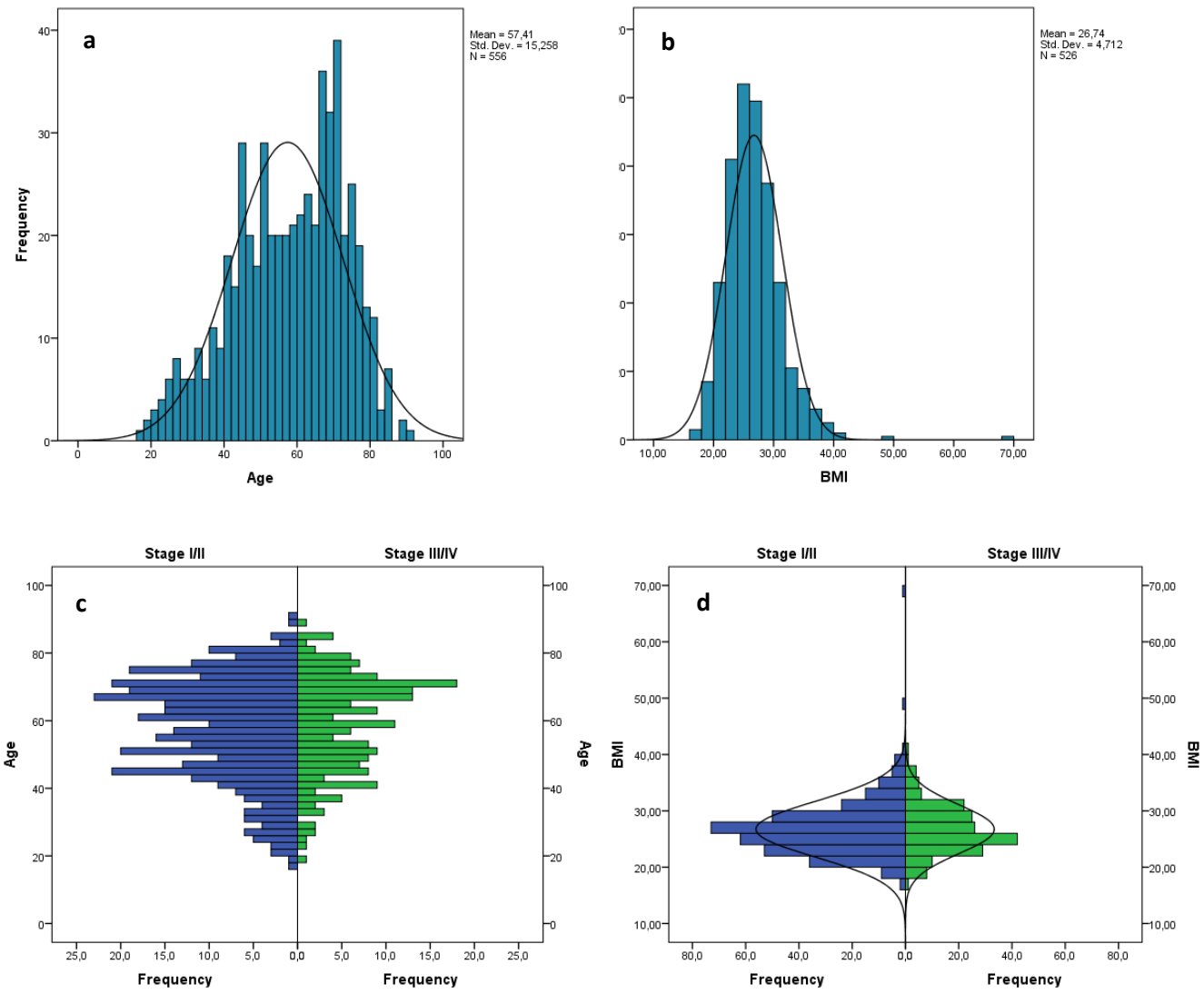


Figure 21: Age and BMI distribution within the complete cohort (a and b) and among early (blue) and late (green) stage patients (c and d)

Pigment traits, naevus count and UV damage

The general characteristics, pigment traits, naevus count and UV damage are summarized in Table 4. Hair colour was the only variable with showed a significant difference between early and late stage patients. All other variables were similar between early and late stage patients. The characteristics of the complete cohort were as follows. Only few patients had red or black hair, with 6% and 5.9% respectively. The majority of patients had either brown (44.3%) or blonde (43.8%) hair. Most patients were blue-eyed with 39.5%, followed by patients with brown eyes (24.1%), grey (19.9%) or green (16.5%) eyes. The most common skin type according to the Fitzpatrick scale among the patients was skin type II with 56.5%, followed by skin type III (35.7%) and skin type I (6.4%). None of the patients had a Fitzpatrick skin type IV, V or

VI. Almost half of the patients indicated their skin reaction after sun exposure as “mostly burned, light tan” (49.3%), followed by “seldom burned, strong tan” (23.6%), “always burned, no tan” (17.6%) and only 9.5% reported they would never burn and always tan. About one third of the patients (35.7%) had between 11 and 30 naevi on their body, 24.2% had up to 10 naevi, followed by 19% with a total body naevus count of 31-50 naevi. Only 6.9% of the patients had more than 100 naevi on their body. The vast majority of patients (80.4%) had no dysplastic naevus, 1-5 dysplastic naevi were reported for 17.3% of patients and only few patients had 6-10 (1%), 11-20 (0.8%) or more than 20 (0.4%) dysplastic naevi on their body. The three categories for UV damage, none, light and severe were almost evenly distributed among the population with 26.1%, 37.3% and 36.7% respectively. Only very view patients (2.3%) had a field cancerisation.

The hair colour was recorded for 546 patients with 10 missing values, the eye colour was recorded for 552 patients with 4 missing values. The skin type was not recorded for 71 patients and the skin reaction was missing for 6 patients. The naevus count was almost completely available with 2 missing values, the dysplastic naevus count was not available for 77 patients, data regarding UV damage was missing for 65 patients and regarding the presence of a field cancerisation on the capillitium for 85 patients.

Table 4: Comparison of general and pigment traits between early and late stage melanoma patients.

	All pts		St I/II		St III/IV		p*
	n	%	n	%	n	%	
Total, n	556	100	365	100	191	100	
Sex							0.494
female	248	44.6	159	43.6	89	46.6	
male	308	55.4	206	56.4	102	53.4	
Age median	59		58		59		0.173
range	17-91		17-91		19-89		
BMI median	26		26		26		0.993
range	17-70		17-70		18-42		
German parents							0.640
yes	508	93.2	335	93.6	173	92.5	
others	37	6.8	23	6.4	14	7.5	
na	11		7		4		
Hair colour							**0.018**
red	33	6.0	21	5.8	12	6.4	
blonde	239	43.8	170	47.4	69	36.9	
brown	242	44.3	143	39.8	99	52.9	
black	32	5.9	25	7.0	7	3.7	
na	10		6		4		
Eye colour							0.499
blue	218	39.5	141	39.0	77	40.5	
grey	110	19.9	70	19.3	40	21.1	
green	91	16.5	66	18.2	25	13.2	
brown	133	24.1	85	23.5	48	25.3	
na	4		3		1		
Skin type (Fitzpatrick) n, %							0.610
I	31	6.4	18	5.6	13	8.0	
II	274	56.5	178	55.3	96	58.9	
III	173	35.7	121	37.6	52	31.9	
IV	6	1.2	4	1.2	2	1.2	
V	1	0.2	1	0.3	0	0	
na	71		43		28		
Skin reaction							0.933
always burned, no tan	97	17.6	62	17.1	35	18.6	
mostly burned, light tan	271	49.3	178	49.2	93	49.5	
seldom burned, strong tan	130	23.6	86	23.8	44	23.4	
never burned, always tan	52	9.5	36	9.9	16	8.5	
na	6		3		3		
UV damage							0.070
none	128	26.1	82	25.1	46	28.0	
light	183	37.3	112	34.3	71	43.3	
severe	180	36.7	133	40.7	47	28.7	
na	65		38		27		
Field cancerization							0.076
yes	11	2.3	10	3.2	1	0.6	
no	460	97.7	300	96.8	160	99.4	
na	85		55		30		
Naevus count							0.495
0-10	134	24.2	93	25.5	41	21.7	
11-30	198	35.7	122	33.4	76	40.2	
31-50	105	19.0	74	20.3	31	16.4	
51-100	79	14.3	51	14.0	28	14.8	
>100	38	6.9	25	6.8	13	6.9	
na	2		0		2		
Dysplastic naevus count							0.496
0	385	80.4	250	78.6	135	83.9	
1-5	83	17.3	61	19.2	22	13.7	
6-10	5	1.0	4	1.3	1	0.6	
11-20	4	0.8	2	0.6	2	1.2	
more than 20	2	0.4	1	0.3	1	0.6	
na	77		47		30		

*Pearson Chi-Square Asymp.Sig (2-sided)

Personal and family medical history

The data is summarized in Table 5. There were no significant differences between early and late stage melanoma patients. A small subset of patients with 6.7% reported a history of a second cancer in addition to the malignant melanoma. The history of psoriasis was rare with 3.9%. A family history of melanoma affecting first degree relatives was reported for 5.4% of patients. When taken all cancer types together, more than half of the patients (52.9%) had a history of cancer in their families.

The information about the personal history of a second cancer was available for all patients. Data regarding the history of psoriasis, the family history of melanoma and the family history of cancer was missing in 21, 19 and 21 cases respectively.

Table 5: Comparison of the medical history between early and late stage melanoma patients

	All pts		**St I/II**		**St III/IV**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	365	100	191	100	
Second cancer							0.540
yes	37	6.7	26	7.1	11	5.8	
no	519	93.3	339	92.9	180	94.2	
na	0		0		0		
History of Psoriasis							0.579
yes	21	3.9	15	4.3	6	3.3	
no	514	96.1	337	95.7	177	96.7	
na	21		13		8		
Family history of MM							0.123
yes	29	5.4	23	6.5	6	3.3	
no	508	94.6	332	93.5	176	96.7	
na	19		10		9		
Family history of Cancer							0.089
yes	283	52.9	195	55.6	88	47.8	
no	252	47.1	156	44.4	96	52.2	
na	21		14		7		

*Pearson Chi-Square Asymp.Sig (2-sided)

Sun behaviour data

The data is summarized in Table 6. There was no significant difference between early and late stage melanoma patients. The majority of patients experienced one sunburn per year in their childhood (44.6%), followed by 24.7% of patients who had less than one sunburn per year and those patients with sunburns several times a year (22.0%). Less than 10% of the patients remembered no sunburn in their childhood (8.7%). The use of sunbeds was very rare among the melanoma patients from Tübingen. Nearly 90% indicated they would never visit a solarium. Less than 10% used sunbeds occasionally and only around 4% of the patients visited a solarium on a regular base, either a few weeks before the holiday, monthly or on a weekly base. About one third of the patients noted they would never go on sun holidays. Most of the patients went less than one time a year (37.0%) or one to twice a year (24.9%) on sun holidays. Around 5% of the patients went on sun holidays more frequently during the year. Most patients applied sunscreen either several times a week (35.1%) or on a daily base (20.8%). Around one third indicated they would seldom use sunscreen and 10.8% stated they never use sunscreen. About half of the patients were solely located indoors during their working hours. Around 30% of the patients spent up to two hours a day outside, with 10.0% less than 30min a day, 11.8% of the patients 30-60min a day and 9.8% one to two hours a day respectively. Around 20% of the patients spent more than 2 hours outside during their occupation, with 6.7% of the patients spending 2-4 hours a day outside, 5.5% of patients with 4-6 hours a day and 6.7% with more than 6 hours a day. In terms of leisure sun exposure, the majority of patients spent 2-4 hours a day outside (33.2%), followed by 26.0% of patients who spent 1-2 hours outside, 17% spent 30-60min outside, 14.4% spent 4-6 hours outside, 5.0% spent less than 30 min a day outside, 3.5% of the patients more than 6 hours a day. Only 0.9% of patients stated they would stay completely indoors during their leisure time.

Questionnaire based information of the sun behaviour was recorded for all melanoma patients in 6 categories: the frequency of sunburns during childhood, the use of sunbeds, the frequency of sun holidays during the year, the regular use of sunscreen and the estimated time of occupational and leisure sun exposure. Missing values were apparent in 5 to 15 cases of the 556 patients per category.

Table 6: Comparison of the sun behaviour between early and late stage melanoma patients

	All pts		**St I/II**		**St III/IV**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	365	100	191	100	
Sunburns (age < 20 ys)							0.658
no sunburns	48	8.7	28	7.7	20	10.6	
less than 1x/year	136	24.7	93	25.7	43	22.8	
1x/year	246	44.6	161	44.5	85	45.0	
several times a year	121	22.0	80	22.1	41	21.7	
na	5		3		2		
Use of Solarium							0.578
never	476	87.2	309	86.1	167	89.3	
occasional	48	8.8	35	9.7	13	7.0	
few weeks before holiday	8	1.5	5	1.4	3	1.6	
less than 1x/month	2	0.4	2	0.6	1	0.5	
monthly	6	1.1	5	1.4	3	1.6	
weekly	6	1.1	3	0.8	4		
na	10		6				
Sun holidays							0.662
never	182	33.0	114	31.4	68	36.2	
less than 1x/year	204	37.0	133	36.6	71	37.8	
1-2x/year	137	24.9	96	26.4	41	21.8	
2x/year	15	2.7	11	3.0	4	2.1	
more frequent than 2x/year	13	2.4	9	2.5	4	2.1	
na	5		2		3		
Use of sunscreens							0.056
never	59	10.8	33	9.2	26	13.9	
seldom	182	33.3	111	30.8	71	38.0	
several times a week	192	35.1	136	37.8	56	29.9	
daily	114	20.8	80	22.2	34	18.2	
na	9		5		4		
Occupational sun exposure	268	49.5	170	47.6	98	53.3	0.105
only indoors	54	10.0	39	10.9	15	8.2	
less than 30 min a day	64	11.8	42	11.8	22	12.0	
30-60 min a day	53	9.8	39	10.9	14	7.6	
1-2 hours a day	36	6.7	28	7.8	8	4.3	
2-4 hours a day	30	5.5	14	3.9	16	8.7	
4-6 hours a day	36	6.7	25	7.0	11	6.0	
more than 6 hours a day	15		8		7		
na							
Leisure sun exposure							0.583
only indoors	5	0.9	5	1.4	0	0	
less than 30 min a day	27	5.0	18	5.0	9	4.9	
30-60 min a day	92	17.0	55	15.4	37	20.0	
1-2 hours a day	141	26.0	94	26.3	47	25.4	
2-4 hours a day	180	33.2	119	33.3	61	33.0	
4-6 hours a day	78	14.4	52	14.6	26	14.1	
more than 6 hours a day	19	3.5	14	3.9	5	2.7	
na	14		8		6		

*Pearson Chi-Square Asymp.Sig (2-sided)

Genetic data

The presence of one or more germline variants was assessed for all 556 patients in the genes coding for MC1R, PGC-1a and PGC-1b. The majority of patients had one or more variants in the gene coding for MC1R (77.3%), 22.7% of the patients had two wild type alleles. The overall presence of any *MC1R* variant was not different between stage I/II and stage III/IV patients. The sub-analyses of *MC1R* variants – distinguishing between r-variants, R-variants and wt variants, revealed significant differences as described later and presented in (Figure 25). A variant in the gene coding for PGC-1a was less frequently observed, 87.2% had a wild type, 12.8% of the patients had one or more variants. The gene coding for PGC-1b was without any variants in 76.8% of the melanoma patients, 23.2% had one or more variants (Table 7).

Table 7: Comparison of genetic variants between early and late stage melanoma patients

	All pts		**St I/II**		**St III/IV**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	365	100	191	100	
MC1R**							0.626
wt	126	22.7	85	23.3	41	21.5	
variant	430	77.3	280	76.7	150	78.5	
na							
MC1R r alleles							**0.004**
0	288	51.8	187	51.2	101	52.9	
1	217	39.0	134	36.7	83	43.5	
2	51	9.2	44	12.1	7	3.7	
MC1R R alleles							0.772
0	310	55.8	207	56.7	103	53.9	
1	207	37.2	132	36.2	75	39.3	
2	39	7.0	26	7.1	13	6.8	
MC1R WT alleles							**0.028**
0	174	31.3	126	34.5	48	25.1	
1	256	46.0	154	42.2	102	53.4	
2	126	22.7	85	23.3	41	21.5	
***MC1R* Genotype**							**0.019**
WT/WT	126	22.7	85	23.3	41	21.5	
WT/r	133	23.9	78	21.4	55	28.8	
WT/R	123	22.1	76	20.8	47	24.6	
r/r	51	9.2	44	12.1	7	3.7	
r/R	84	15.1	56	15.3	28	14.7	
R/R	39	7.0	26	7.1	13	6.8	
PGC-1a							0.870
wt	485	87.2	319	87.4	166	86.9	
variant	71	12.8	46	12.6	25	13.1	
na							
PGC-1b							0.885
wt	427	76.8	281	77.0	146	76.4	
variant	129	23.2	84	23.0	45	23.6	
na							

*Pearson Chi-Square Asymp.Sig (2-sided)

**deeper analyses with data of the *MC1R* locus were performed, considering the numbers of r, R or WT alleles respectively, and the specific MC1R genotype

Tumour specific data

The tumour specific data are presented in Table 8. The differences between early and late stage patients are discussed in the next chapter. The median tumour thickness of all melanoma patients was 1.5mm. The category 1.01mm to 2.0mm was most frequent with 41.0%, followed by the categories below 1mm and 2.01-4mm with 24.5% and 24.3%. A tumour thickness of above 4mm was recorded for 10.2% of patients. The main subtype was superficial spreading melanoma with 61.2%, followed by 17.9% of patients with nodular melanoma. Lentigo maligna melanoma was about as frequent as acral lentiginous melanoma with 5.2% and 5.6%. Other melanoma subtypes were summarized with 10.2% of cases. Most melanomas were localized on the trunk (38.3%), followed by the lower extremities (26.8%), the upper extremities (18.2%) and the head and neck region (15.2%). Other localisations were recorded in 1.5% of the cases. A primary melanoma was not detectable in 4.2% of the melanoma patients. These patients were initially diagnosed with melanoma metastases and were summarized as patients with unknown primary. A Clark level 4 was found in 70.8% of the cases, followed by 17.7% with Clark level 3. Few melanomas had a Clark level 2 (7.1%) or Clark level 5 (4.3%) and no Clark level 1 was recorded in this cohort. The histopathology report described ulceration in 72.5% of the cases and signs of regression in 14.2% of the melanoma specimens. An association to a pre-existing naevus was reported for 19.5% of melanomas (Table 8).

Tumour specific data were recorded for each melanoma patient. Missing cases were found as follows: The tumour thickness was missing in 37 cases, the melanoma subtype in 54 cases, the primary localisation in 23 cases. The presence or absence of an unknown primary tumour was specified in all but in 14 cases. Details of the histopathology report such as indication of the Clark level, presence of ulceration, signs of regression and an association of the melanoma to a pre-existing naevus were missing in 93, 112, 126 and 135 cases respectively.

Table 8: Comparison of tumour specific data between early and late stage melanoma patients

	All pts		St I/II		St III/IV		p*
	n	%	n	%	n	%	
Total, n	556	100	365	100	191	100	
Tumour thickness							
median	1.5mm		1.3mm		2.1mm		**<0.001**
<1,0mm	127	24.5	108	29.8	19	12.1	**<0.001**
1,01-2,0mm	213	41.0	156	43.1	57	36.3	
2,01-4mm	126	24.3	68	18.8	58	36.9	
>4mm	53	10.2	30	8.3	23	14.6	
na	37		3		34		
Histology							**0.016**
SSM	307	55.2	224	64.6	83	53.5	
NM	90	16.2	51	14.7	39	25.2	
LMM	26	4.7	21	6.1	5	3.2	
ALM	28	5.0	16	4.6	12	7.7	
others	51	9.2	35	10.1	16	10.3	
na	54		18		36		
Localisation							**0.002**
head and neck	81	15.2	46	12.6	35	20.8	
trunk	204	38.3	150	41.1	54	32.1	
upper extremities	97	18.2	76	20.8	21	12.5	
lower extremities	143	26.8	90	24.7	53	31.5	
others	8	1.5	3	0.8	5	3.0	
na	23		0		23		
Clark level							**<0.001**
2	33	7.1	31	9.4	2	1.5	
3	82	17.7	67	20.4	15	11.2	
4	328	70.8	220	66.9	108	80.6	
5	20	4.3	11	3.3	9	6.7	
na	93		36		57		
Ulceration							**<0.001**
yes	122	27.5	63	20.9	59	41.5	
no	322	72.5	239	79.1	83	58.5	
na	112		63		49		
Regression							0.169
yes	61	14.2	47	15.7	14	10.7	
no	369	85.8	252	84.3	117	89.3	
na	126		66		60		
Naevus-associated MM							0.463
yes	82	19.5	60	20.4	22	17.3	
no	339	80.5	234	79.6	105	82.7	
na	135		71		64		

*Pearson Chi-Square Asymp.Sig (2-sided)

4.3.2. Stage I/II compared with Stage III/IV

The cohort of melanoma patients from Tübingen consisted of 556 patients and was split into two groups to compare phenotypes, sun behaviour and tumour specific data between early and advanced melanoma patients. Early melanoma patients, who were diagnosed with stage I or stage II disease and who did not progress over the time period of follow up, formed the first group of 365 patients. Advanced melanoma patients, who were either initially diagnosed with stage III/stage IV or who progressed and were classified stage III or stage IV at the end of follow up, formed the second group of 191 patients.

The comparison between the two groups in regard of their general data, pigment traits, signs of UV damage and naevus counts are summarized in Table 4. The advanced patients group consisted of slightly more female patients compared to the early stage group (46.6% versus 43.6%), but the difference was not significant. The median age, the BMI and the ancestry were similar between the two groups. The median age was 58 and 59 years respectively and the BMI 26 in both groups. More than 90% of patients had German parents with 93.6% in the early stage group and 92.5% in the late stage group.

A significant difference was observed in regard of the hair colour between the two groups. Patients with blonde hair were more likely to have early stage melanoma, whereas more patients with brown hair had late stage disease. The frequency of blonde hair was 47.4% in the early stage group compared to 36.9% in the late stage group, whereas 39.8% had brown hair in the early stage group compared to 52.9% in the late stage group. This difference was significant with a p-value of 0.018 (Figure 22a). The other pigment traits eye colour and skin type were similar between both groups. No difference was observed between the skin reaction after sun exposure of early versus late stage melanoma patients (Figure 22d). The total body naevus count was recorded in 5 categories. The category 11-30 total body naevus count was most frequent in both groups with 33.4% in early stage and 40.2% in late stage melanoma patients. The distribution between the categories was similar in both cohorts with no significant differences (Figure 22e). Slightly more patients with early stage melanoma disease had a report of dysplastic naevi on their body. While 83.9% of late stage melanoma had no dysplastic naevi, 78.6% of early stage melanoma patients were documented having no dysplastic naevi on their body. However, the differences were not statistically significant (Figure 22f).

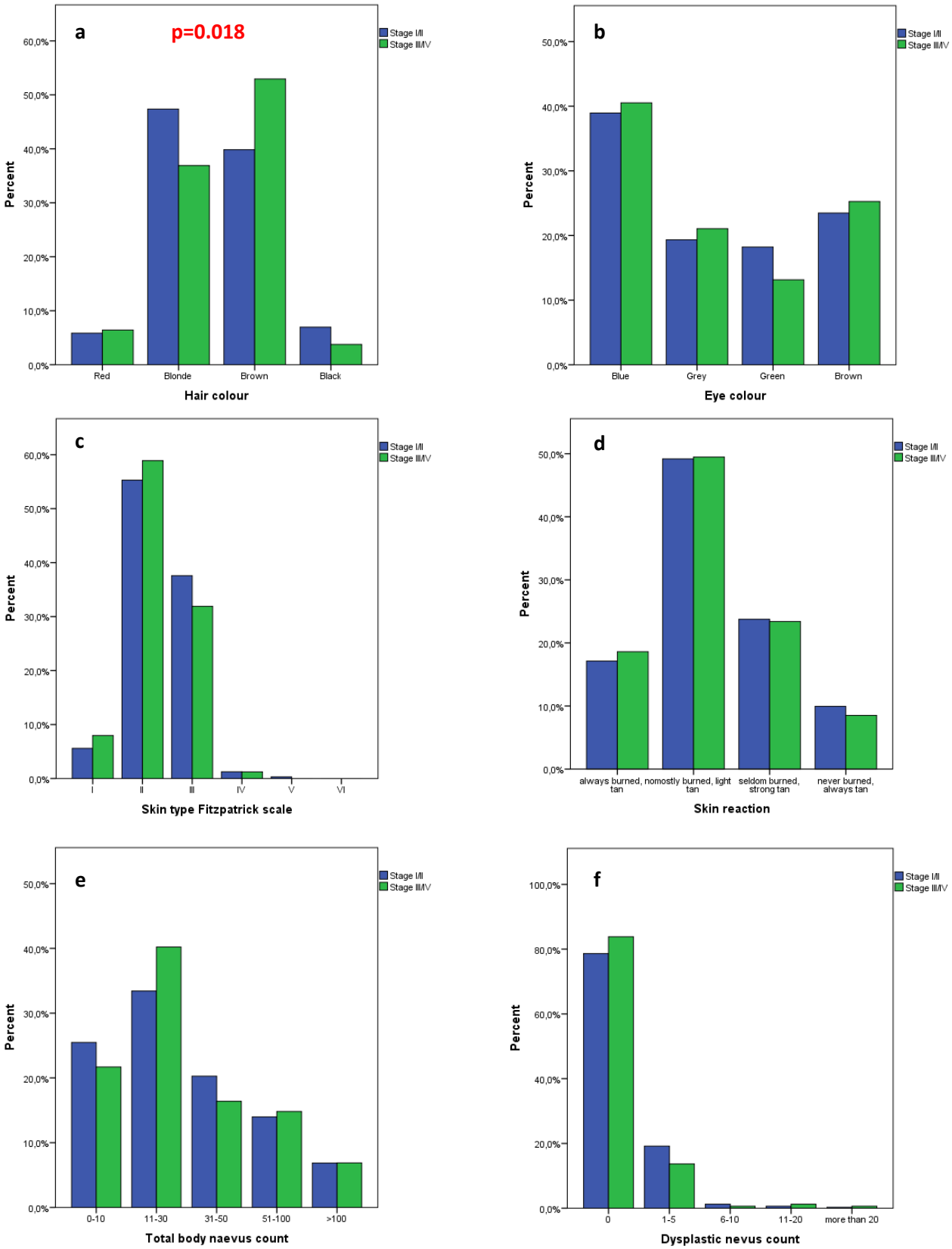


Figure 22: Bar charts displaying the pigment traits of the cohort; a. Hair colour; b. Eye colour; c. Skin type; d. Skin reaction; e. Naevus count; f. Dysplastic Naevus count

Data recorded in regard of the medical history of the patients were

i. the presence of a second cancer other than melanoma

ii. the personal history of psoriasis

iii. the family history of melanoma and

iv. the family history of other cancer types.

None of the variables showed significant differences between the two groups (Table 5). Slightly more patients of the early stage group were affected with a second cancer comprising 7.1% of early stage patients compared to 5.8% of late stage melanoma patients with a second cancer other than melanoma. A history of psoriasis was slightly more frequent in early stage melanoma patients with 4.3% versus 3.3%. A positive family history of melanoma was more frequent in the early stage patients with 6.5% compared to 3.3% of patients in the late stage group. Likewise, more patients of the early stage group had a family member with any other cancer type. These were 55.6% compared to 47.8% of late stage patients with a positive family history of cancer (Figure 23).

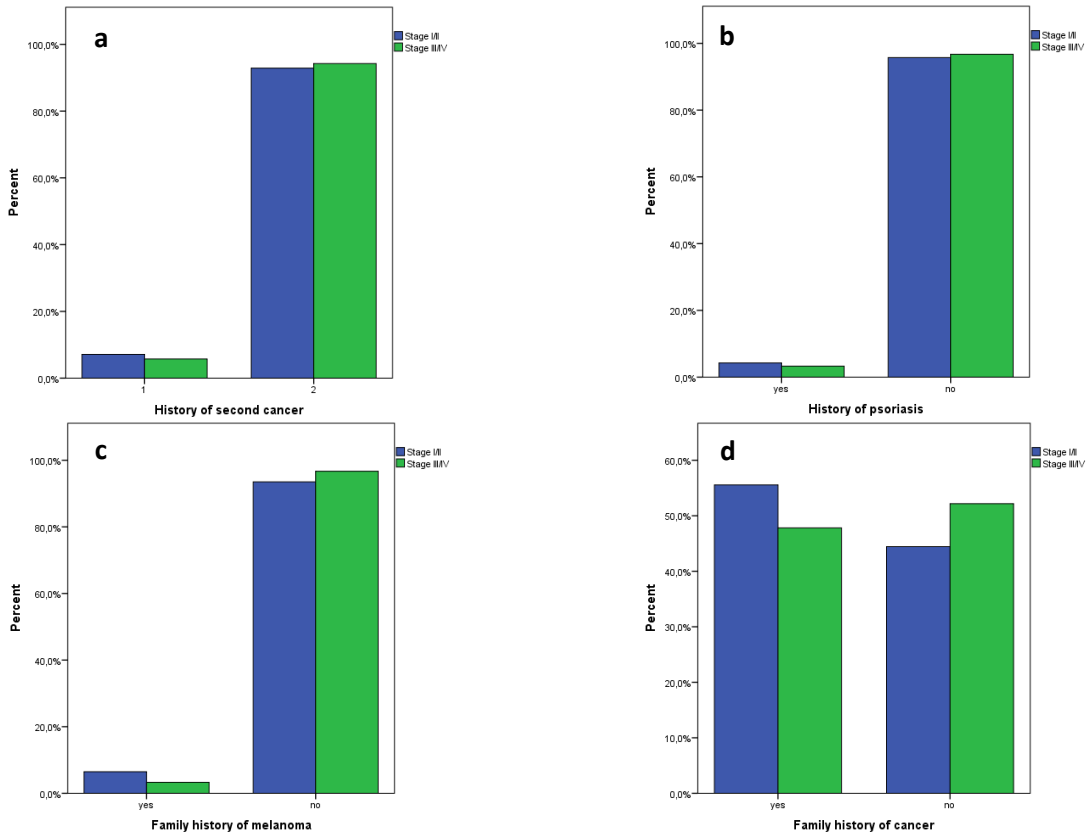


Figure 23: Bar charts displaying the medical history of the cohort; a. Personal history of a second cancer; b. Personal history of psoriasis; c. Family history of melanoma; d. Family history of cancer

Sun behaviour and UV damage data were compared between the two groups. These data consisted of eight different variables: the recall of sunburns in childhood and adolescence, the use of sun beds, frequency of sun holidays, usage of sunscreen, occupational sun exposure, leisure sun exposure, UV damage and field cancerisation. None of the single variables showed significant differences between the early stage melanoma patients and the late stage melanoma patients (Table 6).

Slightly more patients of the late stage group compared to the early stage group indicated they never got sunburned at the age below 20 years (10.6% versus 7.7%). Patients of the early stage group indicated more frequently they got less than 1x/year burned. The other categories 1x/year and several times a year had the same percentage with 44.5% versus 45.0% and 22.1% versus 21.7% respectively (Figure 24a).

Likewise, slightly more of the late stage patient group reported they would never use sun beds with 89.3% compared to 86.1% of the early melanoma stage patients and fewer late stage patients used sun beds occasionally compared to early stage patients. The categories few weeks before holiday, less than 1x/month, monthly and weekly shared nearly the same percentage in both groups (Figure 24b).

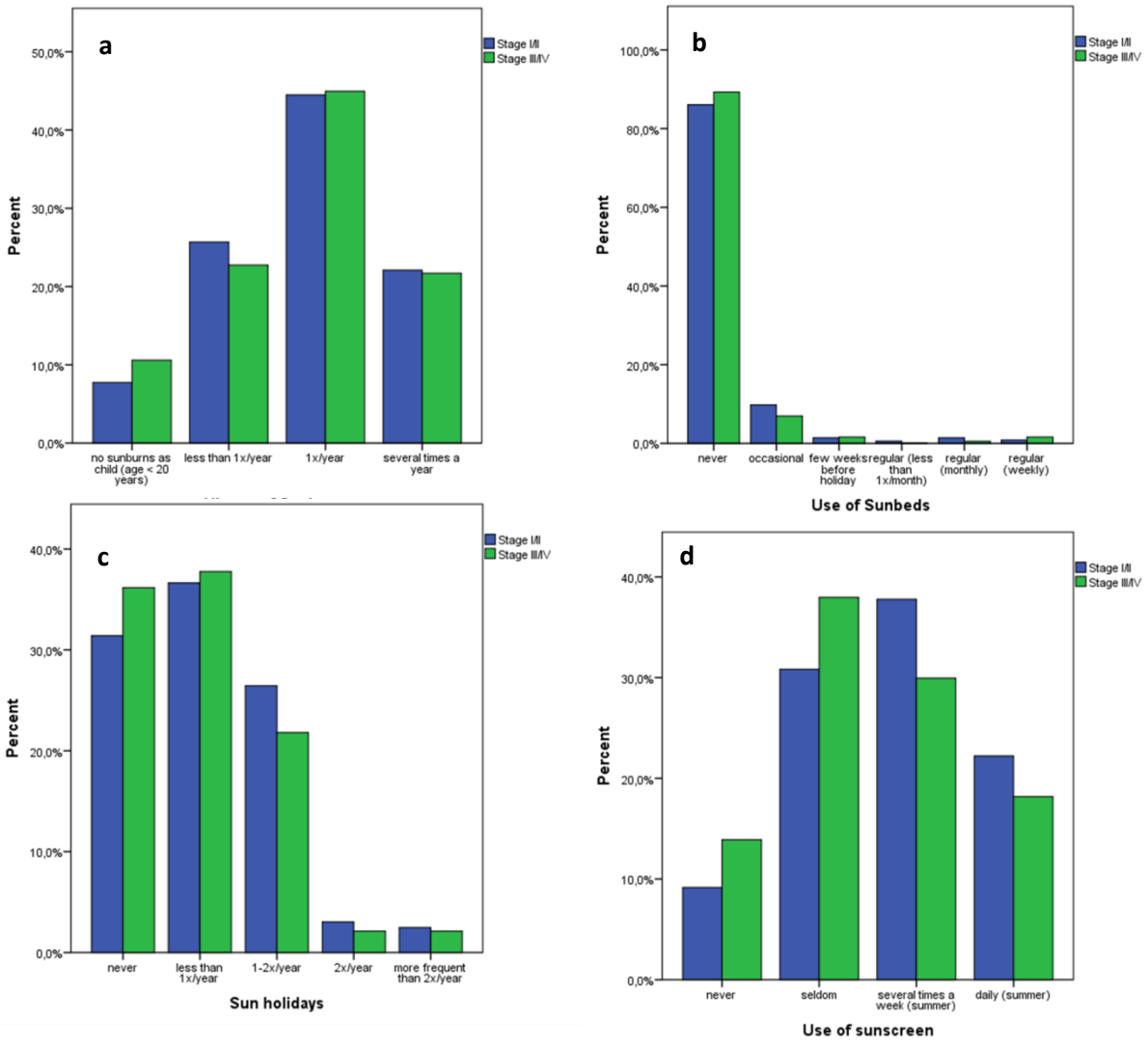
Accordingly, more late stage patients never went on sun holidays with 36.2% compared to 31.4% of the early stage patients (Figure 24c).

