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Melanoma predisposition genes in the Latvian population

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ABSTRACT

Cutaneous melanoma is the most aggressive skin cancer with an increasing incidence worldwide and Latvia as well, and it accounts for the majority of skin cancer related deaths. Early melanoma detection and identification of high risk individuals is important to prevent the disease. Melanoma has a complex aetiology that involves environmental, phenotypic, genetic and epigenetic risk factors.

The **aim** of this study was to investigate the role of high, medium and low melanoma risk genes in melanoma development and survival in the Latvian population. Two different approaches were used for this.

A family study approach was used to study high melanoma risk genes *CDKN2A* and *CDK4* in predisposed individuals, and a case-control study approach was used to study medium (*MC1R*, *MITF*) and low melanoma risk genes (*TP53*, *MDM2*, *PARP1*) in sporadic melanoma patients. The study found melanoma associated variant—6 bp deletion c.–20677_–20682delGTACGC— in the main high melanoma risk gene *CDKN2A* for the first time in the Latvian population. The third Latvian *CDK4* melanoma family with rare variant p.Arg24His was identified confirming *CDK4* as the main familial melanoma risk gene in Latvia so far. Data about Latvian *CDK4* melanoma families were included in an international world-wide *CDK4* family study that demonstrates that *CDK4* melanoma families are phenotypically similar to *CDKN2A* families, and *CDK4* gene needs to be examined when the family is negative for variants in the *CDKN2A* gene.

The highest melanoma risk variant in Latvian sporadic melanoma patients is variant p.Arg151Cys in medium melanoma risk gene *MC1R*. A subset of rare *MC1R* variants is functionally relevant and should be considered as high risk variants in *MC1R* analysis. Carrying two *MC1R* variants is associated with twice as high melanoma risk as carrying one *MC1R* variant, and overall the risk associated with *MC1R* variants is independent from pigmentation phenotype. However, *MC1R* variants have a positive effect on melanoma survival revealing ambiguous effects of *MC1R* variants and the disease. Although variant p.Arg151Cys is the main signal from 16q24.3 associated with melanoma risk, another variant rs4785763 in pseudogene *AFG3L1P* also displays an association with melanoma and is independent from *MC1R*. Medium melanoma risk gene *MITF* variant p.Glu318Lys is not a significant melanoma risk factor in Latvian population. Variants in low melanoma risk genes *TP53* (p.Pro72Arg) and *MDM2* (c.14+309T>G) have a minor effect on melanoma development in the Latvian population, but *PARP1* variant rs2249844 is associated with an increased risk of death. Taken together these data illustrate the complex relations between various melanoma risk genes and their impact on melanoma development and leave space for discoveries of yet other unknown melanoma risk genes and complex interplay between them.

KOPSAVILKUMS

Ādas melanoma ir ļaundabīgākais no visiem ādas audzējiem. Tās incidence visā pasaulē, ieskaitot Latviju, aizvien pieaug, turklāt tā izraisa lielāko daļu nāves gadījumu, kas saistīti ar ādas audzējiem. Liela loma melanomas novēršanā ir agrīnai diagnostikai, kā arī augsta riska indivīdu identificēšanai. Melanomai ir sarežģīta etioloģija, kas ietver gan vides, gan fenotipiskos, ģenētiskos un epiģenētiskos riska faktorus.

Šī pētījuma **mērķis** bija izvērtēt augsta, vidēja un zema melanomas riska gēnu ietekmi uz melanomas attīstību un izdzīvotību Latvijas populācijā. Tā sasniegšanai tika izmantotas divas dažādas pētījuma pieejas.

Ģimeņu ietvaros indivīdiem ar palielinātu melanomas predispozīciju tika pētīti augsta melanomas riska gēni *CDKN2A* un *CDK4*, savukārt pacientu – kontroles personu (*case-control*) pētījuma pieeja tika izmantota, lai analizētu vidēja (*MC1R, MITF*) un zema (*TP53, MDM2, PARP1*) melanomas riska gēnus sporādiskajos pacientos. Pirmo reizi Latvijas populācija tika atrasts *CDKN2A* variants, kas saistīts ar melanomas attīstību – 6 bp delēcija c.–20677_ – 20682delGTACGC. Tika atrasta trešā ģimene Latvijā ar atkārtotiem melanomas saslimšanas gadījumiem un variantu p.Arg24His *CDK4* gēnā, kas apstiprina, ka *CDK4* aizvien ir galvenais riska gēns ģimenēs ar atkārtotiem melanomas saslimšanas gadījumiem Latvijā. Dati par Latvijas *CDK4* ģimenēm tika iekļauti starptautiskā pētījumā par *CDK4* ģimenēm, kas parādīja, ka *CDK4* ģimenes fenotipiski ir līdzīgas *CDKN2A* ģimenēm un gadījumā, ja ģimenē netiek atrastas *CDKN2A* izmaiņas, jāveic *CDK4* analīze.

Galvenais riska variants sporādiskajiem melanomas pacientiem Latvijā ir vidēja melanomas riska gēna *MC1R* variants p.Arg151Cys. Arī daļa reto *MC1R* variantu ir funkcionāli nozīmīgi un *MC1R* variantu analīzē būtu uzskatāmi par augsta riska variantiem. Divi *MC1R* varianti ir saistīti ar divreiz augstāku melanomas risku nekā tikai viens variants, un kopumā *MC1R* variantu veidotais risks ir neatkarīgs no pigmentācijas fenotipa. No otras puses, *MC1R* varianti uzrāda pozitīvu efektu uz melanomas izdzīvotību, tādējādi norādot uz neviennozīmīgo *MC1R* variantu ietekmi uz slimību. Lai gan *MC1R* variants p.Arg151Cys ir galvenais melanomas riska variants 16q24.3 reģionā, tomēr vēl viens šī reģiona variants rs4785763 pseidogēnā *AFG3L1P* uzrāda no *MC1R* neatkarīgu saistību ar melanomu. Vidēja melanomas riska gēna variants p.Glu318Lys nav nozīmīgs melanomas riska faktors Latvijas populācijā. Zema melanomas riska gēnu variantiem – p.Pro72Arg *TP53* gēnā un c.14+309T>G *MDM2* gēnā – ir minimāla ietekme uz melanomas attīstību Latvijas populācijā, bet *PARP1* variants rs2249844 ir saistīts ar paaugstinātu nāves risku. Kopumā rezultāti ilustrē sarežģīto mijiedarbību starp dažādiem melanomas riska gēniem un melanomas attīstību, kā arī norāda uz iespēju nākotnē atklāt vēl citus jaunus melanomas riska gēnus, kā arī dažādas mijiedarbības to starpā.

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ABBREVIATIONS

5'UTR	5' untranslated region
95% CI	95% confidence interval
aa	Amino acid/acids
α-MSH	α-melanocyte stimulating hormone
AC	Adenylate cyclase
ACD, *ACD*	Adrenocortical dysplasia protein homolog (also known as TPP1), gene
AFG3L1P, *AFG3L1P*	AFG3 like matrix AAA peptidase subunit 1, pseudogene
AGR3, *AGR3*	Anterior gradient 3 protein, gene
AIM1, *AIM1*	Melanoma antigen AIM1, gene
AMS	Atypical mole syndrome
ARNT, *ARNT*	Aryl hydrocarbon receptor nuclear translocator, gene
ASIP, *ASIP*	Agouti signaling protein, gene
ASXL1/2, *ASXL1/2*	Additional sex comb-like proteins ASXL1 and ASXL2, genes
ATM, *ATM*	ATM serine/threonine kinase, gene
B	*Stratum basale*
BAP1, *BAP1*	BRCA1-associated protein 1, gene
BARD1, *BARD1*	BRCA1-associated RING domain protein 1, gene
BHK	Baby hamster kidney cells
BM	Basement membrane
BRAF, *BRAF*	Serine/threonine-protein kinase B-Raf, gene
BRCA1/2, *BRCA1/2*	Tumour suppressor breast cancer protein 1 and 2, genes
cAMP	Cyclic adenosine monophosphate
CASP8, *CASP8*	Caspase 8, gene
CCND1, *CCND1*	Cyclin D1, gene
CDK4, *CDK4*	Cyclin-dependent kinase 4, gene
CDK6, *CDK6*	Cyclin-dependent kinase 6, gene
CDK10, *CDK10*	Cyclin-dependent kinase 10, gene
CDKAL1, *CDKAL1*	CDK5 regulatory subunit associated protein 1 like 1 protein, gene
CDKN2A, *CDKN2A*	Cyclin-dependent kinase inhibitor 2A, gene
CDKN2B, *CDKN2B*	Cyclin-dependent kinase inhibitor 2B, gene
CDKN2B-AS1, *CDKN2B-AS1*	Cyclin-dependent kinase inhibitor 2B antisense RNA 1, gene
c-Fos	Protooncogene *c-Fos*
CLPTM1L, *CLPTM1L*	Cleft lip and palate associated transmembrane protein 1 like protein, gene
CREB	cAMP response-element binding protein
CTD	C terminal domain
CYP1B1, *CYP1B1*	Cytochrome P450 family 1 subfamily B member 1, gene
D	Dermis
DCT	Dopachrome tautomerase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2F	Transcription factor E2F
Elk1/4	ETS transcription factors Elk1 and Elk4
ERCC5 (XPG), *ERCC5*	ERCC excision repair 5, endonuclease, gene
ETS	E-twenty six transcription factors
F	Fibroblasts
FAMMM	Familial atypical multiple mole melanoma syndrome
FoxK1/K2	Forkhead transcription factors FoxK1 and FoxK2
FTO, *FTO*	Alpha-ketoglutarate dependent dioxygenase, gene
G	*Stratum granulosum*
G1	Gap1 phase of the cell cycle
G2	Gap2 phase of the cell cycle
GAR1	Ribonucleoprotein GAR1
GEM	Genes, Environment, and Melanoma consortium
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
GWAS	Genome wide association study
HBM	Host cell factor 1 binding domain
HCF1	Host cell factor 1
HeLa	Cervical adenocarcinoma cell line
HERC2, *HERC2*	HECT and RLD domain containing E3 ubiquitin protein ligase 2, gene
HIF1A	Hypoxia-inducible factor 1-alpha
IARC	International Agency for Research on Cancer
IRF4, *IRF4*	Interferon regulatory factor 4, gene
K	Keratinocytes
kbp	Kilobase pairs
kDa	Kilodalton
M	Mitosis phase of the cell cycle or melanocytes
MAF	Minor allele frequency
MAFF, *MAFF*	MAF bZIP transcription factor F, gene
MATP, *MATP*	Membrane-associated transporter protein, gene
MBAIT	Melanocytic BAP1-mutated atypical intradermal tumour
MC1R, *MC1R*	Melanocortin 1 receptor, gene
MC2R, *MC2R*	Melanocortin 2 receptor, gene

MC3R, *MC3R*	Melanocortin 3 receptor, gene
MC4R, *MC4R*	Melanocortin 4 receptor, gene
MC5R, *MC5R*	Melanocortin 5 receptor, gene
MDM2, *MDM2*	Mouse double minute 2 homolog, gene
MITF, *MITF*	Melanogenesis associated transcription factor, gene
MM	Malignant mesothelioma
MPM	Multiple primary melanomas
mRNA	Messenger RNA
M-SKIP	Melanocortin-1 receptor gene, Skin cancer and Phenotypic characteristics project
MTAP, *MTAP*	Methylthioadenosine phosphorylase, gene
MTS1, *MTS1*	Multiple tumour suppressor 1, gene
MX2, *MX2*	MX dynamin like GTPase 2, gene
MYH7B, *MYH7B*	Myosin heavy chain 7B, gene
NCOA6, *NCOA6*	Nuclear receptor coactivator 6, gene
NER	Nucleotide excision repair
NHP2	H/ACA ribonucleoprotein complex subunit 2
NLS	Nuclear localisation signals
NOP10	Nucleolar protein 10
NRAS, *NRAS*	Neuroblastoma RAS viral oncogene homolog, GTPase, gene
NRHC	Non-red hair colour phenotype
OB1/2	Oligonucleotide/ oligosaccharide-binding N terminal domains of POT1
OCA2, *OCA2*	OCA2 melanosomal transmembrane protein, gene
OR	Odds ratio
OMIM	Online Mendelian Inheritance in Man
p	P-value
PARP1, *PARP1*	Poly(ADP-ribose) polymerase 1, gene
p14ARF	Alternate reading frame protein encoded by *CDKN2A*
p16INK4A	Tumour suppressor protein encoded by *CDKN2A*
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
PIGU, *PIGU*	Phosphatidylinositol glycan anchor biosynthesis class U protein, gene
PKA	Protein kinase A
PLA2G6, *PLA2G6*	Phospholipase A2 group VI, gene
POLE, *POLE*	Catalytic subunit of DNA polymerase epsilon, gene
POT1, *POT1*	Protection of telomeres 1 protein, gene
PTEN, *PTEN*	Phosphatase and tensin homolog protein, gene
RAD23B, *RAD23B*	Nucleotide excision repair protein, gene
RALY, *RALY*	RALY heterogeneous nuclear ribonucleoprotein, gene
RB	Retinoblastoma protein
RCC	Renal cell carcinoma
RHC	Red hair colour phenotype
RMDN2, *RMDN2*	Regulator of microtubule dynamics 2 protein, gene
S	Synthesis phase of the cell cycle or *stratum spinosum*
SC	*Stratum corneum*
SLC45A2, *SLC45A2*	Solute carrier family 45 member 2 protein, gene
STN1 (OBFC1), *STN1*	CST complex subunit STN1, gene
SUMO	Small ubiquitin-like modifier
TAL2, *TAL2*	TAL BHLH Transcription Factor 2, gene
TCF	Ternary complex factors
TERF2IP, *TERF2IP*	Telomeric repeat binding factor 2 interacting protein (also known as RAP1), gene
TERC	Telomerase RNA component
TERT, *TERT*	Telomerase reverse transcriptase, gene
TET2, *TET2*	Tet methylcytosine dioxygenase 2, gene
TINF2, *TINF2*	Telomeric repeat binding factor 1 interacting nuclear factor 2 (also known as TIN2), gene
TMEM38B, *TMEM38B*	Transmembrane protein 38B, gene
TP53, *TP53*	Tumour protein TP53, gene
TPP1, *TPP1*	TIN2-interacting protein (encoded by *ACD*), gene
TRF1 (TERF1), *TRF1*	Telomeric repeat binding factor 1, gene
TRF2 (TERF2), *TRF2*	Telomeric repeat binding factor 2, gene
TYR, *TYR*	Tyrosinase, gene
TYRP1, *TYRP1*	Tyrosinase-related protein 1, gene
UCH	N terminal ubiquitin carboxyl hydrolase domain
UM	Uveal melanoma
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
UVR	Ultraviolet radiation
VDR, *VDR*	Vitamin D receptor, gene
YY1	Ying Yang 1 transcription factor

INTRODUCTION

Cutaneous melanoma is the most aggressive skin cancer that accounts for the majority of skin cancer related deaths. Melanoma is easily treatable in early stages, but is highly metastatic and deadly in advanced stages. Melanoma incidence has steadily increased in the fair-skinned population in the last decades by about 4-5% per year that is mainly explained by socioeconomically linked changes in lifestyle (Erdmann *et al.* 2013). The highest melanoma incidence rates in the world are in New Zealand, Australia and Central and Northern Europe (Ervik *et al.* 2016). Melanoma incidence in Latvia is lower, however numbers are increasing and survival rates are lower than in the most of Europe (Azarjana *et al.* 2013). In the last decade significant advancements have been reached in the therapy of advanced melanoma through development of small molecular inhibitors and immunotherapy, however these therapies are expensive, have serious side effects, and only a selective range of patients respond to the treatment. Therefore early melanoma detection and identification of individuals with increased risk is important to prevent the disease. Melanoma has a complex aetiology that involves environmental, phenotypic, genetic and epigenetic risk factors. At least eight high risk genes associated with familial melanoma (*CDKN2A*, *CDK4*, *BAP1*, *TERT*, *POT1*, *ACD*, *TERF2IP*, *POLE*) have been discovered during the last two decades (Read *et al.* 2016; Potrony *et al.* 2015). Several previous studies have reported the role of genetic variants in *CDKN2A* and *CDK4* on melanoma in the Latvian population mainly in a family setting (Pjanova *et al.* 2009; Pjanova *et al.* 2007; Pjanova *et al.* 2006a; Pjanova *et al.* 2006b; Pjanova *et al.* 2003). *CDKN2A* is the most predominant gene associated with familial melanoma, however, no changes in *CDKN2A* associated with melanoma development in Latvian families have been discovered so far. Previous studies have identified two Latvian melanoma families with a hotspot change p.Arg24His in *CDK4* gene. Though, in other Latvian melanoma families, a genetic component contributing to the disease is yet to be discovered. Besides high melanoma risk genes, at least 30 medium and low melanoma risk loci with a role in both familial and sporadic melanoma have been identified and investigated in various populations, however, so far no such studies have been conducted in the Latvian population.

The aim of this study was to investigate the role of high, medium and low risk predisposition genes in melanoma development and survival in the Latvian population.

The main **objectives** were as follows:

- To analyse comprehensively the *CDKN2A* locus, including also the promoter region and introns as well as a deletion screening of the gene, as well as to analyse the exon 2 of the *CDK4* gene in Latvian melanoma patients with a family history of melanoma (paper I);
- To examine jointly the clinical phenotype of melanoma prone families with *CDK4* codon 24 germline variant, including possible modifying effects of *MC1R* variants, with the intent to inform genetic counselling internationally (paper II);
- To investigate the prevalence and type of *MC1R* variants among melanoma patients and control persons in the Latvian population and perform functional analyses of previously uncharacterized rare *MC1R* variants (paper III);
- To examine relations between variants residing in the 16q24.3 region and melanoma (paper IV);

- To test the hypothesis that *MC1R* variants are associated with melanoma survival (paper V);
- To evaluate the prevalence of medium melanoma risk gene *MITF* variant p.Glu318Lys in Latvian melanoma patients and controls (paper VI);
- To evaluate the prevalence of low melanoma risk gene variants, in particular, *TP53* (p.Pro72Arg) and *MDM2* (c.14+309T>G), in the Latvian population (paper VII);
- To analyse the role of *PARP1* gene variants in melanoma development and survival (paper VIII).

1. LITERATURE REVIEW

1.1. Cutaneous melanoma

1.1.1. Melanoma etiology

Cutaneous melanoma (from here forward—'melanoma') is a malignant skin neoplasm that develops from melanocytes. While the majority of melanomas develop in the skin, it can also develop in the eye (uvea, conjunctiva, ciliary body), meninges and on mucosal surfaces. In most cases melanomas are heavily pigmented, however, they can also be amelanotic (Garbe *et al.* 2016).

Melanocytes are pigment producing cells that embryonically develop from ectodermal neural crest cells, and can be found throughout the human body—epidermis, iris, hair, rectum, inner ear, nervous system and heart (Schadendorf *et al.* 2015; Cichorek *et al.* 2013; Brito & Kos 2008; Tachibana 1999). Melanocytes in these histologically different sites can give rise to diverse types of melanoma, however, in 'whites' the most common form is cutaneous melanoma. A simplified model of development of melanoma starts with a benign lesion, termed melanocytic naevus (plural naevi) that subsequently evolves to a dysplastic naevus, then melanoma *in situ* and lastly, invasive melanoma (Shain & Bastian 2016) (Figure 1). However, melanoma can also arise *de novo* without a pre-existent skin lesion.

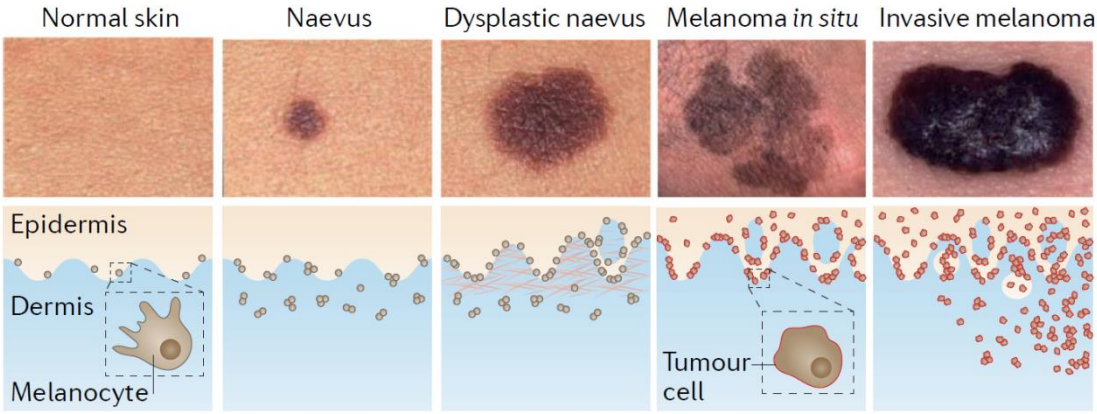

Figure 1. Schematic and photographic representation of cutaneous melanoma development from a melanocytic naevus. From left to right: the first row—clinical photographs of normal skin, a freestanding naevus, a dysplastic naevus, melanoma in situ and invasive melanoma; the second row—schematic illustrations of main histological features associated with each step. Melanocytes are located in the basal layer of epidermis. A naevus is a benign proliferation of melanocytes with very low likelihood for melanoma development. A dysplastic naevus is at least 5 mm in diameter with variable pigmentation, asymmetry and/or irregular borders and histologically displays architectural disorder. Melanoma *in situ* is an irregular proliferation of melanocytes with enlarged nuclei within the epidermis. In the invasive melanoma stage melanocytes leave the epithelium of the epidermis and enter the dermis or submucosa (adapted after Shain & Bastian 2016).

1.1.2. Melanoma incidence, survival and mortality

Melanoma has shown a steady increase in incidence in fair-skinned population in the last decades by about 4-5% per year (Erdmann *et al.* 2013; Godar 2011; MacKie *et al.* 2009; de Vries *et al.* 2003). This rapid increase is mainly explained by greater exposure to ultraviolet radiation (UVR) due to socioeconomically linked changes in recreational habits

and lifestyle—people from high latitude countries more often travel to lower latitudes and experience intensive solar exposure especially for non-acclimatised light skin (Erdmann *et al.* 2013; MacKie *et al.* 2009). It has been shown that short intermittent burning episodes are a greater melanoma risk factor than continuous exposure thus explaining harmful effects of such an episodic and intensive sun exposure (Chang *et al.* 2009; Elwood & Gallagher 1998). The highest melanoma incidence rates in the world are in New Zealand and Australia (age-standardized incidence rates per 100,000—35.8 and 34.9, respectively) followed by Central and Northern European countries Switzerland, The Netherlands, Denmark, Norway and Sweden (20.3, 19.4, 19.2, 18.8 and 18.0). Melanoma incidence is also high in the United Kingdom and USA (14.4 and 14.3, respectively) (Ervik *et al.* 2016; Ferlay *et al.* 2015) (Figure 2).

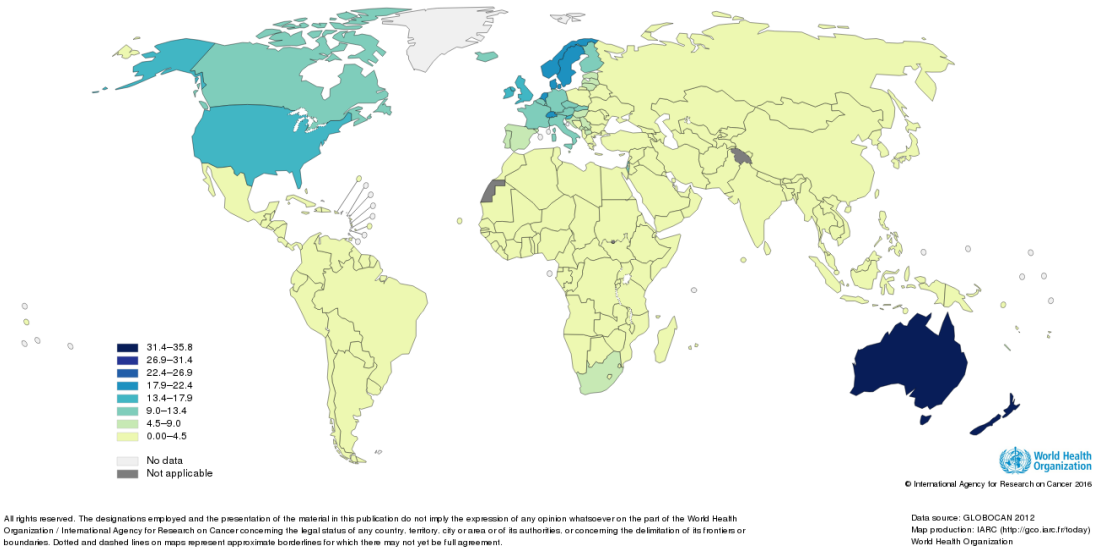

Figure 2. Estimated age-standardized incidence rates of melanoma (both sexes) worldwide (GLOBOCAN 2012 data) (Ervik *et al.* 2016; Ferlay *et al.* 2015).

In the recent years some stabilisation in melanoma incidence rates have been observed in Australia, New Zealand, North America and Northern European countries, especially in younger individuals, that is mostly attributed to public health campaigns and growing awareness of melanoma as well as improvement in early detection (Erdmann *et al.* 2013; Karim-Kos *et al.* 2008; Coory *et al.* 2006; de Vries *et al.* 2003).

Melanoma incidence in Latvia on Europe scale is comparatively low, however, numbers are increasing. Data from the largest oncological hospital in the country Oncology Centre of Latvia shows an increase from 5.1 new melanoma cases per 100,000 inhabitants in 1998 to 7.8 new melanoma cases in 2008 (Azarjana *et al.* 2013).

In the recent couple decades melanoma survival has improved in most of Europe and nowadays 5-year age-standardised relative survival reaches 80-90%, however, in Eastern Europe it is only 50-75% (De Angelis *et al.* 2014; Tryggvadottir *et al.* 2010; Karim-Kos *et al.* 2008; Lasithiotakis *et al.* 2007; Lindholm *et al.* 2004; Smith *et al.* 1998a). Survival rates in Europe for women are higher than for men (86.6% and 79.2%, respectively). In Latvia 5-year age-standardised relative melanoma survival is 65.1% that is lower than survival rate in Lithuania (69.2%) and Estonia (72.1%) (De Angelis *et al.* 2014).

Melanoma mortality rates have mostly stabilized or even declined in North and Central Europe, North America and Australia, however, they are still increasing in Southern

and Eastern Europe (Erickson & Driscoll 2010; Karim-Kos *et al.* 2008; Coory *et al.* 2006; Baade & Coory 2005; de Vries *et al.* 2003; La Vecchia *et al.* 1999). The highest melanoma mortality in the world is in Australia and New Zealand (estimated age-standardised rate per 100,000 is 5.9 for men and 2.4 for women), but lowest in South-Central Asia (0.2 and 0.1, respectively). Altogether melanoma mortality is higher for men and almost 2/3 of deaths from melanoma occur in more developed regions (mortality rates 2.0 and 1.2) than in less developed regions (mortality rates 0.4 and 0.3 for males and females, respectively) (Ferlay *et al.* 2015).

1.1.3. Melanoma risk factors

Melanoma as all cancers is a heterogeneous disease with a complex aetiology that involves environmental, phenotypic, genetic and epigenetic risk factors. The main melanoma environmental risk factor is UVR, both solar and artificial. Solar light consists of a continuous spectrum of electromagnetic radiation: ultraviolet, visible and infrared. UVR light wavelength ranges from 100 to 400 nm and is divided into three bands: UVA (315–400 nm), UVB (280–315 nm) and UVC (100–280 nm). The stratospheric ozone layer absorbs practically all UVC radiation as well as approximately 95% UVB and 5% of UVA. As a result about 5% UVB and 95% UVA reaches the Earth's surface (El Ghissassi *et al.* 2009). Studies using platyfish (*Xiphophorus maculatus*) and swordtails (*Xiphophorus heUeri*) hybrids in the late 1980s showed that UVB can induce melanoma development (Nairn *et al.* 1996; Setlow *et al.* 1989). UVB mechanism of action is direct DNA damaging by formation of dimeric photoproducts, namely, cyclobutane pyrimidine dimers between adjacent thymine or cytosine residues and pyrimidine (6-4) pyrimidone photoproducts among adjacent pyrimidine residues. If not repaired by cellular reparation systems, these DNA lesions can subsequently result in so called 'UVR signature mutations'—C to T and CC to TT transitions (Cadet *et al.* 2005; Matsumura & Ananthaswamy 2004). Data about UVA involvement in melanoma development, however, has been conflicting. It has been shown that UVA can induce melanomas in *Xiphophorus* hybrid models and a focal melanocytic hyperplasia that is a putative melanoma precursor in opossum models (Ley 2001; Setlow *et al.* 1993). These findings led to thinking that UVA might be an even more effective and important melanoma inducer than UVB due to its larger abundance on the Earth's surface (Setlow *et al.* 1993). Yet other studies showed that UVA has very small—if any—effect on melanoma induction (Mitchell *et al.* 2010; De Fabo *et al.* 2004; Robinson *et al.* 2000). However, current opinion is that, UVA has an indirect effect on melanoma development via induction of reactive oxygen species (Matsumura & Ananthaswamy 2004; Petersen *et al.* 2000), oxidative DNA modifications (Kielbassa *et al.* 1997) as well as *in vitro* DNA strand breaks in human keratinocytes and skin fibroblasts (Greinert *et al.* 2012; Wischermann *et al.* 2008) that might lead to chromosomal aberrations. To sum up—both UVA and UVB can promote melanoma development and it applies both to solar UVR and indoor UVR sources—mainly tanning beds. Tanning beds became very popular in Europe and USA in 1980s, however, studies now show that they undoubtedly are associated with increased risk of melanoma (Boniol *et al.* 2012). Since 2009 all solar UVR as well as UVR-emitting tanning devices are classified by the International Agency for Research on Cancer (IARC) as 'carcinogenic to humans (group 1)' (El Ghissassi *et al.* 2009).

The harmful effects of UVR vary depending on the timing and pattern of exposure. There is a clear correlation between the frequency of melanoma and geographical latitudes due to differences in UVR levels. Closer to the equator the sun is higher in the sky and UVR

travels a shorter distance with a more direct angle through atmosphere layers (including protective ozone) thus UVR exposure on the planet's surface is more intense. The average increase in UVB per one geographic latitude degree towards the equator is 3% (Rigel *et al.* 1999). Thereby, the closer to equator, the higher UVR exposure is and skin cancer frequency increases. The most illustrative fact is that the highest melanoma rate in the fair-skinned population in the world is in Australia and New Zealand that lie close to the equator—between latitudes 10° and 45° south while, for example, European countries where melanoma incidence is lower are located further from equator—between latitudes 80° and 35° north.

Although the total UVR level is an important melanoma risk factor, studies show that the risk is higher when a person experiences relatively rare, intermittent sun exposure events, typically associated with recreational activities. Risk is especially higher for indoor workers as well as if experienced in a young age (Chen *et al.* 2013; Gandini *et al.* 2005a; Walter *et al.* 1999; Nelemans *et al.* 1993). On the other hand, intermittent sun exposure has been shown to increase survival from melanoma, possibly due to some vitamin D mediated histological or intracellular effects (Berwick *et al.* 2005). Interestingly, some studies on chronic sun exposure suggest that it might have a protective effect on melanoma development (Walter *et al.* 1999; White *et al.* 1994). To sum up—clearly UVR is involved in melanoma development, however, the mechanism of action is complex and varies depending of individual situations and other factors involved.

Another important melanoma risk factor is sunburns. Painful sunburns are associated not only with melanoma but also with other skin cancers (squamous and basal cell carcinomas) before the age of 20 years. For older individuals (20-40 years), the association remains statistically significant only for melanoma (Kennedy *et al.* 2003). The strongest association between painful sunburns and melanoma risk is when they are experienced in early childhood (before 6 years) (Oliveria *et al.* 2006; Kennedy *et al.* 2003).

UVR is the main but not only environmental melanoma risk factor. Cancer in general is associated with many chemical, biological and other environmental risk factors. Studies demonstrate that people exposed to industrial pollutants, e.g., vinyl chloride (Guarneri & Guarneri 2014; Langard *et al.* 2000), volatile organic compounds (Boeglin *et al.* 2006) and polychlorinated biphenyls (Gallagher *et al.* 2011) as well as ionizing radiation are at increased melanoma risk (Fink & Bates 2005). On the question of extrinsic biological risk factors, Merrill *et al.* (2015) suggests human papillomavirus as a possible reason for growing melanoma incidence due to the fact that it is found in more than half of melanoma biopsies.

The impact of environmental factors very much depends on each individual's phenotypical and physiological characteristics. A pale skin that hardly tans and easily sunburns, red or blond hair, blue eyes and an increased amount of freckles are typically associated with increased melanoma risk (Gandini *et al.* 2005b; Gandini *et al.* 2005c). However, the major phenotypical risk factor that often is accompanied by above mentioned factors is an increased number of naevi as well as a presence of dysplastic or atypical naevi (Berwick *et al.* 2016; Goldstein & Tucker 2013; Tucker & Goldstein 2003; Clark *et al.* 1978). Meta-analysis results show that persons who carry more than 100 common naevi have almost seven times higher risk of to develop melanoma than persons with less than 15 naevi (relative risk=6.89; 95% CI=4.63–10.25) (Gandini *et al.* 2005b). Melanoma risk doubles with every increase of 25 naevi and is also higher when naevi are large (> 5 mm) (Markovic *et al.* 2007). Dysplastic naevi are another major melanoma risk factor. The International Agency for Research on Cancer (IARC) characterizes dysplastic naevi as slightly raised, irregularly pigmented lesions with vague borders, asymmetric shape, >0.5 cm in the greatest dimension (IARC 2011). These lesions are especially noteworthy in familial setting where atypical mole

syndrome (AMS) (also known as dysplastic naevus syndrome or familial atypical multiple mole and melanoma syndrome (FAMMM)) is often observed (Berwick *et al.* 2016; Clark *et al.* 1978). AMS is characterized by the presence of at least two dysplastic naevi, more than 100 common naevi as well as the presence of naevi on abnormal body sites, and together with family history of melanoma this phenotype is an especially strong melanoma risk factor (Berwick *et al.* 2016; Clark *et al.* 1978).

Skin pigmentation is primarily determined by melanocytes that are functionally connected to dermal fibroblasts and epidermal keratinocytes (Yamaguchi *et al.* 2007). Melanocytes contain melanosomes—pigment-filled organelles—that are later transported to keratinocytes (Yamaguchi *et al.* 2007; Van Den Bossche *et al.* 2006). In keratinocytes of lightly pigmented skin melanosomes are clustered above the nuclei, but in dark skin the heavily pigmented melanosomes are distributed more evenly throughout the cell ensuring maximal UVR absorption (Yamaguchi *et al.* 2007) (Figure 3).

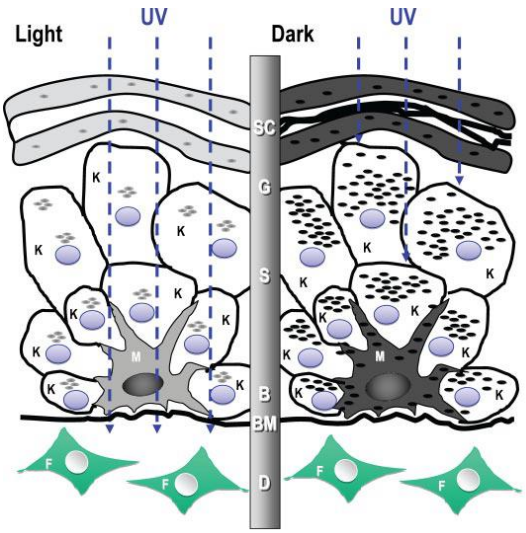

Figure 3. Schematic representations of lightly and dark pigmented human skin structures. Middle column: SC—*stratum corneum*; G—*stratum granulosum*; S—*stratum spinosum*; B—*stratum basale*; BM—basement membrane; D—*dermis*. Cell types: K—keratinocytes; M—melanocytes; F—fibroblasts. Melanin granules depicted as shaded ovals (Yamaguchi *et al.* 2007).

The role of genetics in melanoma development mainly manifests itself through the accumulation of numerous, random somatic genetic aberrations in many cellular pathways (Zhang *et al.* 2016). However, approximately 10% of melanoma cases occur in a familial setting as recurrent cases thus showing the role of germline inheritance in melanoma development (Read *et al.* 2016; Potrony *et al.* 2015). Melanoma risk genes are divided in three groups: high, medium and low risk genes. The first two high melanoma risk genes were discovered more than 30 years ago: cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) (Hussussian *et al.* 1994; Cannon-Albright *et al.* 1992) and cyclin-dependent kinase 4 gene (*CDK4*) (Zuo *et al.* 1996). However, variants in these genes, depending on the study population, are found in only 20-60% of all melanoma families (Goldstein *et al.* 2007). In the recent years due to the rapid progress in next-generation sequencing technologies significant advancement in new melanoma risk gene identification has been reached. Analysis of high-density melanoma families has revealed several new high melanoma risk genes: BRCA1-associated protein 1 gene (*BAP1*), protection of telomeres 1 gene (*POT1*), adrenocortical

dysplasia protein homolog gene (*ACD*), telomeric repeat binding factor 2 interacting protein gene (*TERF2IP*), telomerase reverse transcriptase (*TERT*), catalytic subunit of DNA polymerase epsilon (*POLE*) (Read *et al.* 2016; Aoude *et al.* 2015a; Potrony *et al.* 2015). Still, variants in each of these genes have been found in a relatively small number of melanoma families and no disease causing variant has been found in about half of melanoma families (Read *et al.* 2016). Therefore most likely there could be other currently unknown high melanoma risk genes or disease predisposition might be explained by combinatory effect of lower risk alleles (Goldstein *et al.* 2017). However, most melanoma cases are sporadic and gain an influence from a complex interaction of medium and low risk genetic factors. So far, three genes have been classified as medium melanoma risk genes: melanocortin 1 receptor gene (*MC1R*), melanogenesis associated transcription factor gene (*MITF*) and solute carrier family 45 member 2 gene (*SLC45A2*) (Read *et al.* 2016; Potrony *et al.* 2015). Both of them are involved in pigmentation regulation. There are also at least 30 loci involved in many cellular functions, namely, pigmentation regulation, naevi formation, cell cycle regulation, DNA repair, telomere processes and other functions, that have been identified as low melanoma risk loci (Read *et al.* 2016).

1.2. Genetic risk factors of melanoma

1.2.1. High melanoma risk genes

Cyclin-dependent kinase inhibitor 2A gene—*CDKN2A*

Cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) (OMIM 600160) was the first discovered melanoma risk gene. It is located on chromosome 9 at position 9p21.3 and has four exons that encode two tumour suppressor proteins—p16INK4A and p14ARF via alternative reading frames and splicing (Mao *et al.* 1995; Quelle *et al.* 1995; Stone *et al.* 1995; Kamb *et al.* 1994a; Serrano *et al.* 1993) (Figure 4).

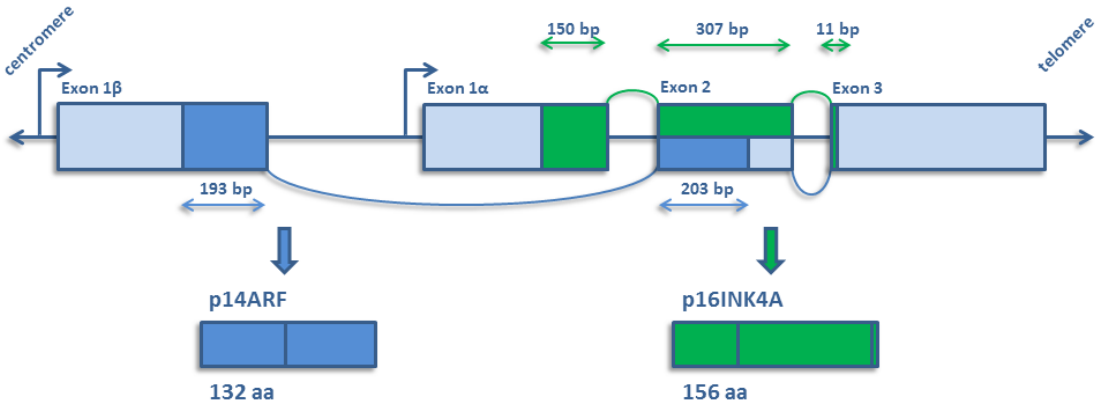

Figure 4. Schematic representation of the human cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) locus on chromosome 9p21. *CDKN2A* contains four exons—exon 1α, exon 1β, exon 2 and exon 3 (coloured boxes) that encodes two tumour suppressor proteins via alternative reading frames and splicing. The protein p16INK4A consists of 156 amino acid residues and is produced from transcript of exon 1α, exon 2 and exon 3 (green boxes). The protein p14ARF consists of 132 amino acid residues and is produced from another transcript of exon 1β, exon 2 and exon 3 (dark blue boxes).

One of the main cancer hallmarks is the dysregulation of cell cycle that results in uncontrolled cell proliferation and an inability to undergo differentiation and/or apoptosis (Hanahan & Weinberg 2011). The cell cycle is classically divided into 4 phases: gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M) with check-points between them. The transition from G1 to S phase is controlled by the cyclin-dependent serine/threonine kinases (Sherr & Roberts 2004). Altogether, the human genome contains >20 genes encoding cyclin-dependent kinases that act as cell cycle regulators in the various stages of the cell cycle (Malumbres *et al.* 2009). In the active state cyclin-dependent kinase is a holoenzyme that is composed of a regulatory subunit, cyclin, and a catalytic subunit, termed a cyclin-dependent kinase (Malumbres & Barbacid 2005). Cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) together with cyclin D phosphorylate the retinoblastoma protein (RB) that allows for cell cycle transition through the G1 checkpoint (Malumbres & Barbacid 2005). The protein p16INK4A binds CDK4/6 thus inhibiting RB phosphorylation and arresting the cell cycle in the G1 phase (Lukas *et al.* 1995; Sherr & Roberts 1995). The protein p14ARF inhibits the cell cycle in G1 and also the G2/M phase by binding and degrading TP53 inhibitor mouse double minute 2 homolog (MDM2), thus allowing TP53 to induce cell growth arrest or apoptosis (Stott *et al.* 1998; Zhang *et al.* 1998; Quelle *et al.* 1995).

The first evidence of 9p locus involvement in melanoma development was the loss of the short arm or even full copy of chromosome 9 in dysplastic naevi and melanomas (Cowan *et al.* 1988). Cannon-Albright with colleagues in 1992 were the first who, using linkage analysis, showed 9p21 locus association with familial melanoma (Cannon-Albright *et al.* 1992) that was later confirmed in other studies (Goldstein *et al.* 1994; Gruis *et al.* 1993; Nancarrow *et al.* 1993). Besides, large 9p21 germline deletion was found in a patient with multiple primary melanomas (MPM) and atypical naevi thus further showing 9p role in melanoma development (Petty *et al.* 1993). Several studies also found that more than half of melanoma cell lines contain mutations in the 9p21 locus and there was localized so called multiple tumour suppressor 1 gene (*MTS1*), now known as *CDKN2A* (Kamb *et al.* 1994a; Nobori *et al.* 1994; Weaver-Feldhaus *et al.* 1994; Fountain *et al.* 1992). Besides, a diverse range of mutations affecting 9p21 locus was also found in other tumour cell lines i.e. leukaemia (Diaz *et al.* 1990), glioma (Olopade *et al.* 1992), non-small cell lung cancer (Merlo *et al.* 1994), as well as various other lung, breast, brain, bone, skin, bladder, kidney, ovary, and lymphocyte tumour cell lines (Kamb *et al.* 1994a) confirming the importance of this locus in tumour formation and underpinning its function as a tumour suppressor in general.

In 1994, Hussussian with colleagues was the first who reported *CDKN2A* germline genetic variants in melanoma families. They analysed 18 melanoma families with at least two melanoma cases and found six germline variants that were restricted to melanoma cases in nine melanoma families thus allowing to assume that these variants might have caused melanoma (Hussussian *et al.* 1994) and subsequent study also demonstrated evidence for the functional effect of these variants on the protein inhibitory properties (Ranade *et al.* 1995). Since then many similar melanoma family studies have been performed, however, results are very varied partly due to different inclusion criteria for ‘melanoma families’. In general a family can be described as a melanoma family if there are at least three melanoma cases irrespective to a degree of relation, however, in regions with lower sunlight level (such as northern Europe, including Latvia) families with at least two first degree melanoma affected relatives are also classified as a melanoma family (de Snoo & Hayward 2005).