The sunscreen use was lower among the late stage melanoma patients. The percentage of patients who never used sunscreen was 13.9% compared to 9.2% among the early stage melanoma patients. Most of the early stage patients (37.8%) reported they would use sunscreen several times a week while this was reported by 29.9% of the late stage patients, who most frequently indicated (38.0%) they would seldom use sunscreen. These differences reached a borderline p-value of 0.056 (Figure 24d).

There was no statistical difference between the two groups concerning the occupational sun exposure as well as the leisure sun exposure. Regarding the occupational sun exposure, the proportion of patients working exclusively indoors was higher among the late stage patients with 53.3% compared to 47.6% of the early stage patients. The leisure sun exposure was nearly identical between the two groups (Figure 24 e+f).

Severe UV damage was more frequent in early stage melanoma patients with 40.7% versus 28.7% of late stage melanoma patients with severe UV damage. Late stage melanoma patients were more likely to have no or light UV damage (Figure 24g).

Likewise, a field cancerization on the head was more frequent in early stage patients with 3.2% compared to a frequency of 0.6% in late stage melanoma patients (Figure 24 h). However, these differences were not statistically significant.



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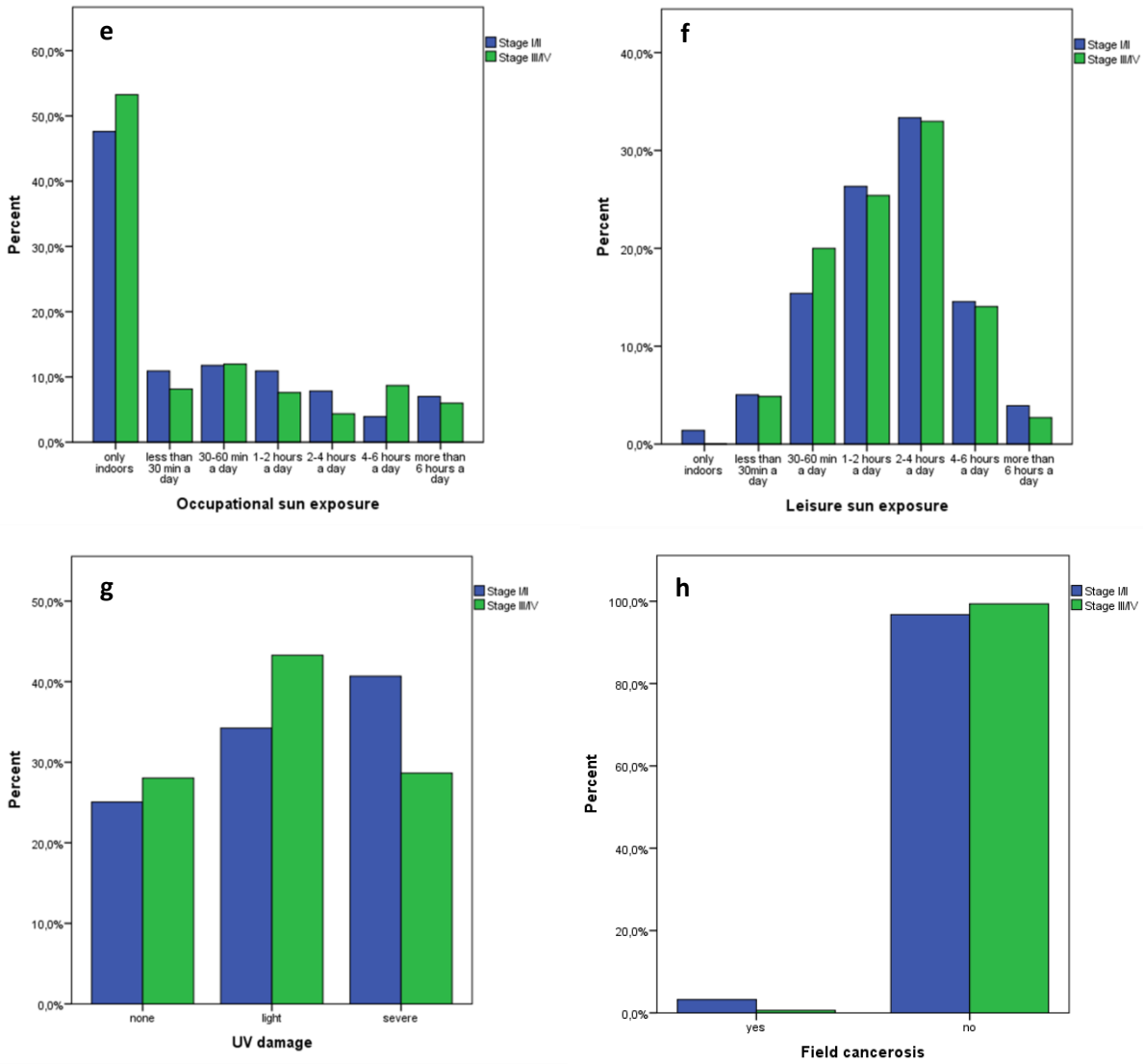


Figure 24: Bar charts displaying data on sun behaviour and UV damage of the cohort; a. History of sunburns; b. Use of sun beds; c. Sun holidays; d. Use of sunscreen; e. Occupational sun exposure; f. Leisure sun exposure; g. UV damage; h. Field cancerisation.

The frequency of genetic germline variants coding for the genes *MC1R*, *PGC-1α* and *PGC-1b* was not significantly different between early stage and late stage melanoma patients. *MC1R* variants were common, 78.5% of the late stage patients and 76.7% of the early stage patients had at least one variant. Further exploration of the type of MC1R variants revealed significant differences between the two groups (Figure 25).

Stage III/IV patients were more likely to have one r-allele and less likely to have two r-alleles compared to Stage I/II patients. Patients with no WT allele were more frequent among Stage I/II patients. The distribution of R-alleles was similar between the two groups. The MC1R genotypes WT/WT, r/r, r/R and R/R were significantly more frequent among Stage I/II, while the genotypes WT/r and WT/R were more frequent among Stage III/IV patients (p=0.019).

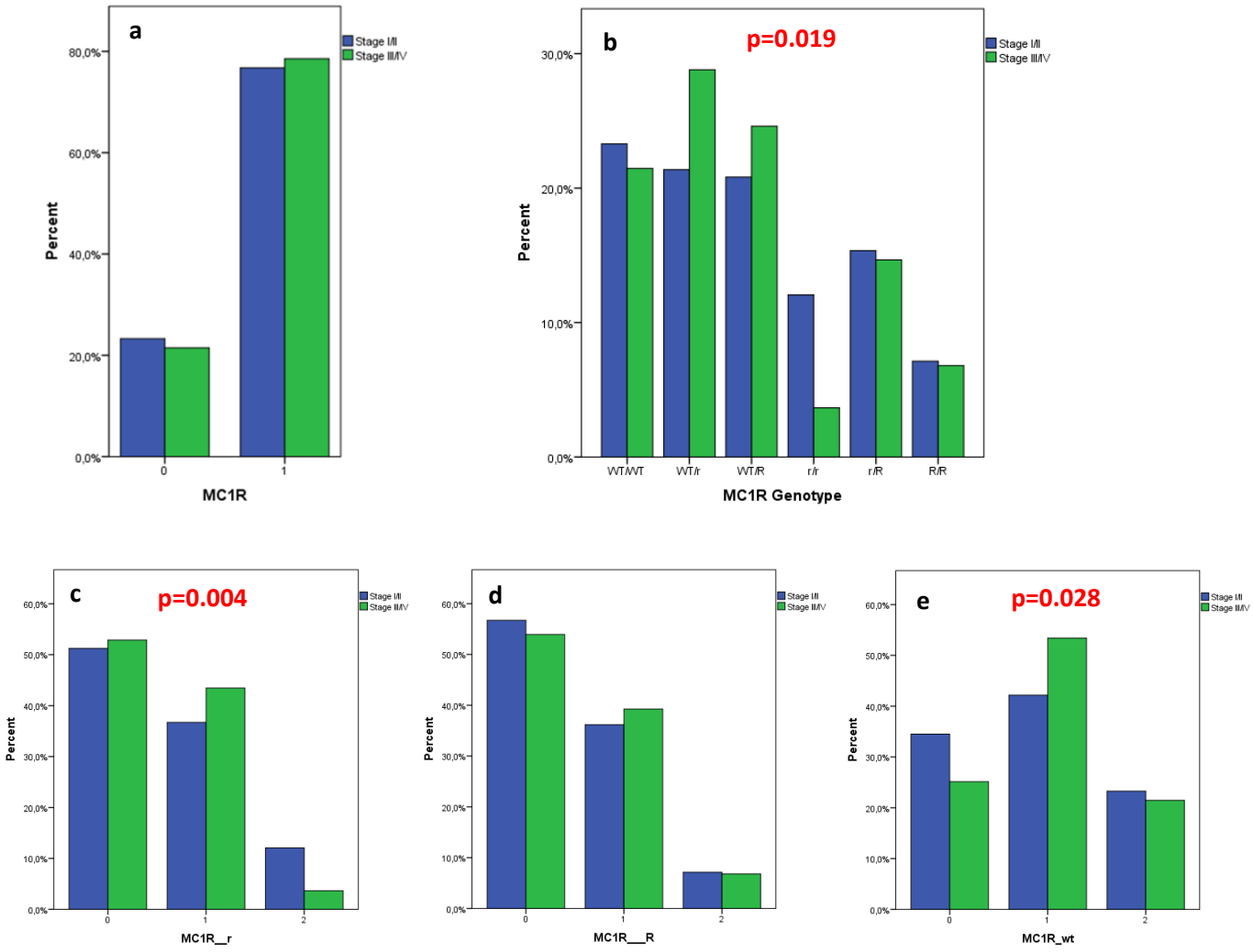


Figure 25: Bar charts displaying a. wildtype versus the presence of at least one variant at the coding region for *MC1R*; b. the distribution of *MC1R* genotypes; c. the presence of *MC1R* r-alleles; c. the presence of *MC1R* R-alleles and e. the presence of *MC1R* WT-alleles in stage I/II versus stage III/IV patients

Variants in the gene coding for PGC-1a were seen in 12.6% of the early stage patients compared to 13.1% of the late stage patients. Variants in the gene coding for PGC-1b were slightly more frequent with 23.0% in early stage and 23.6% in late stage melanoma patients (Figure 26).

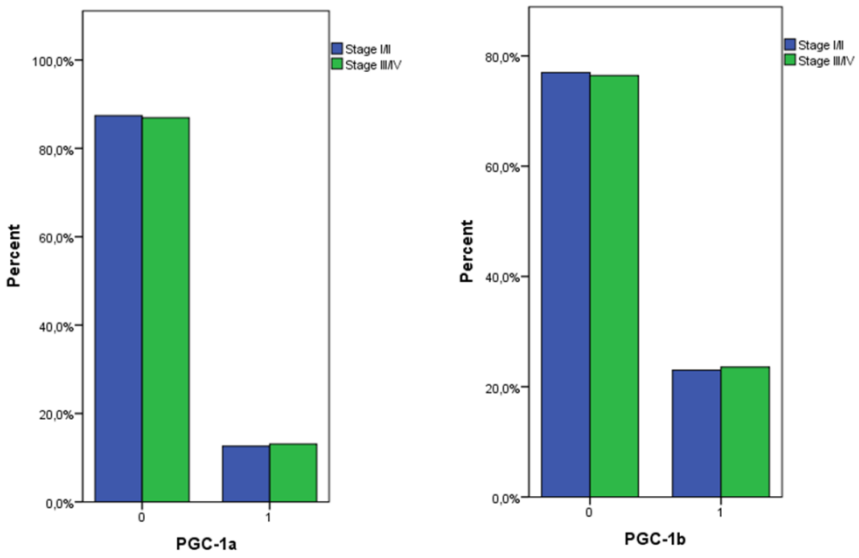


Figure 26: Bar charts displaying genetic data: wildtype versus the presence of at least one variant at the coding regions for a. *PGC-1a* and b. *PGC-1b* in stage I/II versus stage III/IV patients

The following tumour specific data were compared between the two groups: tumour thickness, histology, localisation of the primary tumour, Clark level, ulceration of the primary, signs of regression and the existence of naevus residues in the histological specimen (Table 8).

The median tumour thickness was 2.1mm in the late stage group compared to 1.3mm in the early stage group. This difference was significant with a p-value of <0.001 (Figure 27a). Most patients among the early stage group had a tumour thickness between 1.01 and 2.0mm (43.1%) followed by 29.8% of patients with a tumour thickness below 1.0mm. Interestingly still 30 patients (8.3%) had a tumour thickness of above 4mm without developing any metastases. In parallel 19 patients (12.1%) of the late stage group had initially a low risk tumour with a tumour thickness below 1mm.

The frequent subtype superficial spreading melanoma was more prevalent among the early stage patients with 64.6% compared to 53.5% among the late stage patients (Figure 27b). Likewise, 6.1% of early stage patients had a lentigo maligna melanoma compared to 3.2% of late stage patients. The subtypes nodular melanoma and acral lentiginous melanoma were more frequent among the late stage patients with 25.2% versus 14.7% and 7.7% versus 4.6% respectively (p= 0.016).

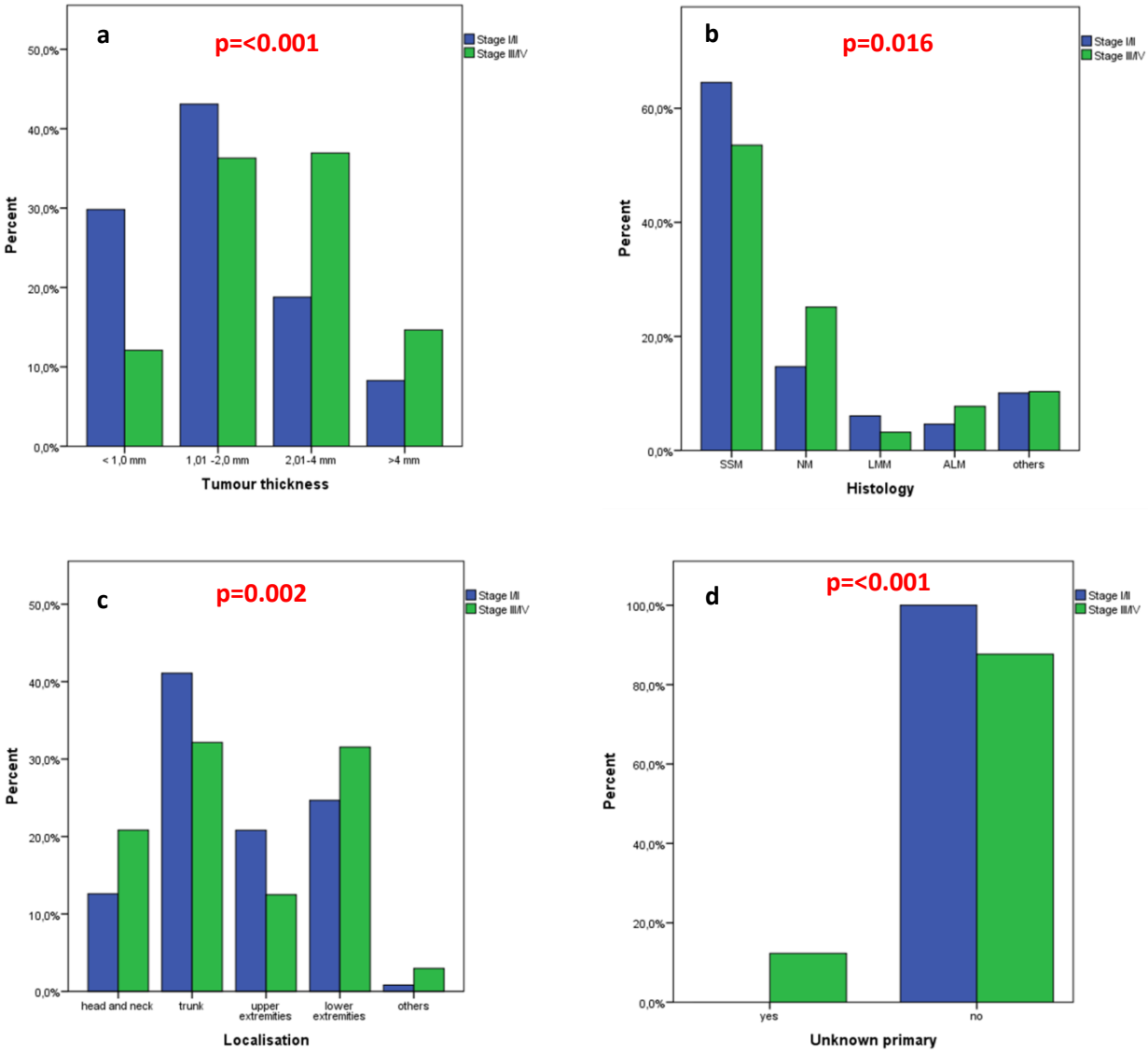
The localisation head and neck and lower extremities were more frequent among late stage patients with 20.8% versus 12.6% and 31.5% versus 24.7% respectively (Figure 27c). The most common localisation among the early stage patients was the trunk with 41.1% compared to 32.1% among late stage patients (p=0.002).

Per definition all cases with unknown primary were included in the late stage group where they contributed with 12.3% of all cases (Figure 27d).

Early stage patients had lower Clark levels compared to late stage patients (Figure 27e). The majority of late stage patients (80.6%) had a Clark level 4 compared to 66.9% of early stage patients (p=<0.001).

Ulcerated primaries were reported in 20.9% of the cases among early stage melanomas compared to 41.5% of the tumours of late stage patients (p=<0.001) (Figure 27f).

Signs of regression were reported more frequently in early stage patients with 15.7% compared to 10.7% in specimens of late stage patients (Figure 27g). This difference was not significant with a p-value of 0.169. An association with a pre-existing naevus was seen in about one fifth in both groups, slightly more in early stage melanoma patients (20.4%) compared to late stage patients (17.3%) (Figure 27h). The difference was not significant (p=0.463).



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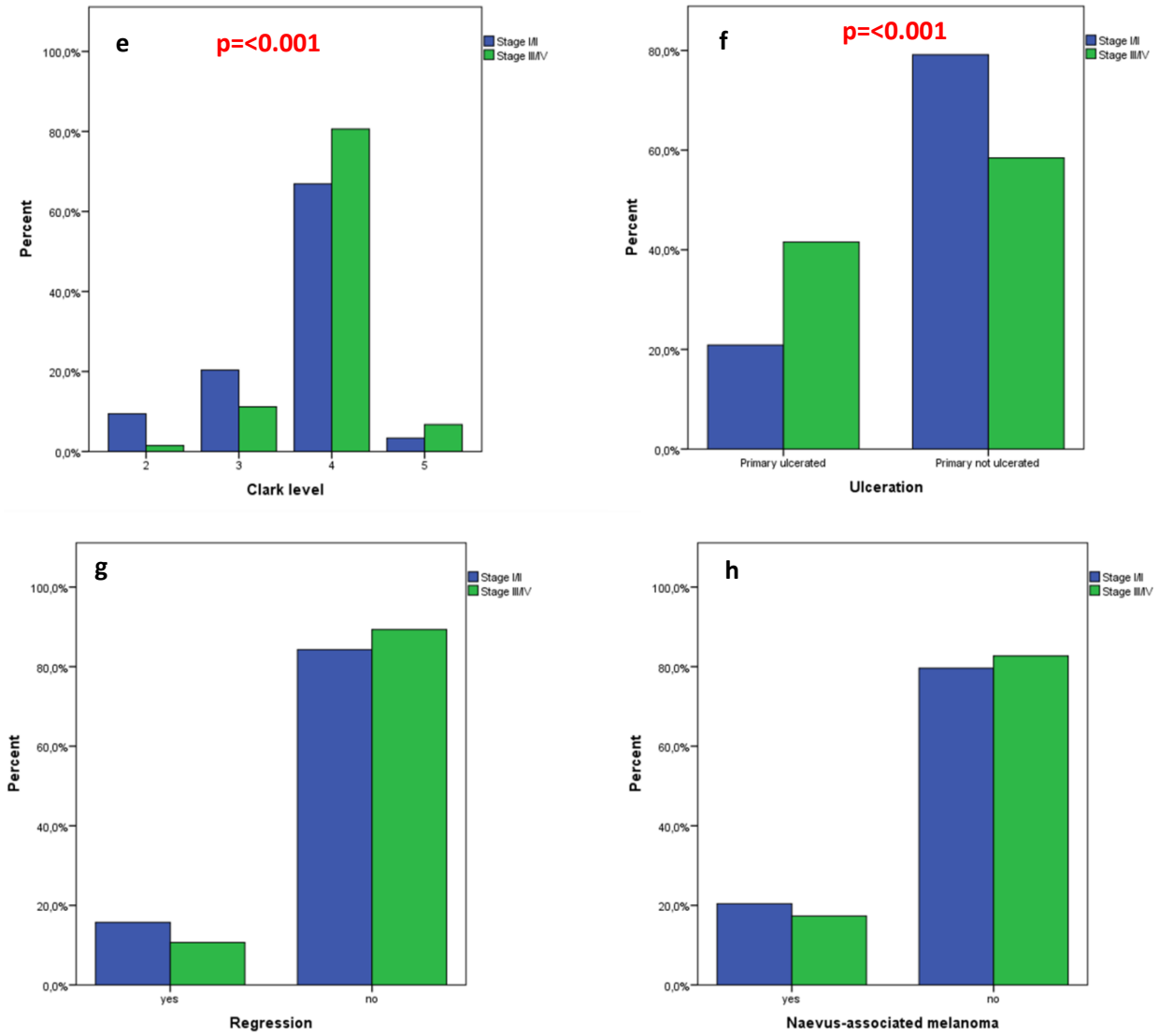


Figure 27: Bar charts displaying the tumour specific data of the cohort; a. Tumour thickness; b. Histology; c. Localisation; d. Unknown primary; e. Clark level; f. Ulceration, g. Regression, h. Naevus associated melanoma

4.3.3. Melanoma Survivors compared with Fatalities

In a next step those patients who survived were compared to those who died of melanoma, independent of the stage of their disease. This was done to focus on those patients who did not only enter a prognostic unfavourable stage but who ultimately died of their disease.

Of all 556 melanoma patients, 64 patients died from melanoma at the end of the follow up time and 492 patients were still alive or died of other causes. All previously assessed variables were analysed between these two groups of patients, the melanoma survivors and those who died.

General data, data pigment traits and the total body naevus count of the patients are summarized in Table 9.

The gender distribution was similar between the two groups. Patients who died were slightly younger with a median age of 54 compared to 60, but this difference was not significant. Patients of both groups had the same median BMI of 26. The percentage of patients with German parents was 93.4% and 92.1% in the two groups.

Blonde-haired patients were more prevalent among survivors than among those who died, whereas patients with brown hair were most common among patients who died. However, this difference was not significant (p= 0.848).

The eye colour was similar between both groups, notably for patients with blue, grey and brown eyes. Patients with green eyes were less frequent among patients who died of melanoma than among those who survived (10.9% versus 17.2%), however this was based on small numbers. Overall the difference of the eye colour between the cohorts was not significant (p=0.605).

A Fitzpatrick skin type II was most frequent among those patients who died with 69.6% compared to 54.8% of patients among the survivors. Among patients who survived a slightly higher proportion had skin type III with 37.1% compared to 25.0% among those who died. Again, this was not significant (p= 0.290)

According to the distribution of the skin type, more patients among the survivors had a higher tanning ability. Fewer patients among those who survived indicated they would always burned or mostly burned and have a light tan after sun exposure compared to those who died in the

course of their disease (17.2% versus 21.0% and 48.8% versus 53.2% respectively). Accordingly, 24.4% of the survivors stated they would seldom burned with a strong tan compared to 17.7% of those who died and 9.6% indicated they would never burned and always tan compared to 8.1%. The differences were not significant (p=0.608).

Severe UV damage was less frequent among melanoma patients who died with 28.1% versus 37.8% of patients with severe UV damage among patients who survived (p=0.286).

In line with this none of the melanoma patients who died had a field cancerisation with the presence of multiple actinic keratosis compared to 11 patients among those who survived (p=0.213).

The distribution of the 5 different naevus count categories was similar between those melanoma patients who survived and those who died. The same was true for the count of dysplastic naevi.

Table 9: Comparison of general and pigment traits between patients who survived and patients who died from melanoma

	All pts		**Alive or dead of other cause**		**Died from melanoma**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	492	100	64	100	
Sex							0.904
female	248	44.6	219	44.5	29	45.3	
male	308	55.4	273	55.5	35	54.7	
Age							0.294
median, range	59, 17-91		60 17 - 91		54 19 - 70		
BMI	26,		26		26		0.705
median, range	17-70		17 - 70		18 - 42		
German parents							0.700
yes	508	93.2	450	93.4	58	92.1	
others	37	6.8	32	6.6	5	7.9	
na	11		10		1		
Hair colour							0.848
red	33	6.0	29	6.0	4	6.6	
blonde	239	43.8	215	44.3	24	39.3	
brown	242	44.3	212	43.7	30	49.2	
black	32	5.9	29	6.0	3	4.9	
na	10		7				
Eye colour							0.605
blue	218	39.5	192	39.3	26	40.6	
grey	110	19.9	95	19.5	15	23.4	
green	91	16.5	84	17.2	7	10.9	
brown	133	24.1	117	24.0	16	25.0	
na	4		4				
Skin type (Fitzpatrick) n, %							0.290
I	31	6.4	28	6.5	3	5.4	
II	274	56.5	235	54.8	39	69.6	
III	173	35.7	159	37.1	14	25.0	
IV	6	1.2	6	1.4	0	0	
V	1	0.2	1	0.2	0	0	
na	71		63		8		
Skin reaction							0.608
always burned, no tan	97	17.6	84	17.2	13	21.0	
mostly burned, light tan	271	49.3	238	48.8	33	53.2	
seldom burned, strong tan	130	23.6	119	24.4	11	17.7	
never burned, always tan	52	9.5	47	9.6	5	8.1	
na	6		4		2		
UV damage							0.286
none	128	26.1	113	26.0	15	26.3	
light	183	37.3	157	36.2	26	45.6	
severe	180	36.7	164	37.8	16	28.1	
na	65		58		7		
Field cancerosis							0.213
yes	11	2.3	11	2.7	0	100	
no	460	97.7	403	97.3	57	0	
na	85		78		7		
Naevus count							0.376
0-10	134	24.2	124	25.3	10	15.6	
11-30	198	35.7	173	35.3	25	39.1	
31-50	105	19.0	94	19.2	11	17.2	
51-100	79	14.3	67	13.7	12	18.8	
>100	38	6.9	32	6.5	6	9.4	
na	2		2		0		
Dysplastic naevus count							0.701
0	385	80.4	342	80.7	43	78.2	
1-5	83	17.3	71	16.7	12	21.8	
6-10	5	1.0	5	1.2	0		
11-20	4	0.8	4	0.9	0		
more than 20	2	0.4	2	0.5	0		
na	77		68		9		

*Pearson Chi-Square Asymp.Sig (2-sided)

Data on the medical history of the patients and the medical family history are presented in Table 10. No significant differences were observed between the groups.

Of the 64 patients who died from melanoma 6.3% had a second cancer and 3.2% had a history of psoriasis. In the group of the 492 survivors there were 6.7% who had a second cancer and 4.0% with a personal history of psoriasis.

A positive family history of melanoma was reported by 3.3% of patients who died from melanoma and by 5.7% of the survivors.

Around half of the patients had a family member with a positive history of any other type of cancer, slightly more among the survivors with 53.8% compared to 45.9% among those patients who died (Table 10).

Table 10: Comparison of the medical history between patients who survived and patients who died from melanoma

	All pts		**Alive or dead of other cause**		**died from melanoma**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	492	100	64	100	
Second cancer							0.890
yes	37	6.7	33	6.7	4	6.3	
no	519	93.3	459	93.3	60	93.8	
na	0		0		0		
History of Psoriasis							0.763
yes	21	3.9	19	4.0	2	3.2	
no	514	96.1	454	96.0	60	96.8	
na	21		19		2		
Family history of MM							0.452
yes	29	5.4	27	5.7	2	3.3	
no	508	94.6	450	94.3	58	96.7	
na	19		15		4		
Family history of Cancer							0.245
yes	283	52.9	255	53.8	28	45.9	
no	252	47.1	219	46.2	33	54.1	
na	21		18		3		

*Pearson Chi-Square Asymp.Sig (2-sided)

Sun behaviour data of melanoma patients who died at the end of the follow up time compared to those who survived are presented in Table 11. There were no significant differences between the two groups.

Around half of the patients among the survivors and among those who died from melanoma indicated they got sunburned once a year, 22.4% and 18.8% respectively got sunburned several times a year, 25.3% and 20.3% less than once a year and 8.6% versus 9.4% never got sunburned. The usage of sunbeds was uncommon among melanoma patients, 87.4% of the survivors and 85.5% of those who died never went to a solarium. About one third of the melanoma patients indicated they would never go on sun holidays, 33.6% of the survivors and 28.6% of those who died of melanoma, 36.9% and 38.1% went less than one time a year, 24.6% and 27.0% between one to two times a year, 2.7% and 3.2% two times a year, and 2.3% versus 3.2% stated they would go more frequently than two times a year on a sun holiday. Regular sunscreen use was slightly less common among those patients who died. Among those patients who died 12.9% used sunscreen on a daily base compared to 21.9% among the survivors, 33.9% versus 35.3% used sunscreen several times a week. The majority of patients who later died of melanoma used seldom or never sunscreen with 45.2% and 8.1% compared to 31.8% and 11.1%. About half of the patients spent their work hours completely indoors, slightly more patients who died with 56.7% compared to 48.6% of those who survived. The categories less than 30 min a day, 30-60 min a day, 1-2 hours a day, 2-4 hours a day, 4-6 hours a day and more than 6 hours a day were evenly distributed among both groups. The same similar distribution was true for the leisure sun exposure. Very few patients, 5 among the survivors and none of those who died spent their leisure time completely indoors, 5.0% versus 4.8% spend less than 30min a day outside, 17.1% and 15.9% were 30-60min a day outside, 26.1% and 25.4% between 1 and 2 hours, 32.6% and 38.1% between 2 and 4 hours, 14.2% versus 15.9% between 4 and 6 hours and 19 patients (4%) of the survivors were more than 6 hours a day outside. This was stated by none of the patients who died.

Table 11: Comparison of the sun behaviour between patients who survived and patients who died from melanoma

	All pts		**Alive or dead of other cause**		**died from melanoma**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	492	100	64	100	
Sunburns (age < 20 ys)							0.634
no sunburns	48	8.7	42	8.6	6	9.4	
less than 1x/year	136	24.7	123	25.3	13	20.3	
1x/year	246	44.6	213	43.7	33	51.6	
several times a year	121	22.0	109	22.4	12	18.8	
na	5		5				
Use of Solarium							0.886
never	476	87.2	423	87.4	53	85.5	
occasional	48	8.8	41	8.5	7	11.3	
few weeks before holiday	8	1.5	7	1.4	1	1.6	
less than 1x/month	2	0.4	2	0.4	0	0	
monthly	6	1.1	6	1.2	0	0	
weekly	6	1.1	5	1.0	1	1.6	
na	10		8		2		
Sun holidays							0.934
never	182	33.0	164	33.6	18	28.6	
less than 1x/year	204	37.0	180	36.9	24	38.1	
1-2x/year	137	24.9	120	24.6	17	27.0	
2x/year	15	2.7	13	2.7	2	3.2	
more frequent than 2x/year	13	2.4	11	2.3	2	3.2	
na	5		4		1		
Use of sunscreen							0.133
never	59	10.8	54	11.1	5	8.1	
seldom	182	33.3	154	31.8	28	45.2	
several times a week	192	35.1	171	35.3	21	33.9	
daily	114	20.8	106	21.9	8	12.9	
na	9		7		2		
Occupational sun exposure							0.434
only indoors	268	49.5	234	48.6	34	56.7	
less than 30 min a day	54	10.0	49	10.2	5	8.3	
30-60 min a day	64	11.8	54	11.2	10	16.7	
1-2 hours a day	53	9.8	50	10.4	3	5.0	
2-4 hours a day	36	6.7	32	6.7	4	6.7	
4-6 hours a day	30	5.5	29	6.0	1	1.7	
more than 6 hours a day	36	6.7	33	6.9	3	5.0	
na	15		11		4		
Leisure sun exposure							0.698
only indoors	5	0.9	5	1.0	0	0	
less than 30 min a day	27	5.0	24	5.0	3	4.8	
30-60 min a day	92	17.0	82	17.1	10	15.9	
1-2 hours a day	141	26.0	125	26.1	16	25.4	
2-4 hours a day	180	33.2	156	32.6	24	38.1	
4-6 hours a day	78	14.4	68	14.2	10	15.9	
more than 6 hours a day	19	3.5	19	4.0	0	0	
na	14		13		1		

*Pearson Chi-Square Asymp.Sig (2-sided)

Genetic data of those patients who died compared to those who survived are presented in Table 12. There was no significant difference between the two groups. Slightly more of the fatalities had a wild type at the *MC1R* locus with 28.1% compared to 22.0% but this difference was not significant. Further exploration of *MC1R* variants, considering r alleles, R alleles and the different MC1R genotypes revealed no significant differences as well.

Among the patients who died from melanoma 85.9% had no variant at the coding gene for PGC-1a and 79.7% had no variant at the coding gene for PGC-1b, compared to 87.4% and 76.4% respectively.

Table 12: Comparison of genetic variants between patients who survived and patients who died from melanoma

	All pts		**Alive or dead of other cause**		**died from melanoma**		**p**
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	492	100	64	100	
MC1R							0.267
wt	126	22.7	108	22.0	18	28.1	
variant	430	77.3	384	78.0	46	71.9	
na							
MC1R r alleles							0.087
0	288	51.8	248	50.4	40	62.5	
1	217	39.0	195	39.6	22	34.4	
2	51	9.2	49	10.0	2	3.1	
MC1R R alleles							0.829
0	310	55.8	276	56.1	34	53.1	
1	207	37.2	181	36.8	26	40.6	
2	39	7.0	35	7.1	4	6.3	
MC1R WT alleles							0.197
0	174	31.3	160	32.5	14	21.9	
1	256	46.0	224	45.5	32	50.0	
2	126	22.7	108	22.0	18	28.1	
***MC1R* Genotype**							0.355
WT/WT	126	22.7	108	22.0	18	28.1	
WT/r	133	23.9	119	24.2	14	21.9	
WT/R	123	22.1	105	21.3	18	28.1	
r/r	51	9.2	49	10.0	2	3.1	
r/R	84	15.1	76	15.4	8	12.5	
R/R	39	7.0	35	7.1	4	6.3	
PGC-1a							0.742
wt	485	87.2	430	87.4	55	85.9	
variant	71	12.8	62	12.6	9	14.1	
na							
PGC-1b							0.561
wt	427	76.8	376	76.4	51	79.7	
variant	129	23.2	116	23.6	13	20.3	
na							

*Pearson Chi-Square Asymp.Sig (2-sided)

**deeper analyses with data of the *MC1R* locus were performed, considering the numbers of r, R or WT alleles respectively, and the specific *MC1R* genotype

Tumour specific data of the melanoma patients are summarized in Table 9 for both groups. A significant difference between the two groups was observed for the variables ”history of an unknown primary” and “ulceration of the primary tumour” (Table 13).

Patients who died from melanoma had a median tumour thickness of 1.9mm compared to 1.5mm among the survivors. The proportion of thick melanomas above 4mm and of melanomas between 2.01 and 4mm tumour thickness was higher in comparison to the group of melanoma survivors with 16.4% versus 9.5% and 32.7% versus to 23.3%. Tumours with a thickness between 1.01mm and 2.0mm were most frequent among the survivors with 42.0% compared to 32.7% among patients who died of their melanoma. Still 18.2% (10 patients) among those who died had an initially favourable tumour thickness below 1mm.

The distribution of melanoma subtypes was similar between both groups. Superficial spreading melanoma was most frequent with 61.1% among the survivors and 61.5% among those who died. There were slightly more patients with nodular melanomas among those who died with 23.1% versus 17.3% among the survivors, the same was true for acral lentiginous melanomas with 7.7% versus 5.3%. Only one patient (1.9%) of those who died had a lentigo maligna melanoma compared to 5.6% of the survivors.

The localisation of the primary tumour did not differ significantly between the two groups. The trunk was the most common localisation with 41.4% among those who died and 37.9% among the survivors, followed by the lower extremities with 27.6% and 26.7%, head and neck with 15.5% and 15.2% and the upper extremities with 12.1% versus 18.9%.

A primary tumour was not detectable in 9.4% of those patients who ultimately died of their disease while only 3.6% of those who survived were diagnosed with an unknown primary. This difference was significant with a p-value of 0.030.

Patients who died had higher Clark levels. Clark level 4 was reported for 84.4% in this group compared to 69.4% among the survivors. Primary tumours of the survivors had more frequently a Clark level 3 or Clark level 2 with 18.7% versus 8.9% and 7.7% versus 2.2% compared to patients from the fatality group.

An ulcerated primary was found in nearly half of those patients who died (48.1%) compared to only 24.7% of patients who survived. This difference was significant with a p-value of 0.001.

Signs of regression in the histological specimen were more often observed among the survivors, with 14.8% compared to 9.1% among patients who died.

Remains of a pre-existing naevus were found in a similar proportion in both groups with 19.6% among survivors and 18.2% among the group with fatalities.

Table 13: Comparison of tumour specific data between patients who survived and patients who died from melanoma

	All pts		**Alive or dead of other cause**		**died from melanoma**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	556	100	492	100	64	100	
Tumour thickness							
median	1.5mm		1.5mm		1.9mm		0.106
<1,0mm	127	24.5	117	25.2	10	18.2	0.461
1,01-2,0mm	213	41.0	195	42.0	18	32.7	
2,01-4mm	126	24.3	108	23.3	18	32.7	
>4mm	53	10.2	44	9.5	9	16.4	
na	37		28		9		
Histology							0.461
SSM	307	61.2	275	61.1	32	61.5	
NM	90	17.9	78	17.3	12	23.1	
LMM	26	5.2	25	5.6	1	1.9	
ALM	28	5.6	24	5.3	4	7.7	
others	51	10.2	48	10.7	3	5.8	
na	54		42		12		
Localisation							0.530
head and neck	81	15.2	72	15.2	9	15.5	
trunk	204	38.3	180	37.9	24	41.4	
upper extremities	97	18.2	90	18.9	7	12.1	
lower extremities	143	26.8	127	26.7	16	27.6	
others	8	1.5	6	1.3	2	3.4	
na	23		17		6		
Unknown primary							**0.030**
yes	23	4.2	17	3.6	6	9.4	
no	519	95.8	461	96.4	58	90.6	
na	14		14		0		
Clark level							0.159
2	33	7.1	32	7.7	1	2.2	
3	82	17.7	78	18.7	4	8.9	
4	328	70.8	290	69.4	38	84.4	
5	20	4.3	18	4.3	2	4.4	
na	93		74		19		
Ulceration							**0.001**
yes	122	27.5	97	24.7	25	48.1	
no	322	72.5	295	75.3	27	51.9	
na	112		100		12		
Regression							0.307
yes	61	14.2	57	14.8	4	9.1	
no	369	85.8	329	85.2	40	90.9	
na	126		106		20		
Naevus-associated MM							0.819
yes	82	19.5	74	19.6	8	18.2	
no	339	80.5	303	80.4	36	81.8	
na	135		115		20		

*Pearson Chi-Square Asymp.Sig (2-sided)

4.3.4. Survival Analyses

Survival analyses were performed in order to examine the impact of phenotypes and genotypes on the survival probabilities in a time dependent manner.

4.3.4.1. Survival Analyses with previously assessed Variables

The survival probabilities of the 556 melanoma patients were tested for each of the above assessed variables. Significant results in the log rank test, pooled over strata were found for two of the variables. These were the tumour specific variables “Ulceration of the primary” and “Unknown primary”. Patients with an ulcerated primary tumour had an impaired survival probability (p=0.001) (Figure 30c) as well as patients with unknown primary at the time of initial diagnoses (p=0.008) (Figure 30d).

Using the log rank test with pairwise comparison, additionally six variables showed significant results. This was the variable tumour thickness, with an impaired survival of patients with a tumour thickness >4mm compared to those with melanomas between 1.01 and 2.0mm (p=0.040) (Figure 30a).

Among the general and pigment traits, patients with a Skin type II had an impaired survival probability compared to patients with Skin type III (p=0.032). The difference in survival probabilities remained significant after dichotomization of the variable in Skin type I and II versus Skin type III, IV and V (p=0.031).

Among the variables regarding sun behaviour, patients had better survival probabilities who used sunscreen daily in summer compared to those who seldom used sunscreen (p=0.030) and who had a leisure sun exposure of 4-6 hours compared to 2-4 hours (p=0.050). However, dichotomizing of these two variables resulted in not significant differences, indicating the previously detected differences were driven by small subset numbers.

In regard to the MC1R genotypes patients with a r/r genotype had a better survival compared to WT/R types (P=0.040) and patients with two MC1R r-alleles had a better survival than patients with no MC1R r-alleles (p=0.049) (Figure 31b).

The remaining log rank test results (pooled over strata) of the Kaplan Meier survival analyses were as follows:

The assessed variables of general and pigment traits were gender, with no significant differences between males and females (p=0.884), age (p=0.089), BMI (p=0.498), German ancestry (p=0.630), Hair colour (p=0.908), Eye colour (p=0.616), Skin type (p=0.244), Skin reaction (p=0.600), UV damage (p=0.407), Field cancerosis (p=0.262), Naevus count (p=0.439) and Dysplastic naevus count (p=0.675).

The variables concerning medical history were the personal history of a second cancer (p=0.841), the personal history of psoriasis (p=0.649), the family history of melanoma (p=0.566) and the family history of cancer (p=0.275), all without impact on melanoma specific survival.

The sun behaviour data were the frequency of sunburns at the age below 20 years (p=0.469), the use of sunbeds (p=0.904), the frequency of sun holidays (p=0.862), the use of sunscreen (p=0.114), the occupational sun exposure (p=0.528) and the leisure sun exposure (p=0.775). All variables showed no significant results in the pooled over strata test.

Survival analyses with the genetic variables presence of any variant compared to wildtype of *MC1R* (p=0.333), *PPARGC1A* (p=0.825) and *PPARGC1B* (p=0.579) showed no survival differences. The number of *MC1R* r alleles, *MC1R* R alleles or *MC1R* WT alleles and the *MC1R* Genotype were likewise without significant difference in the log rank pooled over strata test.

4.3.4.2. Genome-Wide Survival Analyses

In order to screen for genetic markers which might influence survival among the cohort of melanoma patients, all available genetic variants across the 23 chromosomes of each individual melanoma patient were assessed in a genome-wide analyses. The ten variants with the most significant results were further investigated. All variants were single nucleotide polymorphisms (SNPs). Four of these SNPs were located in chromosome 1, three in chromosome 10, two in chromosome 4 and one in chromosome 8. All of these 10 SNPs yielded a p-value ≤ 1e-5 and one SNP reached genome wide significance with a p-value of 1e-10.[204,205] The results of the genome-wide association analysis (GWAS) were illustrated in a Manhattan plot (Figure 28).

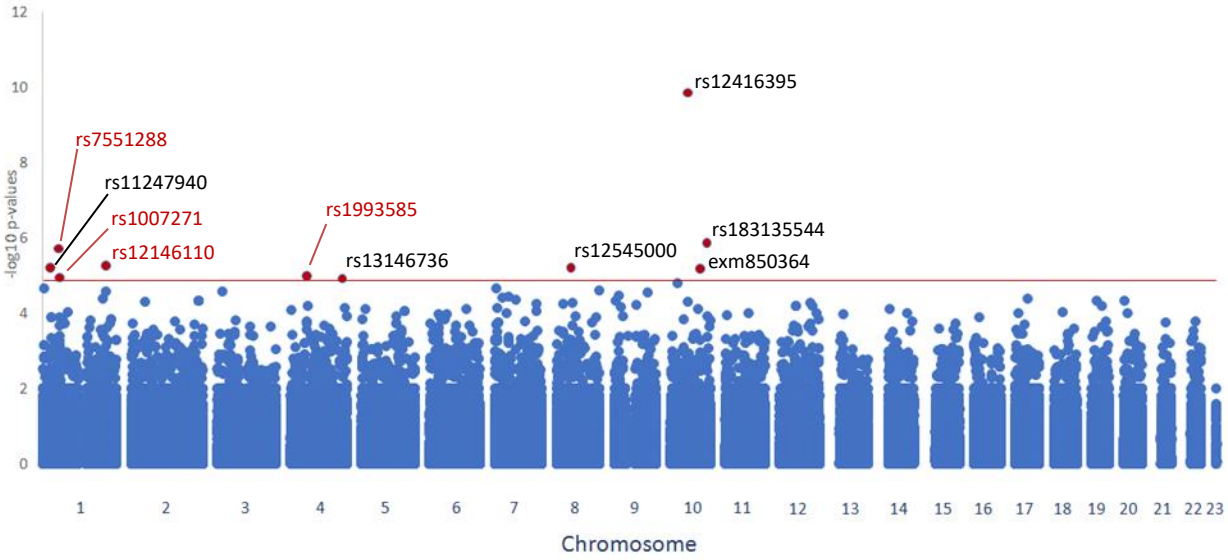


Figure 28: Manhattan Plot illustrating the genome-wide association analyses across all 23 chromosomes for melanoma-specific survival in 556 melanoma patients. The top ten SNPs are presented in red dots with the respective rs numbers (black: most likely artefactual finding, red: potential relevance)

The top 10 SNPs were further explored in regard to their relation to known gene loci, their reported minor allele frequency in dbSNP and the observed frequency of homozygous and heterozygous individuals in the cohort, as described below.

The SNP rs12416395 is located on chromosome 10 and had a reported minor allele of A=0.206. Among the 556 melanoma patients, 77 were heterozygous (A/C) and one patient who died of melanoma was homozygous (A/A). The significant result was driven by this single event. There was no survival difference between wildtype and heterozygous patients at this locus (log rank test p=0.179). The SNP rs183135544, likewise located on chromosome 10, with a reported minor allele frequency of A=0.003, was found among 11 patients. One patient was homozygous (A/A) and died of melanoma and 10 patients were heterozygous (A/G). Again, the significant result was driven by a single case. The SNP **rs7551288** is an intron variant of the gene *DHCR24* located on chromosome 1 with a reported MAF of G=0.386. Homozygous patients (n=96) had the worst survival outcome, wildtype patients (n=176) the best outcome, heterozygous patients (n=284) were in between. The SNP **rs12146110** is again located on chromosome 1 and is an intron variant of the gene *USH2A*. The minor allele frequency was A=0.212, 17 patients were homozygous and 171 patients heterozygous. Homozygous patients had an impaired survival and all homozygous patients who entered Stage IV died of their disease. The

SNP rs12545000 is located on chromosome 8, the minor allele frequency was C=0.126. The significant result was driven by a single case with one homozygous patient who died. The SNP rs11247940 is located on chromosome 1 and the minor allele frequency was C=0.255. Again, the significant result was driven by a single case. The variant exm850364 is a rare variant located on chromosome 10. Only one patient was heterozygous and this patient died. SNP **rs1993585** is located on chromosome 4 with a minor allele frequency of A=0.226. One homozygous patient died and the 95 heterozygous patients had a significant impaired survival compared to the wildtype patients. SNP **rs1007271** is located on chromosome 1 and had a reported minor allele frequency of A=0.472. Heterozygous patients (n=251) had the same survival probabilities than wildtype patients (n=248), however homozygous patients (n=57) had a significant impaired survival (17 events). SNP rs13146736 is located on chromosome 4, the minor allele frequency was T=0.382. Heterozygous patients had an impaired survival compared to wildtype patients, however the homozygous patients had a not coherent improved survival probability, yet driven only by two events. The result was not significant in stage IV.

In total 4 SNPs, rs7551288, rs12146110, rs1993585 and rs1007271 were selected as potentially relevant candidates.

4.3.4.3. Survival Plots of Selected Variables

Specific phenotype and genotype variables were selected to illustrate the respective survival probabilities using a Kaplan Meier plot. The first variable was the phenotype variable “Total body naevus count” as core variable of the second hypothesis of this thesis. Furthermore, variables which were significant or were close to significance in the primary association analyses stage I/II versus stage III/IV were selected. These were the variables Tumour thickness, Histology, Localisation, Unknown primary, Clark level, Ulceration, Hair colour, Use of sunscreen and UV damage. Additionally, the variables Gender and BMI were chosen, as they have been described previously in the literature having a prognostic impact on melanoma survival. Finally, the survival curves with significant results in the pairwise comparisons, skin type and MC1R r-allele and the curves for the SNPs with potential relevance derived from the genome-wide association analyses were selected and presented in Figure 31.

The survival curves for the selected host specific variables are presented in Figure 29, the tumour specific variables are shown in Figure 30.

The survival probabilities for patients with a total body naevus count below 30 naevi compared to patients with a naevus count above 30 naevi are presented in Figure 29a.

The curves overlap with some slight difference 4 years after initial diagnoses, favouring patients with few naevi. Using the log rank test this difference was not statistically significant (p=0.501). Hair colour had no impact on the survival probabilities as shown in Figure 29b. Patients with regular use of sunscreen had a slightly better survival, however this was not statistically significant (p=0.128) (Figure 29c). The same was true for patients with severe sun damage compared to those with light and with no sun damage (p=0.407) (Figure 29d). All patients with the presence of a field cancerisation on the head survived or died of other causes, while all those patients who died from melanoma had no field cancerisation. The survival plot is not presented as all cases were censored and no statistical test could be performed. Female and male patients had the same survival probabilities with overlapping survival curves as shown in Figure 29e (p=0.884). No survival differences were seen for obese versus not obese patients with a BMI threshold of 30 (Figure 29f).

The survival probabilities based on tumour specific factors are presented in Figure 30. The survival curves break up as expected for patients with melanomas of the different tumour thickness groups <1mm, 1.01-2mm, 2.01-4mm, >4mm. The survival difference was not statistically significant in the pooled over strata test (p=0.110) but was significant in the pairwise comparison (p= 0.040) (Figure 30a). The related factor of the histologically diagnosed depth of penetration, the Clark level showed as well no significance in the overall comparison (p=0.273). An ulceration of the primary tumour was associated with a significant decrease of the survival probability as shown in Figure 30c (p=0.001). Patients who were diagnosed initially with metastases of an unknown primary had impaired survival probabilities compared to those with a known primary (Figure 30d, p=0.008). The survival curves of patients stratified for the melanoma subtype did split up as expected but reached no statistical significance (Figure 30e, p=0.576). The different body localisations of the primary tumour showed no impact on survival probabilities as illustrated in Figure 30f (p=0.604).

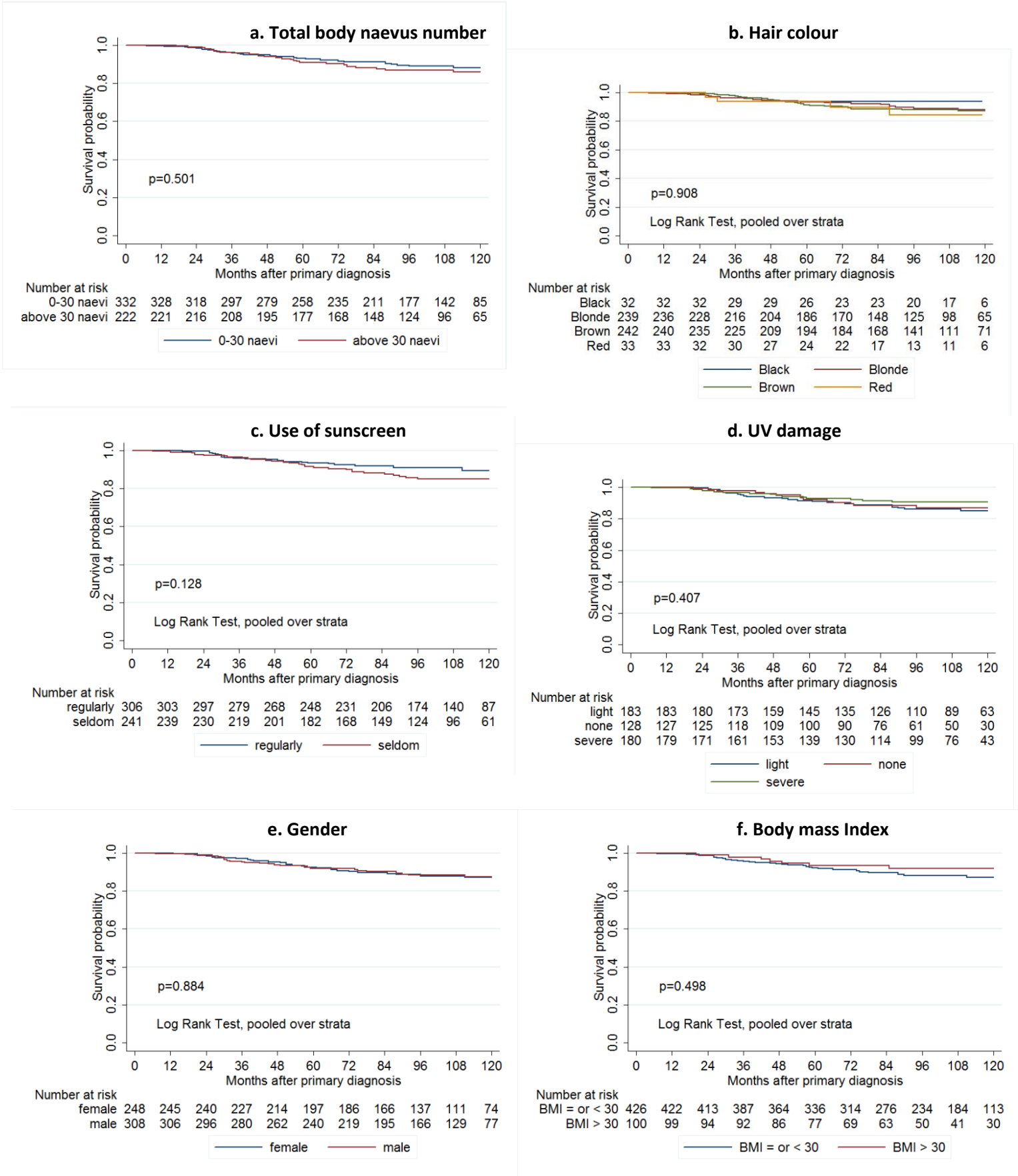


Figure 29: Survival probabilities for patients stratified for host specific variables, a. Total body naevus count, b. Hair colour, c. Use of sunscreen, d. UV damage, e. Gender and f. Body mass index.

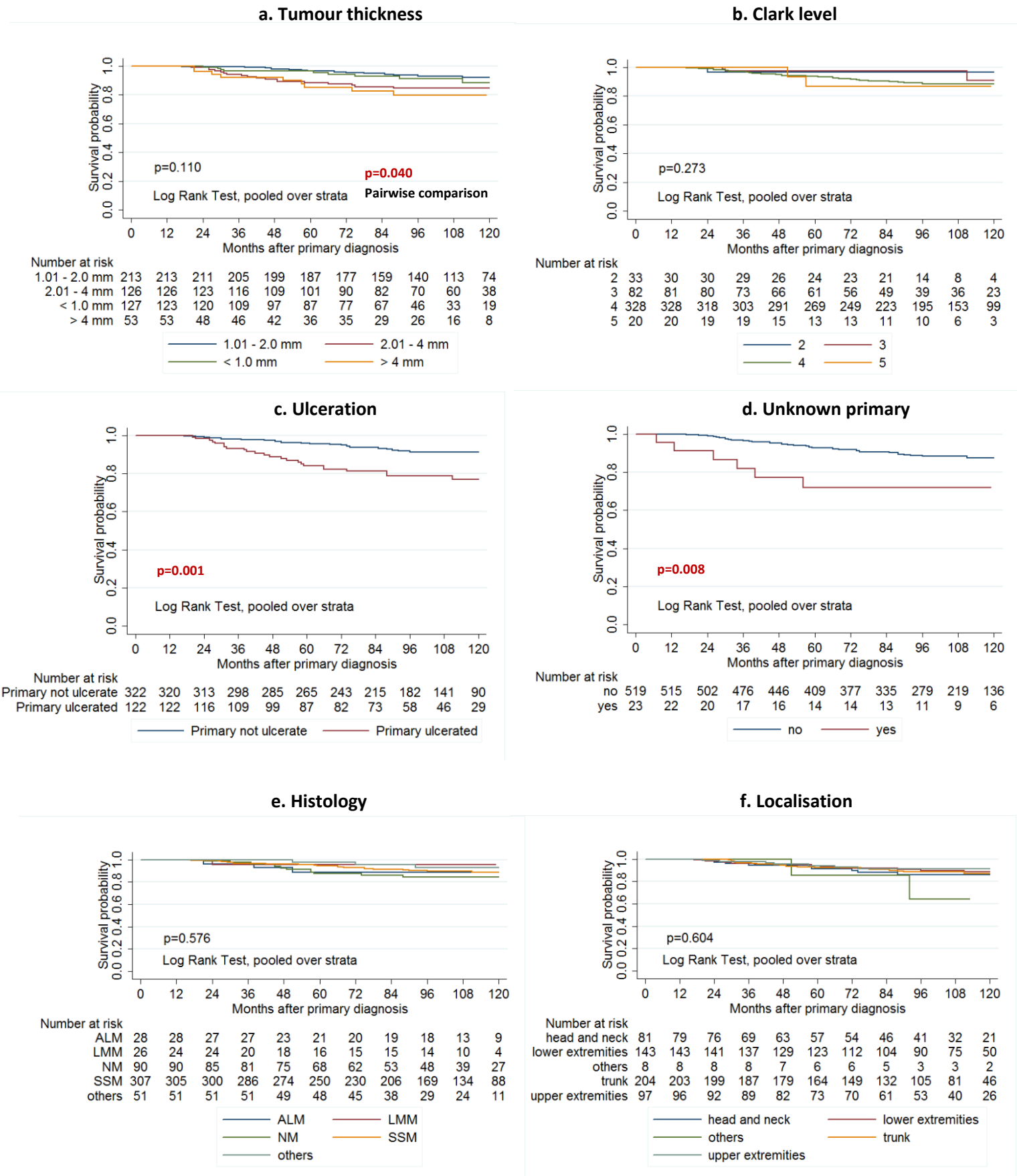


Figure 30: Survival probabilities for patients stratified for tumour specific variables: a. Tumour thickness, b. Clark level, c. Ulceration, d. Unknown primary, e. Histology and f. Localisation.

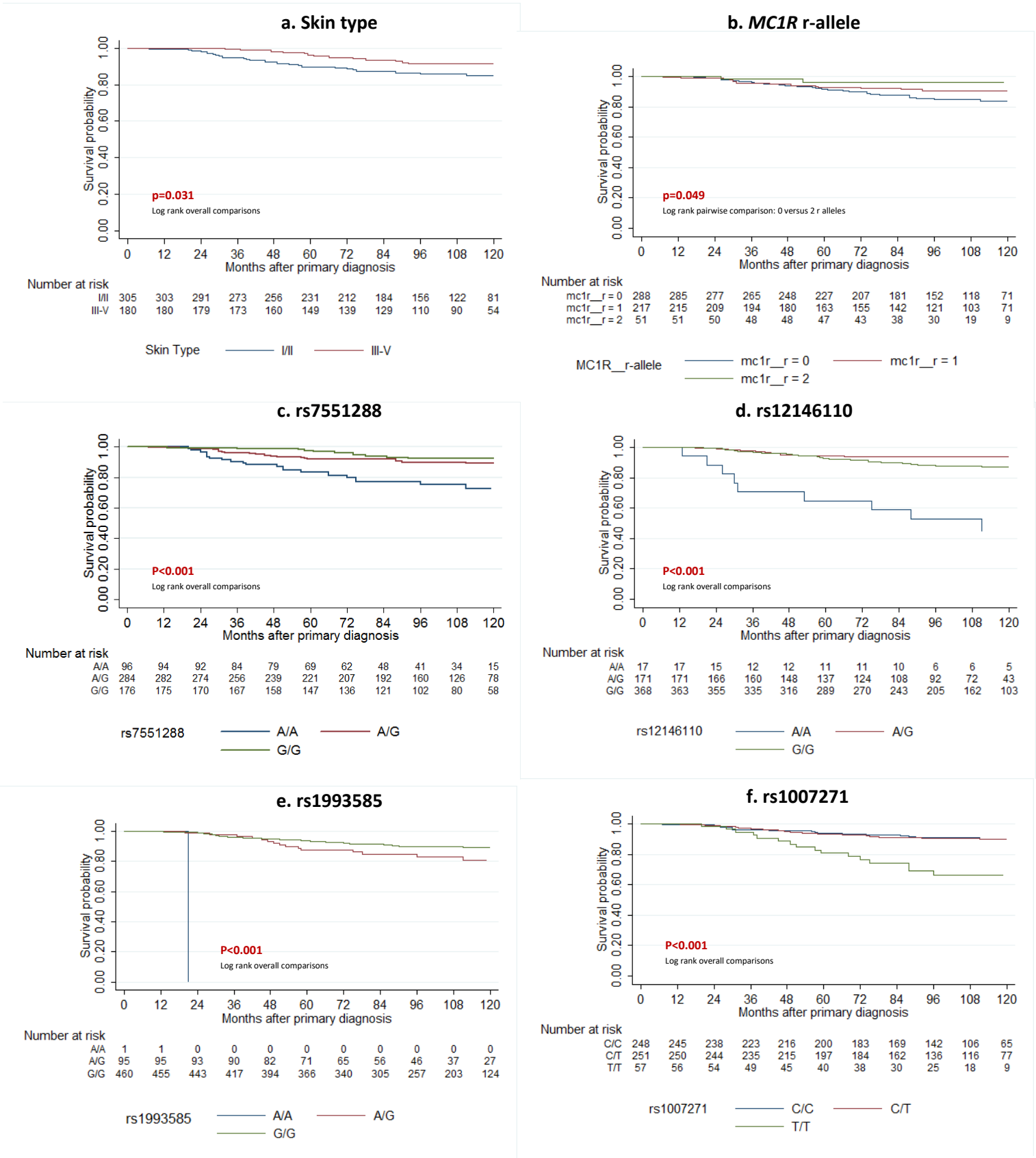


Figure 31: Survival probabilities for patients stratified for a. skin type, b. the presence of a *MC1R* r-allele and for the four SNPs of potential relevance: c. rs7551288, d. rs12146110, e. rs1993585 and f. rs1007271

The Kaplan Meier curves of subsequent analyses, including the pairwise log rank test, the deeper MC1R analyses and of the SNPs derived from the genome-wide association analyses are presented in Figure 31. The survival probabilities of patients according to their skin type yielded a significant result in the pairwise comparison which remained significant after dichotomization of the variable. Patients with a lighter skin type I or II had a worse outcome compared to darker pigmented patients with skin type III or IV (p=0.031) (Figure 31a). The deeper analyses of the MC1R variants, distinguishing between R-alleles and r-alleles among the patients, revealed a significant increasing survival benefit for patients with one or two r-allele(s) (p=0.049) (Figure 31b).

The survival curves presented for the four SNPs rs7551288 (Figure 31c), rs12146110 (Figure 31d), rs1993585 (Figure 31e) and rs1007271 (Figure 31f) revealed diminished survival probabilities of patients homozygous for the respective minor allele variant for all four SNPs. A split up of the survival curves between wildtype patients and heterozygous patients was additionally apparent for the first three SNPs rs7551288, rs12146110 and rs1993585, indicating an additive effect. However, the latter one included only one homozygous patient. The fourth SNP rs1007271 showed only an impact on survival for patients with two minor alleles. The two most interesting SNPs rs7551288 and rs12146110 had both additionally an impact on survival among patients in stage IV (Figure 32).

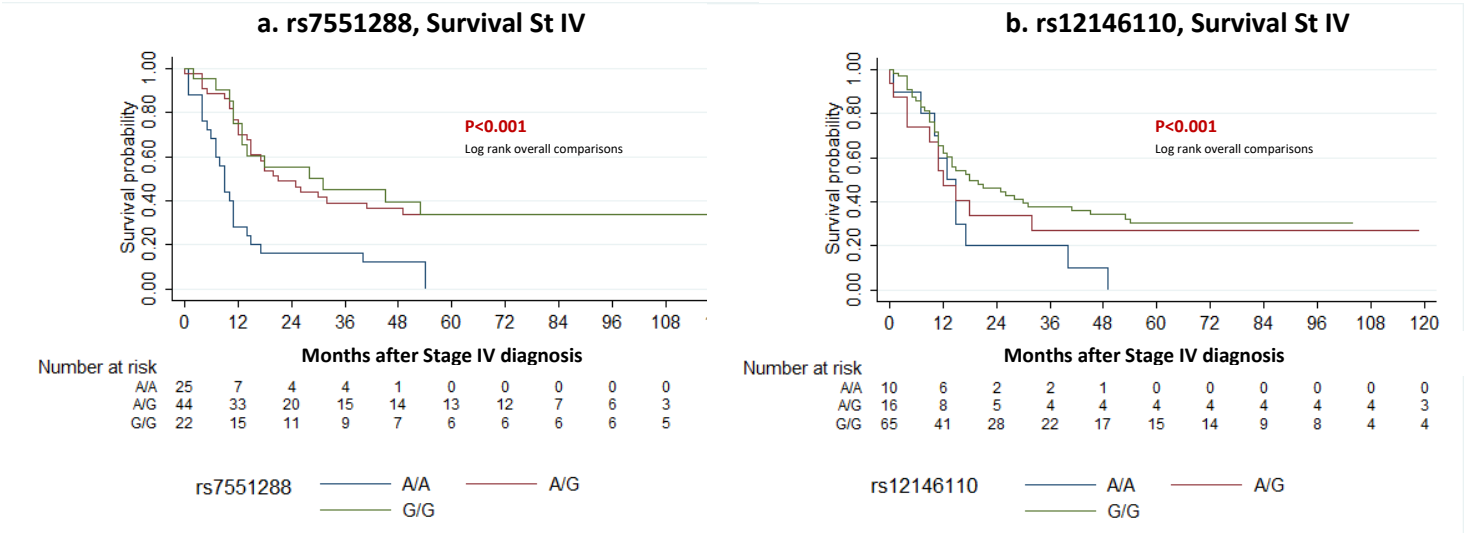


Figure 32: Survival probabilities in Stage IV for patients with the different genotypes at the two best SNPs a. rs7551288 and b. rs12146110

4.4. Discussion

The presented analyses compared the characteristics of early and late stage melanoma patients with an initial focus on phenotypic characteristics and sun behaviour data. Established melanoma specific factors were investigated as well. In a second step SNP array derived genetic markers were additionally analysed in a genome-wide approach. The cohort consisted of 556 melanoma patients who were recruited during their regular appointments in the melanoma outpatient clinic at the University Hospital Tübingen.

The initial hypothesis was that late stage melanoma patients, those patients who ultimately die of their disease would have less naevi than early stage melanoma patients. This was based on a personal observation. This hypothesis was not confirmed in the present cohort. The naevus count between patients who were classified as stage I/II patients at last follow up compared to patients who were classified as stage III/IV patients was similar. The same was true for patients who died of melanoma compared to those who survived. The survival curves of patients with low versus high naevus count were not significantly different. This result is in contradiction to the reported survival benefit for melanoma patients with high naevus numbers by Ribero et al. in 2015.[59] However, the validity of this result was questioned by Autier et al. in respect to statistical concerns and a possible overdiagnosis of screen-detected indolent melanomas.[60] The authors cited a work published by Levell et al. which was investigating a diagnostic drift towards a classification of benign lesions as early melanomas.[206] This trend was explained by subjective criteria in the histological diagnoses in early lesions and the increasing cautiousness of medical practitioners due to the legal consequences of a wrong diagnosis. The assumption that the previously reported survival benefit for patients with a high naevus count was driven by a dilution of patients with actual benign lesions might be supported by the presented data. The cohort from Tübingen comprised relatively few patients with early melanomas as discussed later.