Up to date many *CDKN2A* genetic variants in familial melanoma patients have been found. Most of the p16INK4A variants are missense substitutions in exon 1α and exon 2 that are evenly distributed along practically all protein sequence (exon 3 codes only last 4 amino

acids of the p16INK4A) (Aoude *et al.* 2015b; Goldstein *et al.* 2006; Hussussian *et al.* 1994). At least one change in *CDKN2A* has been identified as a 'hotspot mutation'—24 bp duplication p.Pro11_Ser12insAlaAlaGlySerSerMetGluPr (c.32_33insGGCGGCGGGGAGC AGCATGGAGCC; rs587780668) that repeats two already naturally existing sequences in *CDKN2A* 5'region, extending the protein by eight amino acids while presumably retaining its functionality (Walker *et al.* 1995). This change has been found in several melanoma families in Australia (Flores *et al.* 1997; Walker *et al.* 1995), USA (Goldstein *et al.* 1995) and the United Kingdom (Pollock *et al.* 1998). Haplotype analysis suggests that there have been multiple mutational events causing this duplication, most likely caused by unequal crossing over between naturally occurring 24 bp repeats or polymerase slippage (Pollock *et al.* 1998).

Several of the *CDKN2A* genetic variants are systematically found in distinct geographic regions and populations and are considered 'founders' due to haplotype evidence of common ancestry rather than recurrent hotspot mutations (Goldstein *et al.* 2006). The most common genetic variant in the Netherlands is the 19 bp deletion c.225-243del19 (rs730881674) in *CDKN2A* exon 2, better known as 'p16-Leiden'. It was discovered in melanoma patients coming from a rather isolated Dutch population group near Leiden (de Snoo *et al.* 2008; Gruis *et al.* 1995). This deletion has been found in 90% of Dutch melanoma families with disease associated genetic variants (Goldstein *et al.* 2006).

Another founder variant has been discovered in the Swedish population—an extra arginine insertion in p16INK4A exon 2 in amino acid position 113: p.Arg112_Leu113insArg (c.335_337dupGTC; rs768966657). Similarly as the p16-Leiden variant in the Netherlands, this variant is the most frequent melanoma associated genetic change in Swedish melanoma families—it constitutes 92% of all disease associated variants (Goldstein *et al.* 2006). Interestingly, statistical estimates indicate that this variant arose 98 generations or ~2000 years ago in present-day Sweden region (Hashemi *et al.* 2001; Borg *et al.* 1996).

The missense variant p.Gly101Trp (c.301G>C; rs104894094), also in p16INK4A exon 2, is the most common familial melanoma variant in France, Italy and Spain—its frequency in melanoma families there reaches 60% (Goldstein *et al.* 2006). Similarly to the above mentioned Swedish founder variant, its origination is estimated 97 generations or ~1940 years ago and has a Celtic ancestry (Ciotti *et al.* 2000). Functionally this variant diminishes p16 affinity to CDK4 thus hampering its ability to inhibit CDK4 kinase activity (Reymond & Brent 1995).

In Iceland *CDKN2A* exon 2 variant p.Gly89Asp (c.266G>A; rs137854599) has been found in >2% of Icelandic invasive melanoma patients and is also considered a founder variant that originated in Northern Iceland in the 1600's (Goldstein *et al.* 2008).

In about half of melanoma families in the United Kingdom and Australia the disease is associated with *CDKN2A* exon 2 variants p.Arg24Pro (c.71G>A; rs104894097), p.Leu32Pro (c.95T>C; rs878853650) and p.Met53Ile (c.159G>A; rs104894095), as well as a deep intron variant IVS2-105A>G (c.458-105A>G; rs1060501266) (Lang *et al.* 2007; Goldstein *et al.* 2006; Harland *et al.* 2001; Pollock *et al.* 1998). All three coding variants p.Arg24Pro, p.Leu32Pro and p.Met53Ile are located in highly conserved positions (Goldstein *et al.* 2006; Harland *et al.* 1997). Protein binding assays shows that variant p.Arg24Pro is defective for binding CDK4 and variant p.Met53Ile—both CDK4 and CDK6 (Harland *et al.* 1997).

The deep intron variant IVS2-105A>G creates a false splice donor site producing aberrant mRNA (Harland *et al.* 2001). Interestingly, IVS2-105A>G has also been found in an Italian patient with eight primary melanomas. Haplotype analysis rejected English ancestry

therefore study's authors suggested that this position might be a mutational hotspot (Majore *et al.* 2004).

Besides IVS2-105A>G, other p16INK4A variants that cause aberrant splicing and segregate with melanoma families have also been identified. For example exon 1 splice donor variant p.Gln50Pro (c.149A>C; rs587778189) has been discovered in an American melanoma family without previously detected *CDKN2A* variants. This variant causes either correctly spliced mRNA with nonconservative amino acid change p.Gln50Pro that decreases effectivity of interaction between p16INK4A and CDK4, or aberrantly spliced mRNA leading to truncated p16INK4A suggesting its involvement in melanoma development (Loo *et al.* 2003; Lynch *et al.* 2002). Interestingly, in the same position, another missense amino acid change p.Gln50Arg (c.149A>G; rs587778189) has been observed in melanoma families in Australia (Pollock *et al.* 2001; Walker *et al.* 1995).

Another interesting position is the border between exon 2 and the next intron. Two contiguous variants in the last position of exon 2 and in the first position in the following intron or exon 2 donor splice site c.457G>T (p.Asp153Tyr; rs45476696) and IVS2+1G>T (c.457+1G>T) have been identified in several melanoma families and both of them have been proved to cause aberrant splicing (Loo *et al.* 2003; Rutter *et al.* 2003; Lynch *et al.* 2002; Moskaluk *et al.* 1998; Hussussian *et al.* 1994). The variant IVS2+1G>T has also been discovered in a patient with MPM (MacKie *et al.* 1998). A couple of rare intronic variants also have been demonstrated to have an association with melanoma in English and Australian melanoma families—IVS1+1104C>A (c.150+1104C>A, rs756102261) and IVS1-1104C>G (c.151-1104C>G) (Harland *et al.* 2005a).

Several variants associated with familial melanoma development have also been discovered in the *CDKN2A* promoter and 5'UTR region. The first identified p16INK4A 5'UTR variant with proven functional impact on protein translation was c.-34G>T (rs1800586). This transversion creates a novel AUG translation initiation codon, disrupting normal wild type protein translation. This variant segregates with melanoma in families in Canada, has been found in Australian and USA melanoma families and most likely has ancestry in the British Isles (Eliason *et al.* 2006; Harland *et al.* 2000; Liu *et al.* 1999). Since then several other variants in the p16INK4A promoter region have been identified in English, French, Italian, American and Australian melanoma families—c.-33G>C (rs531597737), c.-191G>A (rs3814960), c.-252A>T (rs538489460), c.-347G>C, c.-493A>T (rs36228834), c.-735G>A (rs3731238), c.-981G>T (rs2811708) nevertheless they showed no segregation with familial melanoma (Goldstein *et al.* 2008; Pollock *et al.* 2001; Harland *et al.* 2000; Soufir *et al.* 1998). Recent studies in Italian, English and French familial as well as sporadic melanoma patients discovered some other novel variants located up to 180 bp upstream from p16INK4A (Andreotti *et al.* 2016; Bisio *et al.* 2010). These two studies also demonstrated that almost half of these variants—c.-21C>T (rs762129503), c.-27_-5del23 (rs757137815), c.-42T>A, c.-56G>T, c.-67G>C and c.-93_-91delAGG—similarly to previously known c.-34G>T (rs1800586) are functionally relevant, while the rest of them—c.-14C>T(rs764244718), c.-20A>G, c.-30G>A (rs780207215), c.-40C>T, c.-25C>T+c.-180G>A (rs144481587+ rs145660371), c.-45G>A, c.-59C>G, c.-87T>A and c.-252A>T (rs538489460), including previously discovered c.-33G>C and c.-191G>A—are functionally neutral (Andreotti *et al.* 2016; Bisio *et al.* 2010).

Altogether variants affecting p14ARF are found less frequently than p16INK4A variants. Some of the genetic variants in *CDKN2A* exon 2 have the potential to modify both gene products, for example, large exon 2 deletion was detected in a French melanoma family, and this deletion leads to truncation of both proteins—p16INK4A and p14ARF (Lesueur *et*

al. 2008). In one Norwegian melanoma family another large germline deletion was detected. This deletion removes exon 1α and half of exon 2 resulting in a truncated p14ARF and loss of p16INK4A (Knappskog *et al.* 2006). In other study a 24 bp deletion within exon 2 was found in a patient with MPM and a family history of melanoma and functional studies showed that this deletion disrupts functional activity of both *CDKN2A* products (Hashemi *et al.* 2002; Hashemi *et al.* 2000). In several families transversion IVS1-1G>C (c.151-1G>C; rs730881677) causing aberrant splicing at the acceptor splice site of *CDKN2A* intron 1 has been found. This variant causes incorrect splicing resulting in both p16INK4A and p14ARF lacking exon 2 (Hocevar *et al.* 2006; Petronzelli *et al.* 2001). First this variant was identified in an Italian family with melanomas, neurofibromas, and multiple dysplastic naevi (Petronzelli *et al.* 2001). Lately this variant has been discovered in another family with melanoma and multiple other tumours and is associated with FAMMM syndrome (Sargen *et al.* 2016). Some exclusively *CDKN2A* exon 1β affecting deletions (Laud *et al.* 2006; Mistry *et al.* 2005; Randerson-Moor *et al.* 2001), insertions (Rizos *et al.* 2001) or splice variants (Harland *et al.* 2005b; Hewitt *et al.* 2002) also have been found in several melanoma families.

Besides the already mentioned *CDKN2A* variants, novel other changes have been found in various studies of different populations (Borroni *et al.* 2017; Burgstaller-Muehlbacher *et al.* 2015; de Torre & Martinez-Escribano 2010; Erlandson *et al.* 2007; Ghiorzo *et al.* 2006; Huber & Ramos 2006; Knappskog *et al.* 2006; Avbelj *et al.* 2003).

Overall *CDKN2A* accounts for about 20% of melanoma associated changes found in melanoma families, however, the number varies from 5% to 72% depending on geographic region and selection criteria used in the particular study (Potrony *et al.* 2015; Goldstein *et al.* 2007). Meanwhile, on a population level *CDKN2A* variant frequency in sporadic melanomas is rather low—it ranges from 0.2% to 3.3% (Harland *et al.* 2014; Nikolaou *et al.* 2011; Goldstein *et al.* 2008; Berwick *et al.* 2006; Aitken *et al.* 1999) and overall probability to detect a germline *CDKN2A* variant in melanoma is <2% except for patients with MPM and/or a family history of melanoma (Harland *et al.* 2014). In a family setting the melanoma risk for *CDKN2A* variant carriers increases with age from 30% by age 50 to 67% by age 80 (Bishop *et al.* 2002) while in the general population risk is much lower—ranging from approximately 14% by age 50 to 28% by age 80 (Begg *et al.* 2005). There are some characteristics that correlate with a positive *CDKN2A* variant status in a melanoma family such as an increased number of family members with melanoma, presence of thick melanomas, an early age of the disease onset, MPM as well as at least one family member with pancreatic cancer (Taylor *et al.* 2016; Pedace *et al.* 2011; van der Rhee *et al.* 2011; Goldstein *et al.* 2007; Goldstein *et al.* 2006). Carriers of *CDKN2A* variants in melanoma families harbour significantly more naevi as well as a higher proportion of atypical naevi than non-carriers (Taylor *et al.* 2017; Florell *et al.* 2004; Bishop *et al.* 2000; Cannon-Albright *et al.* 1994). In addition, variants in *CDKN2A* are associated with atypical mole syndrome in *CDKN2A* families suggesting *CDKN2A*'s role in the development of naevi (Bishop *et al.* 2000; Clark *et al.* 1978). An interesting observation is that pathogenic *CDKN2A* variant carriers within families have a darker skin type and are less likely develop severe burns compared with wild-type or non-pathogenic variant carriers (Taylor *et al.* 2016) highlighting melanoma risk in patients with UVR protective phenotype features.

While pancreatic cancer is the most common type of cancer found in *CDKN2A* melanoma families and changes in *CDKN2A* are also associated with familial pancreatic cancer (Zhen *et al.* 2015; Ghiorzo *et al.* 2012a; Harinck *et al.* 2012; Goldstein *et al.* 2004; Goldstein *et al.* 1995; Whelan *et al.* 1995), *CDKN2A* variants also have been demonstrated to have an association with other types of cancer such as lung, breast, gastro-oesophageal

cancer, malignant mesothelioma (MM), sarcoma and childhood acute lymphoblastic leukaemia (Jouenne *et al.* 2017; Betti *et al.* 2016; Vijayakrishnan *et al.* 2015; Helgadottir *et al.* 2014; Potrony *et al.* 2014; Mukherjee *et al.* 2012; Sherborne *et al.* 2010; Debniak *et al.* 2007; Oldenburg *et al.* 2004; Borg *et al.* 2000).

CDKN2A variants are also associated with MPM. An increased frequency (7-10%) of *CDKN2A* variants has been found in sporadic MPM patients without a family history of melanoma and an increased number of MPM is correlated with higher possibility of carrying a *CDKN2A* variant, however, some studies report frequency close to population level (~3%) (Bruno *et al.* 2016; Harland *et al.* 2014; Helsing *et al.* 2008; Pastorino *et al.* 2008; Berwick *et al.* 2006; Puig *et al.* 2005; Monzon *et al.* 1998).

The *CDKN2A* locus shows association with sporadic melanoma (Barrett *et al.* 2015; Maccioni *et al.* 2013a; Kumar *et al.* 2001). Several common *CDKN2A* coding region variants have been studied with regard to their impact on melanoma risk both within families as well as on a population level. One such is p.Ala148Thr (c.442G>A; rs3731249) in exon 2 of p16INK4A. It has been found in several melanoma families, however, did not fully segregate with the disease (Hussussian *et al.* 1994; Kamb *et al.* 1994b) and results in population level studies are also discrepant. A positive association has been found in Latvian and Polish populations (Pjanova *et al.* 2007; Debniak *et al.* 2005) as well as in Spanish patients with MPM (Puig *et al.* 2005), but no association has been found in English, French, Italian and Icelandic populations (Goldstein *et al.* 2008; Spica *et al.* 2006; Bertram *et al.* 2002). Functionally this variant does not demonstrate an effect on its inhibitory activity (Lilischkis *et al.* 1996; Ranade *et al.* 1995; Reymond & Brent 1995).

A couple of common variants in the *CDKN2A* gene 3'region c.*29G>C, initially known as Nt500C>G (rs11515) and c.*69C>T, initially known as Nt540C>T (rs3088440) have also been studied with their regard to melanoma risk, however, results are inconsistent. Both of these variants have been shown to have an association with shorter disease free survival thereby more aggressive disease nature (Sauroja *et al.* 2000) though in another study c.*69C>T was demonstrated to have an association with improved patient survival (Straume *et al.* 2002). Variant c.*29G>C has been shown to have an association with familial melanoma risk in Australia (Aitken *et al.* 1999) and c.*69C>T has been shown to have an association with sporadic melanoma in Norwegian and Spanish populations (Maccioni *et al.* 2013a; Kumar *et al.* 2001), however, no association for any of these two variants has been found in other population studies (Brazilian, Greek, Icelandic, Latvian, Polish and Spanish population in another study) (Stefanaki *et al.* 2013; Ibarrola-Villava *et al.* 2010; Goldstein *et al.* 2008; Pjanova *et al.* 2007; Debniak *et al.* 2005). To sum-up—meta-analysis from MelGene database shows that c.*69C>T is associated with melanoma while c.*29G>C is not associated (Antonopoulou *et al.* 2015). In addition, genome wide association studies (GWASs) and subsequent genotyping studies demonstrate that variants in the *CDKN2A* locus, also in other genes near *CDKN2A* (for example, methylthioadenosine phosphorylase gene—*MTAP* and cyclin-dependent kinase inhibitor 2B antisense RNA 1 gene—*CDKN2B-AS1*) are associated with melanoma thus showing importance of wider *CDKN2A* locus in melanoma development (Barrett *et al.* 2015; Amos *et al.* 2011; Barrett *et al.* 2011; MacGregor *et al.* 2011; Bishop *et al.* 2009). Some of variants in these regions also have been associated with phenotype features characteristic to high melanoma risk—skin type and development of naevi, especially clinically atypical naevi (diameter >5 mm, irregular border and colour) (Maccioni *et al.* 2013a; Barrett *et al.* 2011; Yang *et al.* 2010; Falchi *et al.* 2009; Florell *et al.* 2004) as well as several histological features, for example, higher intensity of pigmentation (Sargen *et al.* 2015).

Cyclin-dependent kinase 4 gene—*CDK4*

Considering the involvement of p16INK4A in familial melanoma, other molecules involved in the same cell cycle regulation mechanism also appeared to be good melanoma risk gene candidates; research in this area led to the discovery of the second high melanoma risk gene—cyclin-dependent kinase 4 gene (*CDK4*) (OMIM 123829). *CDK4* is located on chromosome 12 in position 12q14.1 (Mitchell *et al.* 1995). It consists of 8 exons, spans a 5kb segment and codes for a 303 amino acid long catalytic subunit of a heterodimeric serine/threonine kinase CDK4 that controls cell cycle G1 phase progression (Zuo *et al.* 1996). CDK4 together with additional protein cyclin D phosphorylates RB, thus releasing transcription factor E2F from the binding with RB and E2F activates transcription of the genes that subsequently leads to cell cycle progression in the synthesis phase (Malumbres & Barbacid 2005). CDK4 is inhibited by p16INK4A that binds to CDK4 preventing its interaction with cyclin D (Serrano *et al.* 1993).

A mutational hotspot in codon 24 of *CDK4* gene exon 2, resulting in substitutions p.Arg24Cys (c.70C>T; rs11547328) or p.Arg24His (c.71G>A; rs104894340) has been identified in several melanoma families across the world (Molven *et al.* 2005). Interestingly, Arg substitution with Cys in CDK4 position 24 initially was found in a somatic melanoma tissue (but not in the patient's blood) (Wölfel *et al.* 1995). It was subsequently discovered that the wild type amino acid Arg24 is located in a region that directly interacts with CDK4 inhibitor p16INK4A and amino acid substitutions caused by these variants functionally impairs CDK4's ability to bind p16INK4A, leading to elevated CDK4 activity and cell cycle progression thus generating dominant oncogene (Coleman *et al.* 1997; Wölfel *et al.* 1995). In addition, substitution p.Arg24Cys as a part of the CDK4 peptide was found to create a tumour-specific neoantigen that is recognized by cytolytic T lymphocytes (Wölfel *et al.* 1995).

Zuo *et al*. (1996) was the first who identified *CDK4* variant p.Arg24Cys that segregated with familial melanoma in two unrelated USA melanoma families without previously detected genetic changes in p16INK4A. Later the same substitution was also found in one Italian patient without familial melanoma history (Ghiorzo *et al.* 2012b). The second *CDK4* codon 24 substitution p.Arg24Hys has been found in two melanoma families in France (Soufir *et al.* 2007; Soufir *et al.* 1998), one in Norway, Australia and the United Kingdom (Molven *et al.* 2005), one in Italy (Majore *et al.* 2008), one in Greece (Nikolaou *et al.* 2011) and two in Latvia (Pjanova *et al.* 2009; Pjanova *et al.* 2007).

BRCA1-associated protein-1 gene—*BAP1*

Since the initial discoveries of high melanoma risk genes *CDKN2A* and *CDK4*, lately other high melanoma risk genes have also been found. One of them is tumour suppressor gene BRCA1-associated protein-1 gene (*BAP1*) (OMIM 603089) that is located on chromosome 3 at position 3p21.1 and consists of 17 exons. It encodes 90 kDa 729 amino acid long nuclear-localized ubiquitin carboxy-terminal hydrolase (Jensen *et al.* 1998). BAP1 contains several physically overlapping but functionally distinct domains and interacting regions (Wang *et al.* 2016) (Figure 5).

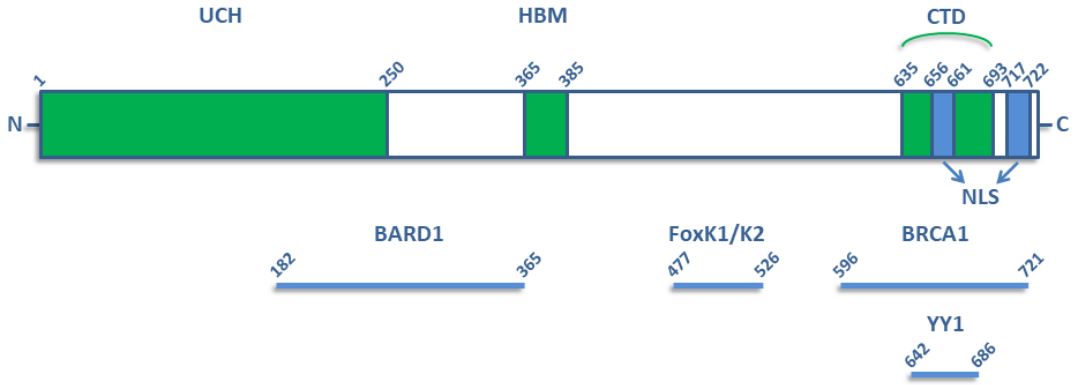

Figure 5. Schematic representation of the human BRCA1-associated protein 1(BAP1) functional domains and interacting regions. UCH—N terminal ubiquitin carboxyl hydrolase domain (1–250); BARD1 binding region (182–365); HBM—host cell factor 1 binding domain (365–385 aa); FoxK1/K2 binding region (477–526); BRCA1 binding region (596–721); CTD (C terminal domain) —ASXL1/2 binding domain (635–693 aa); YY1—Ying Yang 1 binding region (642–686); NLS—nuclear localisation signals (656–661 and 717–722) (adapted after Wang *et al.* 2016).

One of the main functions of BAP1 is tumour suppression through protein deubiquitination, thus regulating a wide range of cell processes, i.e. transcription, cell cycle control, DNA damage repair and cellular differentiation. BAP1 mediated deubiquitination is carried out by the protein N terminal ubiquitin carboxyl hydrolase domain (UCH) (Jensen *et al.* 1998). At molecular level BAP1 acts as a chromatin-associated protein and is involved in formation of various multiprotein complexes with different transcription factors. One of the main BAP1 targets is host cell factor 1 (HCF1). HCF1 is transcriptional factor that modulates chromatine structure and activates other transcription factors such as E2F thus controlling cell cycle progression through the G1/S phase (Tyagi *et al.* 2007). BAP1 regulates cell proliferation by binding and deubiquitinating HCF1 (Machida *et al.* 2009; Misaghi *et al.* 2009). BAP1 also forms protein complexes with other transcription factors, such as, Yin Yang 1 (YY1) (Yu *et al.* 2010), the forkhead transcription factors FoxK1 and FoxK2 (FoxK1/K2) (Okino *et al.* 2015), the additional sex comb-like proteins ASXL1 and ASXL2 (ASXL1/2) (Daou *et al.* 2015) and others that are also involved in cell cycle and proliferation regulation as well as other functions. BAP1 also mediates DNA repair and damage signalling via interaction with several homologous recombination proteins, such as tumour suppressor breast cancer protein 1 (BRCA1) and BRCA1-associated RING domain protein 1 (BARD1) (Jensen *et al.* 1998). BRCA1 forms a heterodimer with BARD1 and this complex acts as an E3 ubiquitin ligase that regulates the DNA damage response (Greenberg *et al.* 2006; Dong *et al.* 2003; Hashizume *et al.* 2001). BAP1 regulates ubiquitination during the DNA damage response and the cell cycle by binding and deubiquitylating BARD1, thus modulating E3 ligase activity of the BRCA1-BARD1 protein complex (Nishikawa *et al.* 2009).

The first evidence about *BAP1*'s role in melanoma was demonstrated by Harbour *et al.* (2010) who discovered inactivating *BAP1* somatic mutations in 84% (26 of 31) of highly metastatic uveal melanomas (UM). In addition, in one patient a germline insertion in *BAP1* exon 12 p.Glu402fsTer2 (c.1318-1319insA) was detected, and the conclusion was drawn that *BAP1* might be implicated in UM metastasis (Harbour *et al.* 2010). Later a germline variant at the *BAP1* intron 6/exon 7 boundary c.438-2A>G (rs587776879) and a variant in exon 16 p.Q684Ter (c.2050C>T; rs387906848) creating a stop codon was discovered in two families with multiple MMs, UM and other types of cancer (Testa *et al.* 2011). In addition, protein

truncating deletions were also found in couple sporadic MM patients with UM history (Testa *et al.* 2011). Concurrently a second study identified *BAP1* germline variants in two families with cutaneous melanocytic neoplasms and UM or cutaneous melanoma—a deletion in exon 13 p.Gln436AsnfsTer135 (c.1305delG; rs587776877) and a variant that removes the acceptor splice acceptor site before exon 17 p.Met687GlufsTer28 (c.2057-2A>G; rs587776878) (Wiesner *et al.* 2011). Since then germline *BAP1* variants have been found in at least 87 families with various types of cancers and that has led to the identification of germline autosomal dominant *BAP1* hereditary cancer predisposition syndrome (OMIM 614327) that clinically is characterized by UM, cutaneous melanoma, renal cell carcinoma (RCC), MM, as well as specific atypical cutaneous melanocytic proliferations—melanocytic *BAP1*-mutated atypical intradermal tumours (MBAITs) (Haugh *et al.* 2017; Betti *et al.* 2016; McDonnell *et al.* 2016; Ohar *et al.* 2016; Cheung *et al.* 2015; Maerker *et al.* 2014; Pilarski *et al.* 2014; Aoude *et al.* 2013a; Cheung *et al.* 2013; Farley *et al.* 2013; Höiom *et al.* 2013; Popova *et al.* 2013; Carbone *et al.* 2012; Njauw *et al.* 2012; Wadt *et al.* 2012; Wiesner *et al.* 2012a; Wiesner *et al.* 2012b; Abdel-Rahman *et al.* 2011). Several other types of cancer also have been identified in these families, for example, basal cell carcinoma and squamous cell carcinoma and it has been suggested to include them in the *BAP1* tumour syndrome (Rawson *et al.* 2017; de la Fouchardière *et al.* 2015; Wadt *et al.* 2015). Thus melanoma is a part of a broader phenotype associated with *BAP1* tumour syndrome—approximately 18% of patients with germline *BAP1* variants have been diagnosed with cutaneous melanoma while frequencies for UM and MM in this tumour syndrome is higher—28% and 22%, respectively (Rai *et al.* 2016). In a study by Njauw *et al.* (2012) only 0.52% (1/193) of patients with a family history of solely cutaneous melanoma carried *BAP1* variant while there were 28.6% (2/7) patients with *BAP1* variants and family history of both cutaneous melanoma and UM (Njauw *et al.* 2012).

So far more than 70 different *BAP1* variants have been discovered in *BAP1* tumour syndrome families and most of them are frameshift or nonsense variants resulting in protein truncation (Haugh *et al.* 2017). At least six variants have been found recurrently in two or more families—one variant p.Arg60Ter (c.178C>T) in *BAP1* exon 4 has been found recurrently in three families from Denmark and USA with UM, cutaneous melanoma, MM and other cancers (Wadt *et al.* 2015; Njauw *et al.* 2012) while the rest of the recurrent variants have been found in two families each (Haugh *et al.* 2017).

A couple of studies have explored *BAP1* variant frequencies in population-based patient samples. An Australian study analysed 66 UM patients and found protein altering *BAP1* variants that might have contributed to disease risk in 3% (2/66) of them (Aoude *et al.* 2013b). In a Finnish population-based sample frequency of UM patients reaches 2% (3/148), however, two of these three patients had a family history of UM (Turunen *et al.* 2016). In sporadic cutaneous melanoma patients germline *BAP1* variant frequency according to data is lower compared to UM patients—no more than 1% (O'Shea *et al.* 2017), that relatively reflects the situation in familial melanoma; *BAP1* variants are more prevalent in UM there as well.

TELOMERASE ASSOCIATED HIGH MELANOMA RISK GENES

Telomerase reverse transcriptase gene—*TERT*

In recent years several genes associated with telomere maintenance (*TERT*, *POT1*, *ACD*, *TERF2IP*) have been identified as high melanoma risk genes and it is estimated that germline variants in these genes might explain ~1% of familial melanoma cases (Potrony *et al.* 2015).

Telomeres are structures at the ends of chromosomes that ensure chromosomal integrity and genomic stability, and in mammals, including humans, they are formed of double stranded tandem 5'-TTAGGG-3' DNA repeats with 3' single stranded overhang that forms a secondary structure called a t-loop, that helps them to be distinguished from damaging DNA breaks (Doksani *et al.* 2013; O'Sullivan & Karlseder 2010; Palm & de Lange 2008; Moyzis *et al.* 1988). Telomeres progressively shorten with each round of cell division leading to cellular or replicative senescence reaching the Hayflick limit (Bodnar *et al.* 1998; Harley *et al.* 1990; Hayflick & Moorhead 1961). Telomeres are elongated by enzyme telomerase or terminal transferase. The telomerase holoenzyme complex includes the enzyme component—reverse transcriptase (TERT), the RNA component (TERC) and several additional ribonucleoproteins (Mason & Perdigones 2013; Greider & Blackburn 1985) (Figure 6). Telomerase activity in most tissues is silenced through TERT promoter regulatory elements leading to telomere shortening and DNA damage while in carcinogenic processes it is upregulated by various transcriptional activators, and telomerase activity is characteristic to most of the cancer types (Shay 2016; Kyo *et al.* 2008; Kim *et al.* 1994). Human telomerase was isolated as a constituent of crude extracts from HeLa cells (Morin 1989), but the catalytic subunit coding gene was identified several years later. The *TERT* gene (OMIM 187270) is located on chromosome 5 at position 5p15.33, spans more than 37 kb, encompasses 16 exons and encodes a 1132-amino acid protein with 127 kDa molecular mass (Wick *et al.* 1999; Kilian *et al.* 1997; Meyerson *et al.* 1997; Nakamura *et al.* 1997).

Complete telomere structures include not only chromosomal DNA, but also its accompanying accessory nucleoproteins involved in telomere maintenance (Figure 6). One of these protein complexes is shelterin that is involved in regulation of practically all functions associated with telomeres—telomerase complex recruitment and interaction with telomeres, telomerase-based telomere maintenance, telomere protection from degradation, aberrant recombination or incorrect DNA repair distinguishing natural chromosome ends from DNA breaks (Palm & de Lange 2008). Shelterin consists of six proteins—protection of telomeres 1 protein (POT1), telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), telomeric repeat binding factor 2 interacting protein (TERF2IP, also known as RAP1), TRF1-interacting nuclear protein 2 (TINF2 or TIN2), and TIN2-interacting protein (TPP1) encoded by adrenocortical dysplasia protein homolog gene (*ACD*) (Heidenreich & Kumar 2017; Palm & de Lange 2008) (Figure 6). POT1 binds to single-stranded TTAGGG repeats at the 3' telomere DNA overhang (Flynn & Zou 2010). TRF1 and TRF2 bind to double-stranded telomere DNA recruiting other shelterin complex proteins to telomeres. TPP1 enhances POT1 binding to telomeres as well as telomerase complex recruitment to telomeres (Zhong *et al.* 2012; Xin *et al.* 2007).

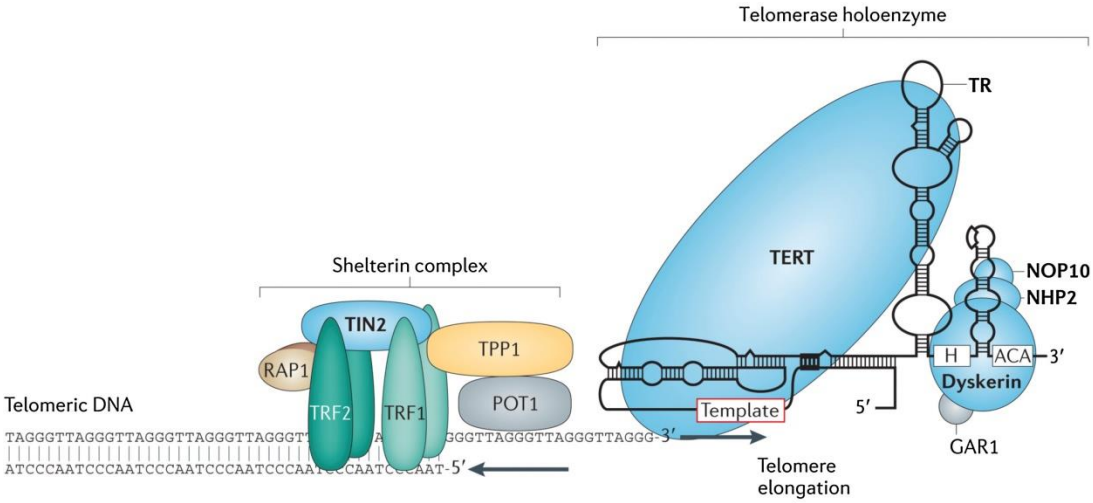

Figure 6. Schematic representation of the telomerase holoenzyme and the shelterin complex relatively to the telomere end structure. Telomerase holoenzyme comprises the enzyme component—reverse transcriptase (TERT) and the RNA component (TERC) containing a 3′ H/ACA box motif that binds dyskerin protein complex with additional proteins H/ACA ribonucleoprotein complex subunit 2 (NHP2), nucleolar protein 10 (NOP10) and ribonucleoprotein GAR1. The shelterin complex contains six protein components—protection of telomeres 1 protein (POT1), telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), telomeric repeat binding factor 2 interacting protein (TERF2IP, also known as RAP1), TRF1-interacting nuclear protein 2 (TINF2 or TIN2), and TIN2-interacting protein (TPP1) (Heidenreich & Kumar 2017).

TERT was the first gene of the telomere maintenance complex components that was found to be associated with familial melanoma. Using linkage analyses and high-throughput sequencing a novel germline noncoding transversion T>G at the -57 position from the translation initiation codon ATG in the *TERT* promoter was identified in a four generation German family with 14 melanoma patients without changes in *CDKN2A* or *CDK4* (Horn *et al.* 2013). The novel variant was identified in all four melanoma patients analysed as well as in one 36 years-old unaffected member of the family with multiple naevi. Patients in this family developed melanoma at an early age and a couple of affected members also had other types of cancer. Variants were not found among 140 sporadic cases and 165 controls as well as in public variant databases (Horn *et al.* 2013).

Functionally variant c.-57T>G (rs878855297) introduces promoter sequence change CCTGAA>CCGGAA creating a new binding site for E-twenty six (ETS) transcription factors of ternary complex factors (TCFs) Elk1 and Elk4 resulting in an approximately twofold increase in *TERT* transcription (Horn *et al.* 2013).

Another study screened *TERT* promoter variant c.-57T>G in 675 melanoma families without changes in other known high melanoma risk genes from the United Kingdom, Denmark, Australia and the Netherlands and the variant was detected in seven-case family from the United Kingdom with MPM and an early disease onset and one case also has seven basal cell carcinomas (Harland *et al.* 2016). None of the 1863 cases and 529 controls carried this variant thus confirming that *TERT* promoter variant c.-57T>G is rare, highly penetrant and associated with early onset melanoma as well as other types of cancer (Harland *et al.* 2016).

In addition to familial melanoma, several variants in *TERT* locus in GWASs and case-control studies have been demonstrated to have an association with sporadic melanoma.

Among them are variants rs2853676 (c.1573+4881A>G), rs2242652 (c.1950+245C>T), rs401681 (c.1316-153G>A), and rs13356727 (g.1312342G>A), the latter two of which have protective effect, and rs13356727 is also associated with decreased number of naevi (Law *et al.* 2012; Barrett *et al.* 2011; Nan *et al.* 2011a; Rafnar *et al.* 2009; Stacey *et al.* 2009).

Interestingly, in one study promoter variant c.-57T>G was also found in several primary melanoma cell lines (3 of 109) as a somatic variant (Heidenreich *et al.* 2014a). Various other *TERT* promoter variants have been found in melanoma cell lines and tissue samples as somatic changes. Horn *et al*. (2013) who initially identified c.-57T>G promoter variant in familial melanoma also screened 168 melanoma cell lines and none of them carried particular promoter variant c.-57T>G, however, they found various recurrent somatic ultraviolet signature variants in the *TERT* core promoter with prevailing hotspot variants c.-124G>A and c.-146G>A that also introduce ETS/TCF binding motifs in 74% (125) of cell lines and confirmed these variants in 85 % (45 of 53) corresponding metastasized tumours. In addition 33% (25 of 77) unrelated formalin-fixed, paraffin-embedded primary melanomas also harboured changes in *TERT* promoter (Horn *et al.* 2013). Further studies strengthened the evidence about the role of somatic *TERT* promoter variants in melanoma development. Huang *et al*. (2013) concurrently examined 70 melanoma cell lines and found the same promoter changes in hotspot positions -124 and -146 in 71% (50) of the total cell lines analysed (Huang *et al.* 2013). It was also observed that *TERT* promoter variants tend to occur together with changes in serine/threonine-protein kinase B-Raf proto-oncogene (*BRAF*), neuroblastoma RAS viral oncogene homolog (*NRAS*) and *CDKN2A* (Nagore *et al.* 2016a; Heidenreich *et al.* 2014a) and are also associated with a more aggressive disease and poorer disease prognosis characterized by faster growing melanomas, increased Breslow thickness, ulceration, high mitotic rate (Nagore *et al.* 2016a; Nagore *et al.* 2016b; Macerola *et al.* 2015; Griewank *et al.* 2014; Heidenreich *et al.* 2014a).

Somatic *TERT* promoter variants, predominantly c.-124G>A and c.-146G>A have also been identified in many other types of malignancies—non-melanoma skin cancer, glioma, medulloblastoma, hepatobiliary, thyroid, urinary tract, endometrial, ovarian, esophagus, lung and other types of cancer thus exposing a wide range of malignancies influenced by changes in *TERT* promoter region (Heidenreich & Kumar 2017; Heidenreich *et al.* 2014b). Germline *TERT* variants have also been observed in several families with dyskeratosis congenita—rare congenital disease with phenotypic characteristics resembling premature ageing. Germline coding variants detected in these families lead to reduced telomerase activity and extremely short telomeres (Basel-Vanagaite *et al.* 2008; Marrone *et al.* 2007; Armanios *et al.* 2005).

Protection of telomeres 1 gene—*POT1*

After *TERT* promoter variant c.-57T>G was discovered in melanoma families, other telomere proteins also became a targets of interest as a possible high melanoma risk genes that was strengthened by variant discovery in *POT1*.

Human *POT1* (OMIM 606478) was cloned in 2001 and was found to be located on chromosome 7 at position 7q31.33 (Baumann & Cech 2001). It is 120 kb long, contains 22 exons with translation starting in exon 6, and encodes at least five different proteins (Baumann *et al.* 2002).

The first rare melanoma-associated germline variants in *POT1* were discovered in two concurrent studies using exome sequencing in individuals from melanoma families without changes in *CDKN2A* and *CDK4* genes (Robles-Espinoza *et al.* 2014; Shi *et al.* 2014). In the first study exome sequencing was performed in 184 individuals from 105 melanoma

families with 2-11 melanoma cases from Australia, the United Kingdom and the Netherlands (Robles-Espinoza *et al.* 2014). Altogether four different *POT1* germline variants in five melanoma families were identified—missense variant Tyr89Cys in five-case melanoma family, splice acceptor variant between exons 17 and 18 c.1687-1G>A (rs587777473) in a six-case melanoma family, missense variant p.Gln94Glu (c.280C>G; rs587777474) in a two-case melanoma family, and p.Arg273Leu (c.818G>A; rs587777476) in one two-case melanoma family. Interestingly, the latter variant was also identified in one early-onset MPM patient from population-based sporadic melanoma cohort. All three missense variants p.Tyr89Cys, p.Gln94Glu and p.Arg273Leu are located in highly conserved oligonucleotide/ oligosaccharide-binding N terminal domains OB1 and OB2 of POT1 resulting in weakened or completely disrupted POT1 binding to telomeric DNA possibly allowing unwanted DNA damage response and/or access by telomerase that leads to increased telomere length that has been associated with cancer development (Haycock *et al.* 2017; Aoude *et al.* 2015b; Robles-Espinoza *et al.* 2014). A couple of tested individuals also developed breast and lung cancer besides melanoma, and some untested family members in these families developed other malignancies thus suggesting *POT1* might have an influence on a wider range of cancers (Robles-Espinoza *et al.* 2014).

In the second study exome sequencing was performed in 101 cases or obligate variant carriers in 56 melanoma families from Italy as well as exome and targeted sequencing data from familial melanoma cases or MPM sporadic cases from the United States (139 cases from 68 families), France (267 cases from 234 families and 157 MPM patients) and Spain (10 cases from 3 families) were used (Shi *et al.* 2014). Three missense variants were discovered in altogether seven Italian melanoma families—variant p.Gln623His (c.1869G>C; rs587777478) in both cases in a two-case melanoma family, p.Arg137His (c.410G>A; rs587777475) in two cases and one healthy person from another two case family, and p.Ser270Asn (c.809G>A; rs587777477) in five Italian melanoma families. Variant p.Ser270Asn was found in all cases or obligate carriers (altogether 11 individuals) from four families and in one melanoma case from two analysed cases in a bilineal melanoma family. Haplotype analysis revealed that p.Ser270Asn is a founder variant—all carriers shared the same haplotype suggesting a common ancestor ~10 generations ago. A couple more *POT1* variants were discovered in families from other populations—variant p.Asp224Asn (c.670G>A; rs202187871) in four cases in a five-case melanoma family from the US (this variant was also found in one sporadic MPM case from Italy) and p.Ala532Pro (c.1594G>C; rs537377921) in one case with available DNA from a three-case family from France (this variant was also identified in one MPM patient with basal cell carcinoma from France) (Shi *et al.* 2014).

Similarly to the study mentioned above, variants p.Arg137His, p.Asp224Asn and p.Ser270Asn are located in OB domains of POT1. Carriers of founder variant p.Ser270Asn had longer telomeres as well as cells displayed rather large heterogeneity in telomere length, demonstrating that this variant also might have some effect on telomere length regulation. Previously longer telomeres have been associated with melanoma development (Burke *et al.* 2013). Variant p.Ala532Pro, which is located near a splice junction, might disrupt splicing, and variant p.Gln623His is located in C-terminus of *POT1*, which contains the TPP1-binding region thus suggesting their role in DNA binding (Shi *et al.* 2014).

Shi *et al*. (2014) also demonstrated an association between *POT1* rare variants and sporadic melanoma with OR=2.1, 95% CI=1.1–4.2, p=0.024 (31/768 cases and 15/768 controls), especially when analysis was restricted to exonic variants with OR=5.4, 95% CI=1.5–29.2; p=0.0021 (16/768 cases and 3/768 controls).

To sum up, altogether 9 variants have been associated with familial melanoma and their location in the POT1 relative to protein domains are depicted in Figure 7.

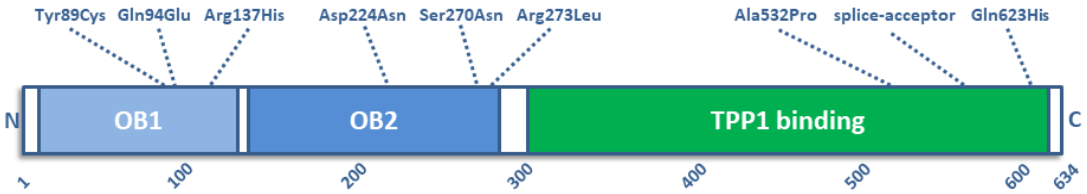

Figure 7. Schematic representation of the human POT1 protein and localization of variants associated with familial melanoma. OB1, OB2—oligonucleotide/oligosaccharide-binding domains 1 and 2, TPP1—shelterin complex protein TPP1 binding domain (adapted after Bainbridge *et al.* 2015).