Overall, the characteristics between early and late stage patients in the presented cohort were remarkably consistent. This was seen for personal and family medical history data as well as for general, pigment traits and the initially assessed genetic markers of the patients. Hair colour was the only trait which was significantly different between early and late melanoma patients. Blonde patients were more frequent among stage I/II patients while brown haired patients formed the largest sub-group among stage III/IV patients. However, this difference was

not significant between those patients who died from melanoma compared to those who survived and likewise not significant in the Kaplan Meier survival analyses. Moreover, there was even a trend for an impaired outcome for patients with blond/red hair compared to those with brown/black hair. This finding went with the impaired survival of patients with skin type II compared to patients with darker skin type III, which was significant in the pairwise log rank test. Contradicting results were previously reported by Davies et al. showing a more favourable outcome for patients with blond hair compared to brown/black hair and for patients having a *MC1R* variant.[202] While the favourable outcome of fair pigmented individuals was not confirmed within the present cohort, an increasing benefit for patients having one or two *MC1R* r-allele(s) was shown. Likewise, patients having a MC1R WT/WT genotype were more frequent among the fatalities with 28% compared to 22% among those who survived, which was in line with the paper published by Davies et al. It is interesting to observe here an exclusive survival benefit for patients with the r allele. This benefit is suggestive to be independent of pigmentation traits considering the impaired outcome of light skinned patients in the present cohort. The small r variants do not as much influence pigmentation traits compared to the R variants. The high penetrance R alleles have shown to have a combined odds ratio for red hair of 63.3 (95% CI 31.9-139.6) compared to the significantly lower odds ratios for r alleles of 5.1 (95% CI 2.5-11.3).[148,207] Possible explanations for the observed survival differences might be a protective biologic effect of the r allele in metastatic disease, e.g. via an improved immune response. Another cause might be the promotion of less aggressive melanomas in patients having a MC1R r allele.

The sun behaviour data was not significantly different between early and late stage patients and between survivors and fatalities. However, a trend for less chronic UV damage was observed among the fatalities. None of the patients who died had a field cancerisation, a condition caused by cumulative sun exposure, compared to 2.7% among those who survived. Severe UV damage was likewise more frequent among those who survived with 38% compared to 28% among those who died. A potential benefit of UV exposure in melanoma patients derived by high Vitamin D levels has been suggested before[208], supporting the favourable outcome of patients with UV damage in the present cohort. However, the results still have to be interpreted with caution as potential confounders of the observed associations have not been examined.

Tumour specific markers were investigated in analogy to the other variables. As expected, the established prognostic factors such as tumour thickness of the primary, histology, localisation, the presence of an unknown primary, Clark level and ulceration showed a highly significant different distribution between stage I/II and stage III/IV patients. However, after comparing the patients who ultimately died of their disease with those who survived only two factors remained statistically significant. These factors were ulceration and the presence of an unknown primary. This is surprising and requires a closer look on the investigated cohort. Low risk patients with a tumour thickness below 1mm were usually not seen at the University Hospital and relatively few of these patients were therefore included in the present cohort study. However, patients with additional risk factors or those who progressed in spite of their thin tumours were followed up in the hospital. This resulted in an unexpected high number of patients with stage III/IV disease (n=19) and fatalities (n=10) among the 127 patients with a tumour thickness below 1mm. On the other side more patients than expected survived among those with a tumour thickness above 4mm. A possible explanation is the fact that patients with rapidly progressive disease were less likely to be followed up in the outpatient clinic, where the patient recruitment for this cohort study was done, but were rather seen as inpatients. Thus, the present cohort represented a cohort of patients excluding a share of indolent melanomas and a part of those with rapidly progressive disease. This specific cohort composition did not facilitate the detection of the established prognostic markers between the groups.

As a last step the influence of survival probabilities by genetic germline variants in the patients was explored using more than 300 000 SNPs in a genome-wide approach. Of the 10 most significant SNPs a set of four SNPs were selected as potentially relevant prognostic variants based on allele frequency and consistency of the minor allele effects. The SNPs rs7551288, rs12146110 and rs1007271 are located on chromosome 1, rs1993585 on chromosome 4. Two SNPs are intron variants of known genes, rs7551288 lies in the gene *DHCR24* and rs12146110 belongs to the gene *USH2A*. These two SNPs did both have not only an impact on overall survival in the complete cohort but also among the patients who developed metastatic disease. The USH2A protein is associated with the Usher syndrome, a disease characterized by impaired hearing and visual loss due to retinitis pigmentosa.[209] A link to melanoma or cancer has not been described so far. The *DHCR24* gene is coding for the 3beta-hydroxysterol delta24-reductase (DHCR24), an enzyme involved in cholesterol synthesis.[210] Expression levels of

DHCR24 were found to be upregulated in higher grade urothelial carcinoma[211] and in melanoma metastases.[212] The progression promoting effect of the protein DHCR24 was found to be associated with protection against oxidative stress-induced apoptosis.[212] The observed intronic variant rs7551288 might regulate the expression of the protein and thereby influence survival and maybe also response to systemic treatments. The variant is common with a minor allele frequency of 39%. Heterozygous patients had an impaired survival, a worse outcome was observed in patients with two minor alleles and all homozygous patients who entered stage IV died of melanoma.

4.5. Conclusions

In conclusion the hypothesis that patients with high naevus number are more frequent among early stage melanoma patients and survivors was not confirmed in this cohort of patients from Germany.

The absence of an ulcerated primary tumour and the presence of an MC1R r allele were associated with an improved survival.

A genome-wide association study revealed four potentially relevant variants predicting survival outcome in melanoma patients. The intronic variant rs7551288 is located in the gene *DHCR24*. DHCR24 is a protein shown to protect against oxidative stress-induced apoptosis underpinning the potential relevance for melanoma progression and prognosis.

5. Comparison of an Australian and German Melanoma Cohort

5.1. Introduction

Melanoma is common among fair skinned populations with the highest reported incidence rates in Australia and New Zealand, both countries on the southern hemisphere with intense daily UV radiation and with inhabitants of European origin.[23] European populations have lower incidence and mortality rates compared to Australia and New Zealand.[3]

Two independent cohorts of melanoma patients of two regions, one from Europe and one from Australia were selected for comparative analyses. Both cohorts consisted of patients with similar Western European ancestry and similar socioeconomic background. Both cohorts were drawn from populations with a similar lifestyle in a western culture but with one major difference: the intensity of the environmental UV radiation. Brisbane is a city located in a sub-tropical region, at southern latitude 27° with an average between 6 and 9 hours sun a day around the year. In contrast Tübingen is located in a tempered climate zone at northern latitude 48° and has an average of 2 hours sun in the winter months and only a period of 2 months with 8 hours sun a day.[213]

The aim of the following analyses was to compare the general characteristics, sun behaviour data and tumour specific variables of the two cohorts with different environmental sun exposure levels.

5.2. Methods

The methods related to the establishment of the two patient cohorts, such as patient recruitment and data management are described in chapter 2.

In order to perform comparative analyses between the Australian and the German cohort of melanoma patients, a shared SPSS file was created. The complete set of melanoma patients from each cohort was selected to be merged into one file.

The following variables were chosen for comparisons:

General characteristics

- Sex
- Age
- BMI
- Ancestry
- Hair colour
- Eye colour
- Skin reaction
- Total body naevus count
- Family history of melanoma

Sun behaviour

- Sunburns
- Use of sunscreen

Genetic data

- MC1R
- PGC-1a
- PGC-1b

Tumour specific data

- Tumour thickness
- Multiple melanomas*
- Histology
- Localisation
- Clark level
- Ulceration
- Regression
- Naevus-associated melanoma

*in case of multiple melanomas, the tumour specific data of the thickest primary melanoma were recorded

Variables which had not the same values where adapted to enable combined analyses as described below.

Hair colour: The variable of the Brisbane cohort had 5 values: red, fair, light brown, dark brown and black. In Tübingen the values were red, blonde, brown and black. The values light and dark brown were combined to brown for the matched variable with the 4 values red, blonde, brown and black.

Eye colour: The variable of the Tübingen cohort had 4 values and distinguished between blue, grey, green and brown. In Brisbane the values were blue/grey, green/hazel and brown. The matched variable had 3 values blue/grey, green/hazel and brown.

Skin reaction: The variable of the Tübingen cohort had 4 values: 1. Always burned, no tan, 2. Mostly burned, light tan, 3. Seldom burned, strong tan and 4. Never burned, always tan. The two values “mostly burned, light tan” and “seldom burned, strong tan” were combined to one value “burn then tan” to match the 3 values of the Brisbane cohort: always burn and never tan, burn then tan, only tan.

Total body naevus count: The variable of the Brisbane cohort was a metric variable with the absolute naevus number, counting naevi from a size of 5mm. In Tübingen naevi were reported from a size of 3mm. The variable of the Tübingen cohort was categorized in 5 groups: 0-10, 11-30, 31-50, 51-100 and more than 100. The same categories were used to group the variable naevus count for patients of the Brisbane cohort.

Family history melanoma: The variables of the Brisbane cohort had 3 values and distinguished between first and second-degree relatives. The variable in Tübingen did only refer to second degree relatives. The matched variable had only 2 values: First degree relatives with melanoma and no first-degree relatives with melanoma.

Sunscreen usage: The variable of the Tübingen cohort had 4 values: never, seldom, several times a week and daily. Variable 3 and 4 were merged to the value more than 50% of the time. The matched variable had the 3 values of the Brisbane cohort: never, less than 50% and more than 50% of the time with sunscreen usage.

Sunburns: The patients of the Tübingen cohort were asked how many sunburns they had per year, while the patients in Brisbane were asked for painful sunburns in their lifetime. The

variable was therefore not directly transferable and only the information if the patient ever experienced sunburns was kept. The matched variable had two values: no, the patient never got sunburned, yes, the patient got sunburned before.

SPSS was used to calculate the frequencies and percentages of the selected variables. The missing variables were indicated with na. The given percentages were calculated without missing cases. The median was calculated for the metric variables, age, BMI and tumour thickness.

Comparisons between the two cohorts were made with crosstabs for the ordinal and nominal variables. A two-sided Pearson Chi-Square test was used to test if the null hypothesis could be rejected.

The normally distributed metric variables BMI and tumour thickness were compared between the cohorts using the parametric t-test of equality of means.

The bimodal distributed metric variable age was compared with the nonparametric Mann-Whitney U test. The significance level to reject the null hypothesis was 0.05 for all statistical tests.

5.3. Results

Comparison of general baseline data and pigmentation traits

A total of 1010 melanoma patients were analysed. The general and pigment characteristics are presented in Table 14. Male patients were more frequent in both cohorts with 246 male patients of 454 patients in Brisbane and 308 male patients of 556 patients in Tübingen. The median age of all patients was 58 with a range from 14 to 91 years. The median age was balanced between the two cohorts with 57.5 and 59 years in Brisbane and Tübingen respectively. The body mass index (BMI) was higher in Brisbane with 27.45 versus 26.25 in Tübingen. This difference was significant with a p-value of 0.003. The vast majority of patients were of European ancestry. Only 6 patients (1.4%) from Brisbane and only 2 patients (0.4%) from Tübingen had another ancestry. The majority of patients from Brisbane were of British/Irish origin while most patients from Tübingen had German parents (data not shown). Brown hair colour was the most frequent hair phenotype in both cohorts with 59.5% and 44.3% of patients from Brisbane and from Tübingen respectively. Patients with blonde hair were about as frequent as

patients with brown hair in Tübingen with 43.8%, whereas only 20.4% of patients had blonde hair in Brisbane. However, the percentage of patients with red hair was more than doubled in the Brisbane cohort with 16.4% compared to 6% in Tübingen (Figure 33a). Patients with green/hazel eyes were more frequent in Brisbane with 27.5% compared to 16.5% of patients with green eyes in Tübingen. The percentage of patients with blue/grey eyes was similar between the cohorts with 58.4% and 59.4% in Brisbane and Tübingen respectively. Less patients had brown eyes in Brisbane (14.1%) compared to Tübingen (24.1%). In terms of skin reaction few patients of both cohorts indicated their skin would only tan after sun exposure. About half of the patients from Brisbane (49.0%) stated their skin reaction as “burn then tan” and 43.2% of the patients as “always burn, never tan”. This most sun sensitive category “always burn, never tan” was only chosen by 17.6% of melanoma patients from Tübingen to describe their skin reaction, while the majority of the patients from Tübingen (72.9%) indicated they would “burn then tan” (Figure 33c). The distribution of the 5 different total body naevus count categories was similar between the patients from Brisbane and the patients from Tübingen. The category “between 11 and 30 naevi” was most common with 37.5% of patients from Brisbane and 35.7% of patients from Tübingen. However, the comparison of this variable has a limitation as only naevi from 5mm were counted in Brisbane while all naevi from 3mm size were counted in Tübingen. A positive family history of melanoma was reported by 37.3% of patients from Brisbane while only 5.4% of patients from Tübingen had a first degree relative with a malignant melanoma (Figure 33d).

Table 14: Comparison of general traits between melanoma patients from Brisbane and Tübingen

	All pts		Brisbane		Tübingen		p*
	n	%	n	%	n	%	
Total, n	1010	100	454	100	556	100	
Sex							0.701
female	456	45.1	208	45.8	248	44.6	
male	554	54.9	246	54.2	308	55.4	
Age							
median, range	58 14 - 91		57.5 14 - 88		59 17 - 91		0.061
BMI							**0.003**
median, range	26.66 16.90 – 69.92		27.45 16.90 – 56.77		26.25 17.16 – 69.92		
European ancestry							0.072
yes	949	99.2	411	98.6	538	99.6	
others	8	0.8	6	1.4	2	0.4	
na	53		37		16		
Hair colour							**<0.001**
red	107	10.7	74	16.4	33	6.0	
blonde	331	33.2	92	20.4	239	43.8	
brown	511	51.2	269	59.5	242	44.3	
black	49	4.9	17	3.8	32	5.9	
na	12		2		10		
Eye colour							**<0.001**
blue/grey	593	58.9	265	58.4	328	59.4	
green/hazel	216	21.5	125	27.5	91	16.5	
brown	197	19.6	64	14.1	133	24.1	
na	4				4		
Skin reaction							**<0.001**
always burn, never tan	291	29.1	194	43.2	97	17.6	
burn then tan	621	62.2	220	49.0	401	72.9	
only tan	87	8.7	35	7.8	52	9.5	
na	11		5		6		
Naevus count**							0.948
0-10	244	24.2	110	24.3	134	24.2	
11-30	368	36.5	170	37.5	198	35.7	
31-50	184	18.3	79	17.4	105	19.0	
51-100	145	14.4	66	14.6	79	14.3	
>100	66	6.6	28	6.2	38	6.9	
na	3		1		2		
Family history of melanoma							**<0.001**
yes(1st degree relatives)	197	19.9	168	37.3	29	5.4	
no	791	80.1	283	62.7	508	94.6	
na	22		3		19		

*Pearson Chi-Square Asymp.Sig (2-sided)

**Naevi were recorded from 5mm size in Brisbane and from 3mm size in Tübingen

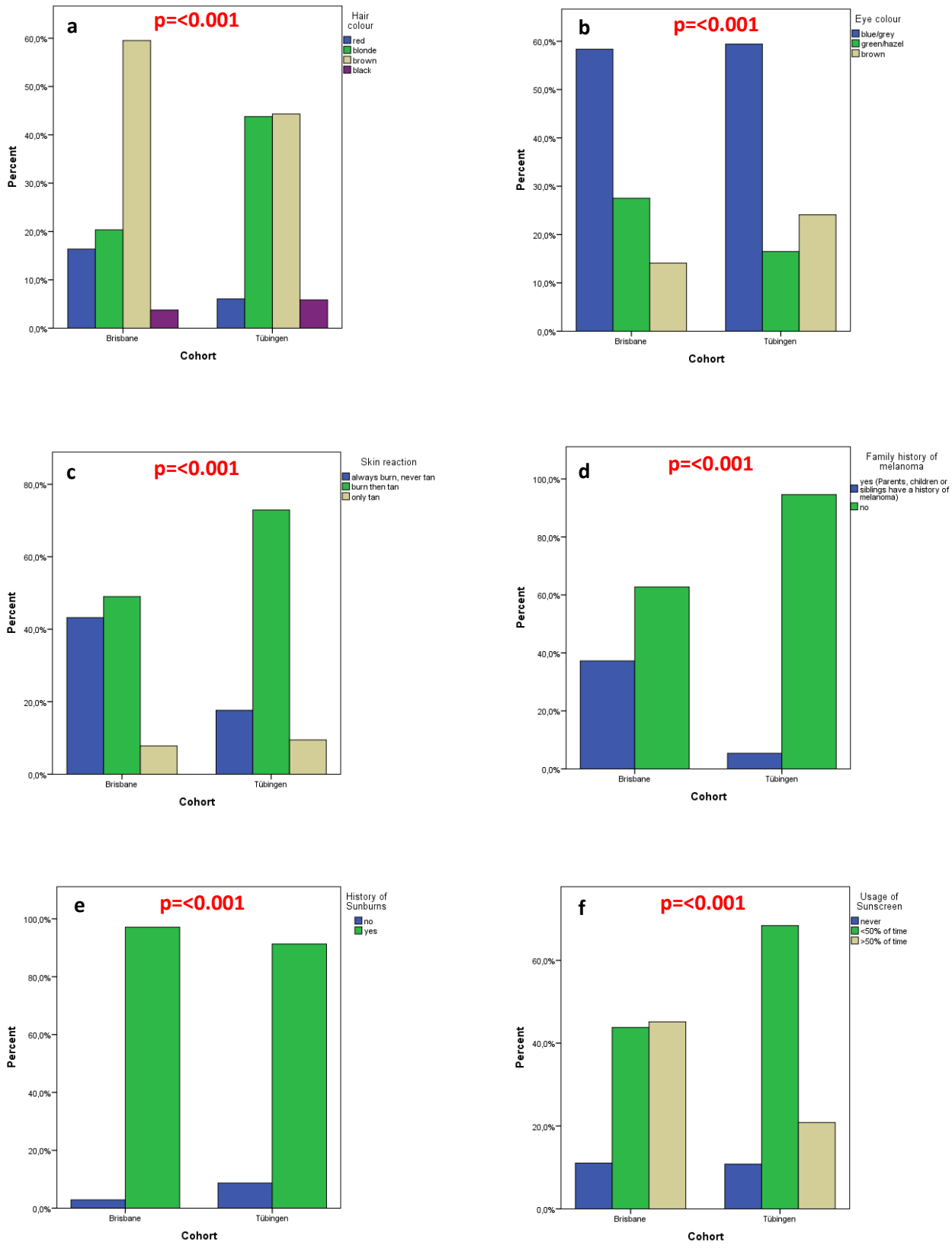


Figure 33: Bar charts illustrating the different frequencies of a. hair colour, b. eye colour, c. skin reaction after sun exposure, d. positive family history of melanoma, e. history of sunburns and f. use of sunscreen in melanoma patients from Brisbane compared to Tübingen.

Comparison of sun behaviour data

The history of sunburns and data concerning the use of sunscreen was available for both cohorts of melanoma patients from Brisbane and Tübingen. The data is presented in Table 15. The majority of patients from both cohorts indicated they had experienced sunburns in their life. Those who never got sunburned were significantly more frequent among the melanoma patients from Tübingen, with 8.7% compared to 2.9% of the patients from Brisbane (Figure 33e). Around 10% of all patients never used sunscreen. Most of the patients from Australia used sunscreen more than 50% of their time (45.1%) while the majority of the patients from Tübingen were applying sunscreen in less than 50% of their time (68.4%) (Figure 33f).

Table 15: Comparison of sun behaviour between melanoma patients from Brisbane and Tübingen

	All pts		**Brisbane**		**Tübingen**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	1010	100	454	100	556	100	
History of sunburns							**<0.001**
no	61	6.1	13	2.9	48	8.7	
yes	939	93.9	436	97.1	503	91.3	
na	10		5		5		
Usage of sunscreen							**<0.001**
never	108	10.9	49	11.1	59	10.8	
<50% of time	568	57.4	194	43.8	374	68.4	
>50% of time	314	31.7	200	45.1	114	20.8	
na	20		11		9		

*Pearson Chi-Square Asymp.Sig (2-sided)

Comparison of genetic data

A principal component analysis was performed to investigate the population structure of the two cohorts.[214] The plot illustrates the ethnical background of the study participants, with PC2 corresponding to North-South and PC1 to East-West in Europe. The plot revealed a partly overlapping but distinct population structure between the melanoma patients from Brisbane and the patients from Tübingen (Figure 34).

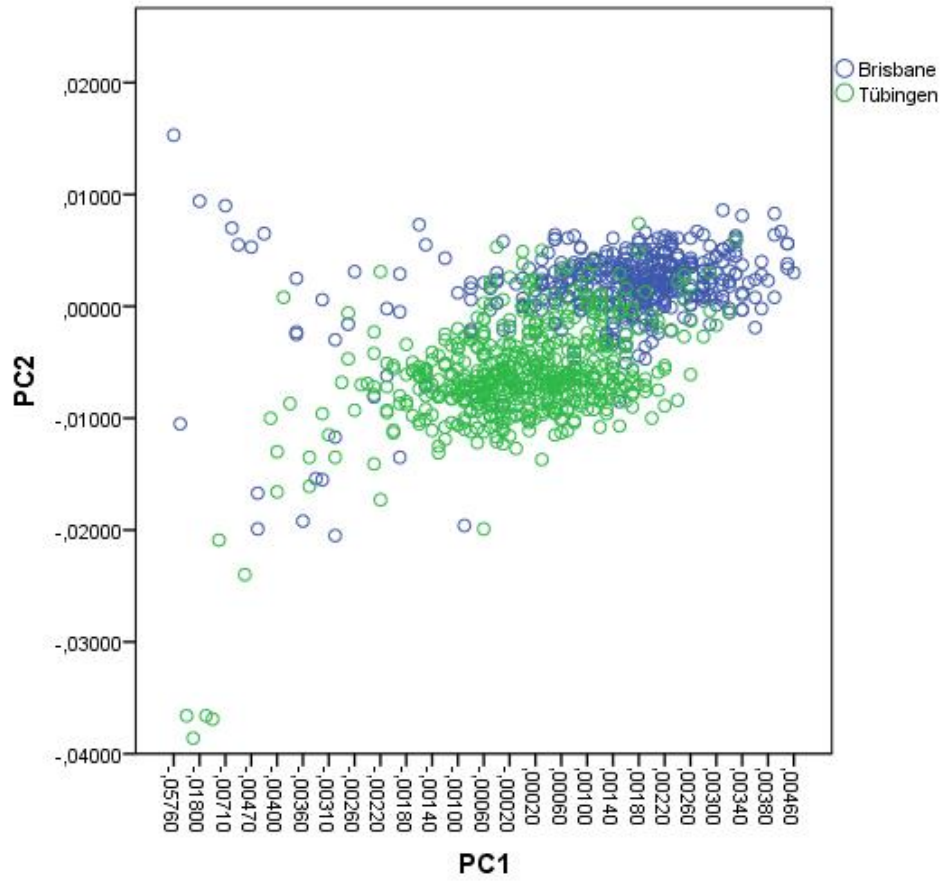


Figure 34: Principal component analysis of the melanoma patients from Brisbane (blue) and Tübingen (green). The variables PC1 and PC2 were calculated using the FlashPCA2 package in R. 29 cases were excluded due to missing or outliner values.

Genetic data was compared for the *MC1R, PGC-1a* and *PGC-1b* locus. The data is presented in Table 16. A significant difference was detected between patients from Brisbane and patients from Tübingen for the locus *MC1R* and *PGC-1a*, but not for *PGC-1b*. Much more patients from Brisbane had at least one variants in the gene locus coding for MC1R (87.6%) compared to patients from Tübingen with a least one *MC1R* variant (77.3%). A deeper analysis of the *MC1R* genotype revealed similar percentages of patients with the WT/r, WT/R and r/r genotype,

however the r/R and R/R genotypes were significantly more frequent among the patients from Brisbane and the WT/WT genotype was more frequent among the patients from Tübingen (p<0.001). This was also reflected by the counts for R-alleles and WT alleles with huge differences between the two cohorts (p<0.001) and a still significant but less pronounced difference between r-alleles counts (p=0.069). The frequency of patients with one or more germline variants in the gene coding for PGC-1a was higher in patients from Tübingen than in patients from Brisbane (12.8% versus 8.8%). Variants in the gene coding for PGC-1b were as frequent in Tübingen as in Brisbane with 76.8% compared to 77.0%.

Table 16: Comparison of genetic data between melanoma patients from Brisbane and Tübingen

	All pts		**Brisbane**		**Tübingen**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	1010	100	454	100	556	100	
MC1R**							**<0.001**
wt	182	18.1	56	12.4	126	22.7	
variant	826	81.9	396	87.6	430	77.3	
na	2		2				
MC1R r alleles							**0.069**
0	496	49.2	208	46.0	288	51.8	
1	426	42.3	209	46.2	217	39.0	
2	86	8.5	35	7.7	51	9.2	
na	2		2				
MC1R R alleles							**<0.001**
0	504	50.0	194	42.9	310	55.8	
1	413	41.0	206	45.6	207	37.2	
2	91	9.0	52	11.5	39	7.0	
na	2		2				
MC1R WT alleles							**<0.001**
0	367	36.4	193	42.7	174	31.3	
1	459	45.5	203	44.9	256	46.0	
2	182	18.1	56	12.4	126	22.7	
na	2		2				
***MC1R* Genotype**							**<0.001**
WT/WT	182	18.1	56	12.4	126	22.7	
WT/r	236	23.4	103	22.8	133	23.9	
WT/R	223	22.1	100	22.1	123	22.1	
r/r	86	8.5	35	7.7	51	9.2	
r/R	190	18.8	106	23.5	84	15.1	
R/R	91	9.0	52	11.5	39	7.0	
na	2		2				
PGC-1a							**0.047**
wt	898	89	413	91.2	485	87.2	
variant	111	11	40	8.8	71	12.8	
na	1		1				
PGC-1b							0.927
wt	776	76.9	349	77.0	427	76.8	
variant	233	23.1	104	23.0	129	23.2	
na	1		1				

*Pearson Chi-Square, Exact Sig. (2-sided)

**deeper analyses with data of the *MC1R* locus were performed, considering the numbers of r, R or WT alleles respectively, and the specific MC1R genotype

Comparison of tumour specific data

Melanoma specific data was available for both cohorts in 8 categories. These categories were the tumour thickness, the history of multiple melanomas in one patient, the histologic subtype of the melanoma, the localisation on the body, the Clark level, the presence of ulceration, signs of regression and of naevus remnants in the histopathological specimen of the primary melanoma. The data is presented in Table 17.

Significant differences were apparent between the two cohorts of melanoma patients from Brisbane and from Tübingen in all of these categories apart from the category localisation of the primary. Around 40% of the patients had their melanoma on the trunk in both cohorts, followed by one quarter of patients with a melanoma on the lower extremities, about 20% on the upper extremities and about 15% at the head and neck (Figure 35a).

The median tumour thickness was 1mm in the cohort of patients from Brisbane compared to 1.5mm in Tübingen. Accordingly, more than half of the patients from Brisbane had thin melanomas with a tumour thickness below 1mm compared to only 24.5% of patients from Tübingen. Most patients from Tübingen had a melanoma with a tumour thickness between 1.01 and 2mm (41.0%). The proportion of thick melanomas above 4mm was similar between the cohorts with 8.5% in Brisbane and 10.2% in Tübingen (Figure 35b).

A Clark level 1 was diagnosed in 34.1% of patients from Brisbane but in none of the patients from Tübingen. The majority of patients from Tübingen had a Clark level 4 (70.8%), followed by a Clark level 3 (17.7%) and a Clark level 2 (7.1%). Patients from Brisbane had a more evenly distribution with 23.4% Clark level 2, 13.2% Clark level 3 and 25.5% Clark level 4. The rate of patients with Clark level 5 melanomas was around 4% in both cohorts.

The most frequent histologic subtype was superficial spreading melanoma with 69.2% among the patients from Brisbane and 61.2% among patients from Tübingen. The second frequent type was nodular melanoma with 13.5% in Brisbane and 17.9% in Tübingen. The subtype lentigo maligna melanoma was much more frequent in Brisbane with 12% compared to 5.2%, whereas acral lentiginous melanoma was more frequent in Tübingen with 5.6% compared to 2.3% in Brisbane (Figure 35c).

Table 17: Comparison of tumour specific data between melanoma patients from Brisbane and Tübingen

	All pts		**Brisbane**		**Tübingen**		p*
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	1010	100	454	100	556	100	
Tumour thickness median	1.35		1.00		1.50		**<0.001**
<1,0mm	259	33.3	132	51.2	127	24.5	**<0.001**
1,01-2,0mm	278	35.8	65	25.2	213	41.0	
2,01-4mm	165	21.2	39	15.1	126	24.3	
>4mm	75	9.7	22	8.5	53	10.2	
na	233		196		37		
Multiple Melanomas							**<0.001**
yes	251	25.0	236	52.6	15	2.7	
no	754	75.0	213	47.4	541	97.3	
na	5		5				
Histology							**<0.001**
SSM	491	63.9	184	69.2	307	61.2	
NM	126	16.4	36	13.5	90	17.9	
LMM	58	7.6	32	12.0	26	5.2	
ALM	34	4.4	6	2.3	28	5.6	
others	59	7.7	8	3.0	51	10.2	
na	242		188		54		
Localisation							0.123
head and neck	135	14.9	54	14.4	81	15.2	
trunk	363	40.0	159	42.5	204	38.3	
upper extremities	167	18.4	70	18.7	97	18.2	
lower extremities	234	25.8	91	24.3	143	26.8	
mucosa	8	0.9	0	0	8	1.5	
na	103		80		23		
Clark level							**<0.001**
1	127	15.2	127	34.1	0	0	
2	120	14.4	87	23.4	33	7.1	
3	131	15.7	49	13.2	82	17.7	
4	423	50.7	95	25.5	328	70.8	
5	34	4.1	14	3.8	20	4.3	
na	175		82		93		
Ulceration							**<0.001**
yes	164	22.1	42	14.1	122	27.5	
no	578	77.9	256	85.9	322	72.5	
na	268		156		112		
Regression							**<0.001**
yes	202	28.1	141	49.0	61	14.2	
no	516	71.9	147	51.0	369	85.8	
na	292		166		126		
Naevus-associated MM							**<0.001**
yes	228	32.6	146	52.3	82	19.5	
no	472	67.4	133	47.7	339	80.5	
na	310		175		135		

*Pearson Chi-Square Asymp.Sig (2-sided)

Ulcerated melanomas which represent a subclass entity with an impaired prognosis, were more frequent in patients from Tübingen, with 27.5% compared to 14.1% patients with ulcerated primaries in Brisbane (Figure 35d).

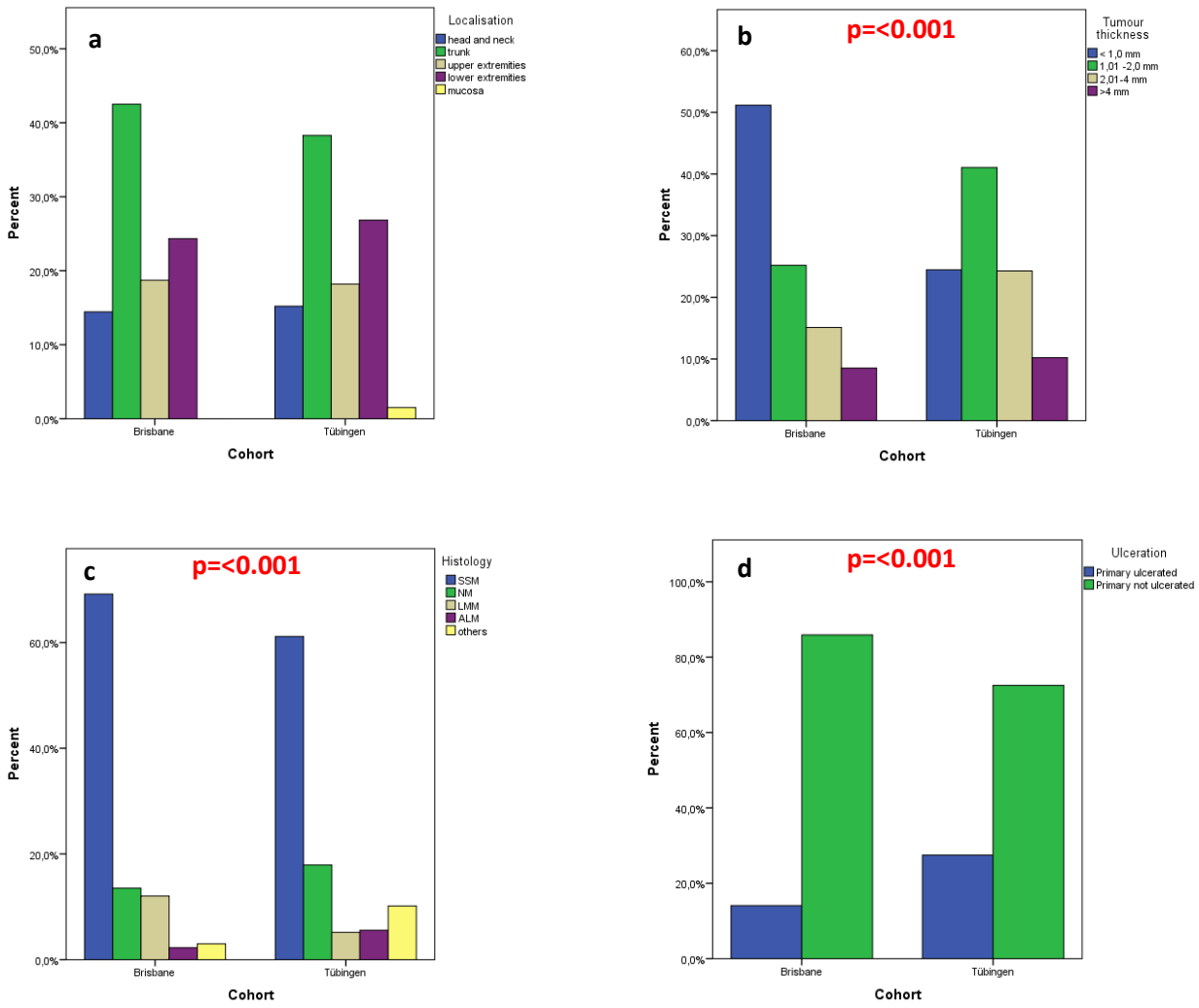


Figure 35: Bar charts illustrating the different frequencies of a. the localisation, b. the tumour thickness of the primary, grouped in 4 categories, c. the histological subtype, and d. the presence of ulceration in the primary tumour in melanoma patients from Brisbane compared to Tübingen.

The most striking difference was the proportion of patients with a history of multiple primary melanomas. More than half of the patients of the cohort from Brisbane (52.6%) had a history of multiple melanomas compared to only 2.7% of patients from Tübingen (Figure 36a).

A remarkable difference was also seen in the frequency of melanomas with signs of regression. About half of the melanoma patients from Brisbane (49.0%) had a melanoma with regression compared to 14.2% of melanoma patients from Tübingen (Figure 36b).

Another divergent variable was the presence of naevus remnants in the histopathological specimen. More than half of the patients from Brisbane (52.3%) had a naevus associated melanoma compared to 19.5% of patients from Tübingen (Figure 36c).