POT1 variants have also been associated with other types of cancer (Calvete *et al.* 2017). Somatic *POT1* variants have been found in 3.5% of chronic lymphocytic leukaemia patients and in 9% of patients with an especially aggressive chronic lymphocytic leukaemia subtype (Ramsay *et al.* 2013). Interestingly, one of the variants p.Gln94Arg was located in codon 94 that was also affected in familial melanoma (variant p.Gln94Glu) (Robles-Espinoza *et al.* 2014; Ramsay *et al.* 2013). Later *POT1* variants were also discovered in familial chronic lymphocytic leukaemia (Speedy *et al.* 2016). *POT1* variants have also been associated with familial glioma (Bainbridge *et al.* 2015). Using whole exome and high-throughput sequencing three different variants with incomplete penetrance were found in three unrelated glioma families—p.Gly95Cys (c.283G>T; rs797045168), p.Glu450Ter (c.1348G>T; rs797045169) and p.Asp617Glufs (c.1851_1852delTA; rs758673417). Variants p.Glu450Ter and p.Asp617Glufs are protein truncating and delete conserved part of the protein C-terminus, which contains the TPP1-binding region (Bainbridge *et al.* 2015). *POT1* variants have also been identified in familial colorectal cancer cases. Three variants that induce premature translation termination were identified—p.Asn75LysfsTer16 (c.219_220insA), p.Arg363Ter (c.1087C>T; rs756198077) and p.Asp617Glufs (previously found in glioma family) (Chubb *et al.* 2016). Finally, a missense *POT1* variant p.Arg117Cys (c.349C>T; rs780936436) as well as other damaging *POT1* variants have been found in *TP53*-negative Li-Fraumeni-like Spanish families with cardiac angiosarcomas and breast angiosarcomas as well as sporadic cardiac angiosarcoma and cardiac sarcoma patients (Calvete *et al.* 2017; Calvete *et al.* 2015). Thus *POT1* variants are involved in tumorigenesis in various tissues.

Adrenocortical dysplasia protein homolog gene—*ACD*

Another protein of the telomere shelterin complex involved in melanoma development is adrenocortical dysplasia protein homolog gene (*ACD*) (OMIM 609377). *ACD* is located on chromosome 16 at position 16q22.1 and encodes 544 amino acid long protein with molecular mass 58 kDa that is often referred to as TPP1 (Liu *et al.* 2004). In the shelterin complex TPP1 enhances POT1 binding to telomeres and helps recruit telomerase complex to telomeres (Zhong *et al.* 2012; Wang *et al.* 2007; Xin *et al.* 2007). Aoude *et al.* (2015c) analysed exome, genome and targeted sequencing data from 601 individuals of 510 melanoma families looking for variants in genes of five shelterin complex components—*ACD*, *TERF2IP*, *TRF1*, *TRF2*, *TINF2*. In six melanoma families five different changes in *ACD* were detected. Four of these variants cosegregated with melanoma. A nonsense variant

p.Gln320Ter (c.958C>T) was found in a six-case Australian family in four of five cases analysed and also one unaffected family member. This variant truncates the protein, disrupting the POT1 and TINF2 binding domains. A missense variant p.Asn249Ser (c.746A>G; rs370512338) was found in two families—in all seven cases available for analysis as well as four unaffected members in a twelve-case Australian family and three of four cases available for analysis in a six-case Danish family. These two families shared the same haplotype suggesting a founder effect of this variant. Another *ACD* missense variant p.Val272Met (c.814G>A; rs780989111) was found in all three cases in an Australian family. All three novel *ACD* variants are located in the POT1 binding domain of ACD. Two additional missense variants p.Ala200Thr (c.598G>A; rs377701284) and p.Ile322Phe (c.964A>T; rs757807430) were discovered in another two families, however, they did not fully segregate with melanoma. None of these variants was detected in any of sporadic cases (n=1669) or controls (n=1590) in a case-control study thus indicating that these are indeed rare variants (Aoude *et al.* 2015c).

Variants in *ACD* have also been associated with other diseases—inherited bone marrow failure where a variant caused telomere shortening (Guo *et al.* 2014), severe dyskeratosis congenita (Hoyeraal-Hreidarsson syndrome) (Kocak *et al.* 2014), familial chronic lymphocytic leukaemia (Speedy *et al.* 2016), and also a somatic variant in leukaemia where it causes increased telomere length (Spinella *et al.* 2015).

Telomeric repeat binding factor 2 interacting protein gene—*TERF2IP*

Simultaneously with *ACD*, another shelterin complex gene—telomeric repeat binding factor 2 interacting protein (*TERF2IP*) (OMIM 605061) was found to be associated with familial melanoma. *TERF2IP* is located on chromosome 16 at position 16q23.1 and encodes 399 amino acid long protein (also known as RAP1) with molecular mass 47 kDa (Li *et al.* 2000). Functionally TERF2IP participates in the repression of homology-directed repair of double-strand chromosomal breaks that might alter telomere length (Sfeir *et al.* 2010).

In the same study mentioned above Aoude *et al.* (2015c) identified four families with four different *TERF2IP* variants (one nonsense and three missense) cosegregating with melanoma. Nonsense variant p.Arg364Ter (c.1090C>T; rs765095939) was found in a four-case melanoma family from the United Kingdom. The variant was detected in one melanoma affected patient and one family member with breast cancer. Two melanoma patients and one unaffected relative were obligate carriers and one melanoma patient could not be analysed. This variant truncates 36 amino acids of the protein C-terminus, disrupting the TRF2 binding domain. A missense variant p.Gln191Arg (c.572A>G) was found in a two-case melanoma family—in both cases who had developed melanoma very early (age 15 and 24) and both also have cervical cancer, as well as in two unaffected family members. This variant was predicted to disrupt TRF2 binding site that might interfere with TERF2IP contribution to the shelterin complex. Two other missense variants p.Met5Ile (c.15G>A) and p.Asp10His (c.28G>C) were detected in two more families, however, their segregation with melanoma was less pronounced. None of these variants were detected in a case-control study (1669 cases and 1590 controls) meaning that they are indeed rare and important in a familial context (Aoude *et al.* 2015c).

Both *ACD* and *TERF2IP* families were associated with MPM and early disease onset; several other types of cancer were also present in these families—breast, lung, cervix, colon, bowel, ovary, and lymphoma suggesting predisposition to a broader spectrum of cancers (Aoude *et al.* 2015c).

Aoude *et al*. (2015c) also detected variants in the three other genes of the shelterin complex but they did not demonstrate an association with melanoma. However, considering all the available data about variants in genes involved in telomere maintenance, this pathway appears to be very important in melanoma predisposition.

Catalytic subunit gene of DNA polymerase epsilon—*POLE*

The gene most recently discovered to be high melanoma risk is *POLE* (OMIM 174762); it is located on chromosome 12 at position 12q24.33 and encodes a central catalytic subunit of DNA polymerase epsilon that is 2285 amino acids long with molecular mass 261 kDa (Li *et al.* 1997) and is involved in DNA repair and replication (Johnson *et al.* 2015).

A melanoma-associated *POLE* variant was discovered using whole-genome or exome sequencing in 87 cases from 34 Australian melanoma families without changes in known high melanoma risk genes. A missense substitution p.Trp347Cys (c.1041G>T) was detected in five of six cases analysed from s seven-case cutaneous melanoma family that also had an eighth melanoma case—UM (Aoude *et al.* 2015a). Two of the affected carriers had MPM, one additionally developed RCC and prostate cancer and one was a UM patient. The only affected individual without the novel *POLE* change developed melanoma at a very late age—94 years. The missense variant p.Trp347Cys is located within the proofreading exonuclease domain of the large subunit of POLE, and a functional study produced evidence showing that this variant would cause an increased mutation rate. Additional population-based melanoma patients (1093 Australian and 150 Danish) were also screened for *POLE* variants and there were ten patients with novel (p.Arg259Cys (c.775C>T), p.Gln352Pro (c.1055A>C), p.Lys425Arg (c.1274A>G) and p.Val460Met (c.1378G>A)) or rare (p.Phe282Ser (c.844C>T; rs138207610), p.Asp287Glu (c.861T>A; rs139075637), p.Arg446Gln (c.1337G>A; rs151273553)) *POLE* variants that were located in the exonuclease domain. Several of these patients had MPM, other types of cancer or a family history of cancer, or they developed melanoma at an early age. Among these patients, a three-case melanoma family was identified where both of the genotyped patients carried another novel missense variant p.Ile515Met (c.1545C>G). In addition, in one Danish family a novel variant three amino acids after the boundary of the exonuclease domain p.Gln520Arg (c.1559A>G) was discovered. This family had several cases of colorectal cancer (Aoude *et al.* 2015a). Another *POLE* variant p.Leu424Val (c.1270C>T; rs483352909) had also previously been found to be associated with familial colorectal cancer (Palles *et al.* 2013) and another variant p.Asn363Lys (c.1089C>T; rs146639652) is suggested to predispose to an even broader spectrum of malignancies i.e. ovarian, endometrial, gastric, pancreatic and brain cancer (Rohlin *et al.* 2014). Altogether these data suggests that *POLE* variants might predispose to a broad spectrum of cancers.

1.2.2. Medium melanoma risk genes

High melanoma risk genes only partly explain melanoma heredity in a familial setting. The rest of the predisposition could be explained either by some yet unknown high penetrance genes or complex interactions of multiple medium and low penetrance risk alleles that are more common in the population (Read *et al.* 2016). In addition, a polygenic heritability component has been found in many sporadic cancers, including melanoma, thus affirming the important contribution of germline variants to the development of sporadic melanoma (Lu *et al.* 2014). However, so far polygenic melanoma risk models utilising information about many medium/low risk variants simultaneously, have contributed little to

conventional phenotypic risk prediction models (Kypreou *et al.* 2016; Penn *et al.* 2014; Cust *et al.* 2013; Fang *et al.* 2013).

Three genes have been defined as medium melanoma risk genes i.e. genes with melanoma predisposing variants that have OR between 2 and 5—melanocortin 1 receptor gene (*MC1R*), melanogenesis associated transcription factor (*MITF*) and solute carrier family 45 member 2 (*SLC45A2*) (Read *et al.* 2016).

Melanocortin 1 receptor gene—*MC1R*

Many of the medium and low melanoma risk genes are involved in pigmentation regulation. So far the most extensively studied medium melanoma risk gene (previously categorized as low risk gene) is melanocortin 1 receptor gene (*MC1R*) (OMIM 155555) that is a key regulator of pigmentation synthesis. Human *MC1R* is located on chromosome 16 at position 16q24.3 (Gantz *et al.* 1994; Chhajlani & Wikberg 1992; Mountjoy *et al.* 1992). The coding region of *MC1R* is 951 bp long and it consists of one exon that encodes a cell membrane receptor of 317 amino acids (Chhajlani & Wikberg 1992). Structurally melanocortin 1 receptor (MC1R) is a typical G-protein coupled receptor (GPCR) that consists of seven transmembrane α spiral domains, extracellular N tail and intracellular C tail (Chhajlani & Wikberg 1992). MC1R belongs to the A-13 family of the rodopshin class of GPCRs that altogether comprises five melanocortin receptors (MC1R, MC2R, MC3R, MC4R and MC5R) that regulates various physiological functions (Rodrigues *et al.* 2015; Cone 2006). MC1R is activated by agonist α-melanocyte stimulating hormone (α-MSH) derived from the preprohormone precursor proopiomelanocortin. The binding of α-MSH to MC1R causes conformational changes in the receptor triggering binding of guanosine triphosphate (GTP) to the G protein α subunit that leads to the activation of adenylate cyclase (AC). AC catalyzes synthesis of the second messenger—cyclic adenosine monophosphate (cAMP). Concentration increase of cAMP activates protein kinase A (PKA) that phosphorylates cAMP response-element binding protein (CREB) leading to the transcriptional activation of melanogenesis associated transcription factor (MITF) (Bertolotto *et al.* 1998; Price *et al.* 1998). MITF stimulates the transcription of several enzymes involved in melanogenesis—tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), dopachrome tautomerase (DCT) and others leading to melanin production in melanocytes (Herraiz *et al.* 2017). There are two types of melanin—black to brown eumelanin and red to yellow pheomelanin. Eumelanin is highly protective against UVR damage while pheomelanin has weaker protective properties. Pheomelanin also contributes to oxidative DNA damage both in the presence and absence of UVR, thus playing a role in cancerogenesis (Napolitano *et al.* 2014; Morgan *et al.* 2013; Mitra *et al.* 2012). Both pigments derive from the same precursor dopaquinone which is formed from tyrosine by TYR (d'Ischia *et al.* 2015; Hirobe 2011). Human skin colour is determined by the ratio of both of these pigments and it largely depends on the availability of amino acid cysteine that is required for pheomelanin but not eumelanin synthesis; thus in the presence of intact MC1R with strong signalling, cysteine reserves are not able to keep pace with melanin synthesis and overall melanin ratio inclines towards eumelanin (Chen *et al.* 2014).

Another important MC1R signalling pathway target is peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α)—a transcription factor involved in the regulation of mitochondrial biogenesis and oxidative metabolism. PGC-1α stimulates MITF expression, resulting in melanin production (Shoag *et al.* 2013), while MITF contributes to PGC-1α overexpression in melanoma, resulting in melanoma cell survival and tumour

progression (Vazquez *et al.* 2013). Thus two MC1R downstream targets PGC-1α and MITF are interconnected in a positive feedback loop (Ronai 2013).

In addition to melanin synthesis regulation, MC1R also protects melanocytes against UVR damage and maintains genomic stability via pathways other than pigmentation. MC1R plays a role in melanocyte protection against oxidative DNA damage and one of the mediators of this process is tumour suppressor TP53 (Kadekaro *et al.* 2012; Kadekaro *et al.* 2010; Song *et al.* 2009; Kadekaro *et al.* 2005). MC1R also activates nucleotide excision repair (NER) pathway that corrects UVR-induced DNA damage (Jarrett *et al.* 2017; Shah & He 2015; Jarrett *et al.* 2014), moreover, NER and melanin synthesis pathways diverge (Wolf Horrell *et al.* 2017). Among other MC1R mediated targets is MAP kinase p38 that promotes melanogenesis and has been shown to inhibit cell proliferation in B16 murine melanoma cells after their treatment with α-MSH (Smalley & Eisen 2000).

MC1R is very polymorphic in the Caucasian population. More than 100 nonsynonymous changes have been found (Pérez Oliva *et al.* 2009). There are also considerable differences in the frequency and distribution of *MC1R* variants among different populations. The highest frequency of nonsynonymous *MC1R* variants is in Great Britain and Ireland populations (57.0 %), followed by the US (47.2%), France (38.4 %), Italy (36.6 %) and Greece (21.3 %) (Gerstenblith *et al.* 2007). In comparison, in African, southern-Asian and other populations where skin pigmentation is comparatively dark only a small number of nonsynonymous *MC1R* variants have been detected, for example, 3.6% in Africa, 5.7 % in India and 8.8 % in Papua New Guinea (Gerstenblith *et al.* 2007; Harding *et al.* 2000). This is uncharacteristic to African populations because usually these populations have higher genomic variation than non-Africans. This phenomenon could be explained by a strong functional constraint on *MC1R* in order to maintain protection against DNA damage by UVR (Harding *et al.* 2000).

Since the last decade of the 20th century, numerous studies in different populations have investigated the relationship between *MC1R* gene variants, pigmentation characteristics and/or melanoma risk.

Initially *MC1R* variants were identified in the British population where their association with pigmentation features (hair colour, skin type) was described (Valverde *et al.* 1995). In this study *MC1R* was sequenced in 30 red-haired persons with fair, sun sensitive skin and 30 dark-haired individuals who tan easily. Nine different *MC1R* variants were found in 21 red-haired individuals while none were found in dark-haired persons. The most often found *MC1R* variants were p.Asp294His (c.880G>C; rs1805009) and p.Val92Met (c.274G>A; rs2228479) (Valverde *et al.* 1995). Shortly after this study an association was discovered between *MC1R* variants p.Asp84Glu (c.252C>A; rs1805006) and p.Val92Met and fair skin and red hair in the US population (Koppula *et al.* 1997). Later, Smith *et al*. (1998b) in an Australian twin study found association between *MC1R* variants p.Arg151Cys (c.451C>A; rs1805007), p.Arg160Trp (c.478C>T; rs1805008), p.Asp294His and red hair. The same three variants together with p.Val92Met were also associated with fair skin (Smith *et al.* 1998b). Similar results were obtained from the Irish population, where an association between red hair and p.Arg151Cys, p.Arg160Trp and p.Asp294His was also found (Smith *et al.* 1998b).

Results from these studies show a link between *MC1R* variants and the red hair, fair skin and poor tanning ability commonly termed as ‘red hair colour’ (RHC) phenotype and it has been confirmed in further larger studies. Four variants p.Asp84Glu, p.Arg151Cys, p.Arg160Trp, p.Asp294His present a strong association with the RHC phenotype and are designated as RHC or *R* variants (Williams *et al.* 2011; Duffy *et al.* 2004; Sturm *et al.* 2003).

Variants p.Arg142His (c.425G>A; rs11547464) and p.Ile155Thr (c.464T>C; rs1110400) are also sometimes termed RHC variants due to a strong association with the RHC phenotype in a familial setting (Duffy *et al.* 2004; Flanagan *et al.* 2000). The p.Val60Leu (c.178G>T; rs1805005), p.Val92Met and p.Arg163Gln (c.488G>A; rs885479) variants present a weaker association with RHC phenotype and therefore are titled 'non-red hair colour' NRHC or *r* variants (Williams *et al.* 2011; Duffy *et al.* 2004; Sturm *et al.* 2003). Variants p.Arg142His and p.Ile155Thr are also often included in the NRHC variant group (Williams *et al.* 2011). In some cases all nonsynonymous *MC1R* variants that are not RHC variants are considered to be NRHC variants (Demenais *et al.* 2010; Kanetsky *et al.* 2010).

Besides hair and skin colour, *MC1R* variants have also been demonstrated to have an association with the amount of freckles (Bastiaens *et al.* 2001a) and sensitivity to sun radiation in persons with non-red hair colour (Healy *et al.* 2000).

These associations with phenotype characteristics can be explained by changes in receptor functional activity caused by genetic variants. Functional and expression studies using heterologous cells transfected with different *MC1R* constructs or primary human melanocytes with germline *MC1R* variants (p.Val60Leu, p.Asp84Glu, p.Val92Met, p.Arg142His, p.Arg151Cys, p.Ile155Thr, p.Arg160Trp, p.Arg163Gln, p.Asp294His) show that all of them impair at least some aspect of receptor functional activity to various degrees—affinity to α-MSH (Ringholm *et al.* 2004; Robinson & Healy 2002; Sánchez-Más *et al.* 2002; Schiöth *et al.* 1999; Frändberg *et al.* 1998), coupling of cAMP (Beaumont *et al.* 2007; Nakayama *et al.* 2006; Newton *et al.* 2005; Ringholm *et al.* 2004; Robinson & Healy 2002; Sánchez-Más *et al.* 2002; Scott *et al.* 2002; Schiöth *et al.* 1999; Frändberg *et al.* 1998) and/or cell-surface expression (Beaumont *et al.* 2007; Sánchez-Laorden *et al.* 2006; Beaumont *et al.* 2005) (Table 1). Often functionally significant *MC1R* variants are called 'loss-of-function' alleles, however, results from different studies are not completely consistent due to differences in experimental design and expression systems (Newton *et al.* 2005) therefore these variants probably have varying degrees of activity (Beaumont *et al.* 2005). In addition, *MC1R* RHC variants act in a dominant negative manner on wild-type receptors due to receptor molecule dimerization (Beaumont *et al.* 2007; Sánchez-Laorden *et al.* 2006; Mandrika *et al.* 2005). The presence of RHC alleles in human primary melanocytes also reduces transcriptional activation of several downstream genes—well known *MC1R* pigmentation regulatory targets *MITF* and *SLC45A2*, as well as proto-oncogene *c-Fos* that confirms the existence of *MC1R* non-pigmentary roles (Newton *et al.* 2007). In addition, functional effects are not limited to the subset of RHC *MC1R* variants—many other less common nonsynonymous *MC1R* variants also impair receptor functional activity (Pérez Oliva *et al.* 2009; Nakayama *et al.* 2006; Sánchez-Más *et al.* 2002; Jiménez-Cervantes *et al.* 2001a; Jiménez-Cervantes *et al.* 2001b; Schiöth *et al.* 1997). *MC1R* variants also hinder DNA repair after UVR damage (Kadekaro *et al.* 2010; Scott *et al.* 2002), therefore the presence of *MC1R* germline variants may promote mutation accumulation and it has been demonstrated that individuals with *MC1R* variants have higher melanoma mutation burden, both non-UVR and UVR signature in coding and non-coding genome regions (Johansson *et al.* 2016; Robles-Espinoza *et al.* 2016).

Table 1. The effect of the *MC1R* RHC variants on the functional activity of the receptor.

Study	**p.Val60Leu** c.178G>T rs1805005	**p.Asp84Glu** c.252C>A rs1805006	**p.Val92Met** c.274G>A rs2228479	**p.Arg142His** c.425G>A rs11547464	**p.Arg151Cys** c.451C>A rs1805007	**p.Ile155Thr** c.464T>C rs1110400	**p.Arg160Trp** c.478C>T rs1805008	**p.Arg163Gln** c.488G>A rs885479	**p.Asp294His** c.880G>C rs1805009
Frändberg *et al.* 1998	-	-	-	-	= α-MSH affinity ↓↓ cAMP	-	-	-	-
Schiöth *et al.* 1999	= α-MSH affinity ↓ cAMP	-	-	↓ α-MSH affinity ↓↓ cAMP	= α-MSH affinity ↓↓ cAMP	-	= α-MSH affinity ↓↓ cAMP	-	↓ α-MSH affinity ↓↓ cAMP
Sánchez-Más *et al*. 2002	-	-	-	-	= α-MSH ↓↓ cAMP	-	-	-	-
Scott *et al*. 2002	-	-	= cAMP	-	↓ cAMP	-	↓ cAMP	-	↓ cAMP
Robinson & Healy 2002	-	-	-	-	= α-MSH affinity ↓↓ cAMP	-	= α-MSH affinity ↓ cAMP	-	= α-MSH affinity ↓↓ cAMP
Ringholm *et al.* 2004	-	↓ α-MSH affinity ↓↓ cAMP	↓↓ α-MSH affinity ↓ cAMP	-	-	-	-	↓ α-MSH affinity = cAMP	↓ α-MSH affinity ↓↓ cAMP
Newton *et al*. 2005	= cAMP	-	-	-	= cAMP	-	= cAMP	-	↓↓ cAMP
Beaumont *et al.* 2005	↓ Cell surface expression	↓↓ Cell surface expression	= Cell surface expression	= Cell surface expression	↓↓ Cell surface expression	↓↓ Cell surface expression	↓↓ Cell surface expression	↓ Cell surface expression	↑ Cell surface expression
Nakayama *et al*. 2006	-	-	= cAMP	-	↓ cAMP	-	-	= cAMP	-
Sánchez-Laorden *et al*. 2006	-	-	-	-	↓ Cell surface expression	-	↓ Cell surface expression	-	↑ Cell surface expression
Beaumont *et al*. 2007	↓ Cell surface expression ↓ cAMP	↓↓ Cell surface expression ↓ cAMP	= Cell surface expression ↑ cAMP	= Cell surface expression ↓ cAMP	↓ Cell surface expression ↓ cAMP	↓ Cell surface expression ↓↓ cAMP	↓ Cell surface expression ↓ cAMP	↓ Cell surface expression ↓ cAMP	= Cell surface expression ↓↓ cAMP

↓↓ completely or almost completely reduces effect, ↓ partially reduces effect compared to wild type receptor, = similar effect to wild type receptor, ↑ increased effect compared to wild type receptor

Many studies in various populations have explored association between individual *MC1R* variants and melanoma risk (Table 2). Meta-analyses have summarized results from different populations, confirming a strong link between *MC1R* RHC and NRHC variants and risk of melanoma (Antonopoulou *et al.* 2015; Pasquali *et al.* 2015; Williams *et al.* 2011) (Table 2). In addition *MC1R* comprising locus 16q24.3 also has reached significance in GWAS (Amos *et al.* 2011). Several studies have grouped various *MC1R* variants together and looked at their pooled effect. Study from the Melanocortin-1 receptor gene, Skin cancer and Phenotypic characteristics (M-SKIP) project shows that the presence of any *MC1R* variant significantly increases risk (OR=1.66, 95% CI=1.41–1.96) and in the presence of two or more variants risk is proportionally higher (OR=2.51, 95% CI=1.83–3.44) (Pasquali *et al.* 2015). When type of *MC1R* variants are taken into consideration, RHC variants (p.Asp84Glu, p.Arg151Cys, p.Arg160Trp, p.Asp294His) show comparatively high melanoma risk with OR=2.44, 95% CI=1.72–3.46, while NRHC variants (p.Val60Leu, p.Val92Met, p.Arg142His, p.Ile155Thr, p.Arg163Gln) increase melanoma risk to a lower extent with OR=1.29, 95% CI=1.10–1.51 (Williams *et al.* 2011). Another study compared RHC variants with NRHC variants as all other non-synonymous variants and discovered that the impact of the presence of any high-risk variant is comparable to presence of two low-risk variants (OR=1.9, 95% CI=1.3–2.8 and OR=1.7, 95% CI=1.0–2.8, respectively) (Kanetsky *et al.* 2010). The same study concluded that the risk was even higher in phenotypically low risk individuals—persons with dark hair (for any high risk variant OR=2.4, 95% CI=1.5–3.6 and two low risk variants OR=1.8, 95% CI=1.0–3.0), dark eyes (for any high risk variant OR=3.2, 95% CI=1.8–5.9) and who tan easily (for one high risk variant OR=2.4, 95% CI=1.6–3.6 and two low risk variants OR=2.0, 95% CI=1.1–3.6). In the presence of one RHC variant the risk is similar for individuals with skin type 1 and 2 (fair skin that does not tan well and burns in sun easily) (OR=2.2, 95% CI=0.9–5.1) and skin type 3 and 4 (darker skin that tans well) (OR=2.3, 95% CI=1.5–3.5) as well as persons with low (OR=2.2, 95% CI=1.5–3.2) and high recreational sun exposure (OR=2.0, 95% CI=1.2–3.3) (Kanetsky *et al.* 2010). A similar large study demonstrates that *MC1R* variants promote a higher risk in persons of European origins with darker pigmentation features. In the presence of any of the nine *MC1R* RHC or NRHC variants the association was strong in individuals with skin type 3 or 4 (OR=1.89, 95% CI=1.29–2.78), no red hair (OR=1.70, 95% CI=1.20–2.42), no freckles (OR=2.39, 95% CI=1.60–3.57) but was not significant in patients with skin type 1 or 2, red hair and/or freckles (Pasquali *et al.* 2015). Meta-analysis results from Raimondi *et al.* (2008) have showed that variants p.Asp84Glu, p.Arg142His, p.Arg151Cys, p.Arg160Trp and p.Asp294His are associated with the RHC phenotype and melanoma risk, p.Val60Leu and p.Val92Met are associated neither with the phenotype nor melanoma while variants p.Ile155T and p.Arg163Gln are associated only with melanoma (Raimondi *et al.* 2008) and altogether these data emphasize non-pigmentary roles of *MC1R* in melanoma development such as DNA repair and oxidative stress response (Wolf Horrell *et al.* 2016; Mitra *et al.* 2012).

Table 2. Individual associations between *MC1R* RHC variants and melanoma risk in various populations.

Study **Population** **Analysis type**[a]	**Measures**	**p.Val60Leu** c.178G>T rs1805005	**p.Asp84Glu** c.252C>A rs1805006	**p.Val92Met** c.274G>A rs2228479	**p.Arg142His** c.425G>A rs11547464	**p.Arg151Cys** c.451C>A rs1805007	**p.Ile155Thr** c.464T>C rs1110400	**p.Arg160Trp** c.478C>T rs1805008	**p.Arg163Gln** c.488G>A rs885479	**p.Asp294His** c.880G>C rs1805009
Gudbjartsson *et al.* 2008 Icelander Per chromosome	OR 95% CI p	1.06 0.89–1.27 0.50	0.97 0.59–1.62 0.92	1.03 0.84–1.28 0.77	-	1.11 0.93–1.33 0.25	1.17 0.68–2.01 0.56	0.87 0.74–1.04 0.12	1.12 0.87–1.44 0.36	1.05 0.69–1.61 0.81
Helsing *et al.* 2012 Norwegian MPM Per chromosome	OR 95% CI p	0.83 0.58–1.17 -	**5.77** **1.97–16.89** -	1.16 0.82–1.64 -	0.54 0.13–2.16 -	**1.80** **1.36–2.37** -	1.99 0.73–5.42 -	1.20 0.90–1.59 -	1.06 0.73–1.55 -	1.83 0.89–3.76 -
Gudbjartsson *et al.* 2008 Swedish Per chromosome	OR 95% CI p	1.10 0.91–1.32 0.33	**2.02** **1.15–3.56** **0.015**	1.03 0.85–1.24 0.76	-	**2.32** **1.77–3.05** **<0.001**	1.2 0.68–2.13 0.52	**1.70** **1.44–2.00** **<0.001**	0.95 0.77–1.18 0.65	**2.16** **1.35–3.46** **0.001**
Höiom *et al.* 2009 Swedish Per individual	OR 95% CI p	**1.54** **1.14–2.08** -	-	1.27 0.95–1.70 -	-	**2.24** **1.70–2.96** -	-	**2.07** **1.60–2.66** -	1.06 0.80–1.42 -	-
Ichii-Jones *et al.* 1998 United Kingdom Per chromosome	OR 95% CI p	-	3.0 0.9–9.6 0.069	-	-	-	-	-	-	-
Debniak *et al.* 2006 Polish Per individual	OR 95% CI p	**1.78** **1.20–2.64** **0.007**	-	-	-	**2.90** **1.82–4.67** **<0.001**	-	**1.76** **1.75–2.62** **0.006**	**2.10** **1.10–3.97** **0.015**	-
Brudnik *et al.* 2009 Polish Per chromosome	OR 95% CI p	1.02 - 0.957	- - 0.314	1.02 0.53–1.96 0.957	1.54 0.91–2.56 0.644	**4.35** **1.41–13.33** **0.006**	0.68 0.11–4.00 0.673	**1.96** **1.02–3.85** **0.041**	1.43 0.43–3.54 0.553	- - 0.155
Mossner *et al.* 2007 German Per individual	OR 95% CI p	0.89 0.62–1.29 0.577	**4.96** **1.06–23.13** **0.032**	0.97 0.65–1.44 0.919	1.74 0.56–5.38 0.406	**1.69** **1.12–2.55** **0.013**	1.97 0.65–5.93 0.283	1.43 0.96–2.12 0.088	0.90 0.55–1.47 0.709	1.42 0.62–3.29 0.525
Scherer *et al.* 2009 German Per chromosome	OR 95% CI p	0.92 0.71–1.2 0.46	**3.24** **1.04–7.69** **<0.01**	1.00 0.74–1.30 0.98	**3.27** **1.66–6.45** **<0.01**	**1.89** **1.41–2.53** **<0.01**	- - **<0.01**	**1.73** **1.30–2.31** **<0.01**	- - 0.53	1.79 0.96–3.35 0.06
Kennedy *et al.* 2001 The Dutch Per chromosome	OR 95% CI p	**1.80** **1.10–3.00** -	**5.30** **1.90–15.10** -	**2.20** **1.30–3.70** -	2.30 0.57–9.50 -	**2.50** **1.40–4.50** -	-	**2.00** **1.30–3.20** -	1.80 0.97–3.50 -	1.90 0.36–9.80 -

Table follows on the next page.

Study **Population** **Analysis type**[a]	**Measures**	**p.Val60Leu** c.178G>T rs1805005	**p.Asp84Glu** c.252C>A rs1805006	**p.Val92Met** c.274G>A rs2228479	**p.Arg142His** c.425G>A rs11547464	**p.Arg151Cys** c.451C>A rs1805007	**p.Ile155Thr** c.464T>C rs1110400	**p.Arg160Trp** c.478C>T rs1805008	**p.Arg163Gln** c.488G>A rs885479	**p.Asp294His** c.880G>C rs1805009
Matichard *et al.* 2004 French Per chromosome	OR 95% CI p	**3.99** **2.03–7.82** **<0.001**	2.16 0.24–26.14 0.41	-	- - 0.07	**6.48** **2.14–19.61** **0.001**	1.44 0.10–20.08 1	**4.73** **1.70–13.18** **0.001**	-	3.84 0.90–22.81 0.058
Guedj *et al.* 2008 French Per chromosome	OR 95% CI p	-	**2.10** **1.04–4.2** **0.04**	-	1.73 0.92–3.28 0.07	**2.35** **1.78–3.11** **<0.001**	-	**1.88** **1.40–2.54** **<0.001**	-	**2.49** **1.67–3.71** **<0.001**
Fernandez *et al.* 2007 Spanish Per individual	OR 95% CI p	**1.77** **1.07–2.92** **0.03**	1.63 0.02–128 1.00	**2.35** **1.07–5.16** **0.03**	0.60 0.10–2.56 0.50	1.47 0.55–3.93 0.50	**7.82** **1.57–75.2** **0.004**	**5.47** **1.10–59.6** **0.03**	2.71 0.87–8.50 0.08	**3.83** **1.14–10.38** **0.05**
Gudbjartsson *et al.* 2008 Spanish Per chromosome	OR 95% CI p	**1.32** **1.03–1.69** **0.027**	1.49 0.28–7.82 0.64	1.05 0.71–1.55 0.80	-	**2.71** **1.63–4.52** **<0.001**	0.83 0.40–1.74 0.62	**1.88** **1.08–3.29** **0.026**	1.44 0.80–2.59 0.23	**2.75** **1.55–4.90** **<0.001**
Scherer *et al.* 2009 Spanish Per chromosome	OR 95% CI p	**1.47** **1.11–1.90** **<0.01**	0.67 0.11–4.00 0.70	1.39 0.92–2.09 0.12	1.88 0.79–4.50 0.08	1.48 0.89–2.46 0.03	- - 0.64	**2.41** **1.25–4.67** **<0.01**	- - 0.37	**2.52** **1.45–4.39** **<0.01**
Ibarrola-Villava *et al.* 2010 Spanish Per chromosome	OR 95% CI p	1.34 0.96–1.87 0.09	0.98 0.16–5.89 0.98	1.18 0.69–2.03 0.54	0.51 0.20–1.31 0.16	1.67 0.83–3.36 0.15	3.35 0.96–11.68 0.06	**4.18** **1.24–14.04** **0.02**	1.88 0.70–5.08 0.21	**3.10** **1.37–7.01** **0.01**
Stratigos *et al.* 2006 Greek Per individual	OR 95% CI p	**2.76** **1.56–4.88** **0.001**	-	1.58 0.64–3.92 0.32	**6.65** **1.33–33.2** **0.021**	**4.18** **1.38–12.70** **0.012**	-	3.17 0.73–13.8 0.125	1.90 0.26–13.9 0.527	-
Fargnoli *et al.* 2006 Italian Per individual	OR 95% CI p	0.67 0.42–1.08 -	-	1.50 0.72–3.11 -	1.43 0.54–3.75 -	**3.14** **1.34–7.36** -	-	1.09 0.48–2.47 -	-	**11.0** **1.42–85.1** **-**
Galore-Haskel *et al.* 2009 Ashkenazi Jews Per individual	OR 95% CI p	1.30 0.80–2.10 0.33	-	1.20 0.60–2.40 0.70	2.70 0.90–8.30 0.09	**2.60** **1.30–5.30** **0.006**	**6.80** **1.40–33.3** **0.024**	1.40 0.60–3.10 0.40	0.80 0.30–2.00 0.60	-
Duffy *et al.* 2010a Australian Per chromosome	OR 95% CI p	1.01 - 0.80	**1.61** - **0.013**	1.07 - 0.53	2.25 - 0.055	**1.58** **-** **<0.001**	-	**1.68** **-** **<0.001**	1.15 - 0.54	**1.42** **-** **0.019**

Table follows on the next page.

Study **Population** **Analysis type**[a]	**Measures**	**p.Val60Leu** c.178G>T rs1805005	**p.Asp84Glu** c.252C>A rs1805006	**p.Val92Met** c.274G>A rs2228479	**p.Arg142His** c.425G>A rs11547464	**p.Arg151Cys** c.451C>A rs1805007	**p.Ile155Thr** c.464T>C rs1110400	**p.Arg160Trp** c.478C>T rs1805008	**p.Arg163Gln** c.488G>A rs885479	**p.Asp294His** c.880G>C rs1805009
Dwyer *et al.* 2004 Australian Tasmanian Per individual	OR 95% CI p	0.75 0.46–1.25 -	2.32 0.63–8.47 -	-	-	1.08 0.67–1.74 -	-	**1.78** **1.08–2.92** -	-	0.51 0.18–1.41 -
Han *et al.* 2006a US Per individual	OR 95% CI p	1.38 0.97–1.96 -	-	**1.56** **1.08–2.27** -	-	**2.49** **1.70–3.64** -	1.99 0.90–4.42 -	**1.59** **1.05–2.41** -	1.69 0.98–2.93 -	**2.66** **1.34–5.28** -
Council *et al.* 2009 US Per chromosome	OR 95% CI p	- - 0.61	- - 0.32	- - 0.51	- - 0.60	- - 0.66	- - 0.46	- - **0.0035**	- - 0.23	- - **0.018**
Nan *et al.* 2011b US Per chromosome	OR 95% CI p	-	-	-	-	**1.63** **1.32-–2.01** **6×10^{-6}**	-	-	-	-
Guan *et al.* 2013 US Per individual	OR 95% CI p	-	-	1.24 1.00–1.53 0.054	-	**1.77** **1.43–2.18** **<0.001**	-	**1.31** **1.05–1.63** **0.016**	-	**1.68** **1.14–2.47** **0.008**
Grazziotin *et al.* 2013 Brazilian Per individual	OR 95% CI p	- - 0.542	- - 0.550	- - 0.159	-	- - 0.188	-	- - 0.386	-	- - 0.576
Williams *et al.* 2011 Meta-analysis Per individual	OR 95% CI p	**1.18** **1.04–1.35** -	**1.67** **1.21–2.30** -	**1.32** **1.04–1.58** -	**2.40** **1.64–3.5** -	**1.93** **1.54–2.41** -	**1.39** **1.05–1.83** -	**1.55** **1.21–1.97** -	**1.21** **1.02–1.4** -	**1.89** **1.39–2.56** -
Williams *et al.* 2011 Meta-analysis Per chromosome	OR 95% CI p	1.10 0.83–1.45 -	**2.24** **2.06–2.45** -	**1.30** **1.08–1.58** -	1.65 0.99–2.73 -	**1.81** **1.51-–2.17** -	**1.73** **1.13–2.67** -	**1.83** **1.59–2.12** -	**1.44** **1.18–1.76** -	**2.12** **1.76–2.54** -
Antonopoulou *et al.* 2015 Meta-analysis Per chromosome	OR 95% CI p	**1.14** **1.03–1.26** **0.0097**	**1.53** **1.26–1.86** **0.00002**	**1.08** **1.00–1.16** **0.042**	**1.47** **1.07–2.02** **0.017**	**1.80** **1.58–2.06** **1.73×10^{-18}**	**1.29** **1.04–1.60** **0.0184**	**1.51** **1.33–1.72** **3.85×10^{-10}**	1.09 0.98–1.21 0.125	**1.89** **1.56–2.28** **5.90×10^{-11}**
Pasquali *et al.* 2015 Meta-analysis Per individual	OR 95% CI p	**1.47** **1.17–1.84** -	**2.74** **1.53–4.89** -	**1.55** **1.30–1.85** -	**2.30** **1.35–3.92** -	**2.32** **1.83–2.95** -	**1.83** **1.16–2.89** -	**2.17** **1.77–2.65** -	**1.53** **1.18–1.98** -	**2.60** **1.97–3.45** -

[a] OR calculated using MAF (per chromosome) or variant frequency per individual

MC1R variants also display various modifying effects on melanoma development. Melanoma patients with *MC1R* variants are younger—the median diagnosis age for patients with two variants (*R*/*R*, *R*/*r* or *r*/*r*) is more than 10 years lower compared to patients with at least one *MC1R* wild-type allele (Johansson *et al.* 2016). *MC1R* variants have been found more often in MPM compared to single melanoma patients, and MPM patients are also more likely to harbour multiple *MC1R* variants (Pastorino *et al.* 2008; Peris *et al.* 2004). *MC1R* variants also increase melanoma risk in a familial setting—a Swedish study shows that melanoma patients from melanoma families negative for *CDKN2A* variants harboured *MC1R* variants more often (54%) compared to controls (33%) (OR=2.4, 95% CI=1.6–3.4), supporting the role of medium and low risk genes in familial melanoma development (Helgadottir *et al.* 2015). *MC1R* also modifies the effect of other melanoma risk genes. In *CDKN2A* positive melanoma families *MC1R* increases *CDKN2A* penetrance, and patients with *MC1R* variants also have an approximately 10 years lower age of disease onset compared to patients with *CDKN2A* variants only (Fargnoli *et al.* 2010; Chaudru *et al.* 2005; Box *et al.* 2001a; van der Velden *et al.* 2001). In addition, as the number of *MC1R* variants increases, the risk becomes more pronounced (Demenais *et al.* 2010; Fargnoli *et al.* 2010) and the same principle can be applied when the type of *MC1R* variants is taken into consideration (Demenais *et al.* 2010). Presence of multiple *MC1R* variants in patients with *CDKN2A* variants has also been associated with MPM development (Goldstein *et al.* 2005). Besides *CDKN2A*, *MC1R* has also demonstrated interaction with other genes. There is some evidence about interplay between *MC1R* variants and germline variant in *TP53* gene p.Pro72Arg (c.215C>G; rs1042522) on melanoma risk (Nan *et al.* 2008; Stefanaki *et al.* 2007). There is also interaction between germline *MC1R* variants and presence of somatic changes in *BRAF*—patients with non-chronic sun-induced damage as well as darker pigmentation characteristics and *MC1R* variants have a higher possibility to develop melanomas with *BRAF* changes and the risk increases proportionally to the number of *MC1R* variants (Thomas *et al.* 2017; Fargnoli *et al.* 2008; Landi *et al.* 2006), however, other studies do not support that (Hacker *et al.* 2010; Thomas *et al.* 2010) and one study observed that the frequency of changes in *BRAF* is even lower in *MC1R* variant carriers (Scherer *et al.* 2010).

Apart from melanoma *MC1R* is also associated with non-melanoma skin cancer—basal cell carcinoma and squamous cell carcinoma (Tagliabue *et al.* 2015; Ferrucci *et al.* 2012; Nan *et al.* 2011b; Brudnik *et al.* 2009; Scherer *et al.* 2008; Han *et al.* 2006a; Dwyer *et al.* 2004; Bastiaens *et al.* 2001b; Box *et al.* 2001b).

Melanogenesis associated transcription factor gene—*MITF*

Another medium melanoma risk gene is melanogenesis associated transcription factor (previously commonly called microphthalmia-associated transcription factor) gene (*MITF*) (OMIM 156845). *MITF* is associated with a couple of overlapping auditory-pigmentary syndromes. An association between *MITF* and the disease was demonstrated for the first time in 1994 when *MITF* variants affecting splice sites were discovered in two families with Waardenburg syndrome type 2A (Read & Newton 1997; Tassabehji *et al.* 1994; Waardenburg 1951). *MITF* variants also have been found in families with the similar Tietz albinism-deafness syndrome (Cortés-González *et al.* 2016; Léger *et al.* 2012; Izumi *et al.* 2008; Smith *et al.* 2000; Amiel *et al.* 1998; Tietz 1963). Recently *MITF* variants have been found in individuals with complex phenotype COMMAD characterized by coloboma, osteopetrosis, microphthalmia, macrocephaly, albinism, and deafness (George *et al.* 2016).

MITF is located on chromosome 3 at position 3p13 and encodes basic helix-loop-helix leucine zipper transcription factor MITF (Tachibana *et al.* 1994). There are at least

seven different human MITF isoforms that are either widely expressed or tissue specific, and they structurally differ in the first exons and promoter: MITF-A (Amae *et al.* 1998), MITF-C (Fuse *et al.* 1999), MITF-H (Steingrimsson *et al.* 1994), MITF-B (Udono *et al.* 2000), MITF-M (Tassabehji *et al.* 1994), MITF-D (Takeda *et al.* 2002) and MITF-J (Hershey & Fisher 2005). MITF-M is almost exclusively expressed in melanocytic lineage (Fuse *et al.* 1999; Amae *et al.* 1998) and is considered a key transcription factor for melanocyte development and differentiation as it regulates roughly a hundred genes involved in many crucial cellular functions i.e. pigmentation synthesis (mostly through cAMP and MC1R pathway, described above), melanocyte development and differentiation, homeostasis, metabolism, oxidative stress, cell cycle and apoptosis—functions that all are also exploited by transformed melanoma cells (Kawakami & Fisher 2017; Hartman & Czyz 2015; Hsiao & Fisher 2014; Yajima *et al.* 2011). It also suppresses melanoma senescence allowing melanoma cell proliferation (Bonet *et al.* 2017; Giuliano *et al.* 2010). MITF is expressed in practically all melanomas and can be detected through all stages of melanoma development (King *et al.* 2001), is somatically amplified or mutated in 10-15% of primary melanomas and 20% of melanoma metastases, and is considered a 'lineage specific' oncogene (Hartman & Czyz 2015; Hsiao & Fisher 2014; Cronin *et al.* 2009; Levy *et al.* 2006; Garraway *et al.* 2005). Depending on MITF expression levels and subsequently activated genes melanomas in general can be divided in two subgroups—'MITF-low' melanomas with high metastatic potential but weakly proliferative cells and 'MITF-high' melanomas that are less metastatic and more proliferative (Kawakami & Fisher 2017; Hoek *et al.* 2006), however, melanoma tumours are heterogeneous and comprise cell subpopulations with various MITF expression levels (Ennen *et al.* 2017; Ennen *et al.* 2015) possibly allowing melanomas to switch between proliferative and metastatic states in a 'rheostat-like' or 'phenotype switching' model (Hoek & Goding 2010; Hoek *et al.* 2008; Carreira *et al.* 2006) or even having simultaneous differential MITF activity thus explaining dynamic and plastic behaviour of melanoma cells (Wellbrock & Arozarena 2015).

Melanoma associated *MITF* germline variant p.Glu318Lys (c.952G>A; rs149617956) was discovered simultaneously in two independent studies using different study approaches (Bertolotto *et al.* 2011; Yokoyama *et al.* 2011). Variant p.Glu318Lys is located at an evolutionarily conserved small ubiquitin-like modifier (SUMO) consensus site (ΨKXE) (Bertolotto *et al.* 2011; Yokoyama *et al.* 2011). Post-translational modification by adding small ubiquitin-like modifiers or SUMOylation is required for repression of MITF transcriptional activity (Murakami & Arnheiter 2005). Functional experiments demonstrate that amino acid substitution from Glu to Lys in codon 318 of *MITF* changes binding affinity for SUMO protein leading to severely impaired MITF SUMOylation, increased MITF transcriptional activity and disrupted up-regulation and down-regulation pattern of MITF target genes. In melanoma and RCC cells mutant MITF protein also displays characteristics associated with enhanced tumorigenic potential—increased clonogenicity, migration and invasion that are somehow opposite to characteristics of 'MITF-high' melanomas; however, mutated MITF protein also has altered transcriptional potential on various target genes and these changes together promote melanoma switching to a more invasive phenotype (Bertolotto *et al.* 2011). Altogether data from functional experiments suggests *MITF* variant p.Glu318Lys has a gain-of-function role in tumorigenesis (Bertolotto *et al.* 2011; Yokoyama *et al.* 2011).

The first study that detected p.Glu318Lys used candidate gene approach proposing *MITF*'s role in genetic predisposition to co-occuring melanoma and RCC (Bertolotto *et al.* 2011). The work was based on emerging evidence of *MITF* as a potential melanoma

oncogene (Garraway *et al.* 2005) and fact that one of the MITF targets—hypoxia-inducible factor 1-alpha (HIF1A) (Buscà *et al.* 2005) is involved in RCC genetic susceptibility (Linehan *et al.* 2010). The authors sequenced *MITF* in 62 French patients with co-occuring melanoma and RCC. A germline missense substitution in *MITF* gene p.Glu318Lys was identified in five of these patients. Further genotyping revealed that the frequency of this variant was at least fivefold higher in all patient groups (patients with melanoma and RCC, patients with melanoma only, and patients with RCC only) compared to 1659 healthy controls. *MITF* variant p.Glu318Lys also co-segregated in three melanoma families analysed (Bertolotto *et al.* 2011).

Concurrently, an Australian study identified the same *MITF* variant in a large melanoma family using whole-genome sequencing approach (Yokoyama *et al*. 2011). Variant p.Glu318Lys was found in three of seven patients in an eight-case melanoma family without changes in *CDKN2A* and *CDK4*. Family analysis was extended to 182 United Kingdom and 88 Australian melanoma families with at least two melanoma cases and without changes in *CDKN2A* and *CDK4* and the variant was identified in 30 of those families, resulting in altogether 31 melanoma families carrying p.Glu318Lys, and the variant fully co-segregated with melanoma in nine families (Yokoyama *et al.* 2011).

Variant p.Glu318Lys is rare—minor allele frequency (MAF) in different populations is consistently lower than 0.01 (Berwick *et al.* 2014; Ghiorzo *et al.* 2013; Bertolotto *et al.* 2011; Yokoyama *et al.* 2011). In a population setting the variant in melanoma patients is found more often than in controls and in most populations the difference is large enough to demonstrate statistically significant association with melanoma. Yokoyama *et al*. (2011) analysed individual and combined effects from Australian and United Kingdom case-control samples, and a joint variant carrier frequency in patients was 0.017 (68 of 3988 cases), corresponding to a 2.2-fold higher melanoma risk (OR=2.19, 95% CI=1.41–3.45, p=0.0003). In addition, the variant is found more often in patients with MPM and a family history of melanoma (Yokoyama *et al.* 2011). Similar results were obtained in the French population by Bertolotto *et al*. (2011), who found that p.Glu318Lys carriers have a 4.8-fold higher melanoma risk (OR=4.78, 95% CI=2.05–11.75, $p=7.8\times10^{-5}$) as well as 5.2-fold higher RCC risk (OR=5.19, 95% CI=1.37–16.87, p=0.008) with a MAF of 0.014 (17 of 603 cases) and 0.015 (5 of 164 cases), respectively. Later, in a study from Italy, 2.85-fold higher melanoma risk (OR=2.85, 95% CI=1.31–6.18, p=0.011) was found, with a MAF of 0.009 (12 of 667 cases) (Ghiorzo *et al.* 2013). The Italian study also highlighted a potential p.Glu318Lys association with not only RCC but also pancreatic cancer. Finally, a large Genes, Environment, and Melanoma (GEM) study from four countries (Australia, Italy, Canada, and the United States) supported p.Glu318Lys as a medium-penetrance melanoma susceptibility variant, with a MAF of 0.014 (44 of 1194 cases) and 1.7-fold melanoma risk (OR=1.7, 95% CI=1.1–2.6, p=0.03) (Berwick *et al.* 2014). In contrast to the previous studies, a study in the Polish population found no association between the *MITF* variant and melanoma risk. Among 748 melanoma patients, there were only two heterozygous p.Glu318Lys carriers, resulting in a MAF of only 0.001 compared to four carriers among 2144 controls and with MAF of 0.0009. This study also found no association between p.Glu318Lys and other types of cancer (kidney, colon, lung, breast, and prostate) (Gromowski *et al.* 2014).

MITF variant p.Glu318Lys has also been demonstrated to have an association with high melanoma risk pigmentation phenotype features such as fair skin, increased number of naevi and freckles (Sturm *et al.* 2014; Yokoyama *et al.* 2011), however, an association also has been found with low melanoma risk phenotype features—non-blue eye colour (Berwick *et al.* 2014; Yokoyama *et al.* 2011), dark hair and contrary to the above mentioned—absence

of naevi (Berwick *et al.* 2014). Affected p.Glu318Lys carriers also more often have nodular melanomas (Ghiorzo *et al.* 2013) and MPM (Sturm *et al.* 2014; Bertolotto *et al.* 2011). In general, p.Glu318Lys' partial co-segregation with disease in familial setting, relatively rare frequency in the population and OR values in the range ~2-5 for association with melanoma fully supports the classification of *MITF* as a medium melanoma risk gene (Bertolotto *et al.* 2011; Yokoyama *et al.* 2011).

Solute carrier family 45 member 2 gene—*SLC45A2*

The third currently known medium melanoma risk gene is solute carrier family 45, member 2 gene (*SLC45A2*) also known as membrane-associated transporter protein gene (*MATP*) or melanoma antigen AIM1 gene (*AIM1*) (OMIM 606202). *SLC45A2* gene is located on chromosome 5 at position 5p13.1 and it encodes a membrane-associated transporter protein that is involved in melanin synthesis (Fukamachi *et al.* 2001; Newton *et al.* 2001). The gene was initially discovered in a person with oculocutaneous albinism who harboured an albinism causing variant within this gene (Newton *et al.* 2001). It was originally identified as a melanocyte differentiation antigen with high expression rate in melanoma cells (Harada *et al.* 2001) and now it is considered a promising target for melanoma immunotherapy (Park *et al.* 2017).

Contrary to the genes and variations within them mentioned in the previous chapters, *SLC45A2* variants are associated with protective effects against melanoma. In many populations, but especially in Southern Europe and Asia, variants in *SLC45A2* coding region as well as promoter are associated with normal skin colour variation and some of the variants, particularly variant p.Phe374Leu (c.1122C>G; rs16891982), are associated with dark pigmentation features (Graf *et al.* 2007; Stokowski *et al.* 2007; Graf *et al.* 2005). While more than 90% of alleles in the European population are Phe, the actual ancestral allele in this position is Leu that reaches almost a 100% frequency in the African population (Yuasa *et al.* 2006; Jackson 2006). Ancestral allele Leu displays a protective effect against melanoma, however, this effect is also evident in fair-skinned individuals that suggests some non-pigmentary effect of this variant (Ibarrola-Villava *et al.* 2012; Ibarrola-Villava *et al.* 2011; Duffy *et al.* 2010a; Fernandez *et al.* 2008; Guedj *et al.* 2008) and it is also supported in meta-analyses (Antonopoulou *et al.* 2015). A couple of studies have demonstrated a positive association between common allele Phe and melanoma risk (Kocarnik *et al.* 2014; Lopez *et al.* 2014). Besides melanoma, *SLC45A2* locus also reaches significance in GWASs of squamous cell carcinoma and basal cell carcinoma (Chahal *et al.* 2016; Stacey *et al.* 2009).

1.2.3. Low melanoma risk genes

Besides high and medium melanoma risk genes, at least 30 low melanoma risk loci have been identified using GWAS as well as case-control study approach (Table 3). Variants in these loci are common in the general population and their risk contributed to melanoma is comparatively low (Read *et al.* 2016). Low melanoma risk variants affect or are located in proximity of genes involved in various cellular functions, namely, cell cycle regulation and tumour suppression (*CDKAL1*, *CCND1*, *CDKN2A*, *CDKN2B*, *CDK10*, *PTEN*, *TP53*), DNA repair (*ATM*, *BRCA1*, *BRCA2*, *ERCC5*, *PARP1*, *RAD23B*), protection of telomeres (*TERT*, *CLPTM1L*, *TRF1*), pigmentation (*TYR*, *TYRP1*, *ASIP*, *OCA2*, *HERC*), metabolism (*FTO*, *VDR*), immunity (*IRF4*, *MX2*, *PLA2G6*), transcription regulation (*CDKN2B-AS1*, *MAFF*, *NCOA6*, *TAL2*), and other functions (*AFG3L1P*, *AGR3*, *ARNT*, *CASP8*, *CYP1B1*, *MTAP*, *MYH7B*, *PIGU*, *RALY*, *RMDN2*, *STN1*, *TET2*, *TMEM38*) (Table 3).

Table 3. Low melanoma risk loci and variants from GWAS, case-control studies and meta-analyses.

Gene (affected or proximal)	Top gene function	Chromosomal localization	Top variants	References
ARNT Aryl hydrocarbon receptor nuclear translocator	Xenobiotic metabolism	1q21.3	**rs7412746**; g.150887995C>T[a] rs3768013; c.228-3281C>T	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Law *et al.* 2015a; Fang *et al.* 2013; Amos *et al.* 2011; MacGregor *et al.* 2011
PARP1 Poly(ADP-ribose) polymerase 1	DNA repair	1q42.12	**rs3219090**; c.1941+118A>G **rs3219125**; c.2406+230A>G **rs1858550**; g.226608104C>A **rs2249844**; g.226413434T>C	Barrett *et al.* 2015; Law *et al.* 2015a; Peña-Chilet *et al.* 2013; MacGregor *et al.* 2011; Zhang *et al.* 2011
CYP1B1, *RMDN2* Cytochrome P450 family 1 subfamily B member 1, regulator of microtubule dynamics 2	Drug metabolism, cytoskeleton	2p22.2	**rs6750047**; g.38049406A>G	Ransohoff *et al.* 2017; Law *et al.* 2015a
CASP8 Caspase 8	Apoptosis	2q33-q34	**rs13016963**; c.942+1150T>C **rs10931936**; c.757+2237T>C rs700635; c.*153G>T rs1045485; c.904G>C; p.Asp302His rs2349073; c.675+8207G>T	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Law *et al.* 2015a; Barrett *et al.* 2011; Li *et al.* 2008a
TET2 Tet methylcytosine dioxygenase 2	Epigenetic regulation	4q24	**rs4698934**; c.-46-15667T>C	Song *et al.* 2014
TERT, *CLPTM1L* Telomerase reverse transcriptase, cleft lip and palate associated transmembrane protein 1 like	Apoptosis, telomeres	5p15.33	**rs401681**; c.1316-153G>A rs2447853; c.892-1079T>C	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Llorca-Cardeñosa *et al.* 2014; Stefanaki *et al.* 2013; Yin *et al.* 2012; Nan *et al.* 2011a; Rafnar *et al.* 2009; Stacey *et al.* 2009
CDKAL1 CDK5 regulatory subunit associated protein 1 like 1	Cell cycle	6p22.3	**rs6914598**; c.1300-34333T>C	Ransohoff *et al.* 2017; Law *et al.* 2015a
IRF4 Interferon regulatory factor 4	Immunity, transcription	6p25-p23	**rs12203592**; c.492+386C>T rs9405705; n.482-4824C>G	Barrett *et al.* 2015; Peña-Chilet *et al.* 2013; Han *et al.* 2011; Kvaskoff *et al.* 2011; Duffy *et al.* 2010b; Newton-Bishop *et al.* 2010
AGR3 Anterior gradient 3	Protein folding	7p21.1	**rs1636744**; g.16984280C>T	Ransohoff *et al.* 2017; Law *et al.* 2015a
TRF1 Telomeric repeat binding factor 1	Telomeres	8q21.11	**rs2981096**; c.887+875A>G	Nan *et al.* 2011a

Table follows on the next page.

Gene (affected or proximal)	Top gene function	Chromosomal localization	Top variants	References
MTAP, *CDKN2A* Methylthioadenosine phosphorylase, cyclin-dependent kinase inhibitor 2A	Polyamine metabolism, cell cycle	9p21.3	**rs7023329**; c.121-185A>G **rs10757257**; c.33+3784G>A **rs4636294**; n.163+20021T>C rs869330; c.33+1837A>G rs1335510; n.163+10021A>C rs2218220; n.163+11735G>A rs751173; n.164-4236G>A rs1341866; g.21771242T>C rs10811629; c.450+2289A>G rs935053; g.21783923G>A rs10757257; c.33+3784G>A rs201131773; c.33+2426_33+2427insAC rs3088440; c.*69C>T rs3731204; c.193+6554A>G	Yu *et al.* 2018; Ransohoff *et al.* 2017; Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Kocarnik *et al.* 2014; Maccioni *et al.* 2013a; Stefanaki *et al.* 2013; Amos *et al.* 2011; Chatzinasiou *et al.* 2011; Kvaskoff *et al.* 2011; MacGregor *et al.* 2011; Gerstenblith *et al.* 2010; Bishop *et al.* 2009; Falchi *et al.* 2009
CDKN2B, *CDKN2B-AS1* Cyclin-dependent kinase inhibitor 2B, CDKN2B antisense RNA 1	Cell cycle	9p21.3	**rs1011970**; n.2158+109G>T	Barrett *et al.* 2015; Maccioni *et al.* 2013a; Bishop *et al.* 2009
TYRP1 Tyrosinase related protein 1	Pigmentation	9p23	**rs1408799**; g.12672097T>C **rs2733832**; c.1261+20C>T rs72706189; g.11877260T>C	Goldstein *et al.* 2017; Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Gibbs *et al.* 2015; Duffy *et al.* 2010a; Gerstenblith *et al.* 2010; Nan *et al.* 2009a; Gudbjartsson *et al.* 2008
TMEM38B, *RAD23B*, *TAL2* Transmembrane protein 38B, nucleotide excision repair protein, TAL BHLH transcription factor 2	Ca regulation, DNA repair, transcription	9q31.2	**rs10739221**; g.106298549T>C	Ransohoff *et al.* 2017; Kypreou *et al.* 2016; Law *et al.* 2015a
PTEN Phosphatase and tensin homolog	Cell cycle	10q23.31	Miscellaneous	Bubien *et al.* 2013; Tan *et al.* 2012
STN1 (*OBFC1*) STN1, CST complex subunit	DNA replication	10q24.33	**rs2995264**; c.229+1442C>T	Ransohoff *et al.* 2017; Law *et al.* 2015a; Iles *et al.* 2014
CCND1 Cyclin D1	Cell cycle	11q13.3	**rs1485993**; g.69547646A>G rs11263498; g.69567999T>C rs11604821; g.69537369G>A rs12422135; g.69378736A>G	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Barrett *et al.* 2011

Table follows on the next page.

Gene (affected or proximal)	Top gene function	Chromosomal localization	Top variants	References
TYR Tyrosinase	Pigmentation	11q14.3	**rs1126809**; c.1205G>A p.Arg402Gln **rs1393350**; c.1185-6895G>A rs1806319; g.89304768T>C rs1847142; c.1366+3452G>A rs10830253; c.1367-268T>G rs17793678; c.820-2846C>T rs12270717; c.819+250T>A rs5021654; c.-533G>C	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Gibbs *et al.* 2015; Ibarrola-Villava *et al.* 2012; Amos *et al.* 2011; Barrett *et al.* 2011; Hu *et al.* 2011; Ibarrola-Villava *et al.* 2011; Duffy *et al.* 2010a; Bishop *et al.* 2009; Council *et al.* 2009; Nan *et al.* 2009a; Gudbjartsson *et al.* 2008
ATM ATM serine/threonine kinase	DNA damage response	11q22.3	**rs1801516**; c.5557G>A; p.Asp1853Asn rs1800054; c.146C>G; p.Ser49Cys rs4753835; c.3284+1670C>T	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Peña-Chilet *et al.* 2013; Barrett *et al.* 2011; Dombernowsky *et al.* 2008
VDR Vitamin D receptor	Metabolism	12q13.11	**rs1544410**; c.1024+283G>A rs731236; c.1056T>C; p.Ile352= rs2228570; c.2T>C; p.Met1Thr rs4516035; c.-1172A>G rs10875712; g.48363253C>G rs4760674; c.-431A>C rs7139166; c.-1680G>C rs11168287; c.-83-8857C>T rs7305032; c.584-276C>T rs7965281; n.498A>G	Antonopoulou *et al.* 2015; Lee & Gyu Song 2015; Zeljic *et al.* 2014; Orlow *et al.* 2012; Randerson-Moor *et al.* 2009; Li *et al.* 2007; Povey *et al.* 2007; Santonocito *et al.* 2007; Hutchinson *et al.* 2000
MDM2 Mouse double minute 2 homolog	Ubiquitination	12q15	**rs2279744**; c.14+309T>G	Thunell *et al.* 2014; Cotignola *et al.* 2012; Firoz *et al.* 2009
BRCA2 Breast cancer type 2 susceptibility protein	DNA repair	13q13.1	Miscellaneous	Yu *et al.* 2018; Tuominen *et al.* 2016; Debniak *et al.* 2008
ERCC5 (*XPG*) ERCC excision repair 5, endonuclease	DNA repair	13q33.1	**rs17655**; c.3310G>C; p.Asp1104His rs4150355; c.2679-1236C>G	Antonopoulou *et al.* 2015; Gonçalves *et al.* 2011; Zhang *et al.* 2011
OCA2, *HERC2* OCA2 melanosomal transmembrane protein, HECT and RLD domain containing E3 ubiquitin protein ligase 2	Pigmentation	15q12-13.1	**rs1800407**; c.1256G>A; p.Arg419Gln **rs1129038**; c.*50G>A **rs12913832**; c.13272+874T>C rs1800401; c.913C>T; p.Arg305Trp rs4778138; c.-22+8550T>C rs547148386; c.1364+25G>T rs150540829; c.1364+112C>A rs145720174; c.5465-870C>T	Ransohoff *et al.* 2017; Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Zhang *et al.* 2013; Amos *et al.* 2011; Duffy *et al.* 2010a; Fernandez *et al.* 2009; Guedj *et al.* 2008; Sturm *et al.* 2008; Jannot *et al.* 2005

Table follows on the next page.

Gene (affected or proximal)	Top gene function	Chromosomal localization	Top variants	References
FTO Alpha-ketoglutarate dependent dioxygenase	Metabolism	16q12.2	**rs16953002**; c.1365-30850G>A **rs12596638**; c.1365-29845G>A rs12933928; c.1239+8130A>G rs12932428; c.1240-7370C>T rs1125338; c.1364+73758C>T rs12599672; c.1365-29598T>A rs12600192; g.54127816C>G	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Iles *et al.* 2013; Li *et al.* 2013
CDK10, AFG3L1P Cyclin-dependent kinase 10, AFG3 like matrix AAA peptidase subunit 1, pseudogene	Cell cycle, unknown	16q24.3	**rs258322**; c.160+171A>G **rs4785763**; n.1682A>C	Ransohoff *et al.* 2017; Antonopoulou *et al.* 2015; Kocarnik *et al.* 2014; Fang *et al.* 2013; Stefanaki *et al.* 2013; Barrett *et al.* 2011; Han *et al.* 2011; Gerstenblith *et al.* 2010; Bishop *et al.* 2009
TP53 Tumour protein TP53	Tumour suppression	17p13.1	**rs1042522**; c.215C>G; p.Pro72Arg	Geng *et al.* 2015; Ye *et al.* 2013; Oliveira *et al.* 2013; Jiang *et al.* 2011; Li *et al.* 2008b; Stefanaki *et al.* 2007; Gwosdz *et al.* 2006; Han *et al.* 2006b; Shen *et al.* 2003
BRCA1 Breast cancer type 1 susceptibility protein	DNA repair	17q21.31	Miscellaneous	Brose *et al.* 2002
ASIP, MYH7B, PIGU, NCOA6, RALY Agouti signaling protein, myosin heavy chain 7B gene, phosphatidylinositol glycan anchor biosynthesis class U gene, nuclear receptor coactivator 6, RALY heterogeneous nuclear ribonucleoprotein	Pigmentation, myosin, cell division, transcription, immunity, splicing	20q11.22	**rs4911414**; g.32729444T>G **rs1015362**; g.32738612C>T rs6058017; c.*25A>G rs910873; c.926+1469C>G rs1885120; c.1714-554C>G rs4911442; c.514+1221C>T rs910871; c.2914+1403G>T rs819133; c.1168-1343A>C rs6059655; c.829-563A>G	Ransohoff *et al.* 2017; Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Maccioni *et al.* 2013b; Stefanaki *et al.* 2013; Helsing *et al.* 2012; Amos *et al.* 2011; Chatzinasiou *et al.* 2011; Ibarrola-Villava *et al.* 2011; Debniak *et al.* 2010; Duffy *et al.* 2010a; Bishop *et al.* 2009; Nan *et al.* 2009a; Brown *et al.* 2008; Gudbjartsson *et al.* 2008
MX2 MX dynamin like GTPase 2	Immunity	21q22.3	**rs45430**; c.-71-2682C>T rs443099; c.-71-5436G>T	Ransohoff *et al.* 2017; Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Gibbs *et al.* 2015; Barrett *et al.* 2011
PLA2G6, MAFF Phospholipase A2 group VI, MAF bZIP transcription factor F	Immunity, transcription	22q13.1	**rs2284063**; c.210-2638T>C **rs6001027**; c.210-3959T>C rs132985; c.209+1754G>A rs738322; c.-45-3528T>C rs3891103; c.610-983G>C rs4608623; c.-899G>T	Antonopoulou *et al.* 2015; Barrett *et al.* 2015; Liu *et al.* 2013; Stefanaki *et al.* 2013; Amos *et al.* 2011; Barrett *et al.* 2011; Gerstenblith *et al.* 2010; Newton-Bishop *et al.* 2010; Bishop *et al.* 2009; Falchi *et al.* 2009

Colouring in **bold** indicates the most important variants in the region; [a] genomic coordinated showed according to genome assembly GRCh38.p7.

2. MATERIALS AND METHODS

2.1. Overview of study subjects

All patients included in the studies were consecutive consenting melanoma patients with histopathologically confirmed cutaneous melanoma recruited at the Riga East University Hospital, Latvian Oncology Centre (LOC) starting from 2001 to 2011. Initially patients were recruited within the framework of specific studies but later in collaboration with Latvian Genome centre and Genome Database of the Latvian population (described in Rovite *et al.* 2018). Control individuals were healthy volunteers recruited through general practitioners and/or participants in the Genome Database of the Latvian population. Information about age, sex, family history of cancers and pigmentation phenotype was obtained from self-report questionnaires from all individuals enrolled in studies. More detailed information about history of familial cancer was then obtained by interview during the visit to dermatologist, and medical confirmation was sought for all relatives reported to have had melanoma. Additional family members of melanoma patients were enrolled in the study by a dermatologist. All participants have signed an informed consent form approved by the Central Medical Ethics Committee of Latvia. Data collection was performed according to Central Medical Ethics Committee protocols No. A-1, A-3 and A-7. All studies were performed according to the Helsinki declaration. All DNA samples were isolated from peripheral blood mononuclear cells using standard phenol–chloroform extraction method either in the melanoma laboratory or centrally at the Genome centre for those included in the Genome Database of the Latvian population. The following paragraphs describe patients and controls included in each study in more detail.

For the comprehensive study of *CDKN2A* locus and *CDK4* exon 2 in Latvian melanoma families (paper I) altogether 20 melanoma patients were selected and patients that were included were recruited at LOC between 2009 and 2011. The inclusion criteria were as follows: i) histopathologically confirmed melanoma; ii) at least one first-degree (n=15) or second-degree (n=5) relative with melanoma. Additional unrelated sporadic melanoma patients without melanoma history in family and healthy controls were included for individual variant genotyping (211 patients and 326 controls for the p14ARF promoter deletion analysis, 309 patients and 150 controls for the *CDK4* variant p.Arg24His, 174 patients and 212 controls for the *CDKN2A* intron variant IVS1+1569T>A and 180 patients and 228 controls for the *CDKN2A* variant IVS2+82C>T analyses).

All members (n=14) of the three Latvian *CDK4* melanoma families whose DNA was available, recruited in this as well as in previous studies between 2001 and 2011, were included in the international worldwide overview study of *CDK4* families (paper II). Two of these families have been reported on before (Pjanova *et al.* 2009; Pjanova *et al.* 2007). Members of the third, novel *CDK4* family were recruited as described above. Families with germline *CDK4* variants were involved in the study either by contacting the authors of published studies or by requests for unpublished families via GenoMEL, the Melanoma Genetics Consortium. Altogether 17 families were recruited in the study, and information about pigmentation phenotype, number of common/clinically atypical naevi, presence/number of MPM, age at melanoma diagnosis, anatomic location (head/neck, limbs, and trunk), histological type (superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo malignant melanoma (LMM), *in situ* melanoma, and melanoma unclassified/classification unknown), non-melanoma cancers as well as *MC1R* variant status was gathered about members of those families. DNA samples were available from 209 persons—62 melanoma

cases, 106 unaffected family members, and 41 spouses, and the DNA analysis of *CDK4* and *MC1R* was performed by each research group individually.

For the *MC1R* variant study in the Latvian population (paper III) 200 melanoma patients and 200 controls recruited between 2007 and 2011 were selected. The inclusion criteria were as follows: i) histopathologically confirmed melanoma; ii) complete information on skin type natural hair colour, eye colour, degree of freckling, and number of moles available. Exclusion criteria: i) no or incomplete information about pigmentation.

For the chromosome 16 variant study (paper IV) 255 melanoma patients and 224 controls recruited between 2007 and 2011 were selected. The inclusion criteria were as follows: i) histopathologically confirmed melanoma; ii) complete information about *MC1R* variants.

The international, collaborative BioGenoMEL study of *MC1R* variants and melanoma survival (paper V) comprised altogether 3060 melanoma patients from 10 cohorts (Barcelona, Essen, Genoa, Leeds, Paris, Philadelphia, Riga, Stockholm, Valencia, Vienna), among them 137 Latvian melanoma patients recruited between 2007 and 2011 who donated DNA samples no later than 2 years after diagnosis with survival data. Analysis of *MC1R* variants was performed by each research group individually and the most often used technique was conventional Sanger sequencing.

The *MITF* study (paper VI) included 477 consecutive consenting melanoma patients recruited between 2001 and 2011 among whom 18 had a family history of melanoma, 11 had relatives with pancreatic cancer, 7 had MPM and 49 had developed melanoma early—before age 35. All of these patients were tested negative for the presence of variants in *CDKN2A* and *CDK4*. Additionally 225 controls were also included in the study.

The study of low melanoma risk variant p.Pro72Arg in *TP53* and c.14+309T>G in *MDM2* in the Latvian populations (paper VII) included altogether 490 consecutive consenting melanoma patients and 356 controls recruited between 2001 and 2011.

The international, collaborative BioGenoMEL study on *PARP1* variant s2249844 (paper VIII) comprises altogether 8599 cases from 10 different cohorts (Athens, Barcelona, Essen, Leeds, Lund, Riga, Stockholm, Tampa, Valencia, Vienna), including 243 cases from the Latvian population recruited between 2003 and 2011. Information about date of diagnosis, date of death, Breslow thickness, tumour site, age at diagnosis and sex were also collected from nine European BioGenoMEL cohorts, including Riga. The complete follow up information was available for 3965 and 175 cases, respectively, and these cases were included in the final analysis.

2.2. Sequencing and genotyping

All primers for DNA fragment amplification, sequencing or genotyping were synthesized by Metabion International AG, Planegg/Steinkirchen, Germany. Detailed PCR conditions for each genomic region of interest are described individually below. Enzymatic clean-up of PCR products before sequencing was performed in 10 µl reaction volume containing 1 µl ExoI, 2 µl SAP/FastAP (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA) and 7 µl PCR product, and incubated for 30 min at 37°C followed by 15 min at 80°C. All sequencing reactions were performed using the ABI PRISM BigDye Terminator v.3.1 cycle sequencing kit in 10 µl reaction volume containing 5 µl H_2O, 2 µl 5x sequencing buffer, 1 µl primer, 1 µl BigDye and 1 µl PCR product in conditions as follows—25 cycles of 30 s at 94°C, 15 s at 53°C and 4 min at 60°C—and analysed on an ABI 3100 Sequencer (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA).

Analysis of sequence electropherograms was performed manually using the Vector NTI software (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Comprehensive *CDKN2A* locus analysis was done in patients with a family history of melanoma and that included analysis of coding, flanking and promoter regions as well as the first and the second intron of the *CDKN2A* gene including deletion screening in locus 9p21. Coding and flanking regions of *CDKN2A* were analysed as described previously (Pjanova *et al.* 2007). Promoter region 1185 bp upstream of *CDKN2A* exon 1α and the first and second intron of *CDKN2A* were amplified as described previously (Harland *et al.* 2005a; Harland *et al.* 2000) and sequenced with the same primers. Locus 9p21 deletion analysis in patients with a family history of melanoma was carried out using the SALSA multiple ligation-dependent probe amplification (MLPA) kit ME024-A1 (MRC-Holland, Amsterdam, the Netherlands) according to manufacturer's instructions. Precise size and location of the deletion was determined by direct sequencing and genotyping. Deletion comprising probe region was amplified and sequenced with primers: forward 5'-CCTAGTCCCGAATCCTCTGG-3' and reverse 5'-CAACCATTCTACGCGAGGAC-3'. An alternative fluorescently labelled forward primer 5'-CTTAGACCGCGCTCAGGACC-3' was designed for deletion genotyping in sporadic patients and controls, and fragments were analysed on an ABI 3100 Sequencer.

The novel *CDKN2A* intronic variant IVS2+82C>T found in familial melanoma patients by sequencing was genotyped in sporadic patients and controls by the restriction fragment length polymorphism (RFLP) method using SmaI restriction enzyme (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA) under conditions recommended by the manufacturer. *CDKN2A* intronic variant IVS1+1569T>A (rs138967562) found in familial melanoma patients by sequencing was genotyped in sporadic patients and controls using custom TaqMan® probe in reaction conditions specified by the manufacturer on a ViiA™ 7 Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA).

The second exon of *CDK4* was analysed as described previously (Pjanova *et al.* 2007). *CDK4* haplotypes in the third p.Arg24His positive newly described melanoma family were determined using four microsatellite markers flanking *CDK4* (D12S305, CDK4M4, CDK4M1, D12S1691), three intragenic variants (rs2270777, rs2069502, rs2069506), one promoter variant (rs2072052), and one tetranucleotide repeat within intron 5 (rs2069504) as described previously (Molven *et al.* 2005).

The entire *MC1R* coding sequence was amplified using primers: forward 5'-GCAGCACCATGAACTAAGCA-3' and reverse 5'-CAGGGTCACACAGGAACCA-3' (Kanetsky *et al.* 2004). PCR was performed in 25 µl reaction volume containing 25 ng of template DNA, 1x Taq buffer, 10% dimethyl sulfoxide (DMSO), 1.5 mM magnesium chloride ($MgCl_2$), 0.24 mM dNTPs, 4 mM of each primer and 1.25 U Taq DNA Polymerase (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA). Cycling conditions were as follows: an initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 50 s, annealing at 61°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Amplicons were sequenced as described above using another set of four primers: forward-1 5'-AACCTGCACTCACCCATGTA-3', reverse-1 5'-CTGCAGGTGATCACGTCAAT-3', forward-2 5'-TCGTCTTCAGCACGCTCTTC-3', and reverse-2 5'-TTTAAGGCCAAAGCCCTGGT-3' (Kanetsky *et al.* 2006).

Chromosome 16 variants rs258322, rs4785763, and rs8059973 were genotyped using TaqMan SNP genotyping assays C_653812_1, C_2875849_10 and C_29970391_10, respectively) on ViiA™ 7 Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions.

The *MITF* sequence region encompassing variant p.Glu318Lys was amplified using primers: forward 5'-CAGGCTCGAGCTCATGGA-3' and reverse 5'-TGGGGACACTATAGGCTTGG-3' (Yokoyama *et al.* 2011). Amplification PCR was performed in 25 µl reaction volume containing 25 ng of template DNA, 1x Taq buffer, 5% DMSO, 1.5 mM $MgCl_2$, 0.24 mM dNTPs, 0.4 mM of each primer and 1.25 U Taq DNA Polymerase (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA). Cycling conditions were as follows: an initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Sequencing was performed as described above using the same primers as for initial fragment amplification.

Fragment of *TP53* exon 4 carrying variant p.Pro72Arg was amplified using primers: forward 5'-ATCTACAGTCCCCCTTGCCG-3' and reverse 5'-GCAACTGACCGTGCAAGTCA-3' (Shen *et al.* 2003). *MDM2* gene intronic promoter region harbouring variant c.14+309T>G was amplified using primers: forward 5'-CGGGAGTTCAGGGTAAAGGT-3' and reverse 5'-AGCAAGTCGGTGCTTACCTG-3' (Bond *et al.* 2004). Both PCRs for *TP53* variant p.Pro72Arg and *MDM2* variant c.14+309T>G were performed in 25 µl reaction volume containing 25 ng of template DNA, 1x Taq buffer, 5% DMSO, 1.5 mM $MgCl_2$, 0.24 mM dNTPs, 0.4 mM of each primer and 1.25 U Taq DNA Polymerase (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA). Cycling conditions were as follows: an initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for *TP53* and 55°C for *MDM2* for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. *TP53* fragments obtained in PCR were cleaved with Bsh1236l (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA) in 20 µl reaction volume containing 1x Buffer R, 5 U enzyme, and 17 µl PCR product at 37°C overnight. Sequencing of the *MDM2* promoter region fragment was performed as described above using the same primers as for initial fragment amplification.

Genotyping of *PARP1* variant s2249844 was performed using TaqMan® assay C__34511379_10 and ABI 7900HT Real-time PCR system according to the manufacturer's instructions (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Construction of *MC1R* variant clones

For functional activity analysis *MC1R* variants with a frequency of less than 1% were selected. Three variants were excluded from the analysis— p.Asp84Glu, as its functional role is already known (Table 1 in the Literature); p.Tyr152*, as it creates a truncated inactivated protein; and p.Asp184His, due to its close position to the variant p.Val188Ile, assuming that their effects might be similar. For the remaining eight variants—p.Phe45Leu, p.Ser83Leu, p.Gly89Arg, p.Thr95Met, p.Asp121Glu, p.Val165Ile, p.Val188Ile, and p.Arg213Trp—expression constructs were created. As a positive control sample, variant p.Arg151Cys construct was also created since it has previously been shown to have reduced cell surface expression and functional activity in the cAMP assay (Table 1 in the Literature). Polymorphic *MC1R* constructs were based on the vector pcDNA3.1+ with human consensus *MC1R* sequence (Missouri S&T cDNA Resource Center, Rolla, MO, USA) and created by site-directed mutagenesis with overlap extension (Ho *et al.* 1989) using iProof High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA, USA) and primers containing polymorphic mismatch or restriction sites (Table 4). For the cAMP assay, amplified PCR fragments were subcloned into the pcDNA5/FRT vector (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). For

the microscopy studies, *MC1R* variants without the stop codon were subcloned into the pGFP2-N3 vector (PerkinElmer™, Waltham, MA, USA). For the microscopy, melanocortin 2 receptor (MC2R) expression construct was used as negative control (Fridmanis *et al.* 2010; Hinkle & Sebag 2009). Sequences of all obtained constructs were verified by plasmid sequencing.

Table 4. Primers used for creation of polymorphic *MC1R* constructs.

Genetic variant/primer name	Primer sequence
p.Phe45Leu (c.133T>C)	5′-GACGGGCTCCTCCTCAGC-3′
	5′-GCTGAGGAGGAGCCCGTC-3′
p.Ser83Leu (c.248C>T)	5′-GCCTTGTTGGACCTGCTGGT-3′
	5′-ACCAGCAGGTCCAACAAGGC-3′
p.Gly89Arg (c.265G>C)	5′-GCTGGTGAGCCGGAGCAACG-3′
	5′-CGTTGCTCCGGCTCACCAGC-3′
p.Thr95Met (c.284C>T)	5′-TGCTGGAGATGGCCGTCAT-3′
	5′-ATGACGGCCATCTCCAGCA-3′
p.Asp121Glu (c.363C>G)	5′-ACAATGTCATTGAGGTGATCAC-3′
	5′-GTGATCACCTCAATGACATTGT-3′
p.Arg151Cys (c.451C>T)	5′-CTACGCACTGTGCTACCACA-3′
	5′-TGTGGTAGCACAGTGCGTAG-3′
p.Val165Ile (c.493G>A)	5′-GGCGAGCCATTGCGGCCAT-3′
	5′-ATGGCCGCAATGGCTCGCC-3′
p.Val188Ile (c.562G>A)	5′-ACGTGGCCATCCTGCTGTGC-3′
	5′-GCACAGCAGGATGGCCACGT-3′
p.Arg213Trp (c.637C>T)	5′-CATGCTGGCCTGGGCCTGC-3′
	5′-GCAGGCCCAGGCCAGCATG-3′
For cAMP assay:	
MC1R NheI	5′-CATA**GCTAGC**CACCATGGCTGTGCAGGGATCCCA-3′
MC1R XhoI	5′-CATA**CTCGAG**TCACCAGGAGCATGTCAGCA-3′
For microscopy studies:	
MC1R EcoRI	5′-CATA**GAATTC**CACCATGGCTGTGCAGGGATCCCA-3′
MC1R HindIII	5′-CATA**AAGCTT**CCAGGAGCATGTCAGCACCT-3′

2.4. Cell culture and transfection

For the *MC1R* variant functional study BHK cells (American Type Culture Collection, Manassas, VA, USA) were grown at 37°C with 5% CO_2 and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum and a penicillin–streptomycin mix (Sigma-Aldrich, St. Louis, MO, USA). When 70–90% confluence was reached, DNA constructs were transfected into the cells using TurboFect Transfection Reagent (Thermo Scientific™, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cells were harvested and assayed 24 h or 48 h after transfection.

2.5. Confocal laser scanning microscopy

For *MC1R* construct expression visualization using confocal laser scanning microscopy, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes and then stained with Alexa Fluor633 labeled wheat germ agglutinin (WGA) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). Cells were examined with a Leica TCS SP2 confocal

microscope (Leica Microsystems, Wetzlar, Germany). For each construct two transfection reactions were performed and three images were obtained from each independent transfection reaction, resulting in a total of six images for each construct.

2.6. cAMP assay

For *MC1R* variant construct cAMP activity assay transiently transfected BHK cells were distributed into a 384-well plate (1×10^4 cells/well) and stimulated with synthetic MC1R agonist NDP-MSH (PolyPeptide Group, Hillerød, Denmark) in serial dilutions (10^{-12} to 10^{-6} M in 1X phosphate-buffered saline, 1% bovine serum albumin, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO, USA)) for 30 minutes at 37°C. The intracellular cAMP level was measured with a LANCE cAMP kit (PerkinElmer™, Waltham, MA, USA) using a Victor3V multilabel reader (PerkinElmer™, Waltham, MA, USA) following the manufacturer's instructions. All experiments were performed in duplicate and repeated three times. Data was analyzed using the GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

2.7. Data processing and statistical analysis

Two-sided Fisher's exact test was used to estimate frequency differences of variants initially found in families in sporadic melanoma patients (paper I), as well as low risk variants (paper VII), and p-value<0.05 was considered to be statistically significant. The test was carried out using GraphPad Prism v.5.04 software (GraphPad Software, La Jolla, CA, USA).

In the *CDK4* family study (paper II) Pearson χ^2 test or the Fisher exact test were used depending on the sample size to compare differences between subject groups (melanoma affected/unaffected *CDK4* positive family members, *CDK4* negative family members and spouses) and categorical variables (melanoma status, presence of clinically atypical naevi, phenotype categories, *MC1R* variants). The non-parametric Mann–Whitney or Kruskal–Wallis tests were used to compare the continuous variable (age at time of diagnosis) with the categorical variables (melanoma status, tumour location, histological type, presence of clinically atypical naevi, *MC1R* variants). For statistical analysis purposes individuals were grouped according to the number of *MC1R* variants carried (consensus *MC1R* sequence, one and two *MC1R* variants) or type of *MC1R* variants carried (RHC (p.Asp84Glu, p.Arg142His, p.Arg151Cys, p.Arg160Trp, p.Asp294His), NRHC (all other non-synonymous *MC1R* variants), and RHC+NRHC variants). Unconditional logistic regression analysis was used to evaluate the presence of atypical naevi depending on melanoma and *CDK4* variant p.Arg24His status when adjusted for age. P-values<0.05 were considered to be statistically significant. Analyses were performed using the IBM Statistical Package for the Social Sciences, v19 (SPSS Inc, Chicago, IL, USA) and SAS v9.1.3 software (SAS Institute Inc, Cary, NC, USA).

In the *MC1R* study in the Latvian population (paper III) two-sided Fisher's exact test was used to evaluate the associations between melanoma status, pigmentation characteristics, and *MC1R* variants using various types of stratification. Unpaired t-test was used to assess the difference in mean age between patients and controls. P-values<0.05 were considered to be statistically significant. For each *MC1R* variant, deviation from Hardy–Weinberg equilibrium was tested in controls as implemented in PLINK version 1.07 (Shaun Purcell, http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al.* 2007). For more detailed analysis of *MC1R* variant association with melanoma risk, individuals were grouped according to number and type of *MC1R* variants. Individuals with no *MC1R* variant or only synonymous

variants were considered as the wild type group. Others were grouped in a following manner: carriers of any *MC1R* variant, carriers of one *MC1R* variant, carriers of multiple *MC1R* variants, carriers of one or more RHC variant (and no NRHC variant), carriers of one or more NRHC variant (and no RHC variant), and carriers of one or more RHC and one or more NRHC variants. The RHC group consisted of previously reported RHC variants p.Asp84Glu, p.Arg151Cys, p.Arg160Trp (Raimondi *et al.* 2008) and additionally variants p.Ser83Leu, p.Gly89Arg, p.Asp121Glu, and p.Arg213Trp that were proved to be functionally relevant within this study. All remaining nonsynonymous variants were considered NRHC variants. Tests were carried out using GraphPad Prism v.5.04 software for Windows (GraphPad Software, La Jolla, CA, USA).