The results of the last two variables had a limitation due to a notable amount of missing values, which were 292 missing values for the variable ´Regression´ and 310 missing values for the variable ´Naevus associated melanoma´.

The data in regard to the number of melanomas in each individual patient however was nearly complete, with 5 missing values for the Brisbane cohort and no missing values for the Tübingen cohort.

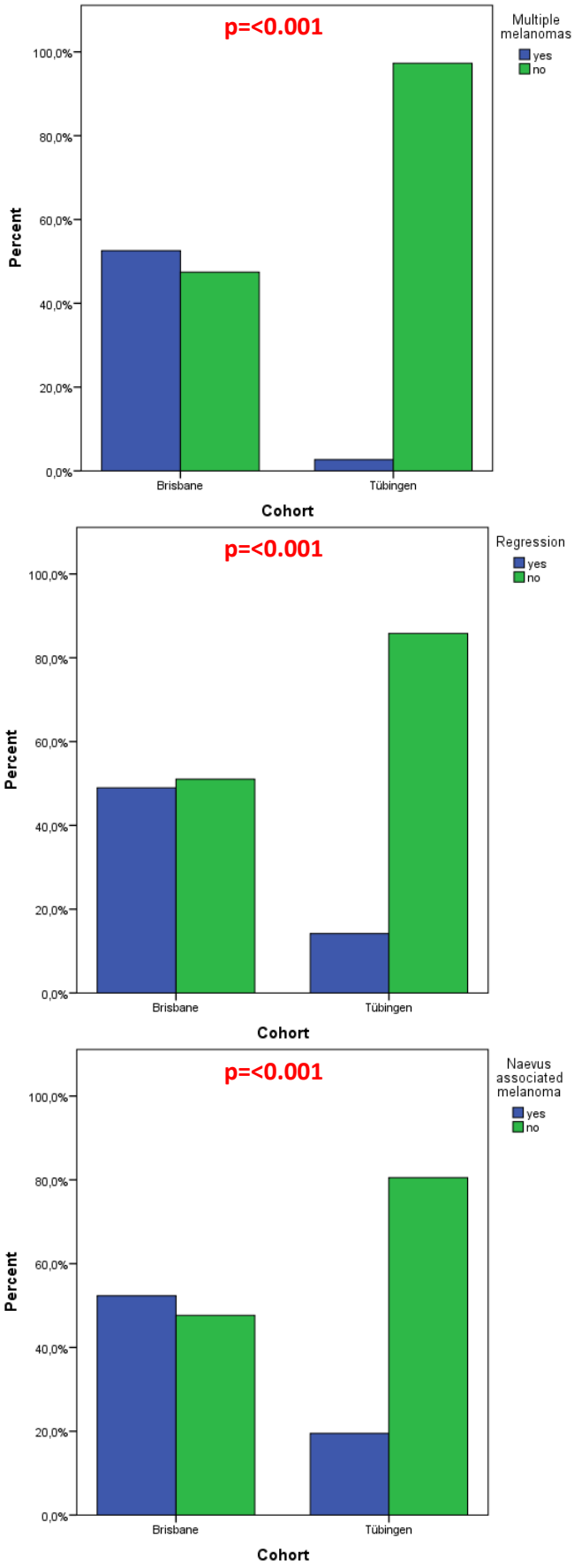


Figure 36: Bar charts illustrating the different frequencies of a. multiple melanomas, b. presence of regression in the primary tumour and c. Naevus associated melanoma in patients from Brisbane compared to Tübingen.

5.3.1. Results after Exclusion of In Situ Melanoma Cases

A bias between the cohorts from Brisbane and from Tübingen was the fact that patients with in situ melanomas were included in the Brisbane cohort while those patients were not included in Tübingen. In order to test the validity of the described results, the analyses were repeated after the exclusion of 127 melanoma in situ cases from Brisbane. The total number of analysed melanoma patients was thus 883, including 327 patients from Brisbane and 556 patients from Tübingen.

The comparison of the general characteristics resulted again in a significant difference between the cohorts for the variables BMI, Hair colour, Eye colour, Skin reaction and Family history of melanoma (Table 18). The median age was 56 after exclusion of in situ cases compared to 57.5 years including in situ cases. The median BMI with 27 remained constant after exclusion of patients with a melanoma in situ.

The share of patients with low naevus numbers went up after exclusion of those patients with in situ cases. The lowest category with 0-10 naevi from 5mm size had a share of 26.7% compared to previous 24.3% and 38.7% of patients were within the second category (11-30 naevi) after exclusion of the in-situ cases compared to 36.8% before.

Patients with an ancestry other than European were more frequent in the Brisbane cohort after exclusion of in situ melanoma cases. The difference towards the Tübingen cohort reached significance (p=0.048).

Table 18: Comparison of general traits between melanoma patients from Brisbane and Tübingen, after exclusion of in situ cases

	All pts		**Brisbane**		**Tübingen**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	883	100	327	100	556	100	
Sex							0.588
female	400	45.3	152	46.5	248	44.6	
male	483	54.7	175	53.5	308	55.4	
Age							
median, range	58 14-91		56 14-88		59 17-91		0.061
BMI							
median, range	26.57 16.90-69.92		27.36 16.90-54.09		26.25 17.16 – 69.92		**0.003**
European ancestry							**0.048**
yes	834	99.2	296	98.3	538	99.6	
others	7	.8	5	1.7	2	0.4	
na	42		26		16		
Hair colour							**<0.001**
red	89	10.2	56	17.2	33	6.0	
blonde	305	35.0	66	20.2	239	43.8	
brown	433	49.7	191	58.6	242	44.3	
black	45	5.2	13	4.0	32	5.9	
na	11		1		10		
Eye colour							**<0.001**
blue/grey	526	59.8	198	60.6	328	59.4	
green/hazel	173	19.7	82	25.1	91	16.5	
brown	180	20.5	47	14.4	133	24.1	
na	4		0		4		
Skin reaction							**<0.001**
always burn, never tan	236	27.0	139	42.9	97	17.6	
burn then tan	560	64.1	159	49.1	401	72.9	
only tan	78	8.9	26	8.0	52	9.5	
na	9		3		6		
Naevus count**							0.621
0-10	221	25.1	87	26.7	134	24.2	
11-30	324	36.8	126	38.7	198	35.7	
31-50	159	18.1	54	16.6	105	19.0	
51-100	120	13.6	41	12.6	79	14.3	
>100	56	6.4	18	5.5	38	6.9	
na	3		1		2		
Family history of mela-noma							**<0.001**
yes(1st degree relatives)	146	16.9	117	36.0	29	5.4	
no	716	83.1	208	64.0	508	94.6	
na	21		2		19		

*Pearson Chi-Square Asymp.Sig (2-sided)

**Naevi were recorded from 5mm size in Brisbane and from 3mm size in Tübingen

The exclusion of in situ melanoma cases had no influence on the results regarding sun behaviour data between the two cohorts (Table 19).

Table 19: Comparison of sun behaviour between melanoma patients from Brisbane and Tübingen, after exclusion of in situ cases

	All pts		**Brisbane**		**Tübingen**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	883	100	327	100	556	100	
History of sunburns							**0.001**
no	57	6.5	9	2.8	48	8.7	
yes	817	93.5	314	97.2	503	91.3	
na	9		4		5		
Usage of sunscreen							**<0.001**
never	92	10.6	33	10.3	59	10.8	
<50% of time	510	58.9	136	42.6	374	68.4	
>50% of time	264	30.5	150	47.0	114	20.8	
na	17		8		9		

*Pearson Chi-Square Asymp.Sig (2-sided)

The results of the genetic data comparisons after exclusion of in situ cases are presented in (Table 20). *PGC-1a* variants reached a slightly higher frequency among the Brisbane cohort after exclusion of the in-situ cases. This finding levelled up to the percentage of patients with *PGC-1a* variants in the Tübingen cohort. The difference was not significant anymore with a p-value of 0.182. The percentages of patients with at least one *PGC-1b* variants did not alter remarkably with the exclusion of in situ cases and was not significant. The count of at least one *MC1R* variant was still significantly different between the groups (p=0.002). The same was true for the MC1R genotype, the count for WT alleles and for R alleles. However, the r allele frequency was not significantly different between the two cohorts any more, with 9.2% of patients having two r alleles in both groups and around half of each group without any r allele (p=0.219).

Table 20: Comparison of genetic data between melanoma patients from Brisbane and Tübingen, after exclusion of in situ cases

	All pts		**Brisbane**		**Tübingen**		**p***
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	883	100	327	100	556	100	
MC1R**							**0.002**
wt	172	19.5	46	14.1	126	22.7	
variant	710	80.5	280	85.9	430	77.3	
na	1		1				
MC1R r alleles							0.219
0	438	49.7	150	46.0	288	51.8	
1	363	41.2	146	44.8	217	39.0	
2	81	9.2	30	9.2	51	9.2	
na	1		1				
MC1R R alleles							**0.004**
0	459	52.0	149	45.7	310	55.8	
1	345	39.1	138	42.3	207	37.2	
2	78	8.8	39	12.0	39	7.0	
na	1		1				
MC1R WT alleles							**<0.001**
0	316	35.8	142	43.6	174	31.3	
1	394	44.7	138	42.3	256	46.0	
2	172	19.5	46	14.1	126	22.7	
na	1		1				
***MC1R* Genotype**							**0.001**
WT/WT	172	19.5	46	14.1	126	22.7	
WT/r	206	23.4	73	22.4	133	23.9	
WT/R	188	21.3	65	19.9	123	22.1	
r/r	81	9.2	30	9.2	51	9.2	
r/R	157	17.8	73	22.4	84	15.1	
R/R	78	8.8	39	12.0	39	7.0	
na	1		1				
PGC-1a							0.182
wt	780	88.3	295	90.2	485	87.2	
variant	103	11.7	32	9.8	71	12.8	
na	0		0		0		
PGC-1b							0.907
wt	677	76.7	250	76.5	427	76.8	
variant	206	23.3	77	23.5	129	23.2	
na	0		0		0		

*Pearson Chi-Square Asymp.Sig (2-sided)

**deeper analyses with data of the *MC1R* locus were performed, considering the numbers of r, R or WT alleles respectively, and the specific MC1R genotype

The tumour specific data were compared for the two cohorts after exclusion of in situ melanoma cases. The results are presented in Table 21.

All variables which were significant in the analyses with the whole cohort remained significant after the exclusion of the 127 in situ melanoma cases. These were the seven variables tumour thickness, multiple melanomas, histology, Clark level, ulceration, regression, naevus associated melanoma.

As expected the median tumour thickness increased from 1.00 to 1.10mm. The presence of multiple melanomas was less frequent after the exclusion of in situ cases with 43.5% compared to 52.6%. The difference was still highly significant compared to the 2.7% of multiple melanoma cases in Tübingen. The histological subtype nodular melanoma had a higher percentage among the Brisbane cohort while lentigo maligna melanomas were less frequent after the exclusion of in situ cases. The difference was still significant compared to Tübingen.

The presence of an ulcerated primary went up as expected after the exclusion of in situ cases. The difference between the cohorts Brisbane and Tübingen was less pronounced but still significant. The percentage of primary tumours with signs of regression was nearly the same after exclusion of in situ melanomas. Naevus-associated melanomas were less frequent after the exclusion of in situ cases. However, the difference between the cohorts Brisbane and Tübingen was still highly significant.

Table 21: Comparison of tumour specific data between melanoma patients from Brisbane and Tübingen, after exclusion of in situ cases

	All pts		**Brisbane**		**Tübingen**		p*
	n	**%**	**n**	**%**	**n**	**%**	
Total, n	883	100	327	100	556	100	
Tumour thickness median	1.40		1.10		1.50		**<0.001**
<1,0 mm	236	31.7	109	48.4	127	24.5	**<0.001**
1,01-2,0 mm	272	36.6	59	26.2	213	41.0	
2,01-4 mm	162	21.8	36	16.0	126	24.3	
>4 mm	74	9.9	21	9.3	53	10.2	
na	139		102		37		
Multiple Melanomas							**<0.001**
yes	155	17.7	140	43.5	15	2.7	
no	723	82.3	182	56.5	541	97.3	
na	5		5				
Histology							**0.046**
SSM	429	63.2	122	68.9	307	61.2	
NM	126	18.6	36	20.3	90	17.9	
LMM	32	4.7	6	3.4	26	5.2	
ALM	34	5.0	6	3.4	28	5.6	
others	58	8.5	7	4.0	51	10.2	
na	204		150		54		
Localisation							0.231
head and neck	111	14.2	30	12.0	81	15.2	
trunk	308	39.3	104	41.4	204	38.3	
upper extremities	143	18.2	46	18.3	97	18.2	
lower extremities	214	27.3	71	28.3	143	26.8	
mucosa	8	1.0	0	0	8	1.5	
na	99		76		23		
Clark level							**<0.001**
1	0	0	0	0	0	0	
2	120	16.9	87	35.5	33	7.1	
3	131	18.5	49	20.0	82	17.7	
4	423	59.7	95	38.8	328	70.8	
5	34	4.8	14	5.7	20	4.3	
na	175		82		93		
Ulceration							**0.028**
yes	163	24.9	41	19.5	122	27.5	
no	491	75.1	169	80.5	322	72.5	
na	229		117		112		
Regression							**<0.001**
yes	158	25.4	97	50.3	61	14.2	
no	465	74.6	96	49.7	369	85.8	
na	260		134		126		
Naevus-associated MM							**<0.001**
yes	162	26.6	80	42.6	82	19.5	
no	447	73.4	108	57.4	339	80.5	
na	274		139		135		

*Pearson Chi-Square Asymp.Sig (2-sided)

5.4. Discussion

The present analyses were based on a two cohorts of melanoma patients, comprising 454 patients from Brisbane and 556 patients from Tübingen. Both cohorts were collected in hospital based outpatient clinics. Age and gender were well balanced between the groups. Patients from Australia had a higher body mass index (BMI) with 27.45 compared to those from Germany (26.25). The lower median BMI for patients in Germany compared to Australia was in accordance with recently published data for BMI measures in the general population of 70 different countries. Australians were reported with a higher BMI compared to Germans.[215]

The comparison of two patient cohorts derived from a) Australia, where the inhabitants have a pre-dominant British/Irish ancestry and b) from South Germany with primarily German origin revealed some expected phenotype differences. Individuals with red hair and green eyes were much more common among Australian melanoma patients, while more German patients had blond hair. Much more patients from Australia were sun sensitive and indicated they would always burn and never tan compared to the German melanoma patients. This precondition along with the higher environmental UV exposure in Australia gave a plausible explanation of the higher rate of sunburns in the personal history of Australian melanoma patients compared to the German patients. The much more frequent use of sunscreen among the Australian melanoma patients could be interpreted based on the higher need of a sun sensitive group in a high UV exposure environment compared to the German group. Another explanation is the positive result of the intensive public sun prevention campaigns in Australia, which were introduced in the 1980is and which resulted in a broad public acceptance of sun protection.[216]

The different frequencies of *MC1R* variants in the two cohorts are in accordance with the different ancestries. *MC1R* variants are found more frequently in individuals with a British/Irish family background compared to individuals from middle Europe. The overall frequency of *MC1R* variants in both cohorts was high. This is in accordance with the increased melanoma risk for individuals with *MC1R* variants.[217]

First degree family members with a positive history of a melanoma were reported from more than a third of the Australian melanoma patients (37.3%) compared to only 5.4% of the German melanoma patients. This huge difference is reflected by the higher incidence rates in Australia in general. The high proportion of familial melanoma in Australia compared to

Germany is questioning the often claimed genetic background of these cases and does rather point towards an environmental effect of UV light as carcinogen in these families.

The tumour specific parameters were remarkably different between the Australian and the German melanoma patients. The median tumour thickness was 1.0mm in the Australian cohort compared to 1.5mm in the German cohort. While the thick melanomas were equally frequent in both groups the share of thin melanomas was much higher in the Brisbane cohort. This finding might be biased by the fact that patients with in situ melanomas and with thin melanomas were less likely included in the German cohort.

The subtypes superficial spreading melanoma (SSM) and lentigo maligna melanoma (LMM) were more frequent among Australian patients, whereas nodular melanoma (NM) and acral lentiginous melanomas (ALM) were more frequent among German patients. These findings correlate with the fact that SSM and LMM belong to the sun induced melanomas while NM and ALM are not considered to be sun induced.[1] Furthermore NM and ALM define the subgroups with an impaired prognosis compared to SSM and LMM.

Ulceration of the primary is an unfavourable primary tumour characteristic and was again more common in the German cohort. In contrast primary melanomas with more favourable prognostic characteristics, such as naevus associated melanoma[218] and melanomas with signs of regression[53] where more frequent among Australian patients.

An extreme difference of patients with multiple primary melanomas (MPM) was observed between the two cohorts. About half of the melanoma patients from Brisbane (52.6%) had more than one melanoma compared to only 2.7% of patients with multiple melanomas from Tübingen. This extreme difference can be partially explained by the study inclusion procedure of the Brisbane cohort. A special interest in multiple melanoma patients in the working group resulted in an intended oversampling of these patients. In the literature, a varying range of melanoma patients who develop secondary melanomas were reported. Studies based on European populations published between 2010 and 2017 found between 3% and 6.5% of patients with MPM. A share of 6.5% patients with multiple melanomas was calculated in 2253 patients drawn from the German Central Malignant Melanoma Registry (Gassenmaier et al. 2017, submitted). A Swiss study reported a rate of 4.6% MPM[219], an Italian study found 6% MPM cases[220] and a large study from the Netherlands including 42,733 patients from a

national, population-based Cancer Registry found 3% patients with two or more melanomas.[221] Another study from the Netherlands focussed on risk factors for multiple melanoma and found cumulative sun exposure and a high naevus density as main contributing risk factors for the 4.7% reported cases of MPM.[222] Studies from the States and from Canada reported between 1.9% and 10% MPM cases. The oldest study retrieved was from 1979 with 5.3% MPM[223], a study from 2005 with 4,484 patients reported 8.6%[224], a small cohort study of 354 patients described 8% MPM and was published in 2006.[225] A study from 2010 with 2,506 melanoma patients found only 1.9% of multiple melanoma cases. Analyses revealed a significant association of MPM with older age and the diagnoses of a superficial spreading melanoma.[226] Three small cohort studies found 7.7%, 5% and 10% of MPM.[55,227,228] A large study from 2012 based on 208,289 patients found a rate of 3.3% MPM, another large study from 2015 including 16,570 patients found a rate of 7.3% MPM.[229,230] In the latter study risk factors associated with MPM were older age, male, white and having a partner. Four studies from Australia were found with reported rates between 2.9% and 17.8% of MPM cases. One study from Sydney was published in 1982 with 2.9% MPM among 90 melanoma patients.[54] Another report from Sydney including 5,250 patients diagnosed between 1983 and 1999 found a rate of 6% MPM.[56] A report from Queensland found a rate of 7% among 29,908 patients diagnosed between 1995 and 2008.[56] Another cohort from Queensland comprising 1,068 patients found a rate of 17.8% MPM and a positive association with elder age and males.[231] Overall, the rates of MPM in different continents showed remarkable differences. Higher rates were more likely seen in smaller cohorts, in those from more recent years and in cohorts from countries with a higher UV exposure. Smaller cohorts have generally a higher risk of a selection bias which could explain an unexpected high or low observed “out-of-range” value. The consistent high rate of MPM in small cohorts however is more likely explained by the fact that those smaller cohorts tend to be closer observed and to be included in a high quality follow up with a higher chance of finding true secondary melanomas as well as over-diagnosed melanomas, both resulting in higher MPM numbers. An increase of MPM rates in recent years might be explained by the generally rising incidence numbers of melanoma. The report of higher MPM rates in regions with higher UV exposure is setting a light on the impact of UV as carcinogen rather than on a genetic predisposition for the development of MPM.

The prognostic impact of MPM is still unclear and conflicting results are reported. While some studies found an impaired survival probability for patients with multiple melanomas[221,231,232]

other studies reported a survival benefit for patients with MPM in general[56] or for those MPM with thick tumours.[57] Survival data were not yet available for the melanoma patients from Brisbane but were available for the patients from Tübingen. None of the 15 patients with multiple melanomas of the cohort of 556 patients died or progressed during follow up. Thus, the present data point towards a more favourable prognosis for patients with multiple melanomas.

Overall, the rate of prognostic favourable tumours was higher among the Brisbane cohort. These more favourable melanomas might include a share of screen detected and indolent melanomas as a result of the successfully conducted secondary prevention campaigns. UV exposure is the most likely cause for the higher rate of prognostic favourable tumours. However, this rate of prognostic favourable melanomas is perishing among the general high incidence rate caused by UV light. The increasing incidence rates of skin cancer worldwide was linked to changed leisure activities and altered sun seeking behaviour of the western populations after world war II. This resulted in a higher risk of UV induced damages of the unprotected skin. However, a generally higher UV exposure caused by a less protective atmosphere is another likely cause for the worldwide skin cancer epidemic of the last decades. Ozone depletion as a cause for the epidemic in skin cancer was mentioned by A. Woodcock, a Respiratory Physician in an editorial[233], but received little attention by Dermatologists so far. One report was published in 1993 discussing the expected increase of melanoma incidence due to the reduction of ozone in the stratosphere.[234] The higher incidence rates of melanoma in New Zealand compared to Australia is noteworthy. Based on the countries latitude a lower melanoma risk would be expected. The higher incidence and mortality rates compared to Australia[24] might be influenced as well by the close proximity to the Antarctic ozone hole. Moreover the recovering of the ozone layer might have contributed to the reported decline in incidence rates in Australia in 2015.[25] The healing in the Antarctic ozone layer was published last year and was traced back to the banning of industrial chlorofluorocarbons as a result of the Montreal Protocol, established in 1987 by the United Nations.[235]

Conclusions

The comparison of melanoma patients from two countries with different environmental sun exposure revealed clear differences.

Melanoma patients from Brisbane had more often primary tumours with favourable prognostic characteristics compared to melanoma patients from Tübingen. This was observed for all variables with known prognostic impact, such as tumour thickness, histological subtypes, ulceration, regression of the primary and the rate of naevus associated melanomas. Furthermore, the rate of familial and multiple melanomas was higher among the patients from Brisbane.

The higher rate of prognostic favourable melanomas in the cohort from Brisbane compared to Germany supports the impact of UV exposure on the development of initially less aggressive melanomas.

6. Association of *PPARGC1A/B* variants with Pigment Traits and Melanoma

6.1. Introduction

PGC-1 alpha and beta are two proteins belonging to the family of Peroxisome proliferator-activated receptors (PPARs) which are metabolic transcriptional co-factors. The first member of this family was discovered in 1998 by Puigserver in brown fat tissue with an elevated expression after cold exposure.[174] A role of PGC-1 in melanocytic pathways was first described by Shoag et al.[181] They found an induction of PGC-1 alpha and beta through a-MSH signalling and an activation of MITF in human melanoma cell lines through PGC-1 alpha and beta. The expression of PGC-1 alpha was found to be regulated by MC1R and BRAF through different pathways and described as essential for mitochondrial biogenesis and function by Torrens-Mas et al.[236] An overexpression of PGC-1 alpha mediated by MITF in melanoma cells increased the mitochondrial energy metabolism and the survival capacities of these cells.[188] The work by Luo et al. showed for the first time an influence of PGC-1 alpha on melanoma metastasis growth in mice.[237] They postulated two roles for PGC-1 alpha, one promoting growth and survival of the tumours and one suppressing the development of metastases.

Based on these findings, an influence of genetic germline variants within the coding regions for PGC-1 alpha and beta on pigmentation traits, melanoma risk and survival in humans was postulated and investigated here, using both the German and Australian datasets.

6.2. Methods

The following analyses were based on two cohort studies, one from Tübingen, Germany and one from Brisbane, Australia.

Patient inclusion, data and material collection and genotyping methods are described in Chapter 2.

The analyses on PGC-1 associations were performed in the following three steps:

Initially a cohort with either extreme high (n=40) or extreme low (n=38) naevus count was selected to explore an association of PGC-1 with naevus count and melanoma risk. The genotyping method was Sanger Sequencing with a selection of 7 SNPs of interest as described below.

The positive results lead to an extension of the cohort with not specifically selected individuals from Brisbane in order to confirm the observed significant association with one of the previously assessed SNPs. The genotyping method was again Sanger Sequencing.

The third step was initiated in parallel to the second step and included an increase of the sample size to 893 individuals from Brisbane and 614 individuals from Tübingen. Furthermore, the genotyping method was changed to a high throughput genotyping method, a chip based array, the Illumina CoreExome Chip. This technique allowed a detection of more than 500 000 SNPs throughout the whole exome plus tagging intron SNPs at one time.

Statistical analyses were performed using R and SPSS. Initial association analyses were done using fishers exact test and the Kruskal-Wallis-test. The final association analyses including the complete cohort were done with logistic regression analyses.

6.2.1. SNP Selection for Sanger Sequencing

A search in the database dbSNP on Pubmed was performed in 2013 to search for SNPs within *PPARGC1B* and *PPARGC1A*, the two coding genes for PGC-1 alpha and PGC-1 beta. A set of inclusion criteria was applied as follows, in order to focus on a relevant selection of SNPs:

- missense changing SNPs
- location in an exon
- minor allele frequency of near or >1%

These criteria were met by 7 SNPs for PGC-1ß and 4 SNPs for PGC-1α. The rs numbers were retrieved and additional information such as the variant associated base change and the resulting amino acid change and position within the protein product and the previously published minor allele frequency (MAF) were recorded (Table 22).

Table 22: Selected SNPs within *PPARGC1B* and *PPARGC1A* for the initial analyses

Gene	Amino acid Position & Change	dbSNP rs number*	Base Change	MAF**	MAF*** updated
PPARGC1B (encoding for PGC-1ß)	Ala203Pro	rs7732671	C/G	0.1028	C=0.1096
	Arg226Gln	rs45520937	A/G	0.0808	A=0.0705
	Val240Ile	rs17572019	A/G	0.0661	A=0.0659
	Arg253Ser	rs11959820	A/C	0.0900	A=0.1048
	Arg291Trp	rs45470697	C/T	0.0188	T=0.0174
	Val312Met	rs77687445	A/C/G	0.0096	A=0.0088
	Ser350Tyr	rs45519432	A/C	0.0197	A=0.0174
PPARGC1A (encoding for PGC-1α)	Leu438Ser	rs35437002	A/G	0.0110	G=0.0106
	Gly482Ser	rs8192678	A/G	0.2911	T=0.2658
	Arg566Cys	rs58772979	C/T	0.0115	A=0.0108
	Thr612Lys (or Met)	rs3736265	A/G/T	0.1094	A=0.1094

* dbSNP rs number = Single Nucleotid Polymorphism Database reference number

** Minor Allele Frequency (Global) data from dbSNP, ***updated 20th Sep 17

The localisation of the retrieved SNPs within the two genes was identified and plotted on a draft of the two genes (Figure 37). The retrieved SNPs for the protein PGC-1 beta were all located in exon 5 of *PPARGC1B*. A previously published intronic SNP (rs32579) by Shoag et al. was located between exon 4 and exon 5. The retrieved SNPs for the protein PGC-1 alpha were located in exon 8 and exon 9 of *PPARGC1B*. Primers were designed around exon 5 of *PPARGC1B*. Sanger Sequencing was performed for 175 individuals of the cohort from Brisbane as described in chapter 2.4.2.

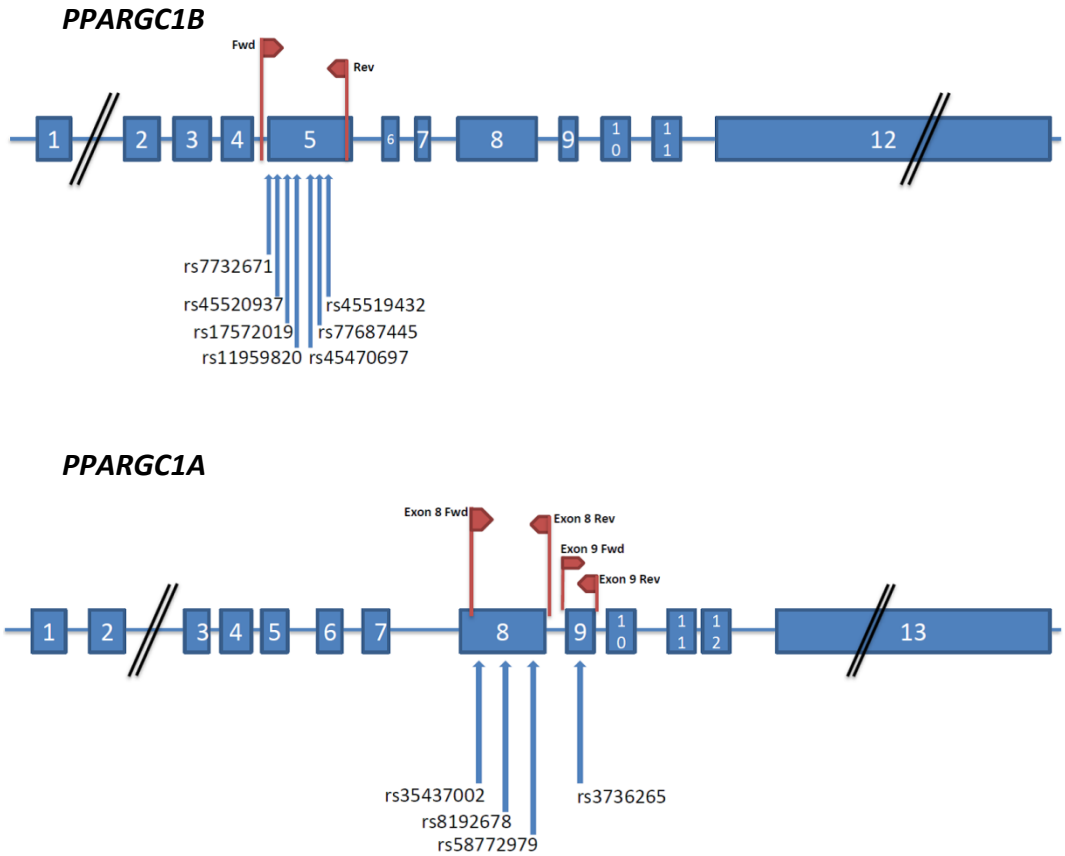


Figure 37: Drafts of the genes *PPARGC1B* and *PPARGC1A* and the localisation of the selected SNPs. The blue boxes represent the exons. The red flags mark the position of the designed primers.

6.3. Results of the Initial Association Analyses based on Sanger Sequencing

A total of 78 individuals from the BNMS were genotyped with Sanger sequencing at the *PPARGC1B* locus. PCR and Sanger sequencing resulted in chromatograms of 671bp long fragments which included the 7 pre-selected SNPs. Variants of the 3 SNPs rs45520937, rs17572019 and rs11959820 were identified in 21 of 78 individuals. The other 4 SNP locations showed no variant in comparison to the reference sequence. The frequencies of the identified minor alleles are presented in Table 23.

Table 23: Frequency of PGC-1b minor alleles in the initial analyses pf 78 individuals

	High NC	**Low NC**	**History of MM**	**No history of MM**	**Total**	**Reported MAF**
Individuals/Alleles	40/80	38/76	35/70	43/86	78/156	
rs number/Amino acid change	**n(%)**	**n(%)**	**n(%)**	**n(%)**	**n(%)**	**%**
rs45520937 Arg226Gln	2 (3)	3 (4)	4 (6)	1 (1)	5 (3)	8
rs17572019 Val240Ile	4 (5)	6 (8)	2 (3)	8 (9)	10 (6)	6
rs11959820 Arg253Ser	7 (9)	1 (1)	4 (6)	4 (5)	8 (5)	9
rs7732671 Ala203Pro	0	0	0	0	0	10
rs45470697 Arg291Trp	0	0	0	0	0	2
rs77687445 Val312Met	0	0	0	0	0	1
rs45519432 Ser350Tyr	0	0	0	0	0	2

NC= naevus count, low naevus count was defined as < 100 naevi, high naevus count ≥ 100 naevi; MM= malignant melanoma. MAF= minor allele frequency.

An association of the retrieved variants with naevus count and melanoma risk was tested. The allele frequencies of each of the three SNPs were plotted against the variables naevus number and history of malignant melanoma in the study cohort. There was a significant difference for rs11959820 (Arg253Ser), with 7 minor alleles found in the high naevus group, while only one

minor allele was found in the low naevus group (p= 0.047, one-tailed fisher´s exact test). Furthermore, there was a difference between the minor allele frequencies for rs45520937 (Arg226Gln) of individuals with a history of malignant melanoma compared to controls, however this was not statistically significant (p= 0.137, one-tailed fisher´s exact test). The results are presented in Figure 38.

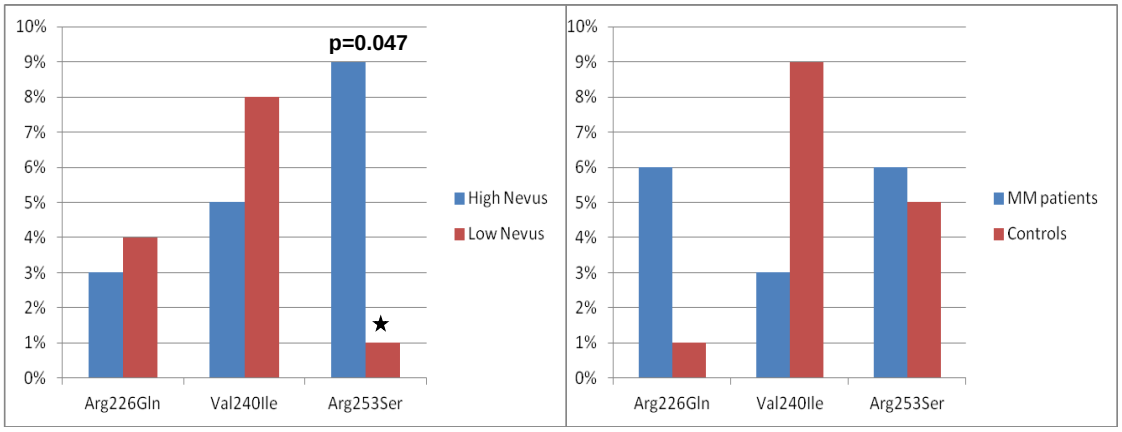


Figure 38: Comparison of the allele frequencies of three assessed PGC-1 SNPs between a. individual with high versus low naevus count and b. melanoma patients versus controls. The difference between the minor allele frequency of rs11959820 (Arg253Ser) in the high versus low naevus count group was significant p= 0.047.

Based on these findings, the study cohort was extended with unselected cases of the Brisbane cohort to a sample size of 175. Allele variants at rs45520937, rs17572019 and rs11959820 were found in a similar number as before. Additionally, 22 new and previously not detected minor allele variants were found for rs7732671 in the extended sample size. This SNP location was located at the beginning of the retrieved PCR fragment resulting in an impaired quality of the chromatogram at that location. This explained the false negative findings for minor variants at that location in the beginning of the analyses.