The functional impact of the *MC1R* variants on the receptor function initially was assessed *in silico* using software tool PolyPhen-2 (Adzhubei *et al.* 2010). Statistical analyses of the confocal microscopy data were performed as described previously (Fridmanis *et al.* 2010). Efficiency of green fluorescent protein (GFP) tagged receptor transport to the cell membrane was measured by calculating GFP and WGA fluorescence intensity ratios at multiple points on the cell using images from confocal microscopy. Using the tool implemented in the Leica Confocal Software (Las AF version 2.6.0), 10 arbitrarily selected linear regions of interest (ROI) were drawn across each image, resulting in 20 points of intersection with the cell membrane and giving at least 120 points for analysis for each construct. A nonparametric Kruskal–Wallis test was applied for uniformity analysis of the GFP/WGA fluorescence ratios per each construct with $\alpha=0.05$. If statistically significant differences between medians were reported, Dunn's multiple comparison test with $\alpha=0.05$ was used to determine which of the data sets were different and those data sets that significantly differed from more than two other data sets were replaced with data acquired from independent repeated experiments. Kruskal–Wallis test with $\alpha=0.05$ was used to compare median values and interquartile ranges of fluorescence ratios from different constructs that was followed by Dunn's multiple comparison test. Modules of differences in rank sums acquired by Dunn's test were arranged in a matrix table that was then used to cluster constructs by their differences in expression on the cell surface. Clustering was carried out with the Euclidean distance method using the MultiExperiment Viewer software version 4.3 (TM4 Development, Boston, MA, USA) (Saeed *et al.* 2003).

In the chromosome 16 variant study (paper IV) MAFs of each variant were estimated using all controls having the genotype information for this variant (224 controls for the *MC1R* gene, 203 for rs258322, 204 for rs8059973 and 205 for rs4785763). In the subsequent statistical analyses, only the variants with $\geq 4\%$ MAF, with at least one homozygote of minor alleles and those that did not significantly deviate from the Hardy-Weinberg equilibrium were considered. Univariate analyses with and without cofactors (age and sex) were carried out by fitting a logistic regression model for each variant and a melanoma case/control indicator. An additive model of the contribution of alleles to the disease was assumed. The significance of a genotype-phenotype association was measured by the Wald test applied to the genotype term with $\alpha=0.05$. Logistic regression models were fitted by the function *glm* in R environment (R Core Team). Permutation tests to obtain empirical p-values of genotype-phenotype associations were carried out. In order to understand potential interactions of variants on chromosome 16, multivariate models with and without cofactors (age and sex) were built by stepwise regression using function *stepAIC* from the R package *MASS* (R Core Team). A generalized linear model was used throughout all multivariate analyses, together with the additive model of allele contributions. Associations of various haplotypes with the disease status were also assessed using the R library *haplo.stats* (Lake *et al*. 2002; Schaid *et al*. 2002).

Linkage disequilibrium (LD) for each pair of associated variants was estimated and the squared correlation coefficient (r^2) was reported using *Ldmatrix* module from the web-based application suite LDlink 2.0 (Machiela *et al*. 2015).

For the *MC1R* and survival study (paper V) *MC1R* variants were classified in 'R' and 'r' variants using methodology that took into account previous existing classifications (Duffy *et al.* 2004), evaluation using *in silico* tools and properties of amino acid in the variant position. Then a numerical score in the range 0–4 was applied for each variant allele, where an 'r' variant allele was given value 1 and an 'R' variant allele was given value 2, thus individuals without *MC1R* variants were scored 0, but individuals with two 'R' variants were scored 4.

Survival time was defined as the period between the date of surgical excision of the primary melanoma and date of death or last date of follow up. Linear regression analysis was performed in order to detect association between *MC1R* variants and tumour thickness in all cohorts using the 'lm' routine in R (R Core Team). Using data from a Leeds cohort Kaplan–Meier analysis was done to evaluate overall survival with respect to hair colour and *MC1R* status using the 'survfit' routine in the 'survival' package in R and Cox's proportional hazards model. Cochran's Q test was used to test for study heterogeneity. Cases with 0.75 mm or thinner tumours were excluded from all analyses assuming that these cases have good prognosis and add little information to the estimation of the effect of predictors of survival.

The hypothesis of association between *MC1R* variants and melanoma survival was initially tested by evaluating hair colour and survival as hair colour is largely determined by *MC1R*. Leeds cohort data was used for these tests. After an association was indeed discovered, the association between *MC1R* status and overall survival was also investigated in each of the 10 data sets. A combined estimate for the nine smaller data sets was created by including the study as a stratification variable in the model. Forest plots were used to compare the hazard ratio estimates across studies. For each research centre hazard ratio estimates for *MC1R* score, for no consensus *MC1R* alleles versus one or more consensus *MC1R* alleles were calculated and a pooled estimate was plotted.

In the BioGenoMEL study of *PARP1* variant s2249844 (paper VIII) survival time was defined as the period between the date of surgical excision of the primary melanoma and date of death or last date of follow-up. As melanoma specific survival data were not available from all groups, in order to exclude non-melanoma related deaths, overall survival time was truncated at 8 years of follow-up. Cox's proportional hazards model was used for multivariable survival analyses using software R (R Core Team). Hazard ratio estimates were calculated for the effect of each of the variants on overall survival and adjusted for sex, tumour site, age of diagnosis and Breslow thickness. An additive genetic model was assumed. Fitted Cox's proportional hazards models were used for each study to estimate per-allele effects and standard errors that were then used for random effects meta-analysis using R software. Several bioinformatic analyses using different tools were also carried out to determine whether rs2249844 has a putative functional effect. Additionally, gene expression levels were analysed with relation to survival, ulceration and presence of angiolymphatic invasion using primary and metastatic formalin-fixed, paraffin-embedded tumour samples from Leeds with the Cox proportional hazards model and Mann–Whitney U-test.

3. RESULTS

The results are presented here as original publications. The author's contribution to the enclosed original publications depicted below each publication.

Paper I. Veinalde R, **Ozola A**, Azarjana K, Molven A, Akslen LA, Doniņa S, Proboka G, Cēma I, Baginskis A, Pjanova D. 2013. **Analysis of Latvian familial melanoma patients shows novel variants in the noncoding regions of *CDKN2A* and that the *CDK4* mutation R24H is a founder mutation.** *Melanoma Res* 23: 221-226.

Performed deletion analysis in melanoma patients, participated in writing, read and approved manuscript.

Paper II. Puntervoll HE, Yang XR, Vetti HH, Bachmann IM, Avril MF, Benfodda M, Catricalà C, Dalle S, Duval-Modeste AB, Ghiorzo P, Grammatico P, Harland M, Hayward NK, Hu HH, Jouary T, Martin-Denavit T, **Ozola A**, Palmer JM, Pastorino L, Pjanova D, Soufir N, Steine SJ, Stratigos AJ, Thomas L, Tinat J, Tsao H, Veinalde R, Tucker MA, Bressac-de Paillerets B, Newton-Bishop JA, Goldstein AM, Akslen LA, Molven A. 2013. **Melanoma prone families with *CDK4* germline mutation: phenotypic profile and associations with MC1R variants.** *J Med Genet* 50: 264-270.

Performed *MC1R* analysis in Latvian *CDK4* families, gathered and summarized data about Latvian *CDK4* melanoma families, read and approved manuscript.

Paper III. Ozola A, Azarjana K, Doniņa S, Proboka G, Mandrika I, Petrovska R, Cēma I, Heisele O, Eņģele L, Streinerte B, Pjanova D. 2013. **Melanoma risk associated with *MC1R* gene variants in Latvia and the functional analysis of rare variants.** *Cancer Genet* 206: 81-91.

Performed *MC1R* sequencing, result interpretation and data analysis, created *MC1R* variant constructs, cAMP measuring, confocal microscopy and statistical analysis of microscopy images, wrote manuscript.

Paper IV. Ozola A, Ruklisa D, Pjanova D. 2018. **Association of the 16q24.3 region gene variants rs1805007 and rs4785763 with heightened risk of melanoma in Latvian population.** *Meta Gene* 18:87-92.

Performed genotyping and result interpretation, wrote manuscript.

Paper V. Davies JR, Randerson-Moor J, Kukalizch K, Harland M, Kumar R, Madhusudan S, Nagore E, Hansson J, Höiom V, Ghiorzo P, Gruis NA, Kanetsky PA, Wendt J, Pjanova D, Puig S, Saiag P, Schadendorf D, Soufir N, Okamoto I, Affleck P, García-Casado Z, Ogbah Z, **Ozola A**, Queirolo P, Sucker A, Barrett JH, van Doorn R, Bishop DT, Newton-Bishop J. 2012. **Inherited variants in the *MC1R* gene and survival from cutaneous melanoma: a BioGenoMEL study.** *Pigment Cell Melanoma Res* 25: 384-394.

Performed *MC1R* sequencing in Latvian melanoma patients, gathered and summarized data about Latvian patients, prepared samples, read and approved manuscript.

Paper VI. Ozola A, Pjanova D. 2015. **The lack of E318K *MITF* germline mutation in Latvian melanoma patients.** *Cancer Genet* 208: 355-356.

Performed sequencing, result interpretation and data analysis, wrote manuscript.

Paper VII. Ozola A, Baginskis A, Azarjana K, Doniņa S, Proboka G, Čēma I, Heisele O, Eņģele L, Štreinerte B, Dace Pjanova. 2014. **Low penetrance melanoma risk gene polymorphisms in Latvian population.** *Environmental and Experimental Biology* 12: 55-56.

Performed genotyping, full result interpretation and data analysis, wrote manuscript.

Paper VIII. Davies JR, Jewell R, Affleck P, Anic GM, Randerson-Moor J, **Ozola A**, Egan KM, Elliott F, García-Casado Z, Hansson J, Harland M, Höiom V, Jian G, Jönsson G, Kumar R, Nagore E, Wendt J, Olsson H, Park JY, Patel P, Pjanova D, Puig S, Schadendorf D, Rachakonda PS, Snowden H, Stratigos AJ, Bafaloukos D, Ogbah Z, Sucker A, Van den Oord JJ, Van Doorn R, Walker C, Okamoto I, Wolter P, Barrett JH, Bishop DT, Newton-Bishop J. 2014. **Inherited variation in the *PARP1* gene and survival from melanoma.** *Int J Cancer* 135: 1625-1633.

Gathered and summarized data about Latvian patients, prepared samples, read and approved manuscript.

3.1. Original paper I. Analysis of Latvian familial melanoma patients shows novel variants in the noncoding regions of *CDKN2A* and that the *CDK4* mutation R24H is a founder mutation.

Analysis of Latvian familial melanoma patients shows novel variants in the noncoding regions of *CDKN2A* and that the *CDK4* mutation R24H is a founder mutation

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Hereditary cutaneous melanoma is associated with mutations in the high-risk *CDKN2A* gene in about 40% of melanoma-prone families. Mutations in the *CDK4* gene are the cause in only a few pedigrees. In this study, we analyzed 20 Latvian familial melanoma probands and carried out a comprehensive analysis of *CDKN2A* including sequencing of its promoter/intronic regions and deletion screening. We also analyzed the critical second exon of the *CDK4* gene. One novel intronic variant (IVS2+82C>T) of the *CDKN2A* gene and a small deletion (c.−20677_−20682delGTACGC) in its promoter region were found. Genotyping of the novel variants in larger melanoma and control groups indicated that the deletion increases the risk of melanoma (odds ratio=6.353, 95% confidence interval: 1.34–30.22, *P*=0.0168). The *CDK4* gene analysis showed a Latvian melanoma family with the mutation R24H carried on the same haplotype as in two previously described Latvian *CDK4*-positive families. Our study suggests that the main risk gene in Latvian families with a strong family history of melanoma is *CDK4* and that most of the other cases analyzed could be sporadic or associated with low-penetrance risk genes. *Melanoma Res* 23:221–226 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: *9p21* locus, *CDK4*, *CDKN2A*, *CDKN2A* promoter, deletion analysis, familial melanoma, high-risk genes, intronic polymorphisms, multiple ligation-dependent probe amplification

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Introduction

A familial predisposition is observed in up to 13% of cutaneous melanoma cases [1]. Melanoma families are considered to be those with three or, in countries with lower overall sunlight exposure, two affected first-degree relatives [2]. Two high-penetrance risk genes associated with familial melanoma identified to date are *CDKN2A* and *CDK4*, both coding for proteins involved in cell cycle regulation [1]. The by far most frequently mutated of these is *CDKN2A*, where mutations in ~40% of familial melanoma cases have been reported [3]. The *CDKN2A* gene is located on chromosome band *9p21* and codes for two alternatively spliced proteins $p16^{INK4a}$ and $p14^{ARF}$ with a role in G1/S phase transition regulation (reviewed in [4]). Only the coding *CDKN2A* regions with an adjacent intronic sequence have been analyzed in Latvian melanoma patients to date. Intriguingly, no high-penetrant *CDKN2A* mutations have been detected. Several polymorphisms [c.−33G>C; c.442G>A (p.A148T) (rs3731249); c.*29G>C (rs11515); c.*69C>T (rs3088440)] have been found, with A148T having a possible association with the disease [5,6].

Notably, two Latvian melanoma families with the *CDK4* mutation R24H have been reported [5,6]. The *CDK4* gene is located on chromosome band *12q4* and codes for a cyclin-dependent kinase 4 (cdk4) that binds to $p16^{INK4a}$ [7]. All mutations found so far affect the binding to $p16^{INK4a}$ and are located in the second exon of the gene, resulting in either an R24H or an R24C amino acid substitution. These mutations have a dominant effect on the possible development of melanoma, as the mutant CDK4 protein is capable of escaping the inhibition of $p16^{INK4a}$, allowing an uncontrolled progression through cell cycle [7]. To date, only 17 melanoma families worldwide have been reported to have mutations in this gene [8].

The aim of the present study was to analyze the *CDKN2A* locus in Latvian familial melanoma patients more comprehensively, including also the promoter region and introns as well as a deletion screening of the gene. Noncoding *CDKN2A* regions have been shown to harbor melanoma-associated mutations [9] and in some families *CDKN2A* gene deletions is the explanation for their melanoma disease [10,11]. We also sequenced exon 2 of the *CDK4* gene.

Methods

Twenty melanoma patients with at least one first-degree ($n=15$) or second-degree ($n=5$) relative with melanoma,

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all with a histologically confirmed diagnosis of the disease, were included in the study. To increase the sample size for genotyping, additional sporadic melanoma patients and control individuals were included (exact numbers of individuals were included, see below next to the results of each analysis). All melanoma patients were recruited at the Riga Eastern Clinical University Hospital, Latvian Oncology Center, between 2009 and 2011. Control individuals were those included in the Latvian Genome Database. All participants received an explanation about the aims of the study and signed an informed consent form approved by the Central Medical Ethics Committee of Latvia.

Genomic DNA was isolated from peripheral blood using standard phenol–chloroform extraction. Coding and flanking regions of the *CDKN2A* gene and the second exon of *CDK4* were analyzed as described previously [5]. The critical promoter region 1185 bp upstream of the gene *CDKN2A* exon 1α was amplified as described by Harland *et al.* [12]. The first and second intron of the *CDKN2A* gene were amplified in eight overlapping fragments each, using the primers and conditions as described by Harland *et al.* [9].

Sequencing was carried out using the ABI PRISM Big Dye Terminator v.3.1 cycle sequencing kit (Life Technologies, Carlsbad, California, USA), followed by analysis on an ABI 3100 Sequencer (Applied Biosystems, Foster City, California, USA). Sequencing was carried out in both directions using the same primers as in the initial amplification of each fragment, except for the promoter region of the gene *CDKN2A/p16^{INK4a}*, where three different primers in each direction were used as described [12].

Haplotype analysis of the *CDK4* region in the R24H mutation-positive family was carried out as described by Molven *et al.* [13] using four microsatellite markers (D12S305, CDK4M4, CDK4M1, D12S1691) flanking the *CDK4* gene, three intragenic single nucleotide polymorphisms (SNPs) (rs2270777, rs2069502, rs2069506), one SNP in the promoter region (rs2072052), and one tetranucleotide repeat within intron 5 of the gene, originally classified as an SNP (rs2069504).

The novel intronic sequence variant IVS2 + 82C > T of *CDKN2A* was genotyped by the restriction fragment length polymorphism method using *Sma*I restriction enzyme under conditions recommended by the manufacturer (Thermo Fisher Scientific Fermentas Inc., Waltham, Massachusetts, USA). For genotyping of the intronic sequence variant IVS1 + 1569T > A (rs138967562), a custom TaqMan probe was designed (primers available on request). The reaction conditions were as specified by the manufacturer and analysis was carried out on a ViiA 7 Real-Time PCR System (Applied Biosystems).

Deletion analysis in the locus *9p21* was carried out using the SALSA multiple ligation-dependent probe amplification (MLPA) kit ME024-A1 (MRC-Holland, Amsterdam, the Netherlands) according to the specifications provided by the manufacturer. Further direct sequencing and genotyping were carried out for the detection of the precise size and location of the detected deletion. For initial amplification of the region surrounding the probe with the deletion, primers were designed as follows: forward 5′-CCTAGTCCCGAATCCTCTGG-3′ and reverse 5′-CAACCATTCTACGCGAGGAC-3′. Sequencing of this fragment was carried out as described before. For genotyping of this region, an alternative fluorescently labeled (FAM) forward primer 5′-CTTAGACCGCGCT CAGGACC-3′ was designed and fragments were analyzed on an ABI 3100 Sequencer (Applied Biosystems). Two-sided Fisher's exact test was used for statistical analysis of the results (GraphPad Prism v.5.04 for Windows; GraphPad Software Inc., La Jolla, California, USA).

Results

Nineteen of the 20 familial melanoma probands analyzed had one relative affected with melanoma. Thirteen of them had a first-degree relative with melanoma, six had a second-degree relative with melanoma, and one patient had four affected relatives (Table 1).

Sequencing of coding and flanking regions of the *CDKN2A* gene showed no disease-associated mutations. Only the three previously known polymorphisms c.442G > A (A148T) (rs3731249), c.*29G > C (rs11515), and c.*69C > T (rs3088440) were observed (Table 1 and Fig. 1). The critical region of the *CDKN2A/p16^{INK4a}* promoter was analyzed in 19 probands, excluding the one with the *CDK4* mutation (see below). Three previously described polymorphisms c.−191A > G (rs3814960), c.−493A > T ($n = 1$), and c.−735G > A in the region of the *CDKN2A/p16^{INK4a}* promoter [12,14] were detected (Table 1 and Fig. 1). Polymorphisms c.−493A > T and c.−735G > A were both detected in the presence of c.−191A > G.

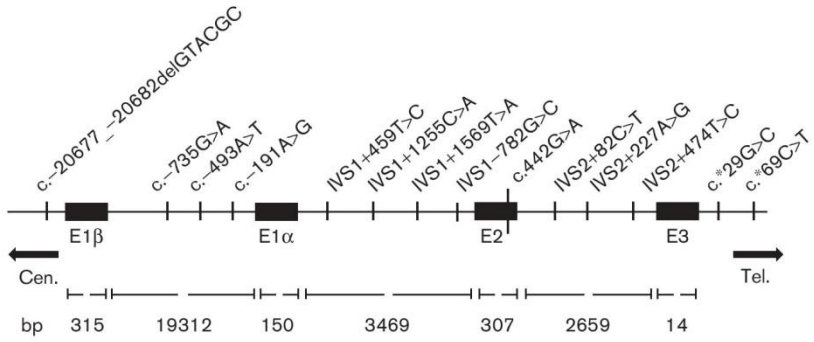
Analysis of the first and second intron of the *CDKN2A* gene was completed for 17 patients. Seven different sequence variants were detected. Five of these [IVS1 + 459T > C (rs3731239), IVS1 + 1255C > A (rs2811708), IVS1–782G > C (rs3731246), IVS2 + 227A > G (rs113886003), IVS2 + 474T > C (rs2518719)] have been described previously and shown to be frequently present also in control individuals, indicating no association with melanoma [9]. Two variants, IVS1 + 1569T > A (rs138967562) and the novel IVS2 + 82C > T, have not been analyzed previously in relation to melanoma (Table 1 and Fig. 1). Notably, in all cases, the polymorphism IVS2 + 474T > C was observed only together with IVS1 + 459T > C, IVS1 + 1255C > A, and promoter variant c.−191A > G,

Table 1 **Description of familial melanoma patients included in the study and results of *CDKN2A* locus analysis**

Number	Sex	Age at primary melanoma diagnosis (years)	Number of melanoma-affected relatives	Other cancers in the family	Variants in *CDKN2A* locus
1	Female	72	1 (sister)	Bladder cancer (father)	WT
2	Female	65	1 (mother)	–	c.–191A>G; c.*69C>T
3	Female	68	1 (brother)	–	c.–191A>G; IVS1+459T>C; IVS1+1255C>A; IVS2+474T>C
4	Female	58	1 (mother's brother)	Prostate cancer (father's brother)	c.–191A>G; IVS1+1255C>A; IVS1–782G>C; c.*29G>C
5	Female	49	1 (mother)	Lung cancer (mother's brother)	c.–191A>G; IVS1+459T>C; c.*69C>T
6	Male	78	1 (father's mother)	–	c.–493A>T; c.–191A>G; IVS1+459T>C; IVS1+1255C>A; IVS1–782G>C; c.442G>A c.*29G>C
7	Female	43	1 (mother)	–	IVS1+459T>C
8	Female	67	1 (mother's sister)	–	c.–191A>G; IVS1+459T>C; IVS1+1255C>A; IVS2+474T>C
9	Male	46	1 (mother's mother)	Breast cancer (sister)	IVS1+1569T>A
10	Male	45	1 (grandfather)	–	IVS1+459T>C
11	Male	67	1 (father)	–	IVS1+459T>C
12	Female	40	1 (father)	Gastric cancer (mother's sister)	c.–20677_–20682delGTACGC
13	Female	60	1 (son)	–	c.–191A>G; IVS1+459T>C
14	Female	71	1 (mother)	Pancreas cancer (sister)	IVS1+459T>C; IVS1+1569T>A
15	Female	55	1 (father)	Pancreas cancer (mother), gynecologic cancer (father's mother), gynecologic cancer (mother's sister)	c.–191A>G; IVS1+1255C>A; IVS1–782G>C; IVS2+82C>T; c.*29G>C
16	Female	49	1 (father's brother)	–	IVS1+459T>C
17	Female	47	1 (mother)	Lung cancer (father), gynecologic cancer (mother's sister)	c.–191A>G; IVS1+459T>C; IVS1+1255C>A; IVS2+474T>C
18	Male	35	1 (sister)	Breast cancer (mother)	c.–735 G>A; c.–191A>G; IVS1+1255C>A; c.442G>A
19	Male	56	1 (father)	–	IVS1+459T>C
20	Male	40	4 (father, father's mother, mother's sister, mother's brother)	Intestinal cancer (father), lung cancer (father's mother's mother), gastric cancer (father's mother's father), kidney cancer (father's mother's brother), kidney cancer (father's mother's brother's daughter)*	WT

*See Fig. 1 for more information.

Fig. 1



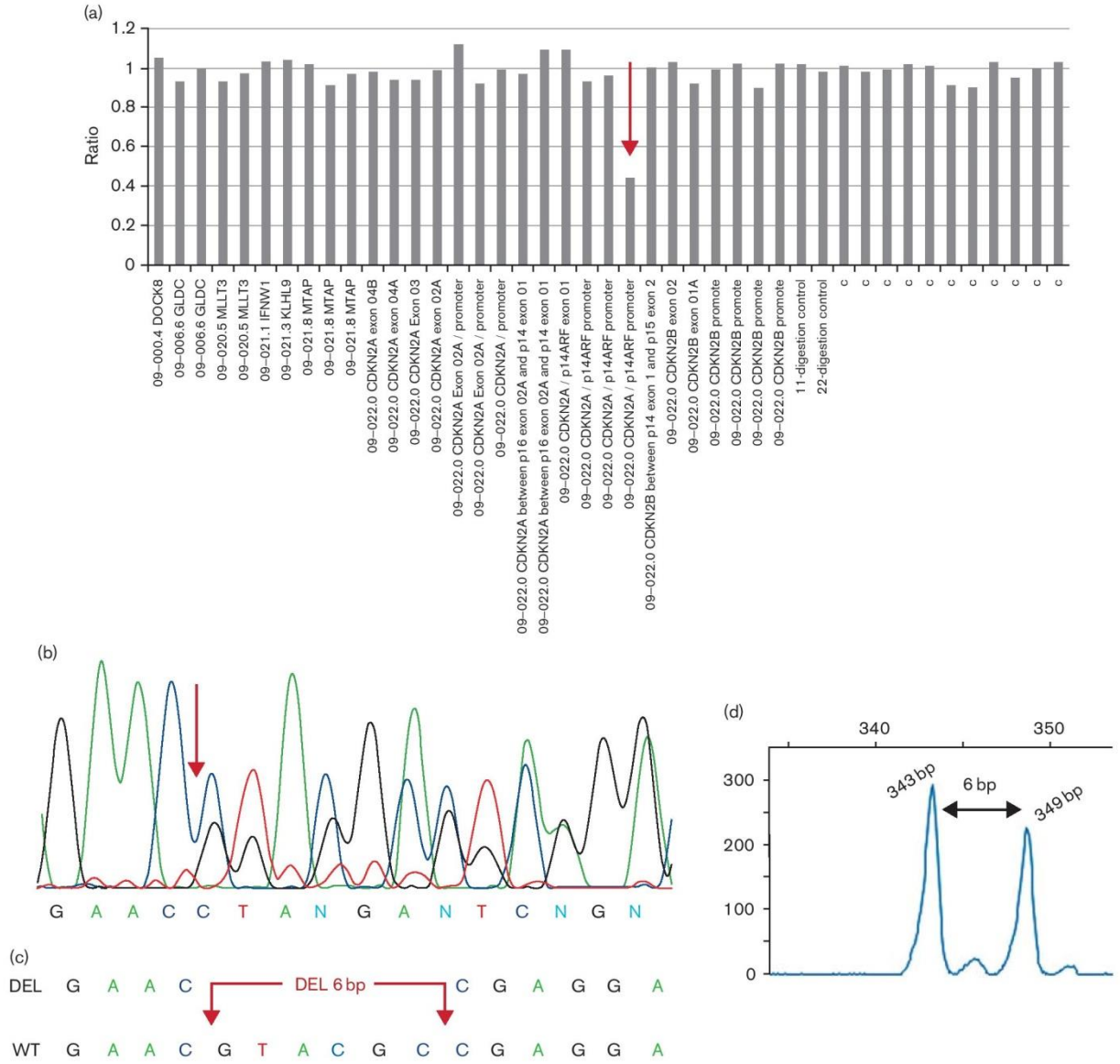
Schematic representation of the sequenced region of the gene *CDKN2A* and detected sequence variants. The region is not depicted at scale – markers below indicate the length of individual fragments in base pairs. Cen., centromere; E1β, E1α, E2, and E3, exons of the *CDKN2A* gene; Tel., telomere.

indicating a possible linkage disequilibrium. Further genotyping results of the sequence variants IVS2 + 82 C > T and IVS1 + 1569T > A in additional sporadic melanoma patients and control individuals were as follows: 0/174 and 1/211 for the IVS1 + 1569T > A variant and 0/180 and 0/228 for the IVS2 + 82C > T variant, respectively, showing that these are rare intronic variants.

Deletion analysis was completed for 10 patients with a family history of melanoma. MLPA showed one patient with a deletion in the probe located in the region of the *CDKN2A/p14*^{ARF} promoter – 1062 bp upstream of the start codon of exon 1β (Fig. 2). Further results from direct sequencing and genotyping showed that this was a 6 bp deletion c.–20677_–20682delGTACGC (Fig. 2). The patient with the deletion was diagnosed with melanoma at the age of 40 years. The father of the patient had been diagnosed with melanoma at the age of 78 years and died at the age of 81 years from lung metastasis.

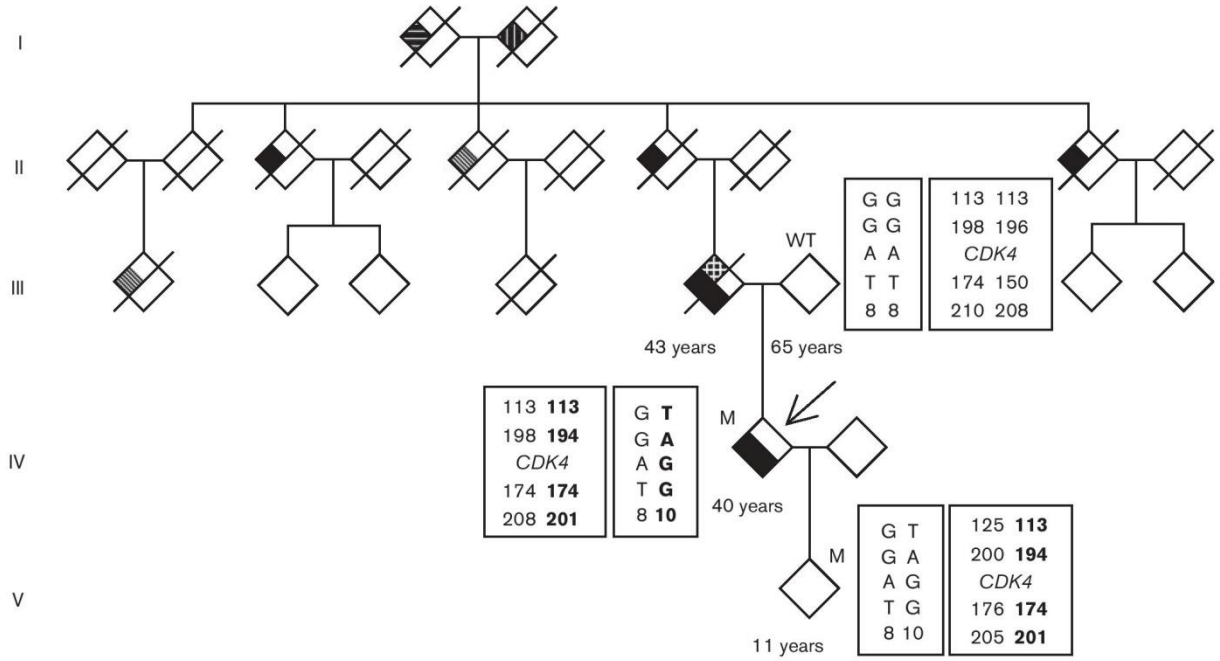
Further genotyping for additional 211 melanoma patients and 326 control individuals indicated the presence of the

Fig. 2



Deletion in the promoter region of the gene *CDKN2A* in a familial melanoma patient. (a) MLPA gene dosage quotients (ratio) in the locus *9p21* in a patient with deletion. The numbers assigned to the MLPA probes are listed and the region where the probe binds is given, c, shows control probes. Arrow denotes the deletion. (b) Fragment of the chromatogram at the area of deletion where the arrow shows the deletion breakpoint. (c) WT (wild-type) allele and allele with the deduced 6 bp deletion c.–20677_–20682delGTACGC. (d) Genotyping results confirming the presence of the 6 bp deletion in the *CDKN2A*^{F} promoter region. MLPA, multiple ligation-dependent probe amplification.

Fig. 3



Pedigree diagram of the novel Latvian family with melanoma and the *CDK4* mutation R24H. Melanoma-affected family members are shown by black quarter-filled symbols with the age of primary melanoma onset below (when known). For healthy individuals analyzed (unfilled symbols), age at the time of the DNA analysis is indicated. Arrow denotes the proband. Mutation status of *CDK4* is indicated as M (carrier) or WT (wild-type). Other malignancies in the family are shown as additional quarter-filled symbols: vertically striped, gastric cancer; horizontally, lung cancer; diagonally, kidney cancer; gridded, intestinal cancer. The deduced *CDK4* haplotypes are shown in the white boxes beside the analyzed members. The haplotype of the allele carrying the mutation is shown in bold. The analyzed SNP markers are, from top to bottom, rs2072052, rs2270777, rs2069502, rs2069506, and rs2069504. For rs2069504, the number of tetranucleotide repeats is given. The analyzed microsatellite markers are, from top to bottom, D12S305, CDK4M4, CDK4M1, and D12S1691, with the allele sizes listed as the number of base pairs. SNP, single nucleotide polymorphism.

same 6 bp deletion in seven melanoma patients and two control individuals. Two-sided Fisher's exact test yielded an odds ratio of = 6.353 and a 95% confidence interval of 1.34–30.22 ($P = 0.0168$).

Analysis of the second exon of the *CDK4* gene showed one patient (no. 20 in Table 1) with the mutation c.71G > A (R24H). This patient was diagnosed with melanoma at the age of 40 years and had four melanoma-affected relatives (Fig. 3). The patient's father was diagnosed with two recurrent melanomas (age 43 and 49 years) and intestinal cancer (age 43 years) and died at the age of 50 years. The patient's grandmother and two of her siblings were diagnosed with melanoma and died at the ages of 88, 56, and 33 years, respectively. In addition to melanoma, multiple other cancers were observed in the family history (Fig. 3). Haplotype analysis of the *CDK4* region showed that the mutation cosegregated with the haplotype determined by the SNPs T-A-G-10-G and microsatellite allele sizes 113-194-174-201 (Fig. 3), consistent with the haplotype for the other two Latvian families with this mutation [6]. Exon 2 of *CDK4* was analyzed in an additional 309 sporadic melanoma patients and 150 control individuals. Neither R24H nor other mutations were found.

Discussion

This study, focusing on Latvian melanoma patients with a family history of the disease, showed several new sequence variants of the *CDKN2A* gene. One novel intronic variant (IVS2 + 82C > T) was detected, in addition to a variant (IVS1 + 1569T > A) not described previously in relation to melanoma. However, after genotyping of a larger group of melanoma patients and control individuals, the status of these variants with respect to the risk of melanoma remains unclarified. Most of the intronic mutations previously associated with the risk of melanoma are localized in the canonical splice donor or acceptor sites [15,16], whereas both analyzed variants are deep intronic. However, deep intronic variants could also affect the efficacy of transcription or splicing [17]. Moreover, as the patients analyzed had a family history of melanoma, we cannot exclude a possibility of them being disease-related causal mutations and further functional studies are required.

MLPA analysis showed the novel deletion c.−20677_−20682delGTACGC in the *CDKN2A/p14^{ARF}* promoter region. Statistical analysis showed a potential association with the disease ($P = 0.0168$), but the power is limited and our result must be confirmed in a larger study. Being only 6 bp in size, this deletion does not resemble other previously detected melanoma-associated deletions in this region, which span several kilobasepairs [10,11]. However, being localized in the promoter region, the deletion could potentially affect the transcription of the gene *CDKN2A/p14^{ARF}* and have an impact on the autoregulatory feedback loop-regulating cell cycle, in which the ability of transcription factors and p53 to bind to the *CDKN2A/p14^{ARF}* promoter is crucial [18].

Analysis of the *CDK4* gene indicated a third Latvian melanoma family with the mutation R24H. Families with a strong melanoma history are very uncommon in Latvia. Interestingly, the three known pedigrees with more than two affected first-degree relatives all carry this mutation [5,6]. Families with the R24H *CDK4* mutation have been shown to carry the mutation on different haplotypes, indicating several independent origins of the mutations worldwide and suggesting that codon 24 is a mutational hot-spot in this gene [13]. Haplotype analysis of the novel *CDK4* family described here showed that it is the same as in the previously described Latvian families [6], indicating a founder effect. Genotyping of additional sporadic melanoma patients and control individuals showed that the mutation is rare in the Latvian population and present only in families with a strong family history of the disease.

Although we have analyzed a limited number of melanoma families, the sample group was heterogeneous and covers different regions of the country, bringing us to the overall conclusion that, so far, *CDK4* appears as the main high-risk gene in Latvia. The most obvious reason for the relatively frequent finding of Latvian *CDK4* melanoma families is the previously mentioned founder effect, which could be particularly strong in the relatively small Latvian population (approximately two million inhabitants). The lack of disease-predisposing *CDKN2A* mutations in Latvia is more surprising. This might indicate that the other analyzed cases of our study are, in fact, sporadic and that segregation in families is a coincidence or an impact from shared environmental factors. Alternatively, familial cases could be influenced by an accumulation of low-risk or intermediate-risk melanoma genes.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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3.2. Original paper II. Melanoma prone families with *CDK4* germline mutation: phenotypic profile and associations with *MC1R* variants

ORIGINAL ARTICLE

Melanoma prone families with *CDK4* germline mutation: phenotypic profile and associations with *MC1R* variants

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ABSTRACT

Background *CDKN2A* and *CDK4* are high risk susceptibility genes for cutaneous malignant melanoma. Melanoma families with *CDKN2A* germline mutations have been extensively characterised, whereas *CDK4* families are rare and lack a systematic investigation of their phenotype.

Methods All known families with *CDK4* germline mutations (n=17) were recruited for the study by contacting the authors of published papers or by requests via the Melanoma Genetics Consortium (GenoMEL). Phenotypic data related to primary melanoma and pigmentation characteristics were collected. The *CDK4* exon 2 and the complete coding region of the *MC1R* gene were sequenced.

Results Eleven families carried the *CDK4* R24H mutation whereas six families had the R24C mutation. The total number of subjects with verified melanoma was 103, with a median age at first melanoma diagnosis of 39 years. Forty-three (41.7%) subjects had developed multiple primary melanomas (MPM). A *CDK4* mutation was found in 89 (including 62 melanoma cases) of 209 tested subjects. *CDK4* positive family members (both melanoma cases and unaffected subjects) were more likely to have clinically atypical nevi than *CDK4* negative family members ($p<0.001$). MPM subjects had a higher frequency of *MC1R* red hair colour variants compared with subjects with one tumour ($p=0.010$).

Conclusion Our study shows that families with *CDK4* germline mutations cannot be distinguished phenotypically from *CDKN2A* melanoma families, which are characterised by early onset of disease, increased occurrence of clinically atypical nevi, and development of MPM. In a clinical setting, the *CDK4* gene should therefore always be examined when a melanoma family tests negative for *CDKN2A* mutation.

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INTRODUCTION

Cutaneous malignant melanoma is characterised by a complex aetiology, involving both genetic and environmental risk factors. Approximately 5–10% of the melanoma cases occur in a familial setting,[1] and two genes have so far been identified as high risk susceptibility genes for the disease: cyclin dependent kinase inhibitor 2A (*CDKN2A*)[2 3] and cyclin dependent kinase 4 (*CDK4*).[4 5]

CDKN2A (MIM 600160) encodes two distinct proteins, $p16^{INK4A}$ and $p14^{ARF}$; both are tumour suppressors involved in cell cycle inhibition through different pathways.[6–8] In studies of melanoma families, the frequency of *CDKN2A* germline mutations is 20–40%, depending on the inclusion criteria.[9] Common features of the *CDKN2A* melanoma families are early onset of disease and an increased risk of developing clinically atypical nevi, multiple primary melanomas (MPMs), and pancreatic cancer.[10 11]

CDK4 (MIM 123829) encodes the catalytic subunit of a heterodimeric Ser/Thr protein kinase, which together with its regulatory subunit (one of the D-type cyclins) is involved in controlling progression through the G1 phase of the cell cycle. Only 12 melanoma prone families with *CDK4* germline mutations have been reported.[4 5 12–18] All mutations are located in codon 24 in exon 2, resulting in either an Arg24His (R24H) or Arg24Cys (R24C) substitution. This changes the $p16^{INK4A}$ binding domain, leading to reduced $p16^{INK4A}$ inhibition of CDK4 kinase activity and, subsequently, to cell cycle progression.[19 20]

Fair skin, red/blonde hair colour, freckling, and sun sensitivity are established pigmentation related risk factors for melanoma development. Pigmentation phenotype is partly regulated by the melanocortin-1 receptor (*MC1R*) gene (MIM 155555), a low risk melanoma susceptibility gene that may act both dependently and independently of ultraviolet radiation to influence melanoma risk.[21 22] *MC1R* encodes a seven-pass transmembrane, G-protein coupled receptor, which is involved in regulation of pheomelanin (yellow/red pigment) and eumelanin (black/brown pigment) production.[23] The *MC1R* locus is highly polymorphic in the Caucasian population,[24] and

certain variants have been associated with the red hair colour (RHC) phenotype; that is, red hair, fair skin, freckling, and the inability to tan.[25 26] It is mainly the RHC variants that have been associated with increased melanoma risk,[27–29] although a link between non-RHC (NRHC) variants and melanoma has also been observed.[30] Studies within *CDKN2A* melanoma families have established that common *MC1R* variants act as modifier genes, in that carrying multiple variants is associated with increased melanoma risk. Several of these studies also reported an association between *MC1R* variants and decreased age at melanoma diagnosis, particularly in MPM patients.[31–35]

A systematic characterisation of melanoma prone families with *CDK4* germline mutation has never been performed. Previous studies of such families have included a limited number of melanoma cases and have mainly served to confirm *CDK4* as a high risk melanoma gene. Here, we report a joint investigation of all 12 published *CDK4* melanoma families along with five unpublished pedigrees. The purpose was to examine the clinical phenotype of these families, including possible modifying effects of *MC1R* variants, with the intent to inform genetic counselling internationally.

METHODS

Recruitment of *CDK4* mutated melanoma families and data collection

Melanoma families with germline *CDK4* mutations were recruited for this study either by contacting the authors of published papers[4 5 12–18] or by requests for unpublished families via GenoMEL, the Melanoma Genetics Consortium (www.genomel.org). Seventeen families, including five unpublished, were enrolled (table 1).

Clinical examinations were performed by dermatologists or specifically trained research nurses, and phenotypic data were collected via a standardised form. Examiners were generally unaware of the genotype of individuals before recording clinical features. Data collected included skin type using the 'Fitzpatrick classification',[36] eye and hair colour, and total number of nevi (>2 mm in diameter). Also the presence of clinically atypical nevi, defined as nevi >5 mm with irregular pigmentation and an irregular or diffuse border,[37] was recorded. Diagnoses of cutaneous melanoma were confirmed by histology reports and/or medical records. For patients with MPMs, the total number of melanomas was recorded; however, age at melanoma diagnosis, anatomic location, and histological type were recorded only for the first three primaries. Anatomic location of the melanomas was categorised into head/neck, limbs, and trunk. Histologic type was recorded as superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo malignant melanoma (LMM), in situ melanoma, and melanoma unclassified/classification unknown. No acral or mucosal melanomas were observed in our material. Information on non-melanoma cancers was collected for the melanoma cases and for those unaffected subjects who either had tested positive for a *CDK4* mutation or were considered as obligate mutation carriers.

Written informed consent was obtained from the family members before enrolment. The study was performed according to the Helsinki declaration.

DNA analysis of *CDK4* and *MC1R*

DNA samples were available from 209 subjects. Altogether, DNA from 62 melanoma cases, 106 unaffected family members (melanoma status not given for three of these; they were considered as unaffected in this study), and 41 spouses were examined for mutations in *CDK4* exon 2 and for variants in the coding region of *MC1R*. The initial DNA analysis was performed by each individual research group, either by direct Sanger sequencing or by single strand conformation polymorphism screening; hence there was slight variation in protocols and primers.[4 5 12–18]

Statistical analysis

Before the statistical analyses, hair and skin colour was grouped because of small sample sizes for these categorical variables: RHC versus all other hair colours, very fair/fair skin colour versus all other skin colours. For comparisons between subject groups (melanoma affected and unaffected *CDK4* positive family members versus *CDK4* negative family members and spouses) and different categorical variables (occurrence of clinically atypical nevi, melanoma status, hair and skin colour, *MC1R* variant distribution), the Pearson χ^2 test or the Fisher exact test were used depending on sample sizes. The non-parametric Mann–Whitney or Kruskal–Wallis tests were used to compare the continuous variable (age at first diagnosis) with the categorical variables (melanoma status, tumour location, histologic type, occurrence of clinically atypical nevi, *MC1R* variant distribution).

All observed *MC1R* variants were recorded, but because many variants were rare, they were grouped before the statistical comparisons with phenotypic data. These comparisons were performed as follows: (1) The distribution of individuals with *MC1R* consensus sequence, one and two *MC1R* variants was compared between the different subject groups (analysis denoted 'Number of *MC1R* variants'). (2) The distribution of individuals with *MC1R* consensus sequence, RHC, NRHC, and RHC+NRHC variants was compared between the different subject groups (analysis denoted 'Type of *MC1R* variants'). We observed no individuals with more than two *MC1R* variants. The RHC variants were defined as D84E, R142H, R151C, R160W, and D294H, all associated with red hair phenotype.[25 26] Other non-synonymous *MC1R* variants were labelled as NRHC. Synonymous *MC1R* variants were excluded from all analyses. When analysing *MC1R* variant distributions, the *CDK4* negative family members and spouses were combined into a single control group.

Unconditional logistic regression analysis was used to assess whether atypical nevi status varied by melanoma affection and *CDK4* carrier status when adjusted for age (age at last examination for unaffected subjects and age at diagnosis for melanoma patients). Statistical analyses were performed using the IBM Statistical Package for the Social Sciences, version 19 (SPSS Inc, Chicago, Illinois, USA) and SAS software (version 9.1.3, SAS Institute Inc, Cary, North Carolina, USA). p values$<$0.05 were considered to represent significant associations. Also p values between 0.05 and 0.10 are shown in the tables.

RESULTS

Seventeen familial melanoma pedigrees with *CDK4* germline mutations (11 with the R24H mutation and six with R24C) were available for this study; 12 previously published and five unpublished (table 1). In these pedigrees, a total of 103 members with cutaneous malignant melanoma (=affected subjects) were recorded. DNA was available for 209 subjects of whom 89 were mutation carriers (62 affected, 27 unaffected), 79 were mutation negative unaffected family members, and the remaining were spouses (table 1). As expected, all spouses had a normal *CDK4* exon 2 sequence. Among the 41 affected subjects for whom DNA was not available, seven were classified as obligate mutation carriers. Of unaffected subjects without available DNA, five were obligate mutation carriers.

Table 1 Overview of the studied melanoma families with *CDK4* germline mutations

Family designation	Laboratory number of family	Mutation	Subjects with verified cutaneous melanoma in pedigree	Total number of subjects with analysed DNA	Mutation positive family members	Mutation negative family members	Spouses	Reference
Norway-1	–	Arg24His	28	108	33	48	27	5
USA-1	8302	Arg24Cys	9	29	12	10	7	4
USA-2	8290	Arg24Cys	6	12	7	5	0	4
UK-1	301	Arg24His	4	5	2	2	1	5
UK-2	1119	Arg24Cys	5	7	4	3	0	Unpublished
Latvia-1	247	Arg24His	5	5	2	3	0	13
Latvia-2	268	Arg24His	5	6	3	3	0	16
Latvia-3	M679	Arg24His	5	3	2	0	1	Unpublished
Australia-1	60007	Arg24His	10	3	2	0	1	5
Italy-1	FM029	Arg24His	4	6	4	1	1	15
Italy-2	501153	Arg24Cys	1	2	1	1	0	18
France-1	759	Arg24His	6	14	9	3	2	12
France-2	–	Arg24His	2	4	3	0	1	14
France-3	–	Arg24Cys	2	1	1	0	0	Unpublished
France-4	14648	Arg24Cys	3	1	1	0	0	Unpublished
France-5	–	Arg24His	2	1	1	0	0	Unpublished
Greece-1	–	Arg24His	6	2	2	0	0	17
Total			103	209	89*	79	41	

*Sixty-two of the mutation positive family members had melanoma and 27 were unaffected.

Phenotypic characteristics of melanoma patients in *CDK4* families

Phenotypic characteristics of the 103 malignant melanoma cases are presented in table 2. Age at first malignant melanoma diagnosis was available for 95 cases and ranged from 18–86 years, with a median age of 39 years. Most cases occurred in the fourth decade of life (31.6%), whereas age of onset above age 60 years was rare (7.4%). There was no statistically significant difference in distribution of age at first diagnosis between males and females, or between cases with and without available DNA.

Forty-three melanoma patients (41.7%) developed more than one primary tumour. The number of primaries ranged from 2–13. Altogether, 217 melanomas were reported for 102 affected subjects (data on the number of melanomas were missing for one subject with MPM). Patients with MPMs showed a significantly lower median age at first diagnosis than patients with single primary melanoma (SPM): 35 and 43 years, respectively (p=0.002). There was no difference in distribution of SPM and MPM by gender.

The melanomas occurred most frequently on the limbs (table 2), and subjects with their first melanoma on this location had a significantly lower age at first diagnosis (33.5 years) than subjects with melanomas located in the head and neck region (45.5 years) (p=0.018). The predominant histologic type was SSM (table 2). Subjects with SSM had a significantly lower median age at first diagnosis than individuals with NM and LMM (p=0.039). The median ages were 36.5, 54, and 64 years, respectively. Ten of the first melanomas were recorded as in situ cases with a median diagnosis age of 33 years.

We further evaluated the occurrence of clinically atypical nevi (table 3). Both affected and unaffected *CDK4* positive subjects showed a significantly higher frequency of atypical nevi (70% and 75%) than the *CDK4* negative subjects (26.5%) (p<0.001). The associations remained significant after age adjustment (affected *CDK4* positive patients: OR 6.08, 95% CI 2.51 to 14.76, p<0.001; unaffected *CDK4* positive subjects: OR 7.37, 95% CI 1.99 to 27.39, p=0.003). The median age at first melanoma diagnosis for the atypical nevi positive patients was significantly lower (32.5 years) than for atypical nevi negative patients (40 years) (p=0.004).

There was no difference in distribution of hair and skin colour between the affected and unaffected *CDK4* positive family members and the *CDK4* negative family members (see online supplementary table 1). We also tested for phenotypic differences between subjects carrying the R24H and R24C mutations. No statistically significant differences were seen with regard to age at first melanoma diagnosis or the occurrence of MPM and clinically atypical nevi (see online supplementary table 2).

Concerning non-melanoma cancers, 33 cases were found in 25 of the 105 subjects where information on other cancers had been specified (see online supplementary table 3). Non-melanoma skin cancers and female related cancers were most frequently observed. Two cases of pancreatic cancer were seen. Ages of onset of the non-melanoma cancers were in a range expected in normal populations.

MC1R variants

Altogether, 15 different *MC1R* variants were observed in our material. Eleven variants predicted non-synonymous amino acid changes (V60L, V60R, D84E, V92M, R142H, R142S, R151C, I155T, R160W, R163Q, D294H), three variants corresponded to synonymous amino acid changes (A166A, Q233Q, T314T), and one was an insertion at the nucleotide level (86insA). V60R and R142S have, to our knowledge, not been reported before. R160W, R151C, and V60L were the most frequently observed variants. Five RHC variants were recorded: D84E, R142H, R151C, R160W, D294H. We classified all remaining non-synonymous *MC1R* variants and 86insA to the NRHC group.

There were no significant differences in *MC1R* variant distribution between the *CDK4* negative family members and spouses. A control group was therefore established consisting of all *CDK4* negative subjects. Comparison of the affected *CDK4* mutation carriers with the *CDK4* negative control group

Table 2 Phenotypic and genotypic characteristics of melanoma cases in families with *CDK4* germline mutations

Variable*	Number	%
Sex (N=103)		
Male	44	42.7
Female	59	57.3
Number of primary melanomas in affected subjects (N=103)		
One	60	58.3
Multiple	43	41.7
Mean	2.1	–
CDK4 mutation status of affected subjects (N=103)		
Subjects (N=77) in R24H families		
CDK4 mutation positive	41	39.8
CDK4 mutation negative	0	0.0
Obligate *CDK4* mutation carriers†	7	6.8
Unknown mutation status†	29	28.2
Subjects (N=26) in R24C families		
CDK4 mutation positive	21	20.4
CDK4 mutation negative	0	0.0
Obligate *CDK4* mutation carriers†	0	0.0
Unknown mutation status†	5	4.9
Age at first melanoma diagnosis (N=95)		
<30 years	20	21.1
30–39 years	30	31.6
40–49 years	24	25.3
50–59 years	14	14.7
≥60 years	7	7.4
Missing data‡	8	–
Mean (years)	40.4	–
Median (years)	39.0	–
Anatomic location (N=140)§		
Head/neck	31	22.1
Limbs	66	47.1
Trunk	43	30.7
Missing data‡	28	–
Anatomic location, first primary melanoma only (N=81)		
Head/neck	17	21.0
Limbs	34	42.0
Trunk	30	37.0
Missing data‡	21	–
Histologic type (N=95)§		
SSM	71	74.7
NM	3	3.2
LMM	1	1.1
In situ melanoma	20	21.1
Melanoma unclassified or classification unknown‡	73	–
Histologic type, first primary melanoma only (N=48)		
SSM	34	70.8
NM	3	6.3
LMM	1	2.1
In situ melanoma	10	20.8
Melanoma unclassified or classification unknown‡	54	–

*One melanoma case was recorded as MPM, but with no information on the actual number of melanomas. It was therefore excluded when calculating the mean number of melanomas and when summarising anatomic location and histologic type.
†DNA was not available for these cases.
‡Missing data are not included in the parentheses (N=) and not included when calculating percentages.
§For persons with MPM, information about the first three registered tumours was recorded.
LMM, lentigo malignant melanoma; MPM, multiple primary melanomas; NM, nodular melanoma; SSM, superficial spreading melanoma.

Table 3 Occurrence of clinically atypical nevi in families with *CDK4* germline mutations

		CDK4 positive family members			
	CDK4 negative family members	Affected		Unaffected	
Clinically atypical nevi*	N=49 (%)	N=50 (%)	p Value†	N=20 (%)	p Value†
Present	13 (26.5)	35 (70.0)	<0.001	15 (75.0)	<0.001
Not present	36 (73.5)	15 (30.0)		5 (25.0)	

*Data on *CDK4* mutation status and clinically atypical nevi were available for 119 subjects.
†*CDK4* negative family members were compared with affected and with unaffected *CDK4* positive family members, respectively.

revealed no significant differences in the *MC1R* variant distribution (table 4). However, when comparing the unaffected *CDK4* mutation carriers with the *CDK4* negative control group, the former group showed a lower number of RHC variants (p=0.012). The unaffected *CDK4* mutation carriers also had a significantly lower number of RHC variants compared with the affected *CDK4* carriers (p=0.042) (table 4).

Finally, we investigated the *MC1R* variant distribution in MPM and SPM subjects (table 4). We found no statistically significant difference in the number of *MC1R* variants, but the MPM subjects were more likely to carry RHC variants (p=0.010). There were no significant associations between age at first melanoma diagnosis and *MC1R* variant distribution (tested in SPM and MPM subjects, both separately and combined). Similarly, there were no significant differences in the *MC1R* variant distribution in subjects with and without clinically atypical nevi.

DISCUSSION

This study presents the largest dataset on melanoma families with *CDK4* germline mutations to date, and is the first systematic evaluation of their phenotype and the influence of *MC1R* variants. We examined 17 families from eight countries that included 103 subjects with a verified melanoma diagnosis. The families carried either an R24H or R24C mutation, and we were not able to reveal any clinical differences between carriers of the two *CDK4* mutations.

Early onset of disease is a characteristic feature of hereditary cancers. In this study, median age at first melanoma diagnosis was 39 years, about 15 years earlier than in the general Caucasian population. Thus, 21.1% of the melanoma patients in the *CDK4* families had been diagnosed before the age of 30 years, whereas only 7.4% were diagnosed at age 60 years or older. Based on all individuals for which clinical information was available at age 50 years (or later), the mutation penetrance at this age was 74.2%. This confirms *CDK4* as a highly penetrant melanoma risk gene. However, since most of the younger mutation carriers are now enrolled in screening programmes where severely dysplastic or borderline lesions are removed, the true lifetime melanoma risk of carrying a *CDK4* germline mutation might be difficult to assess, assuming that such lesions are precursors.

We found that 41.7% of the melanoma subjects developed more than one primary melanoma, a frequency comparable to that observed in families with *CDKN2A* mutations.[11 38 39] Regarding clinically atypical nevi, a significantly higher occurrence

Table 4 Frequencies of *MC1R* variants in families with *CDK4* germline mutations

MC1R variant distribution	*CDK4* negative family members and spouses* N=115 (%)	*CDK4* positive family members† Unaffected N=23 (%)	p Value§	Affected N=60 (%)	p Value§	p Value¶	Number of primary melanomas‡ SPM N=30 (%)	MPM N=30 (%)	p Value**
Number of *MC1R* variants									
0 (consensus sequence)	23 (20.0)	10 (43.5)	0.071	15 (25.0)	NS	NS	11 (36.7)	4 (13.3)	0.070
1 variant	71 (61.7)	10 (43.5)		32 (53.3)			15 (50.0)	17 (56.7)	
2 variants	21 (18.3)	3 (13.0)		13 (21.7)			4 (13.3)	9 (30.0)	
Type of *MC1R* variants									
0 (consensus sequence)	23 (20.0)	10 (43.5)	0.012	15 (25.0)	NS	0.042	11 (36.7)	4 (13.3)	0.010
RHC only	48 (41.7)	3 (13.0)		23 (38.3)			7 (23.3)	16 (53.3)	
NRHC only	31 (27.0)	9 (39.1)		14 (23.3)			10 (33.3)	4 (13.3)	
Both RHC and NRHC	13 (11.3)	1 (4.4)		8 (13.3)			2 (6.7)	6 (20.0)	

**MC1R* data were available for 76 of 79 *CDK4* negative family members and for 39 of 41 spouses. In these groups, the distributions of number and type of *MC1R* variants were very similar, and the two groups were combined into a single control group for the statistical analyses.
†Melanoma status and *MC1R* data were available for 83 of 89 *CDK4* positive family members.
‡The number of primary melanomas and *MC1R* data were available for 60 of the 103 melanoma subjects.
§The control group was compared with unaffected and affected *CDK4* positive family members, respectively.
¶Unaffected mutation carriers were compared with affected mutation carriers.
**Subjects with SPM and MPM were compared with each other with regard to *MC1R* variant distribution.
NS=non-significant p value.
MPM, multiple primary melanomas; NRHC, non-red hair colour; RHC, red hair colour; SPM, single primary melanoma.

was observed in the *CDK4* mutation carriers compared with the *CDK4* negative family members (table 3). Again, this is similar to findings in *CDKN2A* families.[40 41] The presence of clinically atypical nevi has been suggested to be a modifier of melanoma risk in *CDKN2A* mutation carriers,[40 41] and we observed that among affected subjects, the median age at first melanoma diagnosis was 7.5 years lower in atypical nevi positive than in negative family members. On the other hand, the frequency of these nevi was similar in affected and unaffected *CDK4* positive subjects (table 3).

Unfortunately, a high number of the melanoma cases were unclassified with regard to histology, or classification could not be obtained from the patients' records. This mainly concerned the oldest cases, as histology data generally became more complete for more recent cases. Nevertheless, the most frequent histologic type was SSM (74.7%), as in *CDKN2A* families.[39] The relatively high frequency of in situ melanomas (21.1%) may be influenced by increased surveillance of melanoma prone families.

We tried to assess non-melanoma cancers in our material, but encountered some obstacles. Firstly, most participating laboratories had collected anamnestic cancer data only from melanoma cases and *CDK4* positive family members, and not from *CDK4* negative members or spouses. Secondly, the *CDK4* families stemmed from many countries and populations, with varying background incidences and different national registration systems for cancer. Thus, we were prevented from performing formal analyses to test whether the observed incidences of non-melanoma cancers (see online supplementary table 3) were higher than expected. Still, the frequencies of breast cancer and non-melanoma skin cancer might suggest that *CDK4* mutation carriers could be at an increased risk. Sun exposure is an environmental determinant of risk for all skin cancers and an overrepresentation of non-melanoma skin cancer would therefore not be surprising. However, for all observed non-melanoma cancers, the median age of onset was similar to that of sporadic cases, so our data should be interpreted with caution.

When investigating the *MC1R* variant distribution, we observed that unaffected, *CDK4* positive family members had a disproportionally low frequency of RHC variants, suggesting a biological influence. This difference may, however, be related to the smaller number of subjects in the unaffected, *CDK4* positive group. Additionally, these subjects were generally younger (median age 28 years at last examination) than their affected relatives. It is therefore likely that some of the unaffected, *CDK4* positive subjects eventually develop melanoma.

Looking at melanoma cases only, we found that the MPM subjects had a higher frequency of RHC type variants than the SPM subjects (table 4). Moreover, although not reaching statistical significance, MPM subjects also had the highest frequency of any *MC1R* variant (86.7% compared with 63.3% in SPM). These findings are consistent with previous observations in *CDKN2A* melanoma families.[11 38 39] We did not find any modifying effects of *MC1R* variants upon age at disease onset in the *CDK4* families, in contrast to what has been reported for *CDKN2A*.[31–35]

The current study has some limitations. Collection of data and biological material was performed by various groups in several different countries, and the data diverged in completeness. Small sample size due to lack of complete data contributes to low power in some statistical analyses and prohibited us from evaluating each *MC1R* variant separately. Despite these limitations, our study provides results informative for the clinical evaluation of *CDK4* pedigrees.

Melanoma families with *CDK4* germline mutations are very uncommon. However, codon 24 of this gene is likely to be a mutational hotspot and *CDK4* families have been found in various countries, with several independent origins suggested by haplotype analysis.[5 13 15] Our study suggests that *CDK4* melanoma families are phenotypically similar to the *CDKN2A* families with regard to age of melanoma diagnosis, tumour localisation, histological type, and increased incidence of MPM and clinically atypical nevi. A general influence of *MC1R* variants on melanoma risk is seen in both types of melanoma families, although there may be some differences. We therefore conclude that it is not possible to distinguish *CDK4* melanoma families from those with *CDKN2A* mutation based on the phenotype. The clinical implication is that, although *CDK4* mutation carriers are rarely seen, exon 2 of this gene should be examined in melanoma families seeking gene testing whenever tests are negative for *CDKN2A*.

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Contributors HEP performed genotyping, collected all data from the participating research groups, tabulated the results, carried out the statistical analyses, and wrote the first manuscript draft. XRY and AMG provided data on the American families, and participated in the interpretation of data and statistical analyses. SJS, HHV, and IMB carried out genotyping, performed genetic counselling or dermatological examinations, and provided and interpreted clinical data for the Norwegian family. MAT, MH, JAN-B, AO, DP, RV, NKH, JMP, PGr, CC, PGh, LP, MFA, BBdeP, MB, H-HH, NS, TJ, ABD-M, JT, SD, TM-D, LT, AJS, and HT carried out genotyping, performed genetic counselling and dermatological examinations, and provided and interpreted clinical data for all other families included in the study. AM managed and designed the study in collaboration with AMG and LAA. The writing group consisted of HEP, XRY, AMG, LAA, and AM. All other authors have read and commented on the manuscript, and approved the final version.

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3.3. Original paper III. Melanoma risk associated with *MC1R* gene variants in Latvia and the functional analysis of rare variants

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Melanoma risk associated with *MC1R* gene variants in Latvia and the functional analysis of rare variants

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To evaluate the association of melanocortin 1 receptor gene (*MC1R*) variants with melanoma risk in a Latvian population, the *MC1R* gene was sequenced in 200 melanoma patients and 200 control persons. A functional study of previously uncharacterized, rare *MC1R* variants was also performed. In total, 26 different *MC1R* variants, including two novel variants Val165Ile and Val188Ile, were detected. The highest risk of melanoma was associated with the Arg151Cys variant (odds ratio (OR) 4.47, 95% confidence interval (CI) 2.19–9.14, $P < 0.001$). A gene dosage effect was observed, with melanoma risk for carriers of two variants being twice (OR 3.98, 95% CI 2.15–7.38, $P < 0.001$) that of carriers of one variant (OR 1.98, 95% CI 1.26–3.11, $P = 0.003$). After stratification according to the pigmentation phenotype, the risk of melanoma remained in groups with otherwise protective phenotypes. Functional analyses of eight previously uncharacterized *MC1R* variants revealed that a subset of them is functionally relevant. Our results support the contribution of *MC1R* variants to a genetic predisposition to melanoma in Latvia.

Keywords Melanocortin 1 receptor gene, melanoma, rare *MC1R* variants, pigmentation, functionality of *MC1R* variants

Cutaneous melanoma has shown a steady increase in incidence in the white population in the preceding decades, and this trend is likely to continue (1). Melanoma arises through the stepwise transformation of melanocytes within the basal epidermal layer of the skin and involves numerous molecular, cellular, and morphological events (2). Environmental sun exposure and individual's pigmentation phenotype factors have been strongly associated with the risk of melanoma (3). Approximately 10% of melanoma cases occur in a familial setting (4). To date, two genes, cyclin-dependent kinase inhibitor 2A (*CDKN2A*, 9p21.3, OMIM 600160) (5) and cyclin-dependent kinase 4 (*CDK4*, 12q14.1, OMIM 123829) (6), have been associated with high penetrance melanoma susceptibility. However, mutations in these genes account for susceptibility in only 20–57% of melanoma families (7) and have a very low frequency in melanoma patients at a population-based level (8). A genome-wide association study approach highlights several low penetrance melanoma-susceptibility candidate genes, including the melanocortin 1 receptor gene (*MC1R*, 16q24.3, OMIM 155555) (9), which is one of the major human skin pigmentation regulators (10). *MC1R* encodes a seven transmembrane domain, G-protein–coupled receptor of 317 amino acids. The binding of the natural agonist α-melanocyte-stimulating hormone (α-MSH) to a functional MC1R protein activates the intracellular signal cascade that leads to the production of the photoprotective brown or black eumelanin (11).

The *MC1R* gene is highly polymorphic with more than 100 nonsynonymous variants identified so far (12). Four variants—Asp84Glu, Arg151Cys, Arg160Trp, and Asp294His—present strong associations with the so-called red hair color (RHC) phenotype and are designated as RHC or R variants (13). The Arg142His and Ile155Thr variants are also sometimes labeled as RHC variants due to their strong

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association with the RHC phenotype in a familial setting (13,14). Other variants, particularly Val60Leu, Val92Met, and Arg163Gln, present weaker associations with the RHC phenotype and therefore are labeled as NRHC or r variants (13). Regarding *MC1R* variants and melanoma risk, a recently performed meta-analysis summarizes results from different populations, confirming a strong link between *MC1R* variants (especially variants associated with the RHC phenotype) and risk of melanoma (15). Furthermore, *MC1R* variants have been associated with an increased melanoma risk, beyond already known phenotypic risk markers of melanoma, with a higher risk associated with darker hair and skin (16).

Most common *MC1R* variants have also been investigated in relation to their effects on receptor functional activity. Results from these studies demonstrated that MC1R with RHC variants have, to different extents, reduced receptor functional activity in a cyclic adenosine monophosphate (cAMP) assay (17–20) and/or reduced cell surface expression (17,21). However, little is known about the functional activity of other less common MC1R variants.

This is the first study that investigates the prevalence and type of *MC1R* variants among melanoma patients and control persons in a Latvian population and, to our knowledge, in the Eastern European region. In addition, we performed functional analyses of previously uncharacterized rare *MC1R* variants that were detected in our study population.

Materials and methods

Study population

From 2007 to 2011, 200 melanoma patients with histopathologically confirmed cutaneous melanoma were recruited at the Riga Eastern Clinical University Hospital Latvian Oncological Center. The control group consisted of 200 unrelated healthy volunteers without history of melanoma recruited through the general practice of family doctors. All participants enrolled in the study completed a questionnaire about their demographic characteristics and history of personal and familial cancers. Information on skin type according to the Fitzpatrick classification scale (type I: always burns, never tans; type II: burns easily, tans minimally; type III: sometimes burns, slowly tans; type IV: burns minimally, always tans), natural hair color, eye color, degree of freckling, and number of moles were also recorded. Each participant received an explanation of the aims of the study, agreed to participate, and signed an informed consent form approved by the Central Medical Ethical Committee of Latvia.

MC1R sequencing

Genomic DNA was extracted from peripheral blood lymphocytes by a standard phenol–chloroform extraction method. The entire *MC1R* coding sequence was amplified using primers: F 5′-GCA GCA CCA TGA ACT AAG CA-3′, R 5′-CAG GGT CAC ACA GGA ACC A-3′ (Metabion International AG, Martinsried, Germany). Polymerase chain reactions (PCRs) were performed in a 25 μL reaction volume containing 25 ng of template DNA, 1% Taq buffer, 10% dimethyl sulfoxide (DMSO), 1.5 mM magnesium chloride, 0.24 mM dNTPs, 4 μM of each primer and 1.25 U Taq DNA Polymerase (Thermo Scientific Molecular Biology, Waltham, MA). Cycling conditions were as follows: an initial denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 50 seconds, annealing at 61°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. Amplicons were subjected to direct sequencing with four overlapping primers: S1F 5′-AAC CTG CAC TCA CCC ATG TA-3′, S1R 5′-CTG CAG GTG CTC ACG TCA AT-3′, S2F 5′-TCG TCT TCA GCA CGC TCT TC-3′, and S2R 5′-TTT AAG GCC AAA GCC CTG GT-3′. The sequencing reaction was performed using the ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems, Carlsbad, CA) and analyzed on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems). Sequencing analysis was performed and confirmed manually using the Vector NTI (Life Technologies, Carlsbad, Ca).

Construction of *MC1R* variant clones

Variants of the *MC1R* gene with a frequency of less than 1% were analyzed for their effects on receptor functional activity. Variant Asp84Glu was excluded from these analyses as it is one of the RHC variants previously shown to impair receptor function (17,19). Similarly, the analyses excluded variant Tyr152X, which led to a predicted truncated inactivated protein, and variant Asp184His, due to its close position to the variant Val188Ile (assuming that their effects might be similar). For the remaining eight variants—Phe45Leu, Ser83Leu, Gly89Arg, Thr95Met, Asp121Glu, Val165Ile, Val188Ile, and Arg213Trp—expression constructs were generated. In addition, Arg151Cys was included in the study as a control variant since it has previously been shown to have reduced cell surface expression and functional activity in the cAMP assay (17,20,21). All expression constructs were prepared based on the vector pcDNA3.1+, which contains a human consensus *MC1R* sequence (Missouri S&T cDNA Resource Center, Rolla, MO). Polymorphic *MC1R* constructs were obtained by site-directed mutagenesis with overlap extension (22) using iProof High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA) and primers containing polymorphic mismatch or restriction sites (Supplementary Table 1). For the cAMP assay, amplified PCR fragments were subcloned into the pcDNA5/FRT (Life Technologies) between the NheI and XhoI sites. For the microscopy studies, *MC1R* variants without the stop codon were subcloned into the EcoRI and HindIII sites of the pGFP^2-N3 vector (PerkinElmer, Waltham, MA). For the microscopy, an expression construct for the melanocortin 2 receptor (MC2R) was made as an additional negative control that is not naturally transported to the baby hamster kidney (BHK) cell membrane due to the absence of the MC2R accessory protein MRAP (23), as previously described (24). Sequences of all obtained constructs were verified by plasmid sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Kit and ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Cell culture and transfection

BHK cells obtained from the American Type Culture Collection (Manassas, VA) were grown at 37°C with 5% carbon

dioxide and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) fetal calf serum (FCS) and a penicillin–streptomycin mix (Sigma-Aldrich). When the cells reached 70–90% confluence, DNA constructs were transfected into the cells using TurboFect Transfection Reagent (Thermo Scientific Molecular Biology) according to the manufacturer's instructions. The cells were harvested and assayed 24 hours or 48 hours after transfection.

Confocal laser scanning microscopy

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes. Cell membrane and nuclei were stained with Alexa Fluor633 labeled wheat germ agglutinin (WGA) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Life Technologies), respectively. Cells were examined using a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany), as previously described by Fridmanis et al. (24). Briefly, three images were obtained from each independent transfection reaction, and for each construct two transfection reactions were performed, therefore a total of six images were obtained for each construct. The efficiency of green fluorescent protein (GFP)–tagged receptor transport to the cell membrane was measured calculating the GFP/WGA fluorescence intensity ratio at multiple points on the cell membrane. Using the tool implemented in the Leica Confocal Software (Las AF version 2.6.0), 10 arbitrarily selected linear regions of interest (ROI) were drawn across each image, resulting in 20 points of intersection with the cell membrane and giving at least 120 points for analysis for each construct. To avoid ROI selection bias, ROIs were initially selected on the WGA image, and the respective GFP fluorescence intensity from the same ROI was obtained afterward.

cAMP assay

Transiently transfected BHK cells were distributed into a 384-well plate (approximately 1×10^4 cells/well) and stimulated with synthetic MC1R agonist NDP-MSH (PolyPeptide Group, Hillerød, Denmark) at different concentrations ranging from 10^{-12} to 10^{-6} M diluted in 1X phosphate-buffered saline, 1% bovine serum albumin, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 30 minutes at 37°C. The intracellular cAMP level was measured with a LANCE cAMP kit (PerkinElmer) following the manufacturer's instructions and using a Victor3V multilabel reader (PerkinElmer). All experiments were performed in duplicate and repeated three times. Data was analyzed using the GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA).

Statistical analysis

Initially, we evaluated the associations between melanoma and known melanoma risk factors such as pigmentation characteristics using the Fisher exact test. The odds ratios (ORs) and the 95% confidence intervals (CIs) were calculated as well. The difference in mean age between groups was evaluated with an unpaired *t* test. For each *MC1R* variant, deviation from Hardy–Weinberg equilibrium was tested in controls as implemented in PLINK version 1.07 (Shaun Purcell, http://pngu.mgh.harvard.edu/purcell/plink/) (25). In our statistical analyses, all carriers of synonymous variants were pooled with persons with the *MC1R* consensus sequence; this pool was then considered as the wild type group. The association between melanoma risk and each *MC1R* nonsynonymous variant was analyzed separately as well as by combining different variants using the Fisher exact test along with ORs and 95% CIs. In the combination

Table 1 Risk of melanoma associated with phenotypic characteristics in a Latvian population

	Melanoma patients N = 200	Control persons N = 200			
Characteristic	No. (%)	No. (%)	OR	95% CI	*P* value
Hair color					
Black/Brown	85 (42.5)	126 (63.0)	1	-	-
Red/Fair	115 (57.5)	74 (37.0)	2.30	1.54–3.44	<0.001
Skin type					
III/IV	109 (54.5)	167 (83.5)	1	-	-
I/II	91 (45.5)	33 (16.5)	4.23	2.65–6.73	<0.001
Eye color					
Brown/other	52 (26.0)	62 (31.0)	1	-	-
Blue/gray/green	148 (74.0)	138 (69.0)	1.28	0.83–1.98	0.319
Freckles[a]					
None/few	147 (73.5)	167 (83.5)	1	-	-
Some/many	50 (25.0)	33 (16.5)	1.72	1.05–2.82	0.036
NA	3 (1.5)	0 (0)			
Moles[b]					
None/few	77 (38.5)	109 (54.5)	1	-	-
Some/many	117 (58.5)	91 (45.5)	1.82	1.22–2.72	0.004
NA	6 (3.0)	0 (0)			

Abbreviation: NA, not applicable.

[a] "None," no freckles at all; "Few," only a small number of freckles; "Some," a considerable number of freckles; "Many," a very high number of freckles on the face at the end of the summer.

[b] "None," no moles at all; "Few," only a small number of moles; "Some," a considerable number of moles; "Many, " a very high number of moles all across the body regardless of the mole size.

approach, we focused on the following *MC1R* variables: carriers of any *MC1R* variant, carriers of one or multiple (≥2) *MC1R* variants, carriers of one or more RHC variant (and no NRHC variant), carriers of one or more NRHC variant (and no RHC variant), and carriers of one or more RHC and one or more NRHC variants compared with the wild type group. RHC variants included those variants that have been previously shown to cause a significant impairment of receptor function and are strongly associated with red hair and fair skin, that is, the Asp84Glu, Arg151Cys, and Arg160Trp variants (26) and the Ser83Leu, Gly89Arg, Asp121Glu, and Arg213Trp variants that caused an impairment of receptor function similar to that of Arg151Cys in this study. All remaining nonsynonymous variants were considered NRHC variants. In addition, we explored the association between pigmentation characteristics and the presence of *MC1R* variants as well as the association between melanoma and RHC and NRHC variants after stratification according to the pigmentation characteristics. All *P* values cited are two-sided, and a *P* value less than 0.05 is regarded as statistically significant. All statistical analyses were performed using GraphPad Prism software version 5.00 for Windows.

To predict the functional impact of the *MC1R* variants on the receptor function, in silico analysis was performed using the software tool PolyPhen-2 (27). Statistical analyses of the confocal microscopy data were performed as described by Fridmanis et al. (24). A nonparametric Kruskal–Wallis test was first applied for uniformity analysis of the GFP/WGA fluorescence ratios per each construct ($\alpha = 0.05$). If statistically significant differences between medians were reported, Dunn's multiple comparison test ($\alpha = 0.05$) was used to determine which of the data sets were different. Data sets that significantly differed from more than two other data sets were replaced with data acquired from independent repeated experiments. Further, data from different constructs were analyzed by comparing median values and interquartile ranges using the Kruskal–Wallis test ($\alpha = 0.01$) followed by Dunn's multiple comparison test. Modules of differences in rank sums acquired by Dunn's test were arranged in a matrix

Table 2 *MC1R* variants identified in melanoma patients and control persons and each variant's individual association with melanoma risk[a]

		Melanoma patients N = 200	Control persons N = 200			
Nucleotide Change	Amino acid change	No. (%)	No. (%)	OR	95% CI	*P* value
Consensus	None	41 (20.5)	75 (34.1)	-	-	-
Synonymous						
c.399C>T	Cys133Cys	1 (0.5)	0 (0.0)	-	-	-
c.453C>G	Arg151Arg	1 (0.5)	0 (0.0)	-	-	-
c.498G>A	Ala166Ala	1 (0.5)	0 (0.0)	-	-	-
c.699G>A	Gln233Gln	2 (1.0)	2 (1.0)	-	-	-
c.819C>T	Cys273Cys	0 (0.0)	1 (0.5)	-	-	-
c.942A>G	Thr314Thr	64 (32.0)	53 (26.5)	-	-	-
c.948C>T	Ser316Ser	7 (1.8)	7 (1.8)	-	-	-
Nonsynonymous						
Wt[b]	None	47 (23.5)	84 (42.0)	1	-	-
c.133T>C	Phe45Leu	0 (0.0)	1 (0.5)	-	-	-
c.178G>T	Val60Leu	23 (11.5)	12 (6.0)	3.43	1.56–7.50	0.002
c.248C>T	Ser83Leu	2 (1.0)	0 (0)	-	-	-
c.252C>A	Asp84Glu	1 (0.5)	0 (0)	-	-	-
c.265G>C	Gly89Arg	0 (0)	1 (0.5)	-	-	-
c.274G>A	Val92Met	51 (25.5)	34 (17.0)	2.68	1.53–4.70	<0.001
c.284C>T	Thr95Met	1 (0.5)	0 (0)	-	-	-
c.363C>G	Asp121Glu	1 (0.5)	1 (0.5)	1.79	0.11–29.26	1.000
c.425G>A	Arg142His	8 (4.0)	8 (4.0)	1.79	0.63–5.07	0.286
c.451C>T	Arg151Cys	35 (17.5)	14 (7.0)	4.47	2.19–9.14	<0.001
c.456C>A	Tyr152X	1 (0.5)	0 (0)	-	-	-
c.464T>C	Ile155Thr	13 (6.5)	14 (7.0)	1.66	0.72–3.83	0.278
c.478C>T	Arg160Trp	49 (24.5)	35 (17.5)	2.50	1.43–4.39	0.002
c.488G>A	Arg163Gln	16 (8.0)	15 (7.5)	1.91	0.87–4.20	0.151
c.493G>A	Val165Ile	0 (0)	1 (0.5)	-	-	-
c.550G>C	Asp184His	0 (0)	1 (0.5)	-	-	-
c.562G>A	Val188Ile	1 (0.5)	0 (0)	-	-	-
c.637C>T	Arg213Trp	0 (0)	1 (0.5)	-	-	-
Insertion						
c.496_497insG	-	0 (0.0)	1 (0.5)	-	-	-

Abbreviation: Wt, wild type.

[a] Heterozygotes and homozygotes for a particular variant are grouped together (there were six patients homozygous for Thr314Thr, four for Arg160Trp, and one for each Val60Leu, Val92Met, Arg151Cys, Arg163GGln, Cys133Cys, or Arg151Arg variant, as well as three control persons homozygous for Val92Met, three for Thr60Thr, two for Arg160Trp, and one for Arg151Cys).

[b] Wt includes persons with the consensus *MC1R* sequence and carriers of synonymous polymorphisms.

table. The matrix table was used then to cluster constructs by their differences in expression on the cell surface. Clustering was performed with the Euclidean distance method using the MultiExperiment Viewer software version 4.3 (TM4 Development, Boston, MA)(28).

Results

Clinical and pigmentation characteristics of the study population

Overall, 400 persons were included in the study: 200 melanoma patients and 200 control persons. Melanoma patients and the control population did not differ in terms of gender ($P = 0.150$). There were 136 females (68%) and 64 males (32%) in the patient group and 150 females (75%) and 50 males (25%) in the control group. However, a younger mean age was observed in the control group when compared to the melanoma group: 48.7 years (±16.5 y) and 52.6 years (±15.5 y), respectively ($P = 0.012$). The pigmentation characteristics of the study participants as well as the risk of melanoma associated with each pigmentation phenotype are presented in Table 1. In the studied cohort, the strongest melanoma risk factors were fair skin types (OR 4.23, 95% CI 2.65–6.73, $P < 0.001$) and red or fair hair color (OR 2.30, 95% CI 1.54–3.44, $P < 0.001$). Other pigmentation characteristics (e.g., increased numbers of freckles and moles) were lower predictors of melanoma risk. No association with melanoma risk was observed for light eye color (Table 1).

Frequencies of *MC1R* variants and their association with melanoma risk

Altogether, 26 different *MC1R* variants were detected: 18 corresponded to nonsynonymous amino acid substitutions, seven resulted in synonymous changes, and one was an insertion (Table 2). Two of these variants, that is, Val165Ile (c.493G>A) and Val188Ile (c.562G>A), were novel low frequency variants that have not been previously reported. No variant showed a significant departure from Hardy–Weinberg equilibrium.

The frequencies of *MC1R* variants detected in the studied population are listed in Table 2. When nonsynonymous *MC1R* variants were examined individually, four variants (Val60Leu, Val92Met, Arg151Cys, and Arg160Trp) were significantly more frequent in melanoma patients, with the strongest risk for melanoma associated with the Arg151Cys variant (OR 4.47, 95% CI 2.19–9.14, $P < 0.001$) (Table 2). No other variant was individually associated with melanoma risk.

The presence of any *MC1R* variant was associated with a significant increase in melanoma risk when compared with that of the wild type sequence (OR 2.36, 95% CI 1.53–3.63, $P < 0.001$). A gene dosage effect on melanoma risk in carriers of multiple variants was observed, with the OR for one variant equal to 1.98 (95% CI 1.26–3.11, $P = 0.003$) and the OR for two or more variants being twice as high (OR 3.98, 95% CI 2.15–7.38, $P < 0.001$) (Table 3). When both the number and type of variants are considered, a statistically significant increase in melanoma risk was associated with the presence of at least one RHC variant (OR 2.63, 95% CI 1.51–4.58, $P < 0.001$) and at least one NRHC variant (OR 1.90, 95% CI 1.16–3.10, $P = 0.013$). Moreover, the presence of both RHC and NRHC variants together increases melanoma risk approximately 1.4 and approximately 1.9 times more (OR 3.69, 95% CI 1.81–7.53, $P < 0.001$) when compared with the presence of at least one RHC or one NRHC variant, respectively (Table 3).

Association of *MC1R* variants with pigmentation characteristics

The associations among *MC1R* variants and pigmentation characteristics were examined using the control group. The presence of *MC1R* variants was associated with skin type I–II (OR 3.94, 95% CI 1.55–10.05, $P = 0.003$), red or fair hair color (OR 2.52, 95% CI 1.37–4.67, $P = 0.003$), and the presence of freckles (OR 3.94, 95% CI 1.55–10.05, $P = 0.003$). Furthermore, *MC1R* variants were not associated with eye color ($P = 0.642$) or mole count ($P = 0.567$) (Supplementary Table 2).

Associations among *MC1R* variants and melanoma risk after stratification by pigmentation characteristics

Table 4 shows the significant persistence of melanoma risk according to the type of *MC1R* variant after stratification for

Table 3 Associations among number and type of *MC1R* variants and melanoma risk

	Melanoma patients N = 200	Control persons N = 200			
Number and type of *MC1R* variant	No. (%)	No. (%)	OR	95% CI	*P* value
Wt[a]	47 (23.5)	84 (42.0)	1	-	-
Any	153 (76.5)	116 (58.0)	2.36	1.53–3.63	<0.001
1	104 (52.0)	94 (47.0)	1.98	1.26–3.11	0.003
≥2	49 (24.5)	22 (11.0)	3.98	2.15–7.38	<0.001
≥1 RHC[b]	53 (26.5)	36 (18.0)	2.63	1.51–4.58	<0.001
≥1 NRHC[c]	69 (34.5)	65 (32.5)	1.90	1.16–3.10	0.013
≥1 RHC and ≥1 NRHC	31 (15.5)	15 (7.5)	3.69	1.81–7.53	<0.001

[a] Wt includes persons with the consensus *MC1R* sequence and carriers of synonymous polymorphisms.
[b] RHC variants (Asp84Glu, Arg151Cys, Arg160Trp plus Ser83Leu, Gly89Arg, Asp121Glu, and Arg213Trp from the present study, which showed receptor functional impairment similar to that of Arg151Cys).
[c] NRHC variants (all other nonsynonymous variants).