A common presence of the two minor alleles at the SNP locations rs7732671 (Ala203Pro) and rs17572019 (Val240Ile) was detected in the assessed samples, defined as Linkage Disequilibrium between those two SNPs.

Association analyses were performed with the four SNPs rs11959820 (Arg253Ser), rs7732671 (Ala203Pro), rs45520937 (Arg226Gln) and rs17572019 (Val240Ile) in the extended cohort using the Kruskal-Wallis-Test.

The genotype of the individuals for each of the 4 SNPs was plotted against the naevus number. In accordance to the previous analysis, the strongest association was found for the SNP rs11959820, with a C to A transition, leading to an amino acid change from Arginine (R) to Serine (S) at position 253. Individuals with a minor allele at this locus had a trend towards a higher naevus number. However, this association was not significant in the assessed cohort. Furthermore, no association with naevus number could be shown for the other investigated SNPs (Figure 39).

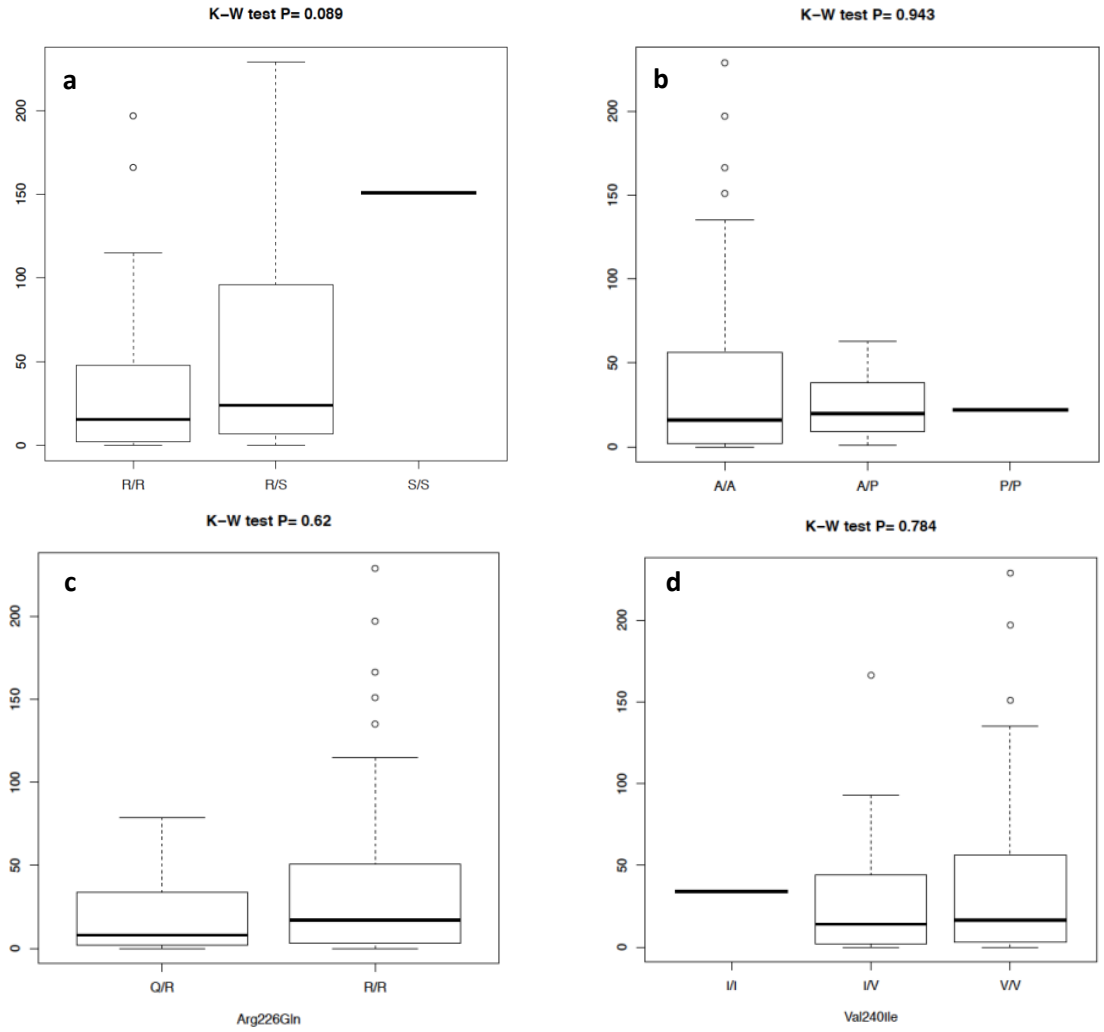


Figure 39: Comparison of naevus number between the different genotypes of the study participants at a. rs11959820 (Arg253Ser), b. rs7732671 (Ala203Pro), c. rs45520937 (Arg226Gln) and d. rs17572019 (Val240Ile). No significant associations were found.

The association of the different genotypes at PGC-1b with melanoma risk was assessed in the extended cohort as well. None of the discovered minor allele variants showed an association with either increased or decreased risk of melanoma development.

To answer the question if PGC-1ß variants would increase or decrease pigment production, the different genotypes were compared with the measured data of skin reflectance. A low value for skin reflectance was obtained from dark skin, which had higher light absorbent properties. The measurement at the sun protected inner forearm was referred to as the constitutive skin colour. The outer, more UV exposed forearm, was referred to as the facultative skin colour. The difference between those two measurements was used as a value for tanning ability. The minor variant at rs45520937 with a G to A transition, inducing an Arginine to Glutamine change at the amino acid position 226, was strongly associated with an increased pigmentation on the inner and on the outer forearm (Figure 40). This association reached statistical significance (p=0.039 and p=0.015). In addition, there was a trend for an increased tanning ability associated with the minor allele at rs45520937. This association was not significant (p=0.058).

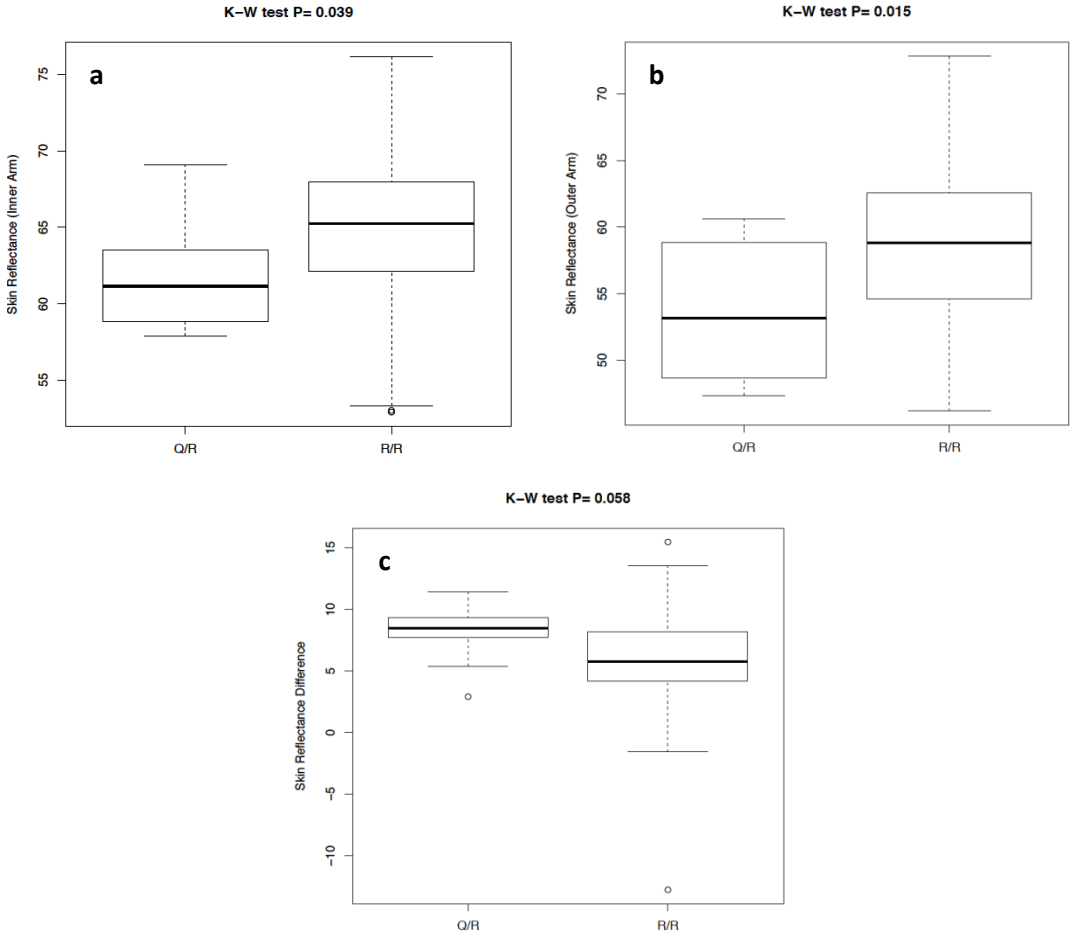


Figure 40: Significant association of rs45520937 (Arg226Gln) with dark skin (low skin reflectance) at the a. inner (p=0.039) and b. the outer forearm (p=0.015) and association with c. an increased tanning ability (p=0.058).

6.4. Results of the Association Analyses based on Chip Array Genotyping

In parallel to the extension of the cohort for Sanger Sequencing the complete cohorts from Brisbane and from Tübingen were genotyped with the Illumina Human CoreExome chip. This included a total of 893 individuals from Brisbane and 614 individuals from Tübingen. Both cohorts included patients with a personal history of melanoma and unaffected individuals. The utilized chip was the Infinium® CoreExome-24 v1.2 BeadChip with a total number of more than 500,000 markers. The Illumina Chip included a total of 19 exon SNPs of the gene *PPARGC1A* and 40 exon SNPs of the gene *PPARGC1B.* The high genotyping quality of the chip results were demonstrated as described below with the retrieved yield of results and with the comparison of the previous Sanger sequence findings.

6.4.1. Sequencing Quality

6.4.1.1. Yield of valid SNP Results

The first analysed samples with the Illumina HumanCoreExome-24 BeadChip included a total number of 232 samples from Germany.

All samples were successfully assessed with a high yield of valid SNP measurements. A total of 545 710 SNPs were analysed per sample. Of these SNPs the mean percentage of failed measurements was <0.00186%, ranging from <0.00137% to 0.0064%. In absolute numbers these were a mean of 471 missing SNPs out of 545 710 per sample with a range of 204 to 3510 SNPs (Figure 41).

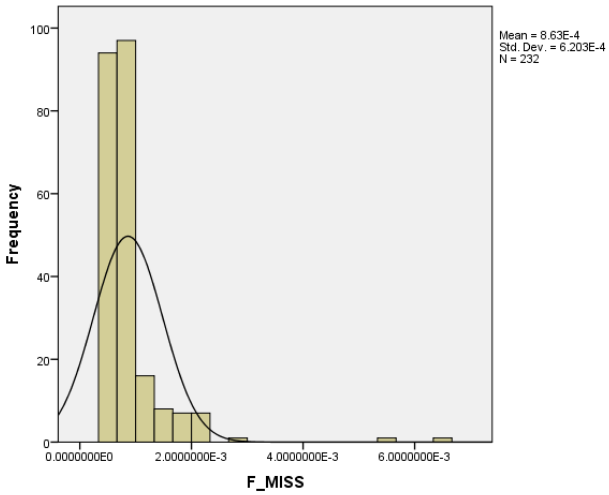


Figure 41: Missing SNPs (%) per 545 710 assessed SNPs of each sample: mean <0.00186%, resulting in a mean of 99.99914% valid SNP results per sample.

6.4.1.2. Comparison of Illumina Sequencing with Sanger Sequencing Results

A total of 175 individuals from Brisbane were genotyped with both methods, Sanger Sequencing and with the Illumina chip. Of the 7 SNPs assessed by Sanger Sequencing all but one SNP were covered by the chip. The SNP which was not covered was rs45520937 with the amino acid change Arg226Gln. All findings of the chip could be confirmed via Sanger Sequencing. One homozygous variant for rs17572019, one homozygous variant and 10 heterozygous variants for rs7732671 were missed by the initial analyses of the chromatograms but were retrospectively identified. The findings are summarized in Table 24.

Table 24: Quality control of SNP Sequencing, Sanger results compared with Illumina Chip results

rs number	AS position	Sanger (n=175)	Illumina (n=175)	Matches/Mismatches
rs7732671	Ala203Pro	156 wildtype 19 heterozygous 0 homozygous	144 wildtype 29 heterozygous 2 homozygous	163/24
rs45520937	Arg226Gln	167 wildtype 7 heterozygous 1 homozygous	-	-
rs17572019	Val240Ile	146 wildtype 28 heterozygous 1 homozygous	145 wildtype 28 heterozygous 2 homozygous	145/1
rs11959820	Arg253Ser	163 wildtype 11 heterozygous 1 homozygous	163 wildtype 11 heterozygous 1 homozygous	163/0
rs45470697	Arg291Trp	175 wildtype	175 wildtype	175/0
rs77687445	Val312Met	175 wildtype	175 wildtype	175/0
rs45519432	Ser350Tyr	175 wildtype	175 wildtype	175/0

6.4.2. Sequencing Results – Frequencies

The frequencies of the minor alleles at the respective SNP localisations for the two analysed cohorts from Brisbane and Tübingen were calculated for the retrieved SNP locations of the gene *PPARGC1A* and of the gene *PPARGC1B*. The results are presented in Table 25 and Table 26. The respective amino acid change for each SNP, the Illumina Map number, rs number, the bases of the variant type and wild type and the published frequencies of the respective minor alleles within the European population were retrieved and recorded.

The cohort from Brisbane had less frequently variants in the gene *PPARGC1A* compared to the cohort from Tübingen. Of the 19 different SNP loci, 15 SNPs had a zero frequency in the Brisbane cohort. Variants were found at 4 SNP loci. Those variants were found in a similar number than previously published frequencies. The frequency of SNP variants for *PPARGC1A* was higher in Tübingen and 6 loci with minor alleles were found (Table 25). A total of 40 exon SNP locations variants from the gene *PPARGC1B* were assessed for minor alleles. Variants were found in both cohorts at 8 different locations in a similar frequency of previously published data (Table 26).

Table 25: Assessed PGC-1a SNPs. Available SNPs located in the PGC-1a coding gene *PPARGC1A*. The amino acid position and change, the rs number (Single-nucleotide polymorphism database reference number) and the MAF (Minor allele frequency) in the European (non-finish) population is given for each SNP. WT = wild-type, VT = variant-type.

Gene	No	Amino acid position and change	Illumina Map Info	rs number	VT/WT	European (Non-Fin) MAF*	MAF Brisbane Cohort (n=893)	MAF Tübingen Cohort (n=614)
PPARGC1A	1.	A774G	exm2072194	rs201337076	C/G	0	0	0
	2.	E733V	exm392323	rs149018936	A/T	3.01e-05	0	0
	3.	R690Q	exm392330	rs148144750	C/T	<0.0011799	0	0
	4.	Q664R	exm392335	rs139102065	C/T	4.521e-05	0	0
	5.	**T612M**	**exm392344**	**rs3736265**	**A/G**	**0.05686**	**0.056**	**0.067**
	6.	R611H	exm392345	rs144103777	C/T	0.002293	0.001	0.008
	7.	Indel	indel.80441		I/D		0	0
	8.	R594S	exm392351	rs148669466	G/T	<0.0011859	0	0
	9.	R580Q	exm392353	rs143326747	C/T	<0.0011213	0	0
	10.	S577L	exm392355	rs17847360	C/T	<0.0015431	0.001	0
	11.	R566C	exm392358	rs58772979	C/T	<0.0016152	0	0.001
	12.	F534L	exm392363	rs199669057	A/G	<0.00118	0	0
	13.	K496Q	exm392367	rs200661963	G/T	<0.00112	0	0.001
	14.	L438S	exm392378	rs35437002	A/G	<0.0015248	0	0.001
	15.	L410I	exm392379	rs34514918	G/T	<0.0014199	0	0
	16.	E369K	exm392383	rs201353657	C/T	7.5e-05	0	0
	17.	E353K	exm392386	rs199902625	C/T	<0.0011055	0	0
	18.	A304V	exm392392	rs200841362	A/G	<0.0011055	0	0
	19.	S74L	exm392419	rs142669571	A/G	0.001414	0.002	0.002

* data from http://exac.broadinstitute.org

Table 26: Assessed PGC-1b SNPs. Available SNPs located in the PGC-1b coding gene PPARGC1B. The amino acid position and change, the rs number (Single-nucleotide polymorphism database reference number) and the MAF (Minor allele frequency) in European (non-finish) population is given for each SNP. WT = wild-type, VT = variant-type.

Gene	No	Amino acid position and change*	Illumina Map Info	rs number	VT/WT	European (Non-Fin) MAF**	MAF Brisbane Cohort (n=893)	MAF Tübingen Cohort (n=614)
PPARGC1B	1.	G31R, G31R, G6R	exm494061	rs45518035	A/G	9.04e-05	0	0.001
	2.	S50N, S50N, S25N	exm494065	rs146830741	A/G	1.502e-05	0	0
	3.	R180H,Silent,Silent	exm494085	rs148461382	A/G	0.087140	0	0
	4.	**A203P, A164P, A139P**	**exm494090**	**rs7732671**	**G/C**	**0.08714**	**0.086**	**0.080**
	5.	T221N, T182N, T157N	exm494093	rs139476696	A/C	0.001991	0.002	0.002
	6.	S222L, S183L, S158L	exm494094	rs75266799	C/T	<0.001214	0	0
	7.	S239N, S200N, S175N	exm494100	rs151338666	A/G	<0.0018256	0.001	0
	8.	S256N, S217N, S192N	exm494105	rs147103978	A/G	1.551e-05	0	0
	9.	P264T, P225T, P200T	exm494107	rs138677083	A/C	0	0	0
	10.	**V279I, V240I, V215I**	**exm494114**	**rs17572019**	**A/G**	**0.0876**	**0.085**	**0.080**
	11.	**R292S, R253S, R228S**	**exm494117**	**rs11959820**	**A/C**	**0.0324**	**0.030**	**0.035**
	12.	R330W, R291W, R266W	exm494124	rs45470697	C/T	6.072e-05	0	0
	13.	V351M, V312M, V287M	exm494127	rs77687445	A/G	6.059e-05	0	0
	14.	T363M, T324M, T299M	exm494128	rs45526537	C/T	7.604e-05	0	0
	15.	R374G, R335G, R310G	exm494131	rs201630695	A/G	<0.0011531	0	0
	16.	S389Y, S350Y, S325Y	exm494136	rs45519432	A/C	6.155e-05	0	0
	17.	R413Q, R374Q, R349Q	exm494142	rs138692598	A/G	9.322e-05	0	0
	18.	R421G, R382G, R357G	exm494143	rs141717472	C/G	0	0	0
	19.	E446V, E407V, E382V	exm494147	rs139404347	A/T	0	0	0
	20.	W451X, W412X, W387X	newrs142816441	rs142816441	A/G	NA	0	0
	21.	R455S, R416S, R391S	exm494149	rs144236856	G/T	4.814e-05	0	0
	22.	V473L, V434L, V409L	exm494151	rs142359633	C/G	0	0	0
	23.	P475H, P436H, P411H	exm494153	rs140114166	A/C	0.004207	0.001	0.003
	24.	R480Q, R441Q, R416Q	exm494157	rs142640674	A/G	<0.0019096	0	0
	25.	S500L, S461L, S436L	exm494161	rs45549037	C/T	9.284e-05	0	0
	26.	E528K, E489K, E464K	exm494168	rs114581116	A/G	2.339e-05	0	0
	27.	G702V, G663V, G638V	exm494186	rs202211888	G/T	<0.0011055	0	0
	28.	P714L, P675L, P650L	exm494188	rs45509002	C/T	3.014e-05	0	0
	29.	T753M, T714M, T689M	exm494194	rs149663552	C/T	0	0	0
	30.	R761C, R722C, R697C	exm494195	rs146710258	C/T	<0.0013017	0	0
	31.	S763N, S724N, S699N	exm494197	rs200739030	A/G	9.053e-05	0	0
	32.	L780V, L741V, L716V	exm494201	rs149078416	C/G	1.51e-05	0.001	0
	33.	R851H, R812H, R787H	exm494213	rs200880286	A/G	1.505e-05	0	0
	34.	R853C, R814C, R789C	exm494214	rs201680092	C/T	7.524e-05	0	0
	35.	R853H, R814H, R789H	exm2091813	rs201238869	A/G	3.011e-05	0	0
	36.	A890T, A851T, A826T	exm494225	rs150637009	A/G	0.002134	0.001	0.001
	37.	R893W, R854W, R829W	exm494226	rs149399240	C/T	<0.0011066	0	0
	38.	R893Q, R854Q, R829Q	exm2091817	rs138772212	A/G	<0.0012137	0	0
	39.	R894Q, R855Q, R830Q	exm494228	rs201959772	A/G	<0.0011838	0	0.001
	40.	I906M, I867M, I842M	exm494232	rs114273610	G/T	1.5e-05	0	0
	41.	**Intronic SNP**		**rs251468**	**A/G**	**NA**	**0.214**	**0.215**
	42.	**Intronic SNP**		**rs32579**	**A/G**	**NA**	**0.233**	**0.223**

* 3 different transcripts of PGC-1b

** data from http://exac.broadinstitute.org

6.4.3. Cohort Description

The different characteristics of the two cohorts from Tübingen and Brisbane are summarized in Table 27. The numbers in regard to the melanoma cases were previously shown in chapter 5. The individuals without a personal history of melanoma were included in the present analyses as well and are listed as controls.

Table 27: Baseline Characteristics of the two cohorts from Tübingen and Brisbane

	Brisbane Cohort						**Tübingen Cohort**					
	MM		**Controls**		**Total**		**MM**		**Controls**		**Total**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
	454	**100**	**439**	**100**	**893**	**100**	**556**	**100**	**58**	**100**	**614**	**100**
Sex												
female	208	45.8	237	54.0	445	49.8	248	44.6	32	55.2	280	54.4
male	246	54.2	202	46.0	448	50.2	308	55.4	26	44.8	334	45.6
Age median, range	58, 14-88		30, 11-76		42, 11-88		59, 17-91		40, 23-81		58, 17-91	
BMI median, range	27, 16.9-56.8		23.9, 17.3-48.8		25.3, 16.9-56.8		26.2, 17.2-69.9		23.1, 17.6-37.0		26.0, 17.2-69.9	
Sun reaction												
always burn, never tan	194	43.2	139	31.9	333	37.6	97	17.6	5	8.6	102	16.8
burn then tan	220	49.0	257	58.9	477	53.9	401	72.9	47	81.0	448	73.7
only tan	35	7.8	40	9.2	75	8.5	52	9.5	6	10.3	58	9.5
na	5		3		8		6		0		6	
Eye colour												
blue/grey	265	58.4	212	48.3	477	53.4	328	59.4	27	46.6	355	58.2
green	125	27.5	126	28.7	251	28.1	91	16.5	8	13.8	99	16.2
brown	64	14.1	101	23.0	165	18.5	133	24.1	23	39.7	156	25.6
na	0		0		0		4		0		4	
Hair colour												
red	74	16.4	31	7.1	105	11.8	33	6.0	2	3.4	35	5.8
blonde	92	20.4	83	18.9	175	19.6	239	43.8	23	39.7	262	43.4
brown	269	59.5	308	70.2	577	64.8	242	44.3	28	48.3	270	44.7
black	17	3.8	17	3.9	34	3.8	32	5.9	5	8.6	37	6.1
na	2				2		10		0		10	
Naevus count*												
0-10	110	24.3	278	63.3	388	43.5	134	24.2	16	27.6	150	24.5
11-30	170	37.5	106	24.1	276	30.9	198	35.7	19	32.8	217	35.5
31-50	79	17.4	29	6.6	108	12.1	105	19.0	10	17.2	115	18.8
51-100	66	14.6	21	4.8	87	9.8	79	14.3	8	13.8	87	14.2
>100	28	6.2	5	1.1	33	3.7	38	6.9	5	8.6	43	7.0
na	1				1		2		0		2	
Family history of melanoma												
yes**	168	37.3	110	25.1	278	31.3	29	5.4	0	0	29	4.9
no	283	62.7	328	74.9	611	68.7	508	94.6	58	100	566	95.1
na	3		1		4		19		0		19	

****Naevi were recorded from 5mm size in Brisbane and from 3mm size in Tübingen

**(1st degree relatives)

6.4.4. Association Analyses

Association analyses were performed including the whole cohorts – melanoma patients and controls – from both countries independently. The analysed SNPs were selected in accordance to their frequency, excluding the very rare variants. This resulted in one exon SNP at the gene *PPARGC1A* and 3 exon SNPs at the gene *PPARGC1B* which were analysed individually in regard to an association with pigment traits, naevus count and melanoma risk. Additionally, the TaqMan genotype data for the intronic SNP rs251468 and the intronic SNP rs32579 were included into the analyses based on two recent publications[238], [Duffy et al., in submission]. The variables of interest were tanning capacity, naevus count and melanoma risk.

The results for the 6 assessed SNPs and their influence on the tanning response in each of the two cohorts are presented in Table 28. A significant increase (estimate 0.64, 95%CI 0.10 - 1.18, p=0.02) of the tanning ability was calculated for individuals from Brisbane having two minor alleles of the intronic SNP rs251468 of *PPARGC1B*. Heterozygous individuals still had a positive estimate for tanning, but this was not significant. A trend for an increased tanning ability was likewise found for the homozygous individuals from Tübingen with overlapping confidence intervals supporting this finding. In contrast a trend towards an impaired tanning ability for individuals with a minor allele at rs11959820 (R253S) was found for both cohorts.

Table 28: Analyses with the selected SNPs of *PPARGC1A* and *PPARGC1B* and the impact on the tanning response in both cohorts using an ordinal regression model

Gene **rs number** Amino acid position and change*	**Cohort**	**Genotype**	**Individuals (n)**	**Estimate (95%CI)**	**P-value**
PPARGC1A **rs3736265** T612M	Brisbane	G/G A/G A/A missing	789 90 5 9	0 (Reference) 0.31 (-0.12 - 0.74) 0.25 (-1.48 - 1.97)	 0.15 0.78
	Tübingen	G/G A/G A/A missing	529 76 3 6	0 (Reference) -0.38 (-0.91 - 0.16) -2.38 (-4.79 - 0.03)	 0.17 0.05
PPARGC1B **rs7732671** A203P, A164P, A139P	Brisbane	C/C C/G G/G missing	741 137 7 8	0 (Reference) -0.03 (-0.38 - 0.33) -0.87 (-2.37 - 0.62)	 0.89 0.25
	Tübingen	C/C C/G G/G missing	516 86 6 6	0 (Reference) 0.31 (-0.22 - 0.83) -0.99 (-2.62 - 0.65)	 0.25 0.24
PPARGC1B **rs17572019** V279I, V240I, V215I	Brisbane	G/G A/G A/A missing	743 135 7 8	0 (Reference) -0.04 (-0.40 - 0.31) -0.87 (-2.37 - 0.62)	 0.81 0.25
	Tübingen	G/G A/G A/A missing	516 86 6 6	0 (Reference) 0.31 (-0.22 - 0.83) -0.99 (-2.62 - 0.65)	 0.25 0.24
PPARGC1B **rs11959820** R292S, R253S, R228S	Brisbane	C/C C/A A/A missing	833 51 1 8	0 (Reference) **-0.53 (-1.01 - 0.02)** 0.91 (-3.02 - 4.83)	 0.06 0.65
	Tübingen	C/C C/A A/A missing	566 42 0 6	0 (Reference) **-0.11 (-0.81 - 0.59)**	 0.75
PPARGC1B **rs251468** Intronic SNP	Brisbane	G/G A/G A/A missing	551 250 58 34	0 (Reference) 0.19 (-0.10 - 0.48) **0.64 (0.10 - 1.18)**	 0.21 **0.02**
	Tübingen	G/G A/G A/A missing	389 180 38 7	0 (Reference) -1.11 (-0.51 - 0.28) **0.57 (-0.19 - 1.32)**	 0.58 **0.14**
PPARGC1B **rs32579** Intronic SNP	Brisbane	G/G A/G A/A missing	470 339 74 8	0 (Reference) 0.31 (-0.17 - 0.80) 0.07 (-0.21 - 0.34)	 0.20 0.63
	Tübingen	G/G A/G A/A missing	336 223 49 6	0 (Reference) -0.05 (-0.43 - 0.33) 0.53 (-0.15 - 1.22)	 0.80 0.13

*3 different transcripts of PGC-1b

The association analyses for total body naevus count are presented in Table 29. The coding SNP rs3736265 (T612M) of the gene *PPARGC1A* was significantly associated with naevus count in the cohort of Brisbane. Individuals with one minor allele had a calculated reduction of naevus counts with an estimate of -0.61 (95%CI -1.03 - -0.18, p=0.005). Although this was not confirmed in the cohort of Tübingen the confidence intervals did still overlap. The association of this SNP with naevus count is illustrated in Figure 42A, showing the high prevalence of minor alleles in individuals with a low naevus count in the Brisbane cohort. Likewise, individuals from Tübingen had higher MAFs within the low naevus count categories, however individuals with more than 100 naevi had again a high MAF. The highest naevus count category (>100 naevi, 3mm Tübingen, 5mm Brisbane) included only 43 individuals in Tübingen and 33 individuals in Brisbane while the other categories included a higher number of individuals with a lower chance of outliers. The previously detected SNP rs11959820 (R253S) of the gene PPARGC1B, assessed with Sanger Sequencing in a smaller cohort with initially significant results, did still show a trend towards an increased naevus count in the cohort of Brisbane. However, this finding was not reproducible in the cohort of Tübingen. Figure 42B is illustrating the increase of the MAF with increasing naevus count in the cohort of Brisbane. The intronic SNP rs32579 in *PPARGC1B* showed a significant reduction of naevus counts for heterozygous individuals in the cohort of Tübingen (estimate -0.48, 95%CI -0.78 - -0.17, p=0.002) with a similar trend for the cohort of Brisbane. This association is illustrated in Figure 42C.

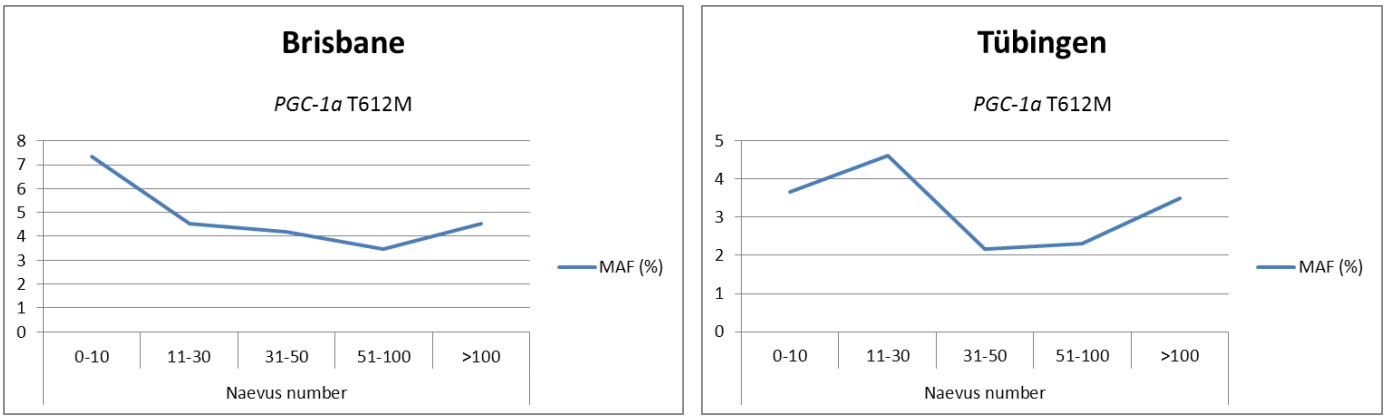
The potential influence on melanoma risk was assessed for the 6 SNPs individually and presented in Table 30. Individuals with a minor allele at SNP rs3736265 (T612M) of the gene *PPARGC1A* had a trend towards a lower risk of melanoma. The result was significant for homozygous individuals from Tübingen, albeit this was based on only 3 cases. However, the risk reduction was congruent for both cohorts in heterozygous and homozygous individuals each with positive estimates. The other assessed SNPs revealed no influence on melanoma risk.