Table 4 Role of *MC1R* variants on melanoma risk after stratification for pigmentation characteristics

Pigmentation characteristics	MC1R variant	Melanoma patients N=200 No. (%)	Control persons N=200 No. (%)	OR	95% CI	P value
Hair color						
Red/fair	Wt[a]	24 (12.0)	21 (10.5)	1	-	-
	Any	91 (45.5)	53 (26.5)	1.50	0.76–2.96	0.294
	≥1 RHC[b]	38 (19.0)	17 (8.5)	1.96	0.86–4.44	0.147
	≥1 NRHC[c]	34 (17.0)	28 (14.0)	1.06	0.49–2.30	1.000
	≥1RHC and 1 NRHC	19 (9.5)	8 (4.0)	2.08	0.75–5.72	0.215
Black/brown	Wt	23 (11.5)	63 (31.5)	1	-	-
	Any	62 (31.0)	63 (31.5)	2.70	1.49–4.88	0.001
	≥1 RHC	15 (7.5)	19 (9.5)	2.16	0.94–4.95	0.082
	≥1 NRHC	35 (17.5)	37 (18.5)	2.59	1.33–5.04	0.005
	≥1RHC and 1 NRHC	12 (6.0)	7 (3.5)	4.70	1.65–13.39	0.006
Skin type						
I/II	Wt	21 (10.5)	6 (3.0)	1	-	-
	Any	70 (35.0)	27 (13.5)	0.74	0.27–2.04	0.631
	≥1 RHC	25 (12.5)	11 (5.5)	0.65	0.21–2.06	0.571
	≥1 NRHC	27 (13.5)	13 (6.5)	0.59	0.19–1.83	0.418
	≥1RHC and 1 NRHC	18 (9.0)	3 (1.5)	1.71	0.37–7.86	0.712
III/IV	Wt	26 (13.0)	78 (39.0)	1	-	-
	Any	83 (41.5)	89 (44.5)	2.80	1.64–4.78	<0.001
	≥1 RHC	28 (14.0)	25 (12.5)	3.36	1.67–6.76	<0.001
	≥1 NRHC	42 (21.0)	52 (26.0)	2.42	1.33–4.43	0.004
	≥1RHC and 1 NRHC	13 (6.5)	12 (6.0)	3.25	1.32–8.01	0.014
Eye color						
Blue/gray/green	Wt	35 (17.5)	56 (28.0)	1	-	-
	Any	113 (56.5)	82 (41.0)	2.21	1.33–3.67	0.002
	≥1 RHC	40 (20.0)	27 (13.5)	2.37	1.24–4.52	0.010
	≥1 NRHC	51 (25.5)	47 (23.5)	1.74	0.97–3.10	0.079
	≥1RHC and 1 NRHC	22 (11.0)	8 (4.0)	4.40	1.77–10.96	0.001
Brown/other	Wt	12 (6.0)	28 (14.0)	1	-	-
	Any	40 (20.0)	34 (17.0)	2.75	1.21–6.21	0.018
	≥1 RHC	13 (6.5)	9 (4.5)	3.37	1.14–9.99	0.033
	≥1 NRHC	18 (9.0)	18 (9.0)	2.33	0.91–5.98	0.101
	≥1RHC and 1 NRHC	9 (4.5)	7 (3.5)	3.00	0.91–9.93	0.125
Freckles[d]						
Some/many	Wt	8 (4.0)	6 (3.0)	1	-	-
	Any	42 (21.0)	27 (13.5)	1.17	0.36–3.74	1.000
	≥1 RHC	14 (7.0)	10 (5.0)	1.05	0.28–3.99	1.000
	≥1 NRHC	14 (7.0)	13 (6.5)	0.81	0.22–2.97	1.000
	≥1RHC and 1 NRHC	14 (7.0)	4 (2.0)	2.63	0.57–12.18	0.267
None/few	Wt	37 (18.5)	78 (39.0)	1	-	-
	Any	110 (55.0)	89 (44.5)	2.61	1.61–4.22	<0.001
	≥1 RHC	39 (19.5)	26 (13.0)	3.16	1.68–5.95	<0.001
	≥1 NRHC	54 (27.0)	52 (26.0)	2.19	1.27–3.78	0.006
	≥1RHC and 1 NRHC	17 (8.5)	11 (5.5)	3.26	1.39–7.65	0.008
	NA	3 (1.5)	0 (0.0)		-	-
Moles[e]						
Some/many	Wt	28 (14.0)	36 (18.0)	1	-	-
	Any	89 (44.5)	55 (27.5)	2.08	1.15–3.78	0.023
	≥1 RHC	29 (14.5)	18 (9.0)	2.07	0.96–4.47	0.084
	≥1 NRHC	42 (21.0)	32 (16.0)	1.69	0.86–3.31	0.172
	≥1RHC and 1 NRHC	18 (9.0)	5 (2.5)	4.63	1.53–14.01	0.007
None/few	Wt	18 (9.0)	48 (24.0)	1	-	-
	Any	59 (29.5)	61 (30.5)	2.58	1.35–4.94	0.005
	≥1 RHC	22 (11.0)	18 (9.0)	3.26	1.43–7.44	0.007
	≥1 NRHC	26 (13.0)	33 (16.5)	2.10	1.00–4.43	0.061
	≥1RHC and 1 NRHC	11 (5.5)	10 (5.0)	2.93	1.07–8.08	0.061
	NA	6 (3.0)	0 (0.0)	-	-	-

[a] Wt includes persons with consensus *MC1R* sequence and carriers of synonymous polymorphisms.

[b] RHC variants (Asp84Glu, Arg151Cys, and Arg160Trp, plus Ser83Leu, Gly89Arg, Asp121Glu, Arg213Trp from the present study, which showed receptor functional impairment similar to that of Arg151Cys).

[c] NRHC variants (all other nonsynonymous variants).

[d] "None," no freckles at all; "Few," only a small number of freckles; "Some," a considerable number of freckles; "Many," a very high number of freckles on the face at the end of the summer.

[e] "None," no moles at all; "Few," only a small number of moles; "Some," a considerable number of moles; "Many, " a very high number of moles all across the body regardless of the mole size.

the pigmentation characteristics. In the studied population, melanoma risk associated with *MC1R* variants appeared to increase significantly in the presence of protective phenotypes such as black or brown hair, skin type III or IV, and decreased amount of freckling. Regarding eye color, the melanoma risk associated with *MC1R* variants was similar in persons with light and dark eye color. A melanoma risk similar to that associated with *MC1R* variants was found in persons with a low or high number of moles (Table 4). The same result was observed when melanoma risk was evaluated according to number of *MC1R* variants (data not shown).

Associations among *MC1R* variants, age of melanoma onset, and tumor characteristics

A statistically significant association between the age of melanoma onset and *MC1R* genotype was found. The median age at diagnosis was lower for noncarriers of *MC1R* variants than for carriers: 49 years and 55 years, respectively ($P = 0.033$). With respect to the anatomic distribution of the tumor, we observed the highest *MC1R* variant frequencies in patients with melanomas on the trunk (36.0%), followed by extremities (29.5%), and head or neck (10%). However, no statistically significant difference was found among these groups ($P = 0.858$). Similarly, no statistically significant difference was found when both the number and type of variants were taken into account (data not shown). We did not identify any relationship between the presence of *MC1R* variants and the Breslow thickness of the tumor. The distribution of variants was similar in tumors below and above the median tumor thickness of 3.0 mm ($P = 0.701$). Similarly, no association was found between tumor ulceration and *MC1R* variants ($P = 0.694$).

Functional studies of rare *MC1R* variants

In our study, 11 *MC1R* variants had a frequency of less than 1% (Table 2). Of these, Asp84Glu has been previously shown to impair receptor functions (17,19), and Tyr152X led to a predicted truncated inactivated protein. Five variants (Phe45Leu, Ser83Leu, Gly89Arg, Thr95Met, and Asp121Glu) were predicted in silico to be possibly damaging for receptor functions, and four others (Val165Ile, Asp184His, Val188Ile, and Arg213Trp) were predicted to be benign. The functional properties of cloned *MC1R*-variant alleles were addressed using transiently transfected BHK cells. First, confocal scanning microscopy of GFP-tagged wild type and variant MC1R proteins (Figure 1) revealed that cell surface expression was strong for wild type MC1R and Phe45Leu, Thr95Met, Val165Ile, and Val188Ile allelic variants, whereas the Asp121Glu and Arg213Trp allelic variants showed markedly reduced and Ser83Leu and Gly89Arg allelic variants showed almost no detectable cell surface expression. The MC1R Arg151Cys variant and MC2R wild type showed significantly reduced or almost no detectable cell surface expression. Quantification of GFP levels in the plasma membrane of the cells confirmed the microscopy results (Figure 2). Next, variant MC1R functions were assessed by the ability of the cloned variants to elevate intracellular cAMP level in response to the agonist NDP-MSH. Only one variant (Gly89Arg) was a complete loss-of-function (LOF) receptor (Figure 3). All other variants showed an increase in cAMP in response to agonist administration, however, each to a different extent. The Thr95Met, Val165Ile, and Val188Ile variants showed essentially similar levels of maximal response as that of the wild type (Figure 3), and similar half maximal effective concentration (EC_{50}) values, except for the Val165Ile variant, for which a slightly increased EC_{50} value was observed (Supplementary Table 3). The Ser83Leu, Asp121Glu, and Arg213Trp variants did show reduced levels of maximal response and slightly increased EC_{50} values in comparison with those of the wild type (Figure 3, Supplementary Table 3). Conversely, the Phe45Leu variant showed a markedly increased level of cAMP response compared with that of the other variants (Figure 3).

Discussion

Numerous studies have shown that *MC1R* is highly polymorphic in the white population (12). In our study, the frequency of *MC1R* variants in the control population (58%) was intermediate compared with the frequencies reported in North European and Mediterranean populations, which were highest in Britain/Ireland and lowest in Greece (29). Similarly, the influence of *MC1R* variants on genetic predisposition to melanoma has been investigated in many association studies (10,13,16,30–35). Most of these studies demonstrated a strong association between *MC1R* variants and the risk of melanoma development, with an OR varying between 2 and 7, depending on the number and type of variants present (15,26,30,33–35). A similar gene dosage effect was observed in the present study, increasing the melanoma risk by a factor of 2 when one variant was present, and by 4 when at least two variants were present (Table 3). Evidence from functional studies indicates that compound heterozygotes and homozygotes of specific MC1R variants have a reduced cAMP response to the α-MSH hormone compared with that of simple heterozygotes (36). Therefore, we postulate that carriers of multiple variants may have a deeply impaired receptor function, but additional studies are needed.

Separate analyses of individual *MC1R* variants showed that four variants were significantly associated with an increased risk of melanoma, namely, Val60Leu, Val92Met, Arg151Cys, and Arg160Trp, with the strongest association being for Arg151Cys (OR 4.47; 95% CI 2.19–9.14, $P < 0.001$). Variants Arg151Cys and Arg160Trp have been strongly linked to melanoma risk in many relevant studies and were associated with the highest risk for melanoma in two recent meta-analyses (15,26). Furthermore, the association of Arg151Cys and Arg160Trp variants with melanoma and their functional impairments of the MC1R protein are well documented (17,20,21). The role of Val60Leu in melanoma development is controversial, as the magnitude of melanoma risk it confers varies among studies. Reports from Australia (10), the Netherlands (31), and Germany (34) have shown a weak or no association with melanoma risk. In contrast, studies of French (33) and Greek (35) populations demonstrated findings similar to those of our study, with Val60Leu serving as a strong predictor of melanoma risk. Similarly, the results for variant Val92Met have been conflicting among various populations and two recently performed

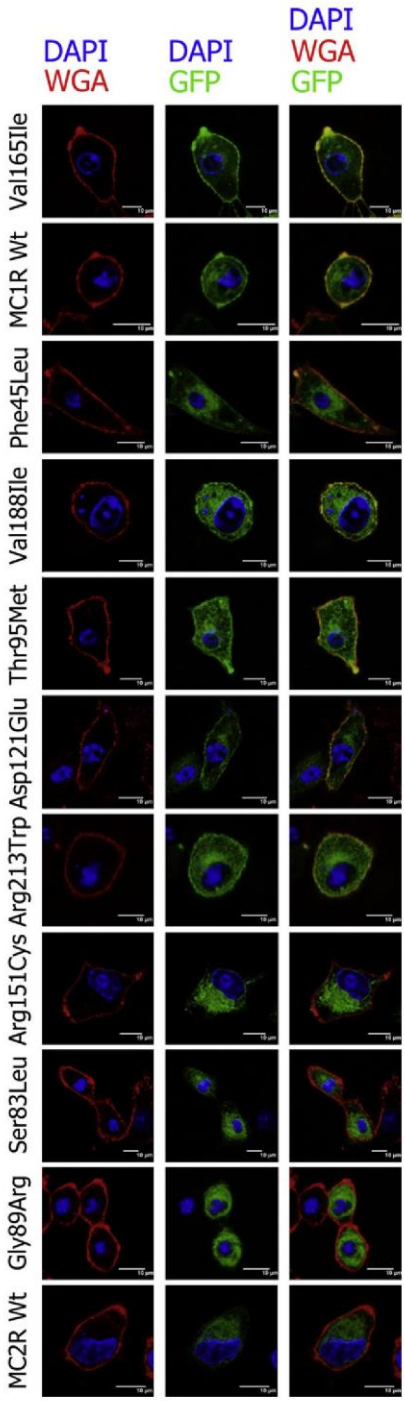

Figure 1 Confocal laser scanning microscopy images of GFP-tagged constructs with wild type MC1R and variant MC1R (Phe45Leu, Ser83Leu, Gly89Arg, Thr95Met, Asp121Glu, Arg151Cys, Val165Ile, Val188Ile, Arg213Trp) in BHK cells. Constructs are ranked from the highest (top) to the lowest (bottom) membrane expression. Wild type MC2R, which is known to be unexpressed on the BHK cell surface without an accessory protein, in this study was used as a negative control for receptor membrane export. The first column represents images of membrane staining with Alexa Fluor 633 conjugated WGA (red). The second column represents fluorescence images of the GFP tag (green). The third column represents a merge of images from cell membrane and GFP expression. Nuclei are always blue (DAPI).

meta-analyses (15,26). Two variants that were previously associated with an important melanoma risk (15,26), Asp84Glu and Asp294His, were detected in only one patient and not in controls or were not found in our population, respectively. Other variants that have been previously linked to melanoma risk, such as Arg142His and Ile155Thr, were not found to have a significant association in our cohort. This could be related to the small size of our affected group.

Several studies have noted that the association of *MC1R* variants with melanoma risk was stronger or limited to persons with protective cutaneous phenotypes, that is, persons with darker hair and darker skin (10,16,31,33). Here, we confirmed the persistent effect of *MC1R* variants on melanoma risk after stratification for the different pigmentation characteristics (Table 4), showing that *MC1R* variants and pigmentation are independent melanoma risk factors. Thus, our results suggest that the *MC1R* genotype provides information about melanoma risk beyond that of the cutaneous phenotype and that the combination of *MC1R* genotype and phenotype data might be important to the prediction of melanoma risk in persons with otherwise protective cutaneous phenotypes.

Previous work has shown that the presence of *MC1R* variants is associated with melanoma thickness, which is one of the main measures of tumor progression (32). However, similar to the study of the Greek population (35), our study found no such association, probably due to a higher median thickness of melanomas in our study. A lower percentage of thin tumors might prohibit us from finding a difference between *MC1R* variant carriers and noncarriers with respect to tumor thickness. Unlike other studies (32,35), our analysis found an association between *MC1R* variants and age of melanoma onset. Carriers of *MC1R* variants were older in our study than in other reports, and this difference cannot be readily explained. However, recently a protective effect of *MC1R* variants was observed on death from melanoma (37).

In the context of association studies, an important question is the classification of different *MC1R* variants, especially those whose frequencies are relatively low and for which it is difficult to evaluate their effect in association studies due to the lack of statistical power. Functional characterization of different *MC1R* variants could solve this problem. At present, however, the functional relevance is mainly determined for more frequently occurring RHC variants showing that these alleles encode for partial LOF receptors with diminished plasma membrane trafficking and ability to activate the cAMP pathway (17–20). However, the degree of residual signaling varies even between different RHC alleles, and it is not known whether other NRHC alleles maintain the same signaling capacity. This situation is further complicated by the fact that, at present, functional characterization is available for only a relatively small number of NRHC variants (12,38–41). In this study, we analyzed eight previously functionally uncharacterized rare *MC1R* variants that were found in a Latvian population. Two among them were novel (Val165Ile and Val188Ile). Four of these eight variants

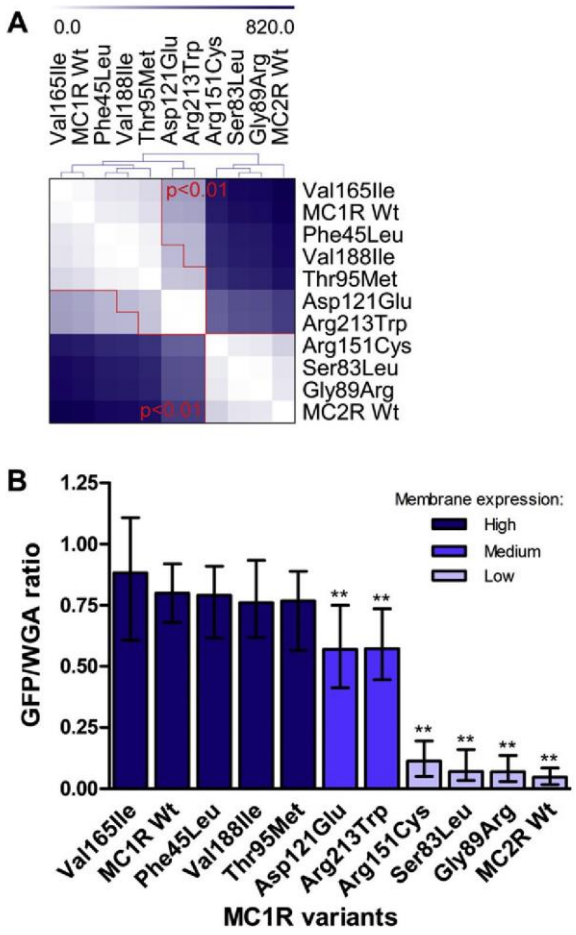

Figure 2 Quantitative results of membrane expression of MC1R variants. (A) A matrix table and MC1R-variant clustering tree for cell membrane export created using rank sums from Dunn's multiple comparison tests. The largest differences in rank sums are shown as dark blue boxes, white boxes correspond to the smallest differences, and color transitions from dark blue to white represent gradual decrease of differences. Red lines indicate borders between cells with significant difference in rank sums ($P < 0.01$) and cells without statistically significant differences in rank sums ($P > 0.01$). (B) A bar graph representing median values with interquartile ranges of GFP/WGA fluorescence intensity ratios at the cell membrane for all analyzed MC1R variants; $^{**}P < 0.01$ compared with wild type MC1R. The results are representative of at least two independent experiments.

(Phe45Leu, Ser83Leu, Gly89Arg, and Thr95Met) are located in the MC1R region that is involved in the formation of a ligand-binding pocket (42). One variant, Asp121Lys (we analyzed a variant in the same sequence position but with a different amino acid substitution, Asp121Glu), has been shown to be directly involved in ligand binding (43). In silico, all five variants participating in ligand binding were predicted to be damaging, whereas the others (Val165Ile, Val188Ile, and Arg213Trp), including both novel variants, were predicted to be benign. However, these predictions were not completely consistent with the results from in vitro analyses. Functional analyses of variants showed that Thr95Met and Val188Ile behaved as wild type variants in terms of both plasma membrane trafficking and signaling via the cAMP pathway. Variant Val165Ile had a cell surface density similar to that of the wild type and only slightly reduced signaling via the cAMP pathway. The other variants displayed varying degrees of LOF, indicating that LOF *MC1R* variants are more common than functionally silent variants, at least in the white population. The behavior of the Ser83Leu variant was reminiscent of that of the strong and frequent variant Arg151Cys, which has been shown to display decreased signaling via the cAMP pathway (20) and reduced cell surface export (21). The LOF of the variant Gly89Arg might also be caused by the reduction of cell surface expression with, accordingly, undetectable cAMP signaling. This phenotype is similar to other previously reported natural variants, such as Leu93Arg (41) and Arg162Pro (38,39). Similarly, the reduced cAMP signaling of variants Asp121Glu and Arg213Trp might be explained by the reduction in cell surface expression, as shown by confocal microscopy. Based on these observations, rare MC1R variants Ser83Leu, Gly89Arg, Asp121Glu, and Arg213Trp should be considered alleles whose residual signaling is comparable to that from RHC alleles. In contrast to all other MC1R variants, the variant Phe45Leu demonstrated a functional cAMP response higher than that of the wild type, with cell surface expression comparable with that of the wild type. The reason for such an increase in the cAMP level is not clear.

The exact mechanism underlying the influence of *MC1R* variants on the development of melanoma is not clear. However, a previous study has shown that MC1R variants, in comparison with the wild type receptor, might have an advantage in early melanoma development due to better proliferation rates and more effective binding of melanoma cells to the extracellular matrix (44). MC1R might also perform some immune-related function, because it is expressed on a variety of immune cells (45).

Our study has several limitations. First, the size of the association study population was small and it did not have the power to detect an association for rare *MC1R* variants. For the same reason, the results from the subgroup analyses should be considered with caution. Although the sample size was not large even for the primary analysis, some of the subgroups were small with limited ability to detect associations. Multiple testing for the association analysis were not performed as a result of the sample size, which might have led to the overestimation of some *P* values. Second, the control participants enrolled in the study on a volunteer basis, which may have caused a selection bias. Another limitation was the self-reported pigmentation characteristics that, due to individual subjectivity, might be inaccurate. Regarding the in vitro analyses of the MC1R variants, our model system consisted of cells without melanocytic origin. Previous reports have shown that the same MC1R variant reduced cAMP levels to distinctive levels in different cell lines, though results from the different cell lines were not conflicting and the overall tendency remained the same (21). Therefore, we believe that our results for the BHK cell system are comparable with data from similar studies. In addition, our results on widely examined Arg151Cys are overall consistent with results from previous research (20,21).

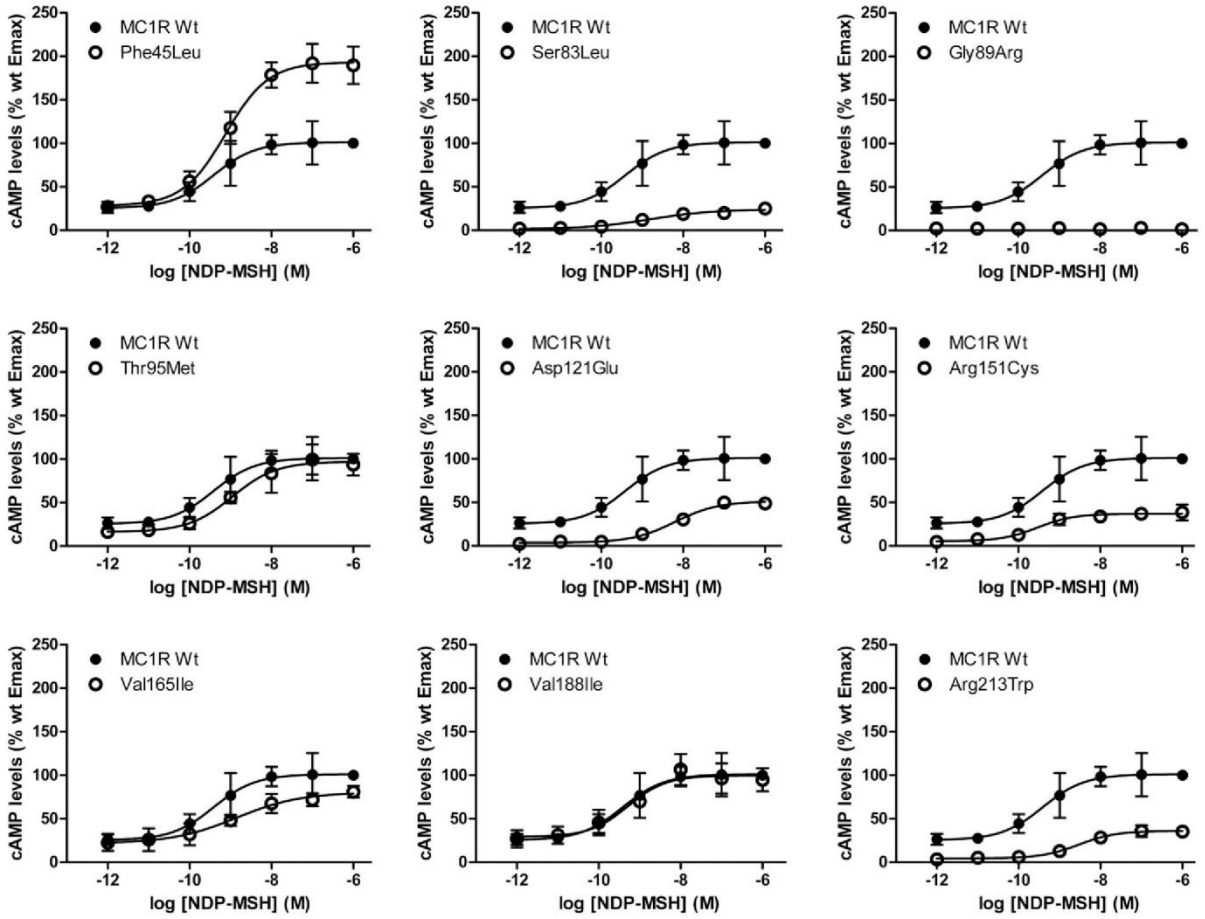

Figure 3 Functional coupling of variant MC1R to the cAMP production. Dose–response curves for induced cAMP production in BHK cells expressing the wild type or variant receptor in response to increasing concentrations of the agonist NDP-MSH. Results are the mean ± standard deviation (SD) of at least three independent experiments performed in duplicate.

In conclusion, for the first time we have determined the occurrence of *MC1R* variants in a Latvian population and evaluated their possible association with melanoma risk. Additionally, we have functionally investigated eight previously uncharacterized *MC1R* variants and demonstrated that a significant subset of these variants are functionally relevant. Our results increase the overall knowledge of the functional properties of MC1R and may help to classify *MC1R* variants more accurately in future studies.

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Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.cancergen.2013.01.002.

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3.4. Original paper IV. Association of the 16q24.3 region gene variants rs1805007 and rs4785763 with heightened risk of melanoma in Latvian population

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Association of the 16q24.3 region gene variants rs1805007 and rs4785763 with heightened risk of melanoma in Latvian population

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ABSTRACT

Chromosome region 16q24.3 has been shown to modify the risk for developing melanoma in genome-wide association studies (GWAS). This region includes at least three SNPs for which significant independent effects on melanoma risk have been demonstrated: rs258322 (*CDK10* intron), rs4785763 (pseudogene *AFG3L1P*), and rs8059973 (flanking 5′UTR of *DBNDD1*). Also variants within the *MC1R* gene, located in the same region, are known to be associated with an increased melanoma risk. However, the exact risk these variants convey has never been estimated in the population of Latvia. Also the haplotypes of the 16q24.3 region and their relationship with melanoma have not been studied in this population before. To elucidate the associations of the variants from the 16q24.3 region with melanoma alongside their mutual interactions, we performed direct sequencing of the *MC1R* gene and genotyped the rs258322, rs4785763, and rs8059973 SNPs. In total, the study subjects included 479 individuals, comprising 255 melanoma patients and 224 controls. Univariate analyses of genotypes showed that only rs1805007 variant from *MC1R* gene, and two chromosome 16 SNPs, rs258322 and rs4785763, were nominally associated with an increased risk of melanoma. Multivariate models built by stepwise regression revealed that the contributions of rs1805007 and rs4785763 to melanoma risk are independent. Haplotype analyses demonstrated that rs1805007 and rs4785763 are independently associated with melanoma, whereas the impact of rs258322 to melanoma risk is related to rs1805007.

1. Introduction

Cutaneous melanoma is a skin cancer that arises from an increased division of pigment-producing cells of neuro-ectodermal origin—melanocytes. The incidence of melanoma is continually increasing in Western countries (Whiteman et al., 2016) as well as in Latvia (Azarjana et al., 2013). According to the European Network of Cancer Registries, the age-adjusted incidence rate of cutaneous melanoma in Latvia is 7.6/100,000 per annum, which is lower than the estimated rate in Europe (11.1/100,000 per annum) (Ferlay et al., 2013). Significant progress in the therapy for advanced melanoma has been reached in recent years—several molecular inhibitors targeting *BRAF* and *MEK* signaling pathways as well as immune checkpoint inhibitors targeting *CTLA4* and *PD1* have been developed. However, an acquired resistance and a short response duration hinders the application of these approaches (Davey et al., 2016; Michielin and Hoeller, 2015), thus bringing prevention and ascertaining of genetic predisposition to the forefront (Corrie et al., 2014).

The most frequently encountered melanoma-associated gene is the cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) located on

Abbreviations: ACD, adrenocortical dysplasia protein homolog gene; *AFG3L1P*, AFG3-like matrix AAA peptidase subunit 1 of the pseudogene; *BAP1*, BRCA1 associated protein-1 gene; BRAF, serine/threonine-protein kinase B-Raf; *CDK4*, cyclin-dependent kinase 4; *CDK10*, cyclin-dependent kinase 10 gene; *CDKN2A*, cyclin-dependent kinase inhibitor 2A gene; CI, confidence interval; CTLA4, cytotoxic T-lymphocyte associated protein 4; *DBNDD1*, dysbindin domain containing 1 gene; GWAS, genome wide association study; LD, linkage disequilibrium; LGDB, Latvian Genome Data Base; MAF, minor allele frequency; *MC1R*, melanocortin 1 receptor gene; MEK, mitogen-activated protein kinase kinase; *MITF*, melanogenesis associated transcription factor gene; OR, odds ratio; p, *p*-value; PD1, programmed death receptor 1; *POT1*, protection of telomeres protein 1 gene; r^2, squared correlation coefficient; rs1110400, SNP p.Ile155Thr in *MC1R*; rs1805007, SNP p.Arg151Cys in *MC1R*; rs1805008, SNP p.Arg160Trp in *MC1R*; rs2228479, SNP p.Val92Met in *MC1R*; rs258322, SNP c.160 + 171A > G in *CDK10*; rs4785763, SNP n.1682A > C in *AFG3L1P*; rs8059973, SNP c.32-3696T > C in *DBNDD1*; *SLC45A2*, solute carrier family 45 member 2 gene; SNP, single nucleotide polymorphism; *TERF2IP*, telomeric repeat-binding factor 2-interacting protein 1 gene; *TERT*, telomerase reverse transcriptase gene

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chromosome 9p21.3. It accounts for 20%–40% of familial melanomas (Read et al., 2016; Goldstein et al., 2006). Another well-known gene that conveys a high melanoma risk is the cyclin-dependent kinase 4 gene (*CDK4*) located on chromosome 12q14.1 (Puntervoll et al., 2013). Several other high-risk genes for melanoma—BRCA1-associated protein-1 gene (*BAP1*), protection of telomeres protein 1 gene (*POT1*), adrenocortical dysplasia protein homolog gene (*ACD*), telomeric repeat-binding factor 2-interacting protein 1 gene (*TERF2IP*), and telomerase reverse transcriptase gene (*TERT*)—have also been identified recently; however, mutations in the latter genes are rare and are found in few families worldwide (Aoude et al., 2015).

At least three genes are considered moderate-risk genes for melanoma: melanogenesis-associated transcription factor gene (*MITF*) located on chromosome 3p13 (Bertolotto et al., 2011; Yokoyama et al., 2011), solute carrier family 45 member 2 gene (*SLC45A2*) located on chromosome 5p13.2 (Fernandez et al., 2008; Guedj et al., 2008), and melanocortin 1 receptor gene (*MC1R*) located on chromosome 16q24.3 (Williams et al., 2011; Raimondi et al., 2008). The latter is highly polymorphic (Pérez Oliva et al., 2009) and encompasses several variants that are associated with an increased melanoma risk and also with various pigmentation traits (Williams et al., 2011; Raimondi et al., 2008). The 16q24.3 region has also been shown to be implicated in modifying melanoma risk in genome-wide association studies (GWAS) (Bishop et al., 2009; Barrett et al., 2011). In these studies, three non-coding SNPs in this region were associated with melanoma—rs258322 (residing within an intron of the cyclin-dependent kinase 10 gene, *CDK10*), rs4785763 (located within the *AFG3*-like matrix AAA peptidase subunit 1 of the pseudogene *AFG3L1P*), and rs8059973 (within flanking 5′UTR of dysbindin domain containing 1 gene, *DBNDD1*) (Bishop et al., 2009; Barrett et al., 2011). However, none of the *MC1R* variants were present on the genotyping arrays used in the GWAS. Moreover, neither the effects nor the significance of the 16q24.3 region variants are known for the Baltic region.

This study aims at gaining an understanding of the associations between the variants residing in the 16q24.3 region and melanoma in the population of Latvia. To achieve this goal, we juxtaposed non-coding variants in the 16q24.3 region with the *MC1R* variants and evaluated the contribution of the *MC1R* variants to the risk conveyed by variants in the 16q24.3 region. Furthermore, we assessed the associations of the haplotypes within the 16q24.3 region with melanoma.

2. Materials and methods

2.1. Study population

We conducted this study using DNA samples and data on cofactors (age and sex) from the Latvian Genome Data Base (LGDB), a government-funded biobank (briefly described in Ciganoka et al. (2011)). All cases and controls included in this study are Latvian nationals and have European ancestry. In addition, the Latvian population has been shown to be genetically homogenous (Pliss et al., 2015). In total, 479 samples were selected, including 224 unrelated healthy volunteers and 255 melanoma patients with histopathologically confirmed cutaneous melanoma (ICD-10 diagnosis code C43).

Written, informed consent was acquired from all LGDB participants. The Central Medical Ethics Committee of Latvia approved the protocols for sample collection. These protocols were No. A-3/2006 and No. A-7/2007, which were part of the project “Creation of Genome Data Base of Latvian population,” and the protocol No. 01-29/2016-1-1 for this particular study.

2.2. Genotyping

The entire coding region of the *MC1R* gene was sequenced as described in Ozola et al. (2013) with at least two-fold coverage (at least one-fold of each strand). Chromosome 16 SNPs—rs258322, rs4785763, and rs8059973—were genotyped using TaqMan SNP Genotyping Assays (C__653812_1_, C__2875849_10 and C__29970391_10, respectively) on ViiA™ 7 Real-Time PCR Instrument (Thermo Scientific Molecular Biology, Waltham, MA) as per manufacturer's instructions. The genotyping included at least two random replicates and a no template control test in each assay.

2.3. Statistical analyses

The minor allele frequency (MAF) of each variant was estimated using all controls having the genotype information for this variant (224 controls for the *MC1R* gene, 203 for rs258322, 204 for rs8059973 and 205 for rs4785763). In the subsequent analyses, we considered only the variants with $\geq 4\%$ MAF and with at least one homozygote of two minor alleles in controls that did not significantly deviate from the Hardy-Weinberg equilibrium. Next, we carried out univariate analyses with and without cofactors (age and sex) by fitting a logistic regression model for each SNP and a melanoma case/control indicator. We assumed an additive model of the contribution of alleles to the disease. We measured the significance of a genotype-phenotype association by the Wald test applied to the genotype term with $\alpha = 0.05$. All logistic regression models were fitted by the function *glm* in R environment. In addition, we carried out a permutation test to obtain empirical *p*-values of genotype-phenotype associations (only individuals without any missing genotypes for all SNPs were considered here and the case/control status for all individuals was permuted 999 times; the minimum *p*-values across all SNPs in a single permuted data set were compared with the real association *p*-value for a SNP).

To gain an understanding of the potential interactions of variants on chromosome 16, we built multivariate models with and without cofactors (age and sex) by stepwise regression. Models were built by the function *stepAIC* from the R package *MASS* starting with a model without any SNPs. We used a generalized linear model throughout all multivariate analyses, together with the additive model of allele contributions.

We also assessed associations of various haplotypes with the disease status. Haplotypes were identified for combinations of SNPs that were either moderately significant within univariate models (empirical *p*-value ≤ 0.1) or were included in any of the multivariate models. Haplotype associations were detected using the R library *haplo.stats* (Lake et al., 2002; Schaid et al., 2002). Initially, genotypes were recoded by functions *geno1to2* and *setupGeno*, and then haplotype association tests were performed by *haplo.glm* (we used logit link for a binomial family of models, and we set the threshold for rare haplotypes to 0.01).

In addition, we estimated a linkage disequilibrium (LD) for each pair of associated variants and reported the squared correlation coefficient (r^2); the computation was based on the European population haplotype data from the 1000 Genomes Project and carried out by the *Ldmatrix* module from the web-based application suite LDlink 2.0 (Machiela and Chanock, 2015).

3. Results

3.1. Demographic characteristics of the study population

The demographic characteristics of the study participants are listed in Table 1. Melanoma patients and controls did not differ significantly in terms of sex (Chi-squared test $p = 0.149$); however, the control group was significantly younger than patients (Mann-Whitney test $p = 2.36 \times 10^{-8}$).

3.2. Genotyping results

Within the *MC1R* gene, we detected 26 different variants: 18 non-synonymous variants, seven synonymous, and one insertion. However, in our set of controls, only four non-synonymous *MC1R* variants (rs2228479, rs1805007, rs1110400, rs1805008) and two of the three

Table 1
Demographic characteristics of melanoma patients and controls included in the study.

		Controls $n = 224$		Patients $n = 255$		p-Value[a]
Mean age		47.2 (± 17.3)		56.2 (± 15.2)		2.36×10^{-8}
Sex	Females	168	(75%)	175	(69%)	0.149
	Males	56	(25%)	80	(31%)	

[a] Group differences were obtained using χ^2 test for age and Mann–Whitney test for sex.

genotyped SNPs on the chromosome 16 (rs258322, rs4785763) matched the inclusion criteria for further analysis (the majority of other variants did not reach ≥4% MAF, while rs8059973 demonstrated a significant deviation from Hardy-Weinberg equilibrium ($p = 0.001$) and a pronounced depletion of heterozygotes).

3.3. Univariate analyses of variant associations with melanoma

All subsequent data analyses were carried out using only individuals with complete genotype information for all polymorphisms included in the analysis according to the criteria described above. Only one variant, rs1805007 or Arg151Cys, c.451C > T, in the *MC1R* gene and two chromosome 16 SNPs, rs258322 (c.160 + 171A > G) and rs4785763 (n.1682A > C), were nominally associated with an increased risk of melanoma (Table 2). According to a permutation test, the associations for rs1805007 in the *MC1R* gene ($p = 0.039$) and for rs258322 ($p = 0.041$) were significant and the association was suggestive for rs4785763 ($p = 0.112$). The associations of rs1805007, rs258322, and rs4785763 remained significant after adjusting for age and sex. A permutation test with cofactors confirmed a significant association for rs1805007 ($p = 0.041$) and somewhat weaker associations for both rs258322 and rs4785763 ($p = 0.066$). The association between melanoma and rs4785763 alleles became more pronounced after the inclusion of cofactors (Table 2).

Table 3
Variants from 16q24 region that were selected by stepwise regression for inclusion in multivariate models*.

Gene	SNP	Model without cofactors		Model with cofactors (age and sex)	
		p-Value	OR (95% CI)	p-Value	OR (95% CI)
MC1R	rs1805007 Arg151Cys c.451C > T	**0.034**	1.99 (1.05–3.75)	**0.044**	1.96 (1.02–3.76)
AFG3L1P	rs4785763 n.1682A > C	0.129	1.26 (0.94–1.69)	0.081	1.32 (0.97–1.80)

p-values < 0.05 are highlighted in bold.
* p-Values and odds ratios reported here were estimated for individual terms of a model.

Table 2
Associations between variants residing in 16q24 region and melanoma risk – univariate analysis of all variants.

Gene	SNP[a,b]	MAF 1000 genomes (EUR) %	Genotypes in controls (n genotyped)	MAF controls %	Genotypes in patients (n genotyped)	MAF patients %	p-Value[c]	OR (95% CI)	Empirical p-value	After adjustment for age and sex		
										p-Value	OR (95% CI)	Empirical p-value
MC1R	rs2228479 Val92Met c.274G > A	6.9	3 AA 34 GA 187 GG (224)	8.9	2 AA 57 GA 196 GG (255)	12.0	0.166	1.36 (0.88–2.09)	0.627	0.149	1.39 (0.89–2.19)	0.568
MC1R	rs1805007 Arg151Cys c.451C > T	7.2	1 TT 16 CT 207 CC (224)	4.0	1 TT 39 CT 215 CC (255)	8.0	**0.008**	2.27 (1.24–4.18)	0.039	**0.009**	2.30 (1.23–4.30)	**0.041**
MC1R	rs1110400 Ile155Thr c.464T > C	0.8	1 CC 16 TC 207 TT (224)	4.0	0 CC 14 TC 241 TT (255)	2.7	0.324	0.70 (0.34–1.43)	0.895	0.322	0.69 (0.33–1.44)	0.883
MC1R	rs1805008 Arg160Trp c.478C > T	6.2	2 TT 45 CT 177 CC (224)	10.9	6 TT 54 CT 195 CC (255)	12.9	0.399	1.19 (0.80–1.77)	0.938	0.345	1.22 (0.81–1.85)	0.912
CDK10 intron	rs258322 c.160 + 171A > G	9.8	1 AA 25 GA 177 GG (203)	6.7	4 AA 52 GA 199 GG (255)	11.8	**0.011**	1.86 (1.16–2.98)	0.041	**0.013**	1.85 (1.14–3.01)	0.066
AFG3L1P	rs4785763 n.1682A > C	29.9	19 AA 103 CA 83 CC (205)	34.4	40 AA 132 CA 83 CC (255)	41.6	**0.023**	1.39 (1.05–1.85)	0.112	**0.013**	1.45 (1.08–1.95)	0.066

p-values < 0.05 are highlighted in bold.
[a] Non-synonymous *MC1R* SNPs and insertion with allele frequency < 4% in controls not included in the statistical analysis: rs61996344 (Phe45Leu, c.133T > C); rs1805005 (Val60Leu, c.178G > T); rs777024553 (Ser83Leu, c.248C > T); rs1805006 (Asp84Glu, c.252C > A); rs34540312 (Gly89Arg, c.265G > C); rs34158934 (Thr95Met, c.284C > T); rs200616835 (Asp121Glu, c.363C > G); rs11547464 (Arg142His, c.425G > A); rs201326893 (Tyr152Ter, c.456C > A); rs885479 (Arg163Gln, c.488G > A); rs762096175 (Val165Ile, c.493G > A); rs780875127 (c.495_496insG); rs530102853 (Asp184His, c.550G > C); rs774680166 (Val188Ile, c.562G > A); rs200000734 (Arg213Trp, c.637C > T).
[b] Synonymous *MC1R* SNPs not included in the statistical analysis: rs201429598 (Cys133=, c.399C > T); rs201827012 (Arg151=, c.453C > G); rs374959395 (Ala166=, c.498G > A); rs146544450 (Gln233=, c.699G > A); rs375813196 (Cys273=, c.819C > T); rs2228478 (Thr314=, c.942A > G); rs151318945 (Ser316=, c.948C > T).
[c] Analyses were carried out using only those individuals who had complete genotype information for all polymorphisms selected for the inclusion in the analysis (255 melanoma patients and 203 controls).

3.4. Multivariate analyses of variant associations with melanoma

We used multivariate models to elucidate whether the associations of rs258322 and rs4785763 are independent from the *MC1R* variants. In the stepwise regression without cofactors two variants were selected: rs1805007 and rs4785763 (Table 3). This model seems to imply that rs4785763 indeed has a moderate effect on the risk of melanoma, and that this effect is orthogonal to rs1805007. Next, we included in the model age and sex. The same two variants were selected: rs1805007 and rs4785763 (Table 3). Thus an independent effect for rs1805007 was confirmed and the independence of the moderate association signal of rs4785763 from rs1805007 was implied. We also estimated the associations between both age and sex and melanoma case/control status from this multivariate model. Age was significantly higher for melanoma patients ($p = 3.8 \times 10^{-8}$, OR = 28.45, 95% CI = 8.63–93.79), and sex also differed between cases and controls ($p = 0.055$, OR = 1.53, 95% CI = 0.99–2.37).

3.5. Haplotype analyses

We explored haplotypes formed by two variants in the 16q24.3 region, rs258322 and rs4785763, and the most strongly associated variant in our study, rs1805007 of the *MC1R* gene (Table 4). We observed a convincing association for the combination of the three alternative alleles A/T/A of rs258322/rs1805007/rs4785763 and a moderate association of G/C/A alleles of the same trio of variants. Both trios involve the alternative allele A of rs4785763, thus again confirming a small independent effect of rs4785763 with respect to rs258322 and rs1805007.

We also looked at the pairwise combinations of these variants and their corresponding haplotypes. As expected, the combination of alternative alleles A/T of rs258322 and rs1805007 was significant and notable associations were yielded by the combinations of T/A and A/A alleles of rs1805007/rs4785763 and rs258322/rs4785763 respectively. LD analyses supported the results of haplotype analyses. According to an LD estimate the effects of rs1805007 and rs4785763 are independent ($r^2 = 0.160$). Meanwhile, rs258322 and rs1805007 work in unison, which is shown both by haplotype associations and by the LD between these variants ($r^2 = 0.602$) (Fig. 1).

4. Discussion

Here, we performed an extended analysis of *MC1R* gene variants and three chromosome 16 variants previously implicated in conveying a risk for melanoma in a case-control cohort representing the population of Latvia. We aimed at assessing both independent and joint associations between these variants and melanoma. Among all univariate analyses, with or without cofactors, the *MC1R* variant rs1805007 exhibited the strongest association. The association of this variant with melanoma has been demonstrated in many populations across the world, with the overall OR = 1.80 (95% CI = 1.58–2.06) according to a meta-analysis of 27 datasets (Antonopoulou et al., 2015). Our results show a larger effect having OR = 2.30 (95% CI = 1.23–4.30); this finding is similar to the observations in Sweden (OR = 2.32, 95% CI = 1.77–3.05) (Gudbjartsson et al., 2008) and France (OR = 2.35, 95% CI = 1.78–3.11) (Guedj et al., 2008). Moreover, the variant rs1805007 retained its significance in multivariate models. In addition, our results suggest the *MC1R* variant rs1805007 can work together with another chromosome 16 variant, rs258322. Even though rs258322 had a small effect in the univariate analysis, it did not enter any multivariate model after the inclusion of rs1805007. This observation seems to confirm related influences on the phenotype. The LD and haplotype analyses also suggested a high correlation between the minor alleles of rs1805007 and rs258322 and melanoma. Demenais et al. (2009) have also explored whether *MC1R* variants might account for the association signals in the 16q24.3 region; they did so using the dataset from Bishop et al. (2009) in which the associations of chromosome 16 variants with melanoma were first discovered. Both their stepwise regression models and haplotype analyses showed that *MC1R* variants rs1805007 and rs1805008 (Arg160Trp, c.478C > T) accounted for the rs258322's association. For variants rs258322 and rs4785763, associations with hair color, skin pigmentation, and tanning ability have been demonstrated in several pigmentation GWASs; however, after the adjustment for *MC1R* variants rs1805007, rs1805008, and rs1805009 (Asp294His, c.880G > C), these associations mostly disappeared (Gerstenblith et al., 2010; Han et al., 2008; Nan et al., 2009).