Table 29: Analyses with the selected SNPs of *PPARGC1A* and *PPARGC1B* and the impact on the total body naevus count in both cohorts using an ordinal regression model

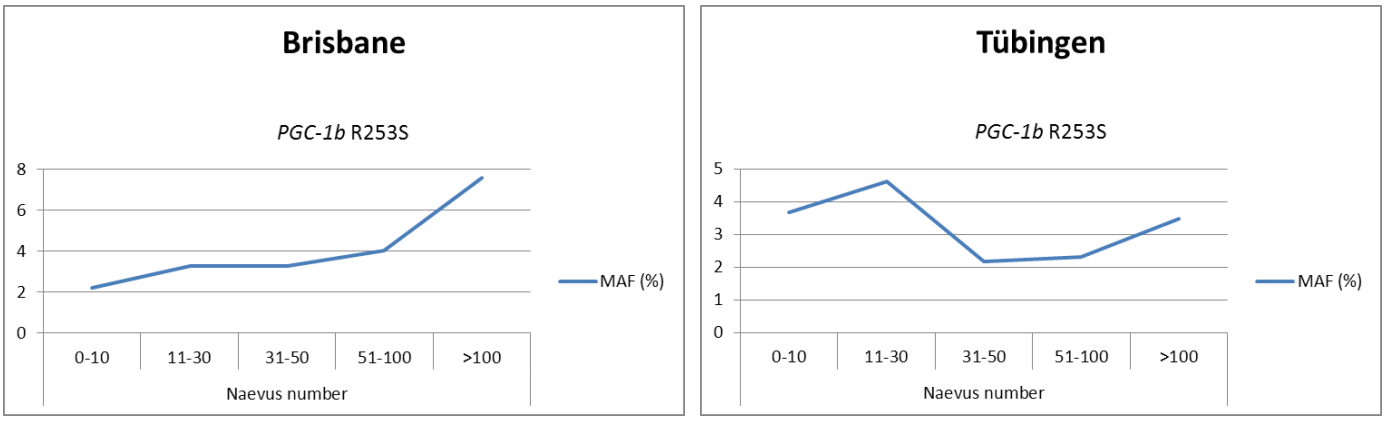
Gene **rs number** Amino acid position and change*	**Cohort**	**Genotype**	**Individuals (n)**	**Estimate (95%CI)**	**P-value**
PPARGC1A **rs3736265** T612M	Brisbane	G/G A/G A/A missing	796 90 5 2	0 (Reference) **-0.61 (-1.03 - -0.18)** -0.28 (-1.92 - 1.37)	 **0.005** 0.74
	Tübingen	G/G A/G A/A missing	533 76 3 2	0 (Reference) **0.02 (-0.41 – 0.45)** 1.60 (-0.43 – 3.63)	 **0.94** 0.12
PPARGC1B **rs7732671** A203P, A164P, A139P	Brisbane	C/C C/G G/G missing	747 138 7 1	0 (Reference) 0.01 (-0.33 – 0.34) 1.15 (-0.17-2.48)	 0.96 0.09
	Tübingen	C/C C/G G/G missing	520 86 6 2	0 (Reference) -0.29 (-0.70 – 0.13) -1.46 (-3.03 – 0.11)	 0.18 0.07
PPARGC1B **rs17572019** V279I, V240I, V215I	Brisbane	G/G A/G A/A missing	749 136 7 1	0 (Reference) 0.01 (-0.33 – 0.35) 1.15 (-0.17 – 2.48)	 0.96 0.09
	Tübingen	G/G A/G A/A missing	520 86 6	0 (Reference) -0.29 (-0.70 -0.13) -1.46 (-3.03 – 0.11)	 0.18 0.07
PPARGC1B **rs11959820** R292S, R253S, R228S	Brisbane	C/C C/A A/A missing	839 52 1 1	0 (Reference) 0.45 (-0.06 – 0.96)	 0.08
	Tübingen	C/C C/A A/A missing	569 43 0 2	0 (Reference) -0.30 (-0.86 – 0.26)	 0.29
PPARGC1B **rs251468** Intronic SNP	Brisbane	G/G A/G A/A missing	554 250 60 29	0 (Reference) -0.10 (-0.38 - 0.18) -0.20 (-0.70 - 0.29)	 0.48 0.42
	Tübingen	G/G A/G A/A missing	391 182 38 3	0 (Reference) -0.26 (-0.58 – 0.06) -0.10 (-0.70 – 0.50)	 0.11 0.74
PPARGC1B **rs32579** Intronic SNP	Brisbane	G/G A/G A/A missing	475 339 76	0 (Reference) **-0.02 (-0.28 – 0-23)** -0.13 (-0.58 – 0.32)	 **0.86** 0.56
	Tübingen	G/G A/G A/A missing	338 225 49 2	0 (Reference) **-0.48 (-0.78 - -0.17)** 0.04 (-0.50 – 0.58)	 **0.002** 0.88

*3 different transcripts of PGC-1b

A



B



C

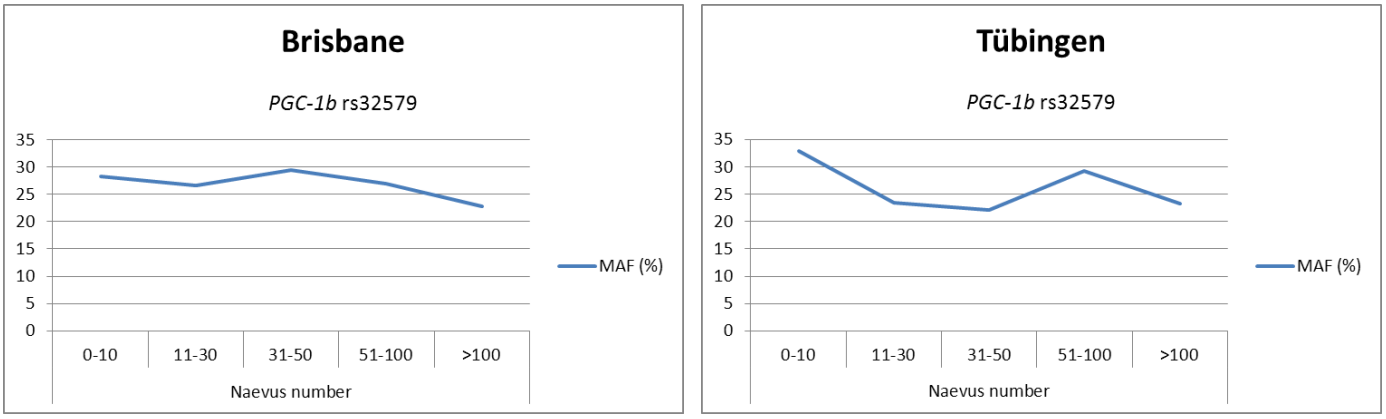


Figure 42: Analyses of the minor allele frequency of three different SNPs in individuals divided in 5 naevus count categories of the two cohorts of Brisbane and Tübingen. **A** rs3736265 (T612M) **B** rs11959820 (R253S) and **C** rs32579 (intronic variant)

Table 30: Analyses with the selected SNPs of *PPARGC1A* and *PPARGC1B* and the impact on melanoma risk in both cohorts using an ordinal regression model

Gene **rs number** Amino acid position and change*	**Cohort**	**Genotype**	**Individuals (n)**	**Estimate (95%CI)**	**P-value**
PPARGC1A **rs3736265** T612M	Brisbane	G/G A/G A/A missing	797 90 5 1	0 (Reference) **0.44 (-0.01 – 0.88)** **0.48 (-1.31 - 2.28)**	 **0.05** **0.60**
	Tübingen	G/G A/G A/A missing	535 76 3 0	0 (Reference) 0.01 (-0.83 – 0.84) **2.99 (0.57 – 5.41)**	 0.99 **0.02**
PPARGC1B **rs7732671** A203P, A164P, A139P	Brisbane	C/C C/G G/G missing	747 139 7 0	0 (Reference) 0.09 (-0.27 – 0.45) -0.24 (-1.75 – 1.26)	 0.63 0.75
	Tübingen	C/C C/G G/G missing	522 86 6 0	0 (Reference) -0.39 (-1.27 – 0.49)	 0.39
PPARGC1B **rs17572019** V279I, V240I, V215I	Brisbane	G/G A/G A/A missing	749 137 7	0 (Reference) 0.09 (-0.28 – 0.45) -0.24 (-1.75 – 1.26)	 0.63 0.75
	Tübingen	G/G A/G A/A missing	522 86 6 0	0 (Reference) -0.39 (-1.27 – 0.49)	 0.39
PPARGC1B **rs11959820** R292S, R253S, R228S	Brisbane	C/C C/A A/A missing	840 52 1 0	0 (Reference) 0.03 (-0.53 – 0.59)	 0.91
	Tübingen	C/C C/A A/A missing	571 43 0 0	0 (Reference) -0.02 (-1.06 – 1.05)	 0.97
PPARGC1B **rs251468** Intronic SNP	Brisbane	G/G A/G A/A missing	555 250 60 28	0 (Reference) 0.19 (-0.11 – 0.49) 0.02 (-0.51 – 0.56)	 0.22 0.93
	Tübingen	G/G A/G A/A missing	393 182 38	0 (Reference) 0.03 (-0.57 – 0.62) -0.66 (-2.12 – 0.81)	 0.93 0.38
PPARGC1B **rs32579** Intronic SNP	Brisbane	G/G A/G A/A missing	475 340 76 2	0 (Reference) 0.05 (-0.23 – 0.33) 0.12 (-0.37 – 0.60)	 0.72 0.64
	Tübingen	G/G A/G A/A missing	475 340 76 2	0 (Reference) 0.06 (-0.50 – 0.62) -0.93 (-2.39 – 0.53)	 0.84 0.21

*3 different transcripts of PGC-1b

6.4.5. Survival Analyses

Survival data were available for the cohort from Tübingen, but not for the Brisbane cohort. Survival analyses based on the single exon SNPs failed to reach validity due to the low overall incidence of variants with only single cases among patients who died from melanoma. Survival analyses considering the presence of any variant at the gene *PPARGC1A* as well as for the gene *PPARGC1B* were performed. However, no significant survival difference between patients with a variant and patients having a wild type at the genes *PPARGC1A* and *PPARGC1B* were detected (Figure not shown).

Recently Li et al. reported an improved survival probability for melanoma patients who were carriers of the minor allele A at rs32579 of the gene *PPARGC1B*. This was investigated in a cohort of 858 melanoma patients from the United States with a total number of 95 melanoma specific deaths. The Hazard rate for melanoma specific death was decreased to 0.64 in the additive model with a p-value of 0.013.

The respective SNP rs32579 was identified within the SNP dataset obtained as tagging SNP from the Illumina CoreExome Chip assay. The survival probability was analysed with the data from the Tübingen cohort using the additive model. A point estimate of 0.84 was calculated indicating a risk reduction for patients with a variant at rs32579. However, this was not significant, the 95% confidence interval ranged from 0.57 to 1.26.

The data of the present study and the data published by Li et al. were used for a meta-analysis performed with R. The test for heterogeneity resulted in a p-value of 0.454, indicating consistency between the two studies. Together the two studies showed a significant result with a summary effect of 0.73 and a 95% confidence interval of 0.47 – 0.99 (p= 6.11e-08). The result is summarized in a Forest plot in Figure 43.

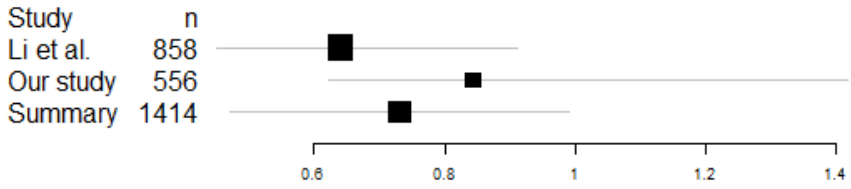


Figure 43: Forest plot of the Hazard Ratios of two studies investigating the impact of *PPARGC1B* rs32579 on melanoma survival.

6.5. Discussion

The presented analyses were based on a large set of SNPs located predominantly in the exons of two genes coding for two proteins, both belonging to one protein family of transcription factors, the peroxisome proliferator-activated receptor gamma coactivator. An interaction of these proteins with pigmentation pathways has been demonstrated[181] as well as an influence in melanoma cells and the development of metastases in mice.[188,237] Based on these findings a positive association of germline variants in the respective genes with pigmentation and melanoma risk and prognosis was assumed. The present analyses focused on exon SNPs which are variants in coding regions. Variants in coding regions result in an altered amino-acid sequence of the respective protein with an optional change of the tertiary protein structure and a potential altered protein function. The assessed variants were previously published and were overall relatively rare in the general population. The effect of these variants on the protein function is unknown. It is interesting to note that all selected exon variants of *PPARGC1B* were located in close proximity at exon 5, assuming a functional relevance of this region. None of the exon variants had an influence on melanoma survival in the investigated cohort of Tübingen (survival data is not yet available for the Brisbane cohort) with the limitation of low MAF of the exon SNPs resulting in only single events among the minor allele carriers. Two exon SNPs of *PPARGC1B* had an influence on pigmentation and naevus count. The SNP rs45520937 (Arg226Gln) was associated with increased pigmentation and tanning ability in individuals from Brisbane. The results were based on Sanger sequencing and were not available for the German cohort. The SNP rs11959820 (R253S) showed in contrast a trend towards an impaired tanning ability for minor allele carriers in Tübingen and Brisbane and a trend towards increased naevus numbers in the Brisbane cohort. However, this was not confirmed in the German cohort. The contrary effect on tanning of this two adjacent SNPs and the lack of confirmation in the second cohort require further studies and these preliminary findings have to be considered with caution.

A rather robust finding was the impact of the *PPARGC1A* SNP rs3736265 (T612M) on naevus count with a significant reduction of naevus counts for minor allele carriers in the Brisbane cohort. This was confirmed by a similar trend in the cohort from Tübingen. A decreased risk for melanoma was likewise observed for both cohorts. The risk reduction was significant for homozygous patients from Tübingen.

Variants within intron regions are more likely to influence protein expression levels. Therefore, an intronic variant in a transcription factor might be even more likely associated with an altered phenotype compared to variants in coding regions. Two intronic SNPs of *PPARGC1B* have been investigated within the present analyses. Both SNPs, rs251468 and rs32579 were associated with increased tanning and decreased naevus count. The results reached significance for rs251468 and tanning in the Brisbane cohort and for rs32579 and naevus count in the Tübingen cohort. The results were in accordance with the published data.[182, Duffy et al. in submission] Additionally a significant impact on melanoma survival with improved survival probabilities for minor allele carriers was demonstrated for rs3257 using the data of the cohort Tübingen and the recently published data in a meta-analysis.

6.6. Conclusions

The present analyses of this large SNP set predominantly located in exons of the two genes *PPARGC1A* and *PPARGC1B* added further evidence for the influence of the transcription factors PGC-1a and PGC-1b on pigmentation, naevus count and melanoma risk and progression. The exon SNP rs3736265 (T612M) of *PPARGC1A* was found to reduce naevus count and melanoma risk. The effects of rs45520937 (Arg226Gln) and rs11959820 (R253S) on tanning and naevus count still have to be confirmed with a larger sample size or in another cohort. The meta-analyses of the present data with data from another group confirmed the reported survival benefit for the intronic SNP rs32579 in *PPARGC1B* and demonstrated how smaller effects can be substantiated by collaborative analyses. Therefore, genetic association analyses should be undertaken in a collaborative way to avoid the publication of false positive or false negative findings derived from small sample sizes.

7. Summary of Findings

The research of the presented work started with a personal observation as a physician working with metastatic melanoma patients: Few metastatic patients seemed to have multiple Naevi. The initial question was: Do metastatic melanoma patients represent a distinct subpopulation in the cohort of melanoma patients?

The field was opened towards four areas: The exploration of dermoscopic naevus pattern in melanoma patients, the comparison of early and late melanoma patients, the comparison of melanoma patients from Brisbane versus Tübingen and genetic association studies with *PPARGC1A* and *PPARGC1B* variants.

Dermoscopy was used to classify the naevus pattern in the patients of the pilot study. The occurrence of an individual dominant pattern was reported previously.[199,239] An individual dominant pattern was found likewise in most of the melanoma patients of the pilot study. However, a recurrent pattern among the melanoma patients was not detectable and further explorations of naevus pattern in melanoma patients were not pursued.

Differences between early and late stage melanoma patients were explored in a well described cohort of 556 patients from Tübingen. These patients were recruited between 2007 and 2011 in a University hospital outpatient setting. The main variable of interest, the total body naevus count was not different between early and late stage patients. This finding was contrasted by a reported survival benefit of patients with high naevus numbers.[59] Beside statistical concerns the results were restricted by a possible amount of screen-detected indolent melanomas.[60] Those patients were not included in the present analysed cohort. Further exploration of phenotype variables, tumour specific and genetic markers revealed a survival impact for the variables Skin type (p=0.031, dichotomized Skin type I/II versus III/IV), Tumour thickness (p=0.040, pairwise comparison), Ulceration (p=0.001), Unknown primary (p=0.008) and the presence of a MC1R r allele (p=0.049). A genome-wide association study enabled the detection of four potentially relevant SNPs associated with survival probability. Three of these SNPs, rs7551288, rs12146110 and rs1007271 are located on chromosome 1, the SNP rs1993585 on chromosome 4. The SNP rs7551288 is an intronic variant of *DHCR24*. The protein DHCR24 was previously reported to impede oxidative stress-induced apoptosis and to be upregulated in melanoma metastases.[212] The SNP rs12146110 is an intronic variant of the gene

coding for USH2A, a protein associated with the Usher syndrome.[209] Both SNPs had a consistent influence on survival in Stage IV as well and might serve as potential predictors for melanoma progression.

Two melanoma cohorts from two regions with fair skinned inhabitants, Queensland, Australia and Southern Germany were investigated. Individuals in Australia are exposed to a higher cumulative UV exposure, and the melanoma incidence and mortality rates are higher compared to Western Europe.[3] The two analysed cohorts were both recruited in a hospital outpatient setting and were well balanced according to age and gender. Phenotypes and genotypes showed characteristic features in relation to the ethnical differences. The melanoma patients from Brisbane had a higher frequency of prognostic favourable tumour characteristics, such as a lower tumour thickness, a high proportion of superficial spreading melanomas, less ulcerated primaries, more frequently a histopathological regression of the primary and a higher rate of naevus associated melanomas compared to the patients from Tübingen. This remained statistical significant after the exclusion of in situ cases from the Brisbane cohort. Furthermore, significantly more patients from Brisbane had a positive family history of melanoma ($p<0.001$) and significantly more patients had multiple melanomas ($p<0.001$) compared to patients from Tübingen.

Genetic association analyses were performed with a set of SNPs located predominantly in exons of PPARGC1A and PPARGC1B. The frequency of PPARGC1A variants was higher among the individuals from Tübingen compared to those from Brisbane, while the PPARGC1B variants were found in a similar frequency in both cohorts. The data revealed a Linkage Disequilibrium for rs7732671 (Ala203Pro) and rs17572019 (Val240Ile). The exon SNP rs3736265 (T612M) of *PPARGC1A* was associated with decreased total body naevus count (estimate -0.61, 95% CI -1.03 - -0.18, p=0.005) and decreased melanoma risk (estimate 2.99, 95% CI 0.57 – 5.41, p=0.02). Furthermore, a significant association of the intronic SNP rs32579 with melanoma survival (p= 6.11e-08) was found based on a meta-analysis with previously published results in a similar cohort. The previously reported associations of both intronic SNPs rs251468 and rs32579 in PPARGC1B with increased tanning ability and decreased naevus count were confirmed.

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8. Final Conclusion

Dermoscopic analyses of naevi from melanoma patients revealed no distinctive pattern and, the detection of a specific naevus pattern in a patient is in turn not feasible for a risk classification. Total body naevus count did not qualify as a prognostic marker in melanoma patients. The genes *DHCR24 and USH2A* will need further investigation and might serve as new marker for melanoma progression. Variants of *PPARGC1A* and *PPARGC1B* have been shown to influence tanning, naevus count, melanoma risk and melanoma progression. Further exploration of other intronic variants within these genes might reveal additional significant associations. Primary melanomas with favourable prognostic characteristics seem to be more common among patients living in an UV intense country with a higher cumulative sun exposure. However, these higher rates of initially less aggressive primaries vanish among the extreme high incidence rates with the world highest melanoma mortality rates in Australia and New Zealand. The depletion and recent healing in the Antarctic ozone layer with altered UV protection levels should be considered in melanoma incidence rate discussions.

In summary, genotypes have an influence on melanoma risk and progression. However, the relevance of genetic variations is low compared to the impact of UV radiation on melanoma. This should urge the efforts towards UV protection, both individually and globally. Early detection and treatment can cure initially less aggressive UV induced melanomas. Both measures are important to reduce the number of melanoma deaths.

9. Funding, work load performed by the candidate and contributions

Funding

Project costs, materials

The DNA collection tubes for the pilot study were funded by the Department of Dermatooncology, Tübingen.

The Brisbane cohort study, Sample shipment costs for the German cohort, DNA extraction and Genotyping was funded in Brisbane by research grant NHMRC APP1062935

Support for the candidate

The candidate received a UQI tuition fee scholarship and a student living stipend for the period of two years in Brisbane with a total value of 24,000 Dollar per year.

The candidate was contracted in Tübingen as physician (100%) until September 2013 and from October 2013 until September 2015 as scientist (30%).

Work load performed by the candidate (AP)

Tübingen 2012-2013

One year prior to commencement in Australia the work involved the planning of the project, the application within the two PhD programs and establishment of the Joint PhD agreement together with the two main supervisors Claus Garbe and Peter Soyer. In 2013 AP wrote the protocol, patient information and consent sheets for the pilot study, obtained the positive ethic vote. AP recruited and investigated all patients of the pilot study. Furthermore, AP attended the required courses and lectures of the PhD program in Germany.

Brisbane 2013-2015

Campus St Lucia, IMB

AP spent the first year mainly at the laboratory at IMB working with the pilot study samples and learning laboratory skills (DNA extraction, PCR, nested PCR, gel extraction, sequence analyses) from Kasturee Jagirdar under the supervision of Rick Sturm. A set of approx. 200 samples of the Brisbane Naevus study were genotyped by AP at the PGC-1b locus, including PCR, Gel extraction, Sequencing reaction for Sanger Sequencing and the variant analyses with the

Sequencher® software. A previously analysed set of approx. 200 patients at the *MC1R* locus was analysed with the Sequencher® software by AP as quality control.

Campus Princess Alexandra Hospital, TRI

AP took part in patient inclusion at the clinical research centre for the Brisbane Naevus study, involving full body examination and imaging with Fotofinder and dermoscopic naevi classification under the supervision of Peter Soyer. The first annual report with preliminary results from the pilot study and PGC-1a association analyses took place in May 2014 in Tübingen. The available material was rated as not sufficient. The decision was made to provide more than 700 blood samples from Tübingen for the project. AP obtained the required ethic vote and organised the shipment to Australia. The DNA extractions and sample preparations were performed by AP together with Hilary Young, a research assistant, with a share of 50% each. The blood related questionnaires were provided from Tübingen via electronic scans and were recorded by AP into SPSS sheets. Preliminary association analyses were performed by AP under the supervision of David Duffy. Survival analyses were performed by AP. Initial results were presented by AP in May 2015 at the Annual Meeting of the Australasian Society for Dermatology Research in Adelaide.

Tübingen 2015-2017

AP re-located to Tübingen in August 2015. AP retrieved previously missing questionnaire files in Germany and completed the German dataset. Data of the Brisbane cohort were supplied from Brisbane in excel sheets via email and vpn. AP transferred all data into SPSS files, performed quality assessments and data clearing. AP conducted the statistical analyses with SPSS based on advice from David Duffy in regard to the appropriate statistical models. AP attended the required courses and lectures of the PhD program in Germany. The writing of the thesis was done by AP under the supervision of Claus Garbe. A presentation of the work is scheduled in October 2017 at the World Congress of Melanoma in Brisbane.

Contributions

University Tübingen	
Prof. Dr. med. Claus Garbe	Supervisor, initiation of Joint PhD arrangement
Prof. Dr. med. Olaf Rieß	PhD committee member
PD Dr. med. Thomas Eigentler	Initiation of the study "Hereditary defects and cutaneous malignant melanoma"
Prof. Dr. med. Benjamin Weide	Establishment of biomarker databank in Tübingen, shipment of samples, advise survival analyses
Ulrike Keim, M.Sc.	Design of survival plots, statistical advise
Prof. Dr. med. Ulrike Leiter	Statistical advise
Katrin Schmidt, M.Sc. and co-workers	Scanning and sending of study questionnaires
Waltraud Rossmann	Data entry German Melanoma Registry
Colleagues Hautklinik Tübingen	Inclusion of study participants into the study "Hereditary defects and cutaneous malignant melanoma"
University Queensland	
Prof. Dr. med. H. Peter Soyer	Supervisor, initiation of Joint PhD arrangement
A/Prof. Rick Sturm	Supervisor, teaching and guidance
A/Prof. David Duffy	Co-Supervisor, statistical analyses and advise
Kasturee Jagirdar, M.Sc	Genotyping Brisbane cohort, lab work advise
Dr. Aaron Smith PhD	Primer design PGC-1b, PhD committee member
Prof. Dr. med. Nikolas Haass	PhD committee chair
Hilary Yong, M.Sc	Assistance with the DNA extractions of the German cohort, TaqMan assays
Katie Lee, B.Sc. (Hons)	Study documentation BNMS, sending of additional information e.g. pathology results
Colleagues TRI/IMB	Inclusion of study participants into the BNMS and documentation

10. Abbreviations

AEGRC	Australian Equine Genetics Research Centre
AJCC	American Joint Committee on Cancer
ALM	Acral lentiginous melanoma
ASIP	Agouti signalling protein
BAP1	encodes BRCA1 associated protein-1
BMI	Body mass index
CDK4	Cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase Inhibitor 2A
CRP	C-reactive protein
csv	comma separated values
dbSNP	Single Nucleotide Polymorphism Database
DCT	tyrosinase-related protein 2/dopachrome tautomerase
DHCR24	24-Dehydrocholesterol Reductase
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleoside triphosphate
EDTA	Ethylendiamintetraacetat
ERRs	estrogen-related receptors
GenoMEL	the Melanoma Genetics Consortium
GNAQ	G protein alpha-subunit
GWAS	Genome-wide association study
HUMARA	human androgen receptor
IMB	Institute for Molecular Bioscience
IRF4	interferon regulatory factor 4
KC	keratinocyte cancers
LDH	Lactate dehydrogenase
LMM	Lentigo maligna melanoma
MAF	Minor allele frequency
MC1R	melanocortin-1 receptor
MIA	melanoma inhibitory activity protein
MITF	microphthalmia transcription factor
MPM	multiple primary melanomas
na	not available
NM	Nodular melanoma

NRF1	nuclear respiratory factor 1
OCA2	oculocutaneous albinism type II
PCR	Polymerase chain reaction
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator
POT1	encodes Protection of telomeres protein 1
POT1	protection of telomeres 1 gene
PRC	PGC-1 related coactivator
Rb	retinoblastoma
ROS	reactive oxygen species
rs number	reference SNP ID number
SLC24A4	solute carrier family 24
SLC45A2	solute carrier family 45, member 2
SNP	single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SSM	Superficial spreading melanoma
Stata	Statistics and data
TE buffer	Tris-EDTA buffer
TNM	tumour, nodes, metastasis
TRI	Translational Research Institute
TYR	tyrosinase
TYRP1	tyrosinase-related protein-1
USH2A	Usherin
UQCCG	UQ Centre for Clinical Genomics
UV	ultraviolet
VPN	virtual private network
VT	variant-type
WT	wild-type
α MSH	α-melanocyte-stimulating hormone

11. Tables

12. Figures

13. References

1 Schadendorf D, Fisher DE, Garbe C *et al.* Melanoma. *Nature reviews. Disease primers* 2015; **1**: 15003.

2 Burnet NG, Jefferies SJ, Benson RJ *et al.* Years of life lost (YLL) from cancer is an important measure of population burden--and should be considered when allocating research funds. *British journal of cancer* 2005; **92**: 241-5.

3 Karimkhani C, Green AC, Nijsten T *et al.* The global burden of melanoma: results from the Global Burden of Disease Study 2015. *The British journal of dermatology* 2017.

4 Clark WH, Jr., From L, Bernardino EA *et al.* The histogenesis and biologic behavior of primary human malignant melanomas of the skin. *Cancer research* 1969; **29**: 705-27.

5 De Schweinitz GE, Shumway EA. Concerning melanoma of the choroid with report of one case of this character and of another exhibiting a pigmented sarcoma of the choroid early in its development. *Transactions of the American Ophthalmological Society* 1905; **10**: 439-51.

6 Duval CW. MELANOMA OF VATER'S DIVERTICULUM AND LOWER PORTION OF COMMON BILE DUCT CAUSING COMPLETE OBSTRUCTION. *The Journal of experimental medicine* 1908; **10**: 465-75.

7 Wigley JE. Naevocarcinoma (Malignant Melanoma). *Proceedings of the Royal Society of Medicine* 1943; **36**: 281.

8 Cherney LS. Nature of the Melanoma. *California and western medicine* 1940; **52**: 155-6.

9 Laidlaw GF, Murray MR. Melanoma Studies: III. A Theory of Pigmented Moles. Their Relation to the Evolution of Hair Follicles. *The American journal of pathology* 1933; **9**: 827-38.7.

10 Moore RF. Melanoma of the Choroid. *Proceedings of the Royal Society of Medicine* 1919; **12**: 60-1.

11 Pfingst AO. Melanosarcoma (Melanoma) of the Choroid Occurring in Brothers. *Transactions of the American Ophthalmological Society* 1921; **19**: 195-204.

12 Neame H. Melanoma of Conjunctiva. *Proceedings of the Royal Society of Medicine* 1927; **20**: 1114.

13 Taussig LR, Torrey FA. Malignant Melanoma: A Statistical and Pathological Review of Thirty-Five Cases. *California and western medicine* 1940; **52**: 15-8.

14 De Cholnoky T. MALIGNANT MELANOMA: A CLINICAL STUDY OF ONE HUNDRED SEVENTEEN CASES. *Annals of surgery* 1941; **113**: 392-410.

15 Pack GT, Perzik SL, Scharnagel IM. THE TREATMENT OF MALIGNANT MELANOMA-REPORT OF 862 CASES. *California medicine* 1947; **66**: 283-7.

16 Lancaster HO, Nelson J. Sunlight as a cause of melanoma; a clinical survey. *The Medical journal of Australia* 1957; **44**: 452-6.

17 Ruttan H. Sunlight and Malignant Melanoma. *Canadian Medical Association journal* 1957; **77**: 136-7.

18 Clark WH, Jr., Reimer RR, Greene M *et al.* Origin of familial malignant melanomas from heritable melanocytic lesions. 'The B-K mole syndrome'. *Archives of dermatology* 1978; **114**: 732-8.

19 Lynch HT, Frichot BC, 3rd, Lynch JF. Familial atypical multiple mole-melanoma syndrome. *Journal of medical genetics* 1978; **15**: 352-6.

20 Whiteman DC, Green AC, Olsen CM. The Growing Burden of Invasive Melanoma: Projections of Incidence Rates and Numbers of New Cases in Six Susceptible Populations through 2031. *The Journal of investigative dermatology* 2016; **136**: 1161-71.

21 Garbe C, Orfanos CE. [Epidemiology of malignant melanoma in West Germany in an international comparison]. *Onkologie* 1989; **12**: 253-62.

22 Katalinic A, Eisemann N, Waldmann A. Skin Cancer Screening in Germany. Documenting Melanoma Incidence and Mortality From 2008 to 2013. *Deutsches Arzteblatt international* 2015; **112**: 629-34.

23 Erdmann F, Lortet-Tieulent J, Schuz J *et al.* International trends in the incidence of malignant melanoma 1953-2008--are recent generations at higher or lower risk? *International journal of cancer* 2013; **132**: 385-400.

24 Sneyd MJ, Cox B. A comparison of trends in melanoma mortality in New Zealand and Australia: the two countries with the highest melanoma incidence and mortality in the world. *BMC cancer* 2013; **13**: 372.

25 Iannacone MR, Youlden DR, Baade PD *et al.* Melanoma incidence trends and survival in adolescents and young adults in Queensland, Australia. *International journal of cancer* 2015; **136**: 603-9.

26 Czarnecki D. The Relentless Rise in the Incidence of Melanoma in Susceptible Australians. *The Journal of investigative dermatology* 2016; **136**: 1912-3.

27 Baade PD, Youlden DR, Youl P *et al.* Assessment of the effect of migration on melanoma incidence trends in Australia between 1982 and 2010 among people under 30. *Acta dermato-venereologica* 2015; **95**: 118-20.

28 Wallingford SC, Iannacone MR, Youlden DR *et al.* Comparison of melanoma incidence and trends among youth under 25 years in Australia and England, 1990-2010. *International journal of cancer* 2015; **137**: 2227-33.

29 Bauer J, Garbe C. Acquired melanocytic nevi as risk factor for melanoma development. A comprehensive review of epidemiological data. *Pigment cell research* 2003; **16**: 297-306.

30 Holman CD, Armstrong BK. Pigmentary traits, ethnic origin, benign nevi, and family history as risk factors for cutaneous malignant melanoma. *Journal of the National Cancer Institute* 1984; **72**: 257-66.

31 Holly EA, Kelly JW, Shpall SN *et al.* Number of melanocytic nevi as a major risk factor for malignant melanoma. *Journal of the American Academy of Dermatology* 1987; **17**: 459-68.

32 Green A, MacLennan R, Siskind V. Common acquired naevi and the risk of malignant melanoma. *International journal of cancer* 1985; **35**: 297-300.

33 Grulich AE, Bataille V, Swerdlow AJ *et al.* Naevi and pigmentary characteristics as risk factors for melanoma in a high-risk population: a case-control study in New South Wales, Australia. *International journal of cancer* 1996; **67**: 485-91.

34 Swerdlow AJ, English J, MacKie RM *et al.* Benign melanocytic naevi as a risk factor for malignant melanoma. *British medical journal (Clinical research ed.)* 1986; **292**: 1555-9.

35 Bliss JM, Ford D, Swerdlow AJ *et al.* Risk of cutaneous melanoma associated with pigmentation characteristics and freckling: systematic overview of 10 case-control studies. The International Melanoma Analysis Group (IMAGE). *International journal of cancer* 1995; **62**: 367-76.

36 Davies JR, Chang YM, Bishop DT *et al.* Development and validation of a melanoma risk score based on pooled data from 16 case-control studies. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2015; **24**: 817-24.

37 Bianchini F, Kaaks R, Vainio H. Overweight, obesity, and cancer risk. *The Lancet. Oncology* 2002; **3**: 565-74.

38 Sergentanis TN, Antoniadis AG, Gogas HJ *et al.* Obesity and risk of malignant melanoma: a meta-analysis of cohort and case-control studies. *European journal of cancer (Oxford, England : 1990)* 2013; **49**: 642-57.

39 Praestegaard C, Kjaer SK, Christensen J *et al.* Obesity and risks for malignant melanoma and non-melanoma skin cancer: results from a large Danish prospective cohort study. *The Journal of investigative dermatology* 2015; **135**: 901-4.

40 Autier P, Dore JF. Influence of sun exposures during childhood and during adulthood on melanoma risk. EPIMEL and EORTC Melanoma Cooperative Group. European Organisation for Research and Treatment of Cancer. *International journal of cancer* 1998; **77**: 533-7.

41 Gallagher RP, Elwood JM, Threlfall WJ *et al.* Socioeconomic status, sunlight exposure, and risk of malignant melanoma: the Western Canada Melanoma Study. *Journal of the National Cancer Institute* 1987; **79**: 647-52.

42 Whiteman DC, Whiteman CA, Green AC. Childhood sun exposure as a risk factor for melanoma: a systematic review of epidemiologic studies. *Cancer causes & control : CCC* 2001; **12**: 69-82.

43 English JS, Swerdlow AJ. The risk of malignant melanoma, internal malignancy and mortality in xeroderma pigmentosum patients. *The British journal of dermatology* 1987; **117**: 457-61.

44 Whiteman DC, Stickley M, Watt P *et al.* Anatomic site, sun exposure, and risk of cutaneous melanoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2006; **24**: 3172-7.

45 McGregor JM, Yu CC, Dublin EA *et al.* Aberrant expression of p53 tumour-suppressor protein in non-melanoma skin cancer. *The British journal of dermatology* 1992; **127**: 463-9.

46 Mitra D, Luo X, Morgan A *et al.* An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. *Nature* 2012; **491**: 449-53.

47 Berger MF, Hodis E, Heffernan TP *et al.* Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature* 2012; **485**: 502-6.

48 Hodis E, Watson IR, Kryukov GV *et al.* A landscape of driver mutations in melanoma. *Cell* 2012; **150**: 251-63.

49 Krauthammer M, Kong Y, Bacchiocchi A *et al.* Exome sequencing identifies recurrent mutations in NF1 and RASopathy genes in sun-exposed melanomas. *Nature genetics* 2015; **47**: 996-1002.

50 Balch CM, Gershenwald JE, Soong SJ *et al.* Final version of 2009 AJCC melanoma staging and classification. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2009; **27**: 6199-206.

51 Gershenwald JE. Melanoma of the Skin. In: *AJCC Cancer Staging Manual* (al. MBAe, ed). 2017; 563-85.

52 Pflugfelder A, Kochs C, Blum A *et al.* Malignant melanoma S3-guideline "diagnosis, therapy and follow-up of melanoma". *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* 2013; **11 Suppl 6**: 1-116, 1-26.

53 Gualano MR, Osella-Abate S, Scaioli G *et al.* Prognostic role of Histologic regression in primary cutaneous melanoma: A Systematic Review and Meta-analysis. *The British journal of dermatology* 2017.

54 Scheibner A, Milton GW, McCarthy WH *et al.* Multiple primary melanoma - a review of 90 cases. *The Australasian journal of dermatology* 1982; **23**: 1-8.

55 Hwa C, Price LS, Belitskaya-Levy I *et al.* Single versus multiple primary melanomas: old questions and new answers. *Cancer* 2012; **118**: 4184-92.

56 Doubrovsky A, Menzies SW. Enhanced survival in patients with multiple primary melanoma. *Archives of dermatology* 2003; **139**: 1013-8.

57 Kricker A, Armstrong BK, Goumas C *et al.* Survival for patients with single and multiple primary melanomas: the genes, environment, and melanoma study. *JAMA dermatology* 2013; **149**: 921-7.

58 Weinstein D, Leininger J, Hamby C *et al.* Diagnostic and prognostic biomarkers in melanoma. *The Journal of clinical and aesthetic dermatology* 2014; **7**: 13-24.

59 Ribero S, Davies JR, Requena C *et al.* High nevus counts confer a favorable prognosis in melanoma patients. *International journal of cancer* 2015; **137**: 1691-8.

60 Autier P, Funck-Brentano E, Aegerter P *et al.* Re: High nevus counts confer a favorable prognosis in melanoma patients by S ribero and co-workers, published in the International Journal of Cancer, 2015 (online 21 march 2015). *International journal of cancer* 2015; **137**: 3006-7.

61 Joosse A, de Vries E, Eckel R *et al.* Gender differences in melanoma survival: female patients have a decreased risk of metastasis. *The Journal of investigative dermatology* 2011; **131**: 719-26.

62 Mervic L, Leiter U, Meier F *et al.* Sex differences in survival of cutaneous melanoma are age dependent: an analysis of 7338 patients. *Melanoma research* 2011; **21**: 244-52.

63 Khosrotehrani K, Dasgupta P, Byrom L *et al.* Melanoma survival is superior in females across all tumour stages but is influenced by age. *Archives of dermatological research* 2015; **307**: 731-40.

64 Joosse A, Collette S, Suciu S *et al.* Sex is an independent prognostic indicator for survival and relapse/progression-free survival in metastasized stage III to IV melanoma: a pooled analysis of five European organisation for research and treatment of cancer randomized controlled trials. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013; **31**: 2337-46.

65 Clement E, Lazar I, Muller C *et al.* Obesity and melanoma: could fat be fueling malignancy? *Pigment cell & melanoma research* 2017; **30**: 294-306.

66 Skowron F, Berard F, Balme B *et al.* Role of obesity on the thickness of primary cutaneous melanoma. *Journal of the European Academy of Dermatology and Venereology : JEADV* 2015; **29**: 262-9.

67 Ward KA, Lazovich D, Hordinsky MK. Germline melanoma susceptibility and prognostic genes: a review of the literature. *Journal of the American Academy of Dermatology* 2012; **67**: 1055-67.

68 Davies H, Bignell GR, Cox C *et al.* Mutations of the BRAF gene in human cancer. *Nature* 2002; **417**: 949-54.

69 Heppt MV, Siepmann T, Engel J *et al.* Prognostic significance of BRAF and NRAS mutations in melanoma: a German study from routine care. *BMC cancer* 2017; **17**: 536.

70 Meckbach D, Bauer J, Pflugfelder A *et al.* Survival according to BRAF-V600 tumor mutations--an analysis of 437 patients with primary melanoma. *PloS one* 2014; **9**: e86194.

71 Thomas NE, Edmiston SN, Alexander A *et al.* Association Between NRAS and BRAF Mutational Status and Melanoma-Specific Survival Among Patients With Higher-Risk Primary Melanoma. *JAMA oncology* 2015; **1**: 359-68.

72 Genomic Classification of Cutaneous Melanoma. *Cell* 2015; **161**: 1681-96.

73 Mort RL, Jackson IJ, Patton EE. The melanocyte lineage in development and disease. *Development (Cambridge, England)* 2015; **142**: 1387.

74 Nishimura EK, Jordan SA, Oshima H *et al.* Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* 2002; **416**: 854-60.

75 Kanitakis J. Anatomy, histology and immunohistochemistry of normal human skin. *European journal of dermatology : EJD* 2002; **12**: 390-9; quiz 400-1.

76 White RM, Zon LI. Melanocytes in development, regeneration, and cancer. *Cell stem cell* 2008; **3**: 242-52.

77 Riley PA. Melanin. *The international journal of biochemistry & cell biology* 1997; **29**: 1235-9.

78 Ando H, Niki Y, Ito M *et al.* Melanosomes are transferred from melanocytes to keratinocytes through the processes of packaging, release, uptake, and dispersion. *The Journal of investigative dermatology* 2012; **132**: 1222-9.

79 Fitzpatrick TB, Breathnach AS. [THE EPIDERMAL MELANIN UNIT SYSTEM]. *Dermatologische Wochenschrift* 1963; **147**: 481-9.

80 Eller MS, Ostrom K, Gilchrest BA. DNA damage enhances melanogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 1996; **93**: 1087-92.

81 Chakraborty AK, Funasaka Y, Slominski A *et al.* Production and release of proopiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: regulation by ultraviolet B. *Biochimica et biophysica acta* 1996; **1313**: 130-8.

82 Cui R, Widlund HR, Feige E *et al.* Central role of p53 in the suntan response and pathologic hyperpigmentation. *Cell* 2007; **128**: 853-64.

83 Bertolotto C. Melanoma: from melanocyte to genetic alterations and clinical options. *Scientifica* 2013; **2013**: 635203.

84 Jagirdar K, Smit DJ, Ainger SA *et al.* Molecular analysis of common polymorphisms within the human Tyrosinase locus and genetic association with pigmentation traits. *Pigment cell & melanoma research* 2014; **27**: 552-64.

85 Thody AJ, Higgins EM, Wakamatsu K *et al.* Pheomelanin as well as eumelanin is present in human epidermis. *The Journal of investigative dermatology* 1991; **97**: 340-4.

86 Valverde P, Healy E, Jackson I *et al.* Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nature genetics* 1995; **11**: 328-30.

87 Palmer JS, Duffy DL, Box NF *et al.* Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? *American journal of human genetics* 2000; **66**: 176-86.

88 Napolitano A, Panzella L, Monfrecola G *et al.* Pheomelanin-induced oxidative stress: bright and dark chemistry bridging red hair phenotype and melanoma. *Pigment cell & melanoma research* 2014; **27**: 721-33.

89 Bastian BC. The molecular pathology of melanoma: an integrated taxonomy of melanocytic neoplasia. *Annual review of pathology* 2014; **9**: 239-71.

90 Piliouras P, Gilmore S, Wurm EM *et al.* New insights in naevogenesis: number, distribution and dermoscopic patterns of naevi in the elderly. *The Australasian journal of dermatology* 2011; **52**: 254-8.

91 Freitas DA, Bonan PR, Sousa AA *et al.* Intramucosal nevus in the oral cavity. *The journal of contemporary dental practice* 2015; **16**: 74-6.

92 Ahn CS, Guerra A, Sangueza OP. Melanocytic Nevi of Special Sites. *The American Journal of dermatopathology* 2016; **38**: 867-81.

93 Hauschild A, Egberts F, Garbe C *et al.* Melanocytic nevi. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* 2011; **9**: 723-34.

94 Metcalf JS. Melanoma: criteria for histological diagnosis and its reporting. *Seminars in oncology* 1996; **23**: 688-92.

95 Farber MJ, Heilman ER, Friedman RJ. Dysplastic nevi. *Dermatologic clinics* 2012; **30**: 389-404.

96 Rosendahl CO, Grant-Kels JM, Que SK. Dysplastic nevus: Fact and fiction. *Journal of the American Academy of Dermatology* 2015; **73**: 507-12.

97 Stolz W, Schmoeckel C, Landthaler M *et al.* Association of early malignant melanoma with nevocytic nevi. *Cancer* 1989; **63**: 550-5.

98 Grichnik JM, Ross AL, Schneider SL *et al.* How, and from which cell sources, do nevi really develop? *Experimental dermatology* 2014; **23**: 310-3.

99 Shain AH, Bastian BC. From melanocytes to melanomas. *Nature reviews. Cancer* 2016; **16**: 345-58.

100 Unna P. Naevi und Naevocarcinoma. *Berl Klin Wochenschr* 1893; **30**: 14-6.

101 Cramer SF. The origin of epidermal melanocytes. Implications for the histogenesis of nevi and melanomas. *Archives of pathology & laboratory medicine* 1991; **115**: 115-9.

102 Zalaudek I, Ferrara G, Argenziano G. Dermoscopy insights into nevogenesis: "Abtropfung" vs "Hochsteigerung". *Archives of dermatology* 2007; **143**: 284.

103 Zalaudek I, Catricala C, Moscarella E *et al.* What dermoscopy tells us about nevogenesis. *The Journal of dermatology* 2011; **38**: 16-24.

104 Shain AH, Yeh I, Kovalyshyn I *et al.* The Genetic Evolution of Melanoma from Precursor Lesions. *The New England journal of medicine* 2015; **373**: 1926-36.

105 Hui P, Perkins A, Glusac E. Assessment of clonality in melanocytic nevi. *Journal of cutaneous pathology* 2001; **28**: 140-4.

106 Robinson WA, Lemon M, Elefanty A *et al.* Human acquired naevi are clonal. *Melanoma research* 1998; **8**: 499-503.

107 Serrano M, Lin AW, McCurrach ME *et al.* Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997; **88**: 593-602.

108 Michaloglou C, Vredeveld LC, Soengas MS *et al.* BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 2005; **436**: 720-4.

109 Pawlikowski JS, McBryan T, van Tuyn J *et al.* Wnt signaling potentiates nevogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 2013; **110**: 16009-14.

110 Davids LM, du Toit E, Kidson SH *et al.* A rare repigmentation pattern in a vitiligo patient: a clue to an epidermal stem-cell reservoir of melanocytes? *Clinical and experimental dermatology* 2009; **34**: 246-8.

111 Van Raamsdonk CD, Deo M. Links between Schwann cells and melanocytes in development and disease. *Pigment cell & melanoma research* 2013; **26**: 634-45.

112 Ross AL, Sanchez MI, Grichnik JM. Molecular nevogenesis. *Dermatology research and practice* 2011; **2011**: 463184.

113 Greene MH, Goldin LR, Clark WH, Jr. *et al.* Familial cutaneous malignant melanoma: autosomal dominant trait possibly linked to the Rh locus. *Proceedings of the National Academy of Sciences of the United States of America* 1983; **80**: 6071-5.

114 Bale SJ, Dracopoli NC, Tucker MA *et al.* Mapping the gene for hereditary cutaneous malignant melanoma-dysplastic nevus to chromosome 1p. *The New England journal of medicine* 1989; **320**: 1367-72.

115 Kefford RF, Salmon J, Shaw HM *et al.* Hereditary melanoma in Australia. Variable association with dysplastic nevi and absence of genetic linkage to chromosome 1p. *Cancer genetics and cytogenetics* 1991; **51**: 45-55.

116 Bataille V, Snieder H, MacGregor AJ *et al.* Genetics of risk factors for melanoma: an adult twin study of nevi and freckles. *Journal of the National Cancer Institute* 2000; **92**: 457-63.

117 Easton DF, Cox GM, Macdonald AM *et al.* Genetic susceptibility to naevi--a twin study. *British journal of cancer* 1991; **64**: 1164-7.

118 Falchi M, Spector TD, Perks U *et al.* Genome-wide search for nevus density shows linkage to two melanoma loci on chromosome 9 and identifies a new QTL on 5q31 in an adult twin cohort. *Human molecular genetics* 2006; **15**: 2975-9.

119 Lee S, Duffy DL, McClenahan P *et al.* Heritability of naevus patterns in an adult twin cohort from the Brisbane Twin Registry: a cross-sectional study. *The British journal of dermatology* 2016; **174**: 356-63.

120 Wachsmuth RC, Gaut RM, Barrett JH *et al.* Heritability and gene-environment interactions for melanocytic nevus density examined in a U.K. adolescent twin study. *The Journal of investigative dermatology* 2001; **117**: 348-52.

121 Zhu G, Montgomery GW, James MR *et al.* A genome-wide scan for naevus count: linkage to CDKN2A and to other chromosome regions. *European journal of human genetics : EJHG* 2007; **15**: 94-102.

122 Kvaskoff M, Whiteman DC, Zhao ZZ *et al.* Polymorphisms in nevus-associated genes MTAP, PLA2G6, and IRF4 and the risk of invasive cutaneous melanoma. *Twin research and human genetics : the official journal of the International Society for Twin Studies* 2011; **14**: 422-32.

123 Gerstenblith MR, Shi J, Landi MT. Genome-wide association studies of pigmentation and skin cancer: a review and meta-analysis. *Pigment cell & melanoma research* 2010; **23**: 587-606.

124 Zhu G, Duffy DL, Eldridge A *et al.* A major quantitative-trait locus for mole density is linked to the familial melanoma gene CDKN2A: a maximum-likelihood combined linkage and association analysis in twins and their sibs. *American journal of human genetics* 1999; **65**: 483-92.

125 Nan H, Xu M, Zhang J *et al.* Genome-wide association study identifies nidogen 1 (NID1) as a susceptibility locus to cutaneous nevi and melanoma risk. *Human molecular genetics* 2011; **20**: 2673-9.

126 Ogbah Z, Badenas C, Harland M *et al.* Evaluation of PAX3 genetic variants and nevus number. *Pigment cell & melanoma research* 2013; **26**: 666-76.

127 Duffy DL, Iles MM, Glass D *et al.* IRF4 variants have age-specific effects on nevus count and predispose to melanoma. *American journal of human genetics* 2010; **87**: 6-16.

128 Damsky WE, Bosenberg M. Melanocytic nevi and melanoma: unraveling a complex relationship. *Oncogene* 2017.

129 Pollock PM, Harper UL, Hansen KS *et al.* High frequency of BRAF mutations in nevi. *Nature genetics* 2003; **33**: 19-20.

130 Yazdi AS, Palmedo G, Flaig MJ *et al.* Mutations of the BRAF gene in benign and malignant melanocytic lesions. *The Journal of investigative dermatology* 2003; **121**: 1160-2.

131 Bastian BC, LeBoit PE, Pinkel D. Mutations and copy number increase of HRAS in Spitz nevi with distinctive histopathological features. *The American journal of pathology* 2000; **157**: 967-72.

132 Roh MR, Eliades P, Gupta S *et al.* Genetics of melanocytic nevi. *Pigment cell & melanoma research* 2015; **28**: 661-72.

133 Tan JM, Tom LN, Jagirdar K *et al.* The BRAF and NRAS mutation prevalence in dermoscopic subtypes of acquired naevi reveals constitutive MAPK pathway activation. *The British journal of dermatology* 2017.

134 Carr J, Mackie RM. Point mutations in the N-ras oncogene in malignant melanoma and congenital naevi. *The British journal of dermatology* 1994; **131**: 72-7.

135 Van Raamsdonk CD, Bezrookove V, Green G *et al.* Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature* 2009; **457**: 599-602.

136 Haenssle HA, Kraus SL, Brehmer F *et al.* Dynamic changes in nevi of a patient with melanoma treated with vemurafenib: importance of sequential dermoscopy. *Archives of dermatology* 2012; **148**: 1183-5.

137 McClenahan P, Lin LL, Tan JM *et al.* BRAFV600E mutation status of involuting and stable nevi in dabrafenib therapy with or without trametinib. *JAMA dermatology* 2014; **150**: 1079-82.

138 Potrony M, Badenas C, Aguilera P *et al.* Update in genetic susceptibility in melanoma. *Annals of translational medicine* 2015; **3**: 210.

139 Hussussian CJ, Struewing JP, Goldstein AM *et al.* Germline p16 mutations in familial melanoma. *Nature genetics* 1994; **8**: 15-21.

140 Nelson AA, Tsao H. Melanoma and genetics. *Clinics in dermatology* 2009; **27**: 46-52.

141 Udayakumar D, Tsao H. Moderate- to low-risk variant alleles of cutaneous malignancies and nevi: lessons from genome-wide association studies. *Genome medicine* 2009; **1**: 95.

142 Robles-Espinoza CD, Harland M, Ramsay AJ *et al.* POT1 loss-of-function variants predispose to familial melanoma. *Nature genetics* 2014; **46**: 478-81.

143 Shi J, Yang XR, Ballew B *et al.* Rare missense variants in POT1 predispose to familial cutaneous malignant melanoma. *Nature genetics* 2014; **46**: 482-6.

144 Aoude LG, Wadt KA, Pritchard AL *et al.* Genetics of familial melanoma: 20 years after CDKN2A. *Pigment cell & melanoma research* 2015; **28**: 148-60.

145 Sturm RA, Duffy DL. Human pigmentation genes under environmental selection. *Genome biology* 2012; **13**: 248.

146 Beaumont KA, Liu YY, Sturm RA. The melanocortin-1 receptor gene polymorphism and association with human skin cancer. *Progress in molecular biology and translational science* 2009; **88**: 85-153.

147 Valverde P, Benedito E, Solano F *et al.* Melatonin antagonizes alpha-melanocyte-stimulating hormone enhancement of melanogenesis in mouse melanoma cells by blocking the hormone-induced accumulation of the c locus tyrosinase. *European journal of biochemistry* 1995; **232**: 257-63.

148 Duffy DL, Box NF, Chen W *et al.* Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. *Human molecular genetics* 2004; **13**: 447-61.

149 Box NF, Duffy DL, Chen W *et al.* MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. *American journal of human genetics* 2001; **69**: 765-73.

150 Raimondi S, Sera F, Gandini S *et al.* MC1R variants, melanoma and red hair color phenotype: a meta-analysis. *International journal of cancer* 2008; **122**: 2753-60.

151 Cheli Y, Ohanna M, Ballotti R *et al.* Fifteen-year quest for microphthalmia-associated transcription factor target genes. *Pigment cell & melanoma research* 2010; **23**: 27-40.

152 Sturm RA, Fox C, McClenahan P *et al.* Phenotypic characterization of nevus and tumor patterns in MITF E318K mutation carrier melanoma patients. *The Journal of investigative dermatology* 2014; **134**: 141-9.

153 Yokoyama S, Woods SL, Boyle GM *et al.* A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. *Nature* 2011; **480**: 99-103.

154 Bonet C, Luciani F, Ottavi JF *et al.* Deciphering the Role of Oncogenic MITFE318K in Senescence Delay and Melanoma Progression. *Journal of the National Cancer Institute* 2017; **109**.

155 Duffy DL, Zhao ZZ, Sturm RA *et al.* Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma. *The Journal of investigative dermatology* 2010; **130**: 520-8.

156 Pena-Chilet M, Blanquer-Maceiras M, Ibarrola-Villava M *et al.* Genetic variants in PARP1 (rs3219090) and IRF4 (rs12203592) genes associated with melanoma susceptibility in a Spanish population. *BMC cancer* 2013; **13**: 160.

157 Han J, Qureshi AA, Nan H *et al.* A germline variant in the interferon regulatory factor 4 gene as a novel skin cancer risk locus. *Cancer research* 2011; **71**: 1533-9.

158 Gibbs DC, Orlow I, Bramson JI *et al.* Association of Interferon Regulatory Factor-4 Polymorphism rs12203592 With Divergent Melanoma Pathways. *Journal of the National Cancer Institute* 2016; **108**.

159 Granovetter M. IRF4 SNP is predictive of melanoma subtypes. *The Lancet. Oncology* 2016; **17**: e96.

160 Gibbs DC, Ward SV, Orlow I *et al.* Functional melanoma-risk variant IRF4 rs12203592 associated with Breslow thickness: a pooled international study of primary melanomas. *The British journal of dermatology* 2017.

161 Potrony M, Rebollo-Morell A, Gimenez-Xavier P *et al.* IRF4 rs12203592 functional variant and melanoma survival. *International journal of cancer* 2017; **140**: 1845-9.

162 Chhabra Y, Yong HXL, Fane ME *et al.* Genetic variation in IRF4 expression modulates growth characteristics, tyrosinase expression and interferon-gamma response in melanocytic cells. *Pigment cell & melanoma research* 2017.

163 Gudbjartsson DF, Sulem P, Stacey SN *et al.* ASIP and TYR pigmentation variants associate with cutaneous melanoma and basal cell carcinoma. *Nature genetics* 2008; **40**: 886-91.

164 Brown KM, Macgregor S, Montgomery GW *et al.* Common sequence variants on 20q11.22 confer melanoma susceptibility. *Nature genetics* 2008; **40**: 838-40.

165 Bishop DT, Demenais F, Iles MM *et al.* Genome-wide association study identifies three loci associated with melanoma risk. *Nature genetics* 2009; **41**: 920-5.

166 Amos CI, Wang LE, Lee JE *et al.* Genome-wide association study identifies novel loci predisposing to cutaneous melanoma. *Human molecular genetics* 2011; **20**: 5012-23.

167 Barrett JH, Iles MM, Harland M *et al.* Genome-wide association study identifies three new melanoma susceptibility loci. *Nature genetics* 2011; **43**: 1108-13.

168 Chatzinasiou F, Lill CM, Kypreou K *et al.* Comprehensive field synopsis and systematic meta-analyses of genetic association studies in cutaneous melanoma. *Journal of the National Cancer Institute* 2011; **103**: 1227-35.

169 Macgregor S, Montgomery GW, Liu JZ *et al.* Genome-wide association study identifies a new melanoma susceptibility locus at 1q21.3. *Nature genetics* 2011; **43**: 1114-8.

170 Schoof N, Iles MM, Bishop DT *et al.* Pathway-based analysis of a melanoma genome-wide association study: analysis of genes related to tumour-immunosuppression. *PloS one* 2011; **6**: e29451.

171 Teerlink C, Farnham J, Allen-Brady K *et al.* A unique genome-wide association analysis in extended Utah high-risk pedigrees identifies a novel melanoma risk variant on chromosome arm 10q. *Human genetics* 2012; **131**: 77-85.

172 Ransohoff KJ, Wu W, Cho HG *et al.* Two-stage genome-wide association study identifies a novel susceptibility locus associated with melanoma. *Oncotarget* 2017; **8**: 17586-92.

173 Kaehler KC, Politz O, Henderson D *et al.* Novel DNA methylation markers with potential prognostic relevance in advanced malignant melanoma identified using COBRA assays. *Melanoma research* 2015; **25**: 225-31.

174 Puigserver P, Wu Z, Park CW *et al.* A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 1998; **92**: 829-39.

175 Lin J, Puigserver P, Donovan J *et al.* Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. *The Journal of biological chemistry* 2002; **277**: 1645-8.

176 Deblois G, St-Pierre J, Giguere V. The PGC-1/ERR signaling axis in cancer. *Oncogene* 2013; **32**: 3483-90.

177 Wu Z, Puigserver P, Andersson U *et al.* Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 1999; **98**: 115-24.

178 Yoon JC, Puigserver P, Chen G *et al.* Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 2001; **413**: 131-8.

179 Michael LF, Wu Z, Cheatham RB *et al.* Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proceedings of the National Academy of Sciences of the United States of America* 2001; **98**: 3820-5.

180 Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *The Journal of clinical investigation* 2006; **116**: 615-22.

181 Shoag J, Haq R, Zhang M *et al.* PGC-1 coactivators regulate MITF and the tanning response. *Molecular cell* 2013; **49**: 145-57.

182 Nan H, Kraft P, Qureshi AA *et al.* Genome-wide association study of tanning phenotype in a population of European ancestry. *The Journal of investigative dermatology* 2009; **129**: 2250-7.

183 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-74.

184 Jones AW, Yao Z, Vicencio JM *et al.* PGC-1 family coactivators and cell fate: roles in cancer, neurodegeneration, cardiovascular disease and retrograde mitochondria-nucleus signalling. *Mitochondrion* 2012; **12**: 86-99.

185 Theodosakis N, Micevic G, Kelly DP *et al.* Mitochondrial function in melanoma. *Archives of biochemistry and biophysics* 2014; **563**: 56-9.

186 Bianchi K, Vandecasteele G, Carli C *et al.* Regulation of Ca2+ signalling and Ca2+-mediated cell death by the transcriptional coactivator PGC-1alpha. *Cell death and differentiation* 2006; **13**: 586-96.

187 St-Pierre J, Drori S, Uldry M *et al.* Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 2006; **127**: 397-408.

188 Vazquez F, Lim JH, Chim H *et al.* PGC1alpha expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress. *Cancer cell* 2013; **23**: 287-301.

189 Ronai Z. The masters talk: the PGC-1alpha-MITF axis as a melanoma energizer. *Pigment cell & melanoma research* 2013.

190 Berwick M, Buller DB, Cust A *et al.* Melanoma Epidemiology and Prevention. *Cancer treatment and research* 2016; **167**: 17-49.

191 Weischer M, Heerfordt IM, Bojesen SE *et al.* CHEK2*1100delC and risk of malignant melanoma: Danish and German studies and meta-analysis. *The Journal of investigative dermatology* 2012; **132**: 299-303.

192 Daley GM, Duffy DL, Pflugfelder A *et al.* GSTP1 does not modify MC1R effects on melanoma risk. *Experimental dermatology* 2016.

193 Cook AL, Chen W, Thurber AE *et al.* Analysis of cultured human melanocytes based on polymorphisms within the SLC45A2/MATP, SLC24A5/NCKX5, and OCA2/P loci. *The Journal of investigative dermatology* 2009; **129**: 392-405.

194 Purcell S, Neale B, Todd-Brown K *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *American journal of human genetics* 2007; **81**: 559-75.

195 R Development Core Team. R: A language and environment for statistical computing. In. Vienna, Austria: R Foundation for Statistical Computing. 2017.

196 Stata W. In. 2017.

197 Beaumont KA, Wong SS, Ainger SA *et al.* Melanocortin MC(1) receptor in human genetics and model systems. *European journal of pharmacology* 2011; **660**: 103-10.

198 Puig-Butille JA, Carrera C, Kumar R *et al.* Distribution of MC1R variants among melanoma subtypes: p.R163Q is associated with lentigo maligna melanoma in a Mediterranean population. *The British journal of dermatology* 2013; **169**: 804-11.

199 Scope A, Burroni M, Agero AL *et al.* Predominant dermoscopic patterns observed among nevi. *Journal of cutaneous medicine and surgery* 2006; **10**: 170-4.

200 Pizzichetta MA, Talamini R, Stanganelli I *et al.* Natural history of atypical and equivocal melanocytic lesions in children: an observational study of 19 cases. *Pediatric dermatology* 2014; **31**: 331-6.

201 Douglas NC, Borgovan T, Carroll MJ *et al.* Dermoscopic naevus patterns in people at high versus moderate/low melanoma risk in Queensland. *The Australasian journal of dermatology* 2011; **52**: 248-53.

202 Davies JR, Randerson-Moor J, Kukalizch K *et al.* Inherited variants in the MC1R gene and survival from cutaneous melanoma: a BioGenoMEL study. *Pigment cell & melanoma research* 2012; **25**: 384-94.

203 Weide B, Richter S, Buttner P *et al.* Serum S100B, lactate dehydrogenase and brain metastasis are prognostic factors in patients with distant melanoma metastasis and systemic therapy. *PloS one* 2013; **8**: e81624.

204 Duggal P, Gillanders EM, Holmes TN *et al.* Establishing an adjusted p-value threshold to control the family-wide type 1 error in genome wide association studies. *BMC genomics* 2008; **9**: 516.

205 Li MX, Yeung JM, Cherny SS *et al.* Evaluating the effective numbers of independent tests and significant p-value thresholds in commercial genotyping arrays and public imputation reference datasets. *Human genetics* 2012; **131**: 747-56.

206 Levell NJ, Beattie CC, Shuster S *et al.* Melanoma epidemic: a midsummer night's dream? *The British journal of dermatology* 2009; **161**: 630-4.

207 Sturm RA, Duffy DL, Box NF *et al.* Genetic association and cellular function of MC1R variant alleles in human pigmentation. *Annals of the New York Academy of Sciences* 2003; **994**: 348-58.

208 Lim A, Shayan R, Varigos G. High serum vitamin D level correlates with better prognostic indicators in primary melanoma: A pilot study. *The Australasian journal of dermatology* 2017.

209 Dad S, Rendtorff ND, Kann E *et al.* Partial USH2A deletions contribute to Usher syndrome in Denmark. *European journal of human genetics : EJHG* 2015; **23**: 1646-51.

210 Zerenturk EJ, Sharpe LJ, Brown AJ. DHCR24 associates strongly with the endoplasmic reticulum beyond predicted membrane domains: implications for the activities of this multi-functional enzyme. *Bioscience reports* 2014; **34**.

211 Lee GT, Ha YS, Jung YS *et al.* DHCR24 is an independent predictor of progression in patients with non-muscle-invasive urothelial carcinoma, and its functional role is involved in the aggressive properties of urothelial carcinoma cells. *Annals of surgical oncology* 2014; **21 Suppl 4**: S538-45.

212 Di Stasi D, Vallacchi V, Campi V *et al.* DHCR24 gene expression is upregulated in melanoma metastases and associated to resistance to oxidative stress-induced apoptosis. *International journal of cancer* 2005; **115**: 224-30.

213 Climate. In. 2017.

214 Abraham G, Inouye M. Fast principal component analysis of large-scale genome-wide data. *PloS one* 2014; **9**: e93766.

215 Masood M, Reidpath DD. Effect of national wealth on BMI: An analysis of 206,266 individuals in 70 low-, middle- and high-income countries. *PloS one* 2017; **12**: e0178928.

216 Montague M, Borland R, Sinclair C. Slip! Slop! Slap! and SunSmart, 1980-2000: Skin cancer control and 20 years of population-based campaigning. *Health education & behavior : the official publication of the Society for Public Health Education* 2001; **28**: 290-305.

217 Sturm RA. Skin colour and skin cancer - MC1R, the genetic link. *Melanoma research* 2002; **12**: 405-16.

218 Cymerman RM, Shao Y, Wang K *et al.* De Novo vs Nevus-Associated Melanomas: Differences in Associations With Prognostic Indicators and Survival. *Journal of the National Cancer Institute* 2016; **108**.

219 Levi F, Randimbison L, Te VC *et al.* High constant incidence rates of second cutaneous melanomas. *International journal of cancer* 2005; **117**: 877-9.

220 de Giorgi V, Rossari S, Papi F *et al.* Multiple primary melanoma: the impact of atypical naevi and follow up. *The British journal of dermatology* 2010; **163**: 1319-22.

221 Pardo LM, van der Leest RJ, de Vries E *et al.* Comparing survival of patients with single or multiple primary melanoma in the Netherlands: 1994-2009. *The British journal of dermatology* 2017; **176**: 531-3.

222 Schuurman MS, de Waal AC, Thijs EJM *et al.* Risk factors for second primary melanoma among Dutch patients with melanoma. *The British journal of dermatology* 2017; **176**: 971-8.

223 Moseley HS, Giuliano AE, Storm FK, 3rd *et al.* Multiple primary melanoma. *Cancer* 1979; **43**: 939-44.

224 Ferrone CR, Ben Porat L, Panageas KS *et al.* Clinicopathological features of and risk factors for multiple primary melanomas. *Jama* 2005; **294**: 1647-54.

225 Titus-Ernstoff L, Perry AE, Spencer SK *et al.* Multiple primary melanoma: two-year results from a population-based study. *Archives of dermatology* 2006; **142**: 433-8.

226 Bower MR, Scoggins CR, Martin RC, 2nd *et al.* Second primary melanomas: incidence and outcome. *The American surgeon* 2010; **76**: 675-81.

227 Pollio A, Tomasi A, Pellacani G *et al.* Multiple primary melanomas versus single melanoma of the head and neck: a comparison of genetic, diagnostic, and therapeutic implications. *Melanoma research* 2014; **24**: 267-72.

228 Krajewski AC, Hart DR, Hieken TJ. Multiple primary melanoma in the elderly. *American journal of surgery* 2016; **211**: 84-8.

229 Jung GW, Weinstock MA. Clinicopathological comparisons of index and second primary melanomas in paediatric and adult populations. *The British journal of dermatology* 2012; **167**: 882-7.

230 Moore MM, Geller AC, Warton EM *et al.* Multiple primary melanomas among 16,570 patients with melanoma diagnosed at Kaiser Permanente Northern California, 1996 to 2011. *Journal of the American Academy of Dermatology* 2015; **73**: 630-6.

231 Rowe CJ, Law MH, Palmer JM *et al.* Survival outcomes in patients with multiple primary melanomas. *Journal of the European Academy of Dermatology and Venereology : JEADV* 2015; **29**: 2120-7.

232 Youlden DR, Baade PD, Soyer HP *et al.* Ten-Year Survival after Multiple Invasive Melanomas Is Worse than after a Single Melanoma: a Population-Based Study. *The Journal of investigative dermatology* 2016; **136**: 2270-6.

233 Woodcock A. The president speaks: prevention is best: lessons from protecting the ozone layer. *Thorax* 2012; **67**: 1028-31.

234 Rauterberg A, Jung EG. [UV exposure, skin cancer and decrease in the ozone layer]. *Therapeutische Umschau. Revue therapeutique* 1993; **50**: 804-7.

235 Solomon S, Ivy DJ, Kinnison D *et al.* Emergence of healing in the Antarctic ozone layer. *Science (New York, N.Y.)* 2016; **353**: 269-74.

236 Torrens-Mas M, Gonzalez-Hedstrom D, Abrisqueta M *et al.* PGC-1alpha in Melanoma: A Key Factor for Antioxidant Response and Mitochondrial Function. *Journal of cellular biochemistry* 2017.

237 Luo C, Lim JH, Lee Y *et al.* A PGC1alpha-mediated transcriptional axis suppresses melanoma metastasis. *Nature* 2016; **537**: 422-6.
238 Li X, Liu H, Amos CI *et al.* A PGC1beta genetic variant associated with nevus count and melanoma mortality. *International journal of cancer* 2017.
239 Zalaudek I, Argenziano G, Mordente I *et al.* Nevus type in dermoscopy is related to skin type in white persons. *Archives of dermatology* 2007; **143**: 351-6.

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