Another variant nominally associated with an increased risk of melanoma in our study was rs4785763. We demonstrated that the association signal of rs4785763 is rather independent from the effect of rs1805007 on melanoma development in the population of Latvia. Variant rs4785763 was selected by stepwise regression, with and without cofactors, alongside the *MC1R* variant rs1805007. The respective haplotype analyses and the rather small LD between these two variants ($r^2 = 0.160$) confirmed this degree of independence. Note that the inclusion of age and sex in both univariate and multivariate regression models enhanced the power of detecting rs4785763 as significant, which implies some calibration of the impact of rs4785763 depending on age and sex. As mentioned above, this variant showed one of the strongest associations with red hair and freckles in a pigmentation GWAS, although after adjusting for *MC1R* variants rs1805007 and rs1805008, this association disappeared (Sulem et al., 2007). Similarly, Demenais et al. (2009) reported that the rs4785763's effect on melanoma risk can be explained by the associations of rs1805007 and rs1805008. However, larger meta-analyses indicate the rs4785763's association with melanoma risk (Antonopoulou et al., 2015; Gerstenblith et al., 2010) and recently a two-stage GWAS (Ransohoff et al., 2017) replicated the associations between rs478563 and melanoma. Variant rs4785763 is located ~80 kb away from *MC1R* and resides within the *AFG3*-like matrix AAA peptidase subunit 1 pseudogene that in turn is orthologous to the murine gene *Afg3l1*. In mice, this gene encodes a subunit of the ATP-dependent proteolytic complex localized in the inner membranes of mitochondria, whereas the human orthologue only produces noncoding mRNA (Kremmidiotis et al., 2001). However, recent evidence suggests that pseudogene products play a variety of roles in cancer biology (Poliseno et al., 2015) and that a genetic variation in these transcripts might have some influence on the activity of pseudogene products that needs further exploration. We cannot provide a single explanation for these discrepancies. One reason might be the specifics of the Latvian population. Genetic studies of the Latvian population have mostly focused on discovering the role of genetic variation in the context of various diseases; still not enough is

Table 4
Haplotypes in 16q24 region associated with an increased risk of melanoma.

SNPs	Haplotype	*p*-Value for association with melanoma	OR (95% CI) for association with melanoma
rs258322/rs1805007/rs4785763	A/T/A	**0.006**	2.60 (1.33–5.09)
rs258322/rs1805007/rs4785763	G/C/A	0.087	1.31 (0.96–1.79)
rs258322/rs1805007	A/T	**0.009**	2.38 (1.24–4.57)
rs1805007/rs4785763	T/A	**0.006**	2.40 (1.29–4.49)
rs258322/rs4785763	A/A	**0.016**	2.12 (1.16–3.89)

p-values < 0.05 are highlighted in bold.

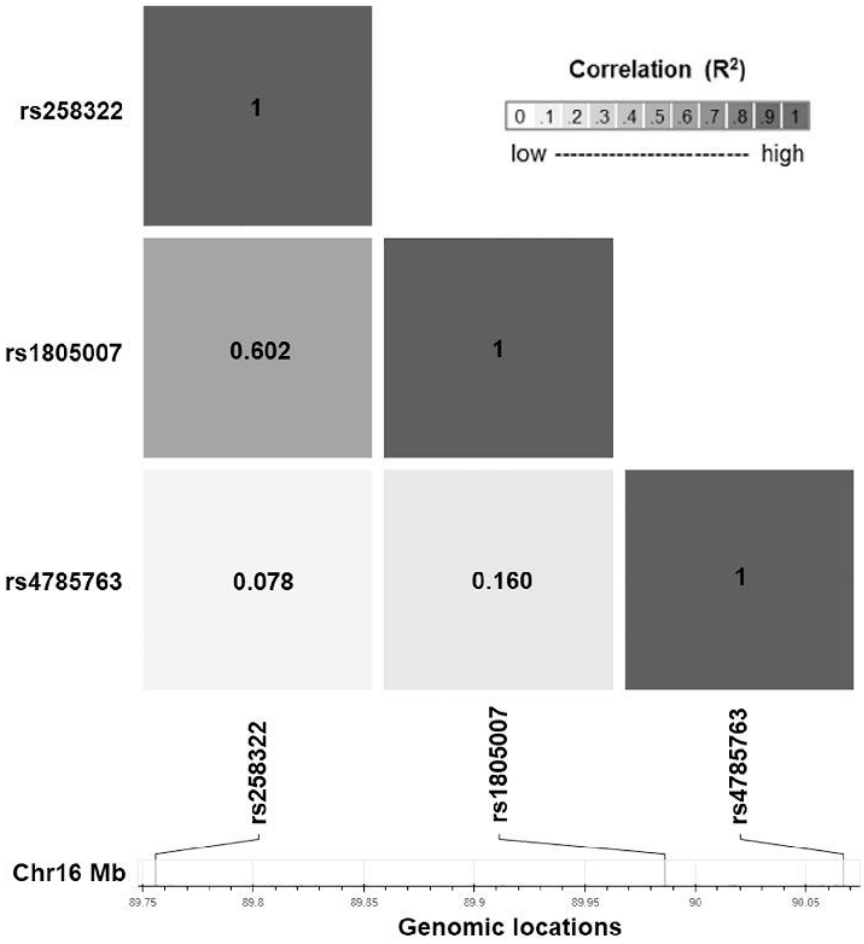

Fig. 1. A heatmap of pairwise LD r^2 values for SNPs rs258322, rs1805007 and rs4785763.

known about the population structure within Latvia. Previous studies of mitochondrial DNA and Y chromosome indicate the intrapopulation homogeneity and similarity to the neighboring populations—Lithuanians, Estonians, Norwegians, and Russians (Pliss et al., 2015; Pliss et al., 2006). Also a study of the genetic makeup of Europeans showed the genetic variation within the Latvian population correlates closely with geography and that Baltic States, Poland, and Western Russia cluster together (Nelis et al., 2009). Individuals from these populations were included neither in the melanoma nor pigmentation GWASs.

This study has several limitations. First, the study sample is rather small for comprehensive haplotype analyses, although it is sufficient for detecting associations of common genetic variants. The size of the cohort implies the overall power for detecting associations is moderate and the effects of some rarer combinations of alleles cannot be discerned with a high resolution. The controls were significantly younger than cases therefore some of the control individuals might develop melanoma in the future. The latter aspect can potentially diminish the power of the study. The strength of our study is the depth of statistical analyses. We have carried out a comprehensive and multifaceted search for potential interactions between genetic variants on chromosome 16 and predisposition to melanoma. Unlike *MC1R* variants, the other chromosome 16 variants we explored have not been previously extensively analyzed in different populations. However, our proposed hypotheses would require additional validation in a larger cohort of samples. To conclude, in the population of Latvia, the main variants from the 16q24.3 region that are associated with melanoma risk are rs1805007 and rs4785763 that operate independently, whereas the smaller effect of rs258322 is related to rs1805007.

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Declarations of interest

None.

Ethical approval

The study protocol was approved by the Central Medical Ethics Committee of Latvia (No. A-3, A-7, and 01-29/2016-1-1). Written informed consent was acquired from all LGDB participants.

Authors' contributions

AO performed genotyping and data analysis. DR performed statistical analyses and interpreted results. DP conceived of the study and participated in design and interpretation. All authors wrote, edited and have approved the final manuscript.

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3.5. Original paper V. Inherited variants in the *MC1R* gene and survival from cutaneous melanoma: a BioGenoMEL study

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ORIGINAL ARTICLE

Inherited variants in the *MC1R* gene and survival from cutaneous melanoma: a BioGenoMEL study

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Summary

Inherited *MC1R* variants modulate MITF transcription factor signaling, which in turn affects tumor cell proliferation, apoptosis, and DNA repair. The aim of this BioGenoMEL collaborative study in 10 melanoma cohorts was to test the hypothesis that inherited variants thereby moderate survival expectation. A survival analysis in the largest cohort (Leeds) was carried out adjusting for factors known to impact on survival. The results were then compared with data from nine smaller cohorts. The absence of any consensus *MC1R* alleles was associated with a significantly lower risk of death in the Leeds set (HR, 0.64; 95% CI, 0.46–0.89) and overall in the 10 data sets (HR, 0.78; 95% CI, 0.65–0.94) with some support from the nine smaller data sets considered together (HR, 0.83; 95% CI, 0.67–1.04). The data are suggestive of a survival benefit for inherited *MC1R* variants in melanoma patients.

Significance

AJCC staging is of strong prognostic value for melanoma patients but only explains a proportion of the variance in survival. In some patients, there is evidence of a host immune response to the tumor both histologically and in the clinical manifestation of vitiligo, so that host/tumor interaction is postulated to be an additional factor which modifies prognosis. It is also likely that interaction between stromal tissues and cancer cells is important, genetically determined, and potentially modifiable. The new consortium BioGenoMEL seeks to bring together distinct patient cohorts to identify genes impacting on host/tumor interaction and therefore outcome, thereby improving our understanding of key biological pathways. This article is the first generated by BioGenoMEL as a consortium pooling data across multiple cohorts and provides evidence for a role for inherited *MC1R* variants in survival.

Introduction

Cutaneous melanoma is predominantly a cancer of white-skinned peoples, and those at increased risk include the very pale skinned (Gandini et al., 2005b), those with many melanocytic nevi (Gandini et al., 2005a), and those with a family history of melanoma (Gandini et al., 2005b). Although both fair hair and blue eyes are associated with increased susceptibility (Gudbjartsson et al., 2008), the pigmentary phenotypes most strongly associated are freckling, red hair, and a tendency to burn in the sun. These latter phenotypes are related to inherited variation in the gene coding for the melanocortin 1 receptor (*MC1R*) (Bishop et al., 2009) and the agouti locus (*ASIP*) (Brown et al., 2008; Gudbjartsson et al., 2008). The MC1R signals through a key pathway within melanocytes via the microphthalmia-associated transcription factor (MITF) to result in pigmentary changes: the default pigmentation is yellow/red (pheomelanin) (Beaumont et al., 2008), and signaling results in more black/brown pigment (eumelanin) synthesis. The agouti protein blocks this signaling, resulting in the default production of yellow/red pigment. It was recognized many years ago that some inherited variants in the *MC1R* gene are associated with red hair, and these have been classified by Duffy et al. (2004) into '*R*' variants and '*r*' variants, strongly and weakly associated with red hair, respectively.

Inherited *MC1R* variants are thought to increase melanoma risk as a result of consequent relative lack of eumelanin, but it is postulated that there are additional non-pigmentary effects (Robinson et al., 2010). The MITF transcription factor, expression of which is regulated by signaling through MC1R, has many target genes in addition to pigment biosynthesis enzymes, including genes that regulate DNA repair (*APEX1*) (Liu et al., 2009), the cell cycle (*CDKN2A, CDK2*) (Du et al., 2004; Loercher et al., 2005), apoptosis (*BCL2*) (McGill et al., 2002), and invasion (*DIA1*) (Carreira et al., 2006). The DNA repair gene apex nuclease 1, also known as apurinic endonuclease *APEX1*), is important in DNA repair responses to reactive oxygen species (ROS) and oxidative DNA base damage (Robinson et al., 2010). Kadekaro et al. (2010) showed that human melanocytes with two red hair color–associated *MC1R* alleles were resistant to α-melanocortin (α-MSH)-mediated DNA repair. The same group had earlier shown that *MC1R* activation mediated reduced oxidative DNA damage in melanocytes when exposed to UV radiation (Song et al., 2009).

The effect of *MC1R* variants on DNA repair and apoptosis may contribute to susceptibility but our hypothesis is that there may be additional effects on survival. There are published data to support this view. Overexpression of DNA repair pathways in melanoma has already been reported to be associated with metastasis and poor patient survival (Jewell et al., 2010; Winnepenninckx et al., 2006). This finding has led to the hypothesis that genetic stability conferred by high expression of DNA repair genes is required for metastasis formation (Sarasin and Kauffmann, 2008). Recent studies have provided support for the view that downstream effects of MITF (via *APEX1*) on apoptosis may be relevant to melanoma: Liu et al. (2009) showed that MITF-positive melanoma cell lines accumulated high levels of APEX1, and in another study, down-regulation of *APEX1* using antisense resulted in apoptosis of melanoma cells in culture (Yang et al., 2005).

In summary, two of the well-established hallmarks of cancer are resistance to apoptosis/cell death and sustained proliferation (Hanahan and Weinberg, 2011); we hypothesized that melanoma cells carrying *MC1R* variants would have less of both, and additionally poorer DNA repair and therefore the patients would have better survival.

We tested this hypothesis by looking at Breslow thickness and overall survival (OS) in 10 melanoma cohorts in relation to *MC1R* genotype. These cohorts were collected by a new consortium called BioGenoMEL (http://www.biogenomel.eu). BioGenoMEL has been created to collaboratively identify small, inherited effects on survival, which potentially have profound biological significance.

Results

Description of the data sets

Table 1 gives the summary characteristics of the cohorts studied. Leeds (the test set) was the largest cohort at 751 eligible cases, the others ranging in size from 137 cases (Riga, Latvia) to 487 cases (Valencia, Spain). Figure 1 shows the Breslow thickness distribution (after exclusion of cases with tumors with thickness 0.75 mm or less): a wider range of thickness was seen particularly in some cohorts, particularly in the Latvian cohort. Figure 1 shows the median age at diagnosis, which was fairly well balanced between cohorts (range 50.0–61.5 yrs). Figure 1 also shows that the greatest difference among the cohorts apart from sample size was in the time period during which participants were recruited. In the combined data set, a strong association was seen between *MC1R* status and hair color (Table S2) as expected (Valverde et al., 1995).

MC1R and tumor thickness

There was a small but significant inverse association between *MC1R* score and log Breslow thickness (estimate −0.02, P-value = 0.03) in cases whose tumor was thicker than 0.75 mm over all 10 cohorts, adjusted for center. This association was weaker, and not statistically significant, when the model was also adjusted for site of primary thickness (estimate −0.02, P-value = 0.1).

Table 1. Hair color, median follow-up, and *MC1R* status of cases in each melanoma cohort

Center	Cases[a]	No. of deaths	Median follow-up (days)	*MC1R* [number (%)][b]							Hair color [number (%)][b]		
				−/−	r/−	R/−	r/r	R/r	R/R	≥1 consensus alleles	No consensus alleles	Black/Brown	Blond	Red
Leeds	751	157	2329	105 (14)	134 (18)	184 (25)	54 (7)	169 (22)	105 (14)	423 (56)	328 (44)	501 (67)	144 (19)	89 (12)
Valencia	487	60	1458	171 (35)	129 (26)	88 (18)	31 (6)	51 (10)	17 (3)	388 (80)	99 (20)	377 (77)	93 (19)	17 (3)
Barcelona	201	30	1058	82 (41)	49 (24)	42 (21)	9 (4)	15 (7)	4 (2)	173 (86)	28 (14)	147 (81)	30 (17)	4 (2)
Genoa	140	18	1759.5	34 (24)	40 (29)	27 (19)	8 (6)	17 (12)	14 (10)	101 (72)	39 (28)	95 (69)	25 (18)	17 (12)
Vienna	159	21	935	43 (27)	52 (33)	33 (21)	8 (5)	16 (10)	7 (4)	128 (81)	31 (19)	111 (70)	38 (24)	10 (6)
Paris	407	88	1127	87 (21)	121 (30)	73 (18)	41 (10)	65 (16)	20 (5)	281 (69)	126 (31)	315 (78)	67 (17)	23 (6)
Essen	218	91	1231.5	44 (20)	51 (23)	58 (27)	12 (5)	40 (18)	13 (6)	153 (70)	65 (30)	–	–	–
Riga	137	44	1018	46 (34)	25 (18)	32 (23)	8 (6)	18 (13)	8 (6)	103 (75)	34 (25)	128 (93)	3 (2)	6 (4)
Stockholm	253	70	2689	45 (18)	49 (19)	77 (30)	14 (6)	37 (15)	31 (12)	171 (68)	82 (32)	43 (17)	195 (77)	15 (6)
Philadelphia	307	36	2132	65 (21)	62 (20)	70 (23)	22 (7)	60 (20)	28 (9)	197 (64)	110 (36)	200 (65)	71 (23)	36 (12)
Total	3060	615	1651	722 (24)	712 (23)	684 (22)	207 (7)	488 (16)	247 (8)	2118 (69)	942 (31)	1917 (68)	666 (24)	217 (8)

[a]Cases with *MC1R*, age, sex, and site information, Breslow thickness data >0.75 mm and a single primary melanoma recruited no more than 2 yrs after diagnosis.
[b]Percentages may not total 100% because of rounding.

Analysis of the Leeds cohort survival data

The Kaplan–Meier curve looking at the relationship between hair color and survival in the Leeds cohort (in the 1397 cases in the cohort who had hair color and were eligible) is shown in Figure 2. The results of this analysis are consistent with the hypothesis: melanoma cases with black/brown hair had poorer outcome than those with blond hair or red hair (log rank test for a significant difference in outcome between the three groups, P = 0.02).

Results from the proportional hazards analysis of hair color, *MC1R* score, and agouti (*ASIP*) status in the Leeds data set are shown in Table 2. It can be seen that hair color in analyses adjusted for factors known to have an effect on outcome (age, sex and tumor thickness) was borderline significant as a determinant of OS (HR, 0.58; 95% CI, 0.35–0.97; P = 0.04, considering red hair compared with black/brown, adjusted for age, sex, Breslow thickness, and site of the primary). *MC1R* status was significantly associated with survival if considered as *MC1R* score (HR per point, 0.82; 95% CI, 0.72–0.94; P = 0.004), no consensus *MC1R* alleles versus one or more consensus alleles (HR, 0.64; 95% CI, 0.46–0.89; P = 0.008), and if *MC1R* score and *ASIP* were included together in a multivariable model (HR, 0.79; 95% CI, 0.69–0.91; P = 0.001).

Analysis of forest plots of the association of *MC1R* with overall survival

Figure 3 shows the forest plots for all the survival data. In the analyses adjusted for age and sex only, the nine other cohorts gave some support to the hypothesis that inheritance of *MC1R* variants was associated with improved outcome (HR, 0.82; 95% CI, 0.66–1.02; P = 0.08 for no consensus *MC1R* alleles versus one or more consensus alleles). This result attained statistical significance with the addition of the Leeds data (HR, 0.77; 95% CI, 0.64–0.93; P = 0.005).

In the analyses adjusted additionally for site of primary and Breslow thickness, we see similar patterns of association in the smaller nine cohorts (HR, 0.83; 95% CI, 0.67–1.04; P = 0.1 for no consensus *MC1R* alleles versus one or more consensus alleles) and in all 10 cohorts combined (HR, 0.78; 95% CI, 0.65–0.94; P = 0.009). This suggests that adjustment for Breslow thickness has only a small overall effect on the association of *MC1R* with outcome. To test this, we compared the results of the same analysis under a univariable model with one adjusted for Breslow thickness only. We found little difference in the estimated hazard ratio under both models (data not shown).

Individually, little change was observed in the magnitude and direction of hazard ratios for most of the cohorts with adjustment for site and thickness. However, in the Philadelphia cohort, the direction of effect was protective in the model adjusted for sex and age only (HR, 0.79; 95% CI, 0.39–1.62) but deleterious in

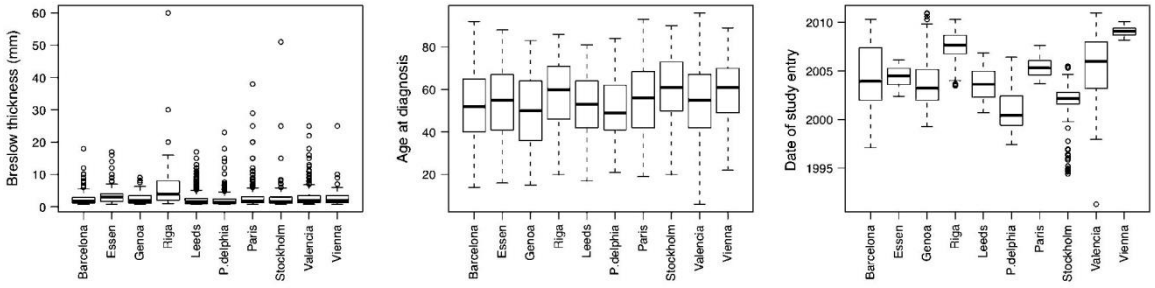

Figure 1. Box plots showing variation of Breslow thickness (thresholded > 0.75 mm), age of diagnosis, and date of study entry in the data taken for analysis from each of the 10 cohorts.

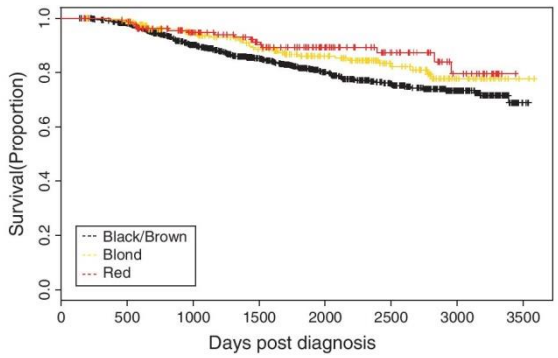

Figure 2. Kaplan–Meier curve showing differences in overall survival by hair color in the Leeds melanoma cohort (Black/brown = 965; Blond = 268; Red = 164; Log rank test, P = 0.02).

the model adjusted for sex, age, site of primary and Breslow thickness (HR, 1.08; 95% CI, 0.52–2.26). There is a significant association between log Breslow thickness and *MC1R* score for these Philadelphia data even when adjusted for site of primary thickness (estimate −0.07, P = 0.02), suggesting that adjusting for Breslow thickness may be obscuring the true relationship between *MC1R* and outcome in the Philadelphia data set even though in the overall analysis it does not.

There is no evidence of significant study heterogeneity between the 10 cohorts in either the model using the *MC1R* score (Cochran's *Q* test for heterogeneity P = 0.2) or the model comparing no consensus *MC1R* alleles versus one or more consensus alleles (Cochran's *Q* test for heterogeneity P = 0.8). However, the clearest support for our hypothesis is seen in the second biggest study (Valencia), and there was also some suggestion of a stronger effect in the Mediterranean countries compared with others.

We tested the effect of ignoring the rare *MC1R* variants on the association of *MC1R* score with outcome and found that this had little effect on our overall conclusions. Details can be found in Supporting information.

Discussion

This study reports a survival analysis of a large cohort of melanoma patients from the UK, which identified an effect of hair color and inherited variants in the *MC1R* gene on OS. We sought to validate these findings in nine additional cohorts within the melanoma genetics consortium BioGenoMEL (http://www.biogenomel.eu).

The identification of hereditary variation moderating host/tumor interaction and therefore survival from cancer is predicted to require multiple large data sets to identify small but biologically important effects. Few or no studies of sufficient power have been performed to

Table 2. Cox's proportional hazards model for overall survival in the Leeds cohort

	Unadjusted HR (95% CI)	P-value	Adjusted[a] HR (95% CI)	P-value
Blond versus black/brown	0.70 (0.49–1.02)	0.06	0.87 (0.60–1.26)	0.5
Red versus black/brown	0.56 (0.34–0.92)	0.02	0.58 (0.35–0.97)	0.04
MC1R score (per point)	0.85 (0.74–0.96)	0.009	0.82 (0.72–0.94)	0.004
MC1R no consensus alleles versus 1 or more consensus alleles	0.65 (0.47–0.90)	0.01	0.64 (0.46–0.89)	0.008
MC1R + ASIP[b]				
MC1R score (per point)	0.82 (0.72–0.94)	0.005	0.79 (0.69–0.91)	0.001
ASIP (per allele)	0.63 (0.44–0.91)	0.01	0.58 (0.40–0.85)	0.005

[a]Adjusted for age, sex, site of primary and Breslow thickness. Cases with tumors 0.75 mm or thinner and cases with multiple primary melanomas were excluded.
[b]MC1R and ASIP are included together as individual terms in the survival model.

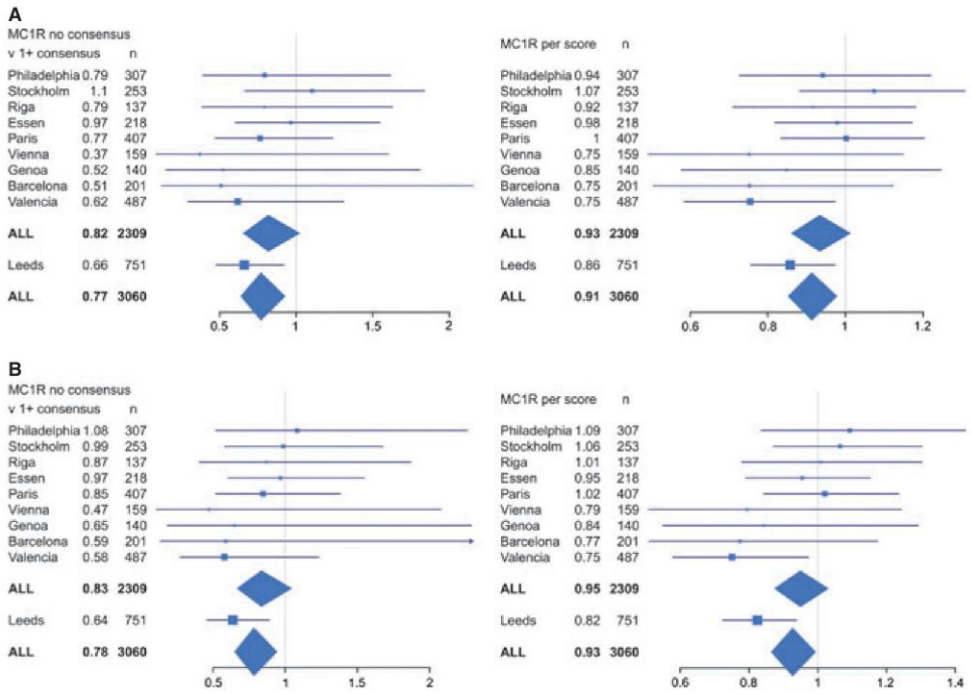

Figure 3. Forest plots of association of *MC1R* score with survival in each of the 10 melanoma cohorts and combined adjusted for (A) sex and age at diagnosis (B) site of primary, sex, age at diagnosis, and Breslow thickness.

date. The strength of this collaborative study is the unique collection of multiple data sets from Europe and the USA, subject to a centralized analysis. A weakness is that although the fact that the majority of melanomas are diagnosed early with excellent survival is very good news in terms of patient outcome, this does reduce the power of the study, in that after exclusion of participants with tumors thinner than 0.75 mm, some of the truncated cohorts were individually small. An analysis of the prevalence of '*R*' *MC1R* variants in the 10 cohorts also shows significant differences among them. This was expected given the known variation in *MC1R* allele frequencies across Europe. The highest proportion with '*R*' variants was seen in UK melanoma patients. High frequencies were also seen in the melanoma patients from Germany and Sweden. The proportion of '*R*' variants in some studies was so low however, these had little individual power to test the hypothesis. Because we could only identify individuals with metastatic disease in a few of our cohorts, we could not comprehensively investigate the effect on the data of excluding individuals who presented with metastatic disease. We are not certain that omitting metastatic cases should be standard in survival analyses that use OS as an end point, given that it is known that there is considerable variation in the survival outcome of cases with stage III disease (Balch et al., 2001, 2010). There were limited data available on stage for the Valencia, Genoa, and Paris cohorts; in this limited data set, we saw that removing stage IV cases had little to no effect. In our analysis, we were unable to take into account effects of drug interventions such as dacarbazine. However no drug used at the time of cohort follow-up has been shown to have survival benefit, so we anticipate this information would not change our conclusions. Analysis was complicated by heterogeneity between each of the cohorts in geographic location, how cases were ascertained to the study and the time period in which cases were recruited. Our analysis assumes that the effect of *MC1R* is similar in each of the cohorts, which is why we stratified baseline risk for each cohort. It is known that average melanoma thickness has decreased over time in European cohorts (Crocetti and Carli, 2003; Garbe et al., 2000; Lipsker et al., 2007), and this could complicate comparisons of the 10 cohorts in our study. However, we did not see any evidence of an association in our own data (mostly collected from 1999 to 2010) between Breslow thickness and year of diagnosis. Cohorts differ in how and how often they obtain follow-up. As the number of deaths is one of the factors that determines power, cohorts that are followed up infrequently are not as informative as they potentially could be. However, because follow-up is continually updated, we anticipate that these cohorts will mature over time and we will have greater power to see associations with other germline variants in future studies.

Another potential weakness of the study is that we did not perform centralized sequencing. Although Sanger sequencing is considered by many to be the 'gold standard' for mutation detection, the possibility of error arising from sequencing at different locations cannot be ruled out. However, we have seen excellent concordance in Sanger sequencing data from different centers, both in this study (sequencing in Leeds and Leiden of Leeds samples) and a previous study in which sequencing of *CDKN2A* across multiple centers within the GenoMEL genetics consortium was shown to be comparable (Harland et al., 2008).

Finally, it is of concern that hair color might be graded differently in different populations, so what might be viewed as 'blond' in Latvia, for example, might be viewed differently in Sweden where most people are pale skinned. Indeed there was little evidence of an effect of hair color on survival in a meta-analysis of the individual nine smaller cohorts overall (data not shown). In collaborative studies of this type, there will always be genetic variation between the populations, which is difficult to correct for; this study was therefore of particular interest because some of that genetic variation was evident as a difference in phenotype. While the main purpose of the study was to investigate *MC1R,* this study also considers the general issues arising from the use of multiple data sets to look at outcome.

The analysis of the data from the largest cohort benefitted from its size, but also SNP typing for the agouti locus as well as *MC1R.* The survival analyses provided strong supportive evidence that increased numbers of *MC1R* '*R*' or '*r*' variants have a protective effect on outcome, consistent with the hypothesis. Although the analysis based upon the number of variant '*R*' or '*r*' alleles is persuasive, there also appeared to be a deleterious effect of inheritance of one or more consensus *MC1R* alleles. Thus, these data suggest that in the presence of physiological signaling via the melanocortin receptor, biological processes downstream of MITF may result in poorer prognosis for melanoma patients. This is putatively through reduced apoptosis mediated by *APEX1*, greater double-strand break DNA repair, and/or increased cellular proliferation.

That there was some evidence in the 10 data sets of a relationship between *MC1R* variants, and thinner tumors is also supportive of the view that reduced proliferative effects may be seen in the presence of variant *MC1R.* We considered the possibility that differences in thickness may also reflect differences in ease of clinical diagnosis for patients with different skin type but published data suggest rather that diagnosis may be more readily missed in the very fair skinned (Cuellar et al., 2009) leading to the converse.

There was support for a protective effect of variant '*R*' or '*r*' *MC1R* on death from melanoma from the other nine cohorts, although overall the result from those nine cohorts was not independently statistically significant. The forest plots in Figure 3 are presented within Europe ordered from the northern latitudes (where blond hair and fair skin are much more prevalent) to Mediterranean countries (where darker hair and skin types are more prevalent, as a result of the inheritance of different patterns of additional pigment genes) and where the proportion of the cohort with '*R*' variants is much smaller. Although there was no statistically significant evidence of heterogeneity between cohorts, examination of the forest plot suggests that the protective effect of the consensus *MC1R* allele might be most obvious in the Mediterranean populations, although overall the Leeds cohort has significantly greater proportions of cases with '*R*' variants than any other cohort. It is not clear therefore whether the limited variation between the cohorts is a result of chance, cohort size, or the co-inheritance of other pigment genes impacting on MC1R signaling.

Inherited *MC1R* variants have previously been suggested to increase the likelihood of somatic *BRAF* mutant tumors (Fargnoli et al., 2008; Landi et al., 2006), so we have considered the possibility that differences in survival associated with germline *MC1R* status might be related to somatic differences between the tumors. The data reported by Landi et al. however were not corroborated by others (Hacker et al., 2010; Thomas et al., 2010), and there are some (albeit small studies) which actually suggest that *BRAF* mutations are associated with an unfavorable prognosis (Long et al., 2011; Si et al., 2012). Although there is no clear evidence to support an effect of somatic tumor variation by *MC1R* status, it is difficult to exclude the possibility. This argues for the need to consider both germline and somatic genetic events in investigating host/tumor interaction, and this is the future intent of BioGenoMEL.

Agnostic genome-wide approaches remain the most likely to identify new biological pathways of relevance, although published results have been mixed. A recent genome-wide association study of 1145 patients with breast cancer, for example, failed to identify such genes (Azzato et al., 2010). A smaller genome-wide study, in 245 patients treated with chemotherapy for small cell lung cancer, however, appears to have identified inherited variation predictive of survival (Wu et al., 2010).

An alternative approach is to take a candidate gene approach but a recent review of 90 candidate gene studies performed in patients with lung cancer confirmed the folly of small-scale studies without validation. Nonetheless, the conclusion of the review was that a small set of potential biomarkers had been identified in this way (Horgan et al., 2011). The effect of inherited genetic variation on outcome from cancer is likely to be relatively small when compared with the effects of variation in somatic genetic changes. That these effects are predicted to be small does not diminish their potential for giving biological insights into host/tumor interaction and therefore approaches to adjuvant therapies.

In conclusion, both genome-wide and candidate gene studies may be needed to identify germline predictors of outcome, but it will clearly be necessary to work collaboratively within consortia such as BioGenoMEL. This will be particularly helpful to avoid a proliferation of small genetic studies producing contradictory results. This study illustrates the value of consortia, but reinforces the need for large studies even within consortia and the potential problems in looking at survival in genetically diverse populations.

Methods

Data collection

The Leeds melanoma cohort

The UK Multicenter Research Ethics Committee (MREC) and the Patient Information Advisory Group (the predecessor of the National Information Governance Board) approved the study. Population-ascertained incident melanoma cases were recruited to a case–control study in a geographically defined area of the UK (Yorkshire and the Northern region south of the River Tyne) (67% participation rate); 960 cases (aged 18–76 yrs) were recruited in the period from September 2000 to December 2005, as described previously (Falchi et al., 2009; Randerson-Moor et al., 2009). Recruitment (and therefore blood sampling) took place wherever possible 3–6 months after diagnosis. Age, sex, natural hair color at age 18 yrs, propensity to burn, ability to tan, skin color of inside upper arm, and freckling as a child using Gallagher's freckle chart (Lee et al., 2005) were self-reported. Recruitment to the cohort has continued beyond 2005, but *MC1R* genotyping is not yet available for the more recent samples. The Leeds cohort contains 1954 cases in total, and *MC1R* genotyping was available for 966 of these cases. After applying the selection criteria applied to all cohorts and listed below in the section on Statistical analysis, 751 cases were eligible for the survival analysis. Information on hair color was available for 1659 cases, and after applying the same selection criteria, 1397 cases were eligible.

BioGenoMEL cohorts

Table 1 shows comparative data on the nine additional cohorts contributed by members of the BioGenoMEL consortium. These cohorts have been collected in the period 1991 to the present day by research groups in Southern Europe [centers in Barcelona, Valencia and Genoa (Goldstein et al., 2007; Pastorino et al., 2008; Scherer et al., 2009)], Central Europe (Vienna), Northern Europe [Paris and Essen (Guedj et al., 2008; Scherer et al., 2009)], far Northern Europe [Riga and Stockholm (Hoiom et al., 2009)], and the USA [Philadelphia (Kanetsky et al., 2010)]. In the Leeds, cohort disease/vital status is established through annual follow-up, inquiry from the GPs, cancer registry data, and by extraction of clinical notes. Attainment of follow-up data was from equivalent sources in other centers. Details can be found in Table S3.

MC1R sequencing

In nine centers, the whole *MC1R* gene was sequenced, and in one center (Sweden), the most common variants were genotyped (see below).

For the Leeds samples, standard PCR techniques were used to amplify the entire 954-nucleotide coding region of the single-exon *MC1R* gene, plus the surrounding untranslated regions, either as a single large amplicon or in smaller overlapping amplicons. Purified PCR products were sequenced using sequencing primers spanning the *MC1R* gene. Sequencing reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) using standard sequencing conditions. The sequencing reaction products were run on an ABI prism 3130XL Genetic Analyzer (Applied Biosystems).

Sequence data were analyzed using SeqScape (Applied Biosystems) or CodonCode Aligner software (CodonCode Corp., Dedham, MA, USA). Compiled sequence data were double scored and checked by a second analyst. *MC1R* polymorphisms were identified by comparison with the *MC1R* consensus sequence (NCBI accession no. NM_002386). Primer sequences for PCR and sequencing are available on request. There was no centralized sequencing between groups, most groups had previously sequenced *MC1R* for other analyses addressed to understanding genetic susceptibility to melanoma (Demenais et al., 2010; Ghiorzo et al., 2009; Kanetsky et al., 2004; Matichard et al., 2004; Scherer et al., 2009). Details can be found in Table S3. Most centers used similar standard sequencing techniques to screen for *MC1R* variants, as described above.

In the Leeds samples, one of 31 plates was genotyped in both Leeds and Leiden to test for errors in the genotyping process. There was 100% concordance between calls for these samples.

Swedish samples were analyzed using a Protease-mediated Allele-Specific Extension (PrASE) method, specific to 21 of the most common *MC1R* variants in European populations (Kaller et al., 2005).

MC1R analysis

As *MC1R* variants are numerous and are thought to have a variable impact on signaling through MITF, the analytic approach was considered carefully. We based the analysis upon a published and widely adopted classification system using the '*r*', '*R*' nomenclature first described by Duffy et al. (2004). Figure 4 shows a flowchart that explains the classification system we implemented, and a detailed explanation is provided in Supporting information.

The classification was turned into a numerical score in the range 0–4 by summing across the two alleles, giving a value of 1 to '*r*' and 2 to '*R*' variants. Thus, individuals with two copies of the consensus sequence (*–/–*) scored 0, and individuals with two '*R*' variants (*R/R*) scored 4. It has been suggested previously that red hair color alleles may act in a recessive manner and that one fully functional copy of *MC1R* may be sufficient to provide normal function (Beaumont et al., 2008). Therefore, we also looked at a second classification system contrasting individuals who have no consensus alleles (*r/r*, *R/r*, *R/R*) with those who have one or more consensus alleles (*–/–*, *r/–*, *R/–*).

Statistical analysis

To test the hypothesis that individuals with *MC1R* variants have thinner tumors, a linear regression analysis was conducted, regressing the natural logarithm of Breslow thickness on the *MC1R* score, adjusting for center, using the 'lm' routine in R 2.10.1 (R Development Core Team, 2010).

We defined survival time as the period between the date of surgical excision of the primary and date of death or last date of follow-up (at which point records were censored). Kaplan–Meier curves were drawn to investigate differences in OS with respect to hair color (classified as black/brown, blond, or red) and *MC1R* status in the Leeds cohort, using the 'survfit' routine in the 'survival' package in R. To test for a significant different in outcome between the three hair color groups in the Leeds cohort, a log rank test was performed using the 'survdiff' routine in the 'survival' package in R.

Multivariate survival analyses were performed using Cox's proportional hazards model. Models were fitted using the 'coxph'

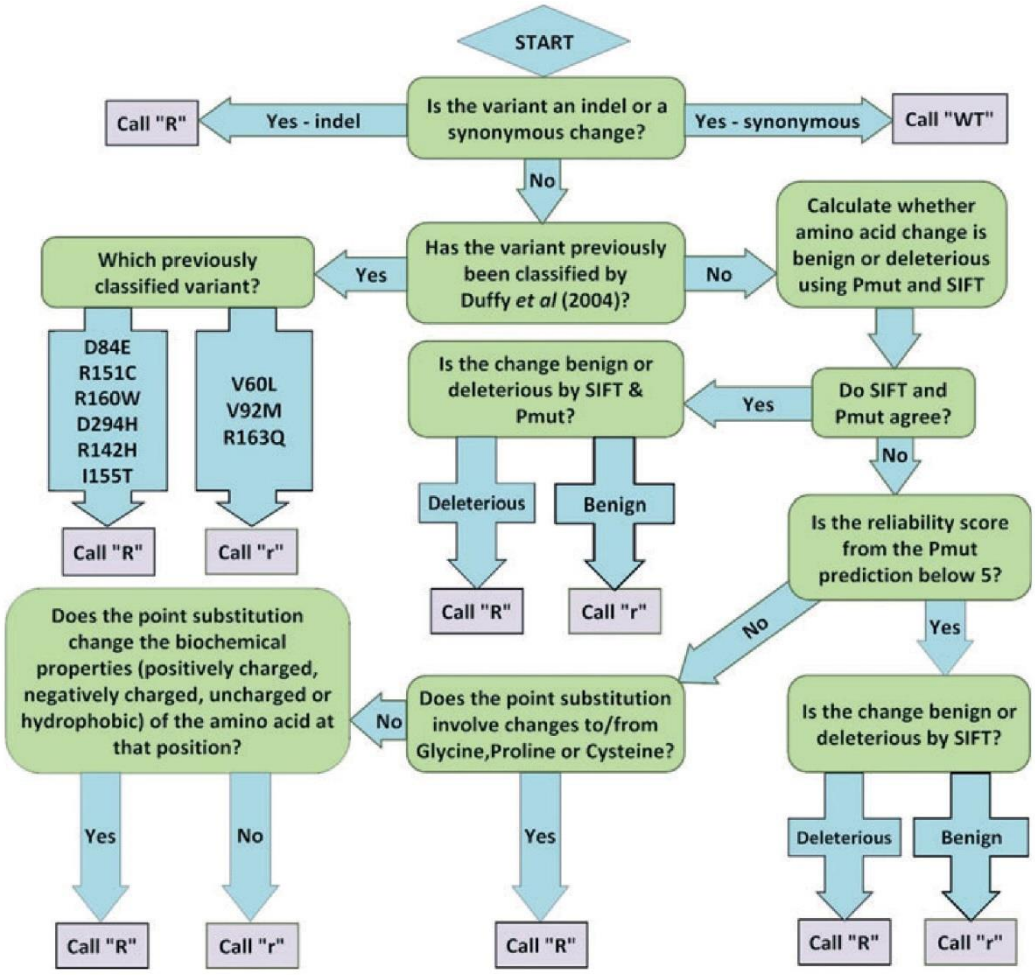

Figure 4. Flowchart showing how *MC1R* variants are called '*r*' or '*R*' using SIFT and PMut to call rare variants (see Methods).

routine in the 'survival' package in R. Hazard ratio estimates were calculated for the effect of hair color and *MC1R* on OS adjusted for Breslow thickness, sex, site of melanoma (head/neck, trunk, limbs or other), and age of diagnosis. We also report models adjusted only for age of diagnosis and sex for the *MC1R* analyses. We did not adjust for tumor ulceration because of incomplete data for this variable across all the cohorts. We could not adjust for AJCC staging for the same reason. We tested the effect of adding stage in three cohorts for which we had data and we found that including stage did not change the association of *MC1R* with outcome. To test for study heterogeneity, we performed Cochran's *Q* test. To do this, we first took relevant point estimates and standard errors for each study from the fitted Cox's proportional hazards models. These data were then used to construct a meta-analysis assuming fixed effects using the 'metaan' function in STATA version 10, which reports the result of Cochran's *Q* test.

We excluded cases with thin tumors (0.75 mm or less) from all analyses on the basis that these cases have an excellent prognosis and add little information to the estimation of the effect of predictors of survival. We did not test for *CDKN2A* mutation carrier status in the 10 melanoma cohorts, but we expect that because the cohorts were not ascertained on the basis of family history that these would be rare and account for 2% of the cases (based on our own unpublished data). A modest number of cases with nodal or metastatic disease were included in the analysis; nodal primaries were assigned a Breslow thickness of 4 mm. We also excluded individuals with multiple primary melanomas and prevalent cases that were recruited in a proportion of the studies. It has been shown that introducing cases into a study a long time after diagnosis (left truncated data) can introduce bias into survival analysis (Cnaan and Ryan, 1989). Each center was therefore asked to provide information of the date of study entry for each case to determine whether the case was recruited within 2 yrs of diagnosis, which we defined as 'incident' as opposed to 'prevalent' cases. We investigated whether it was possible to incorporate prevalent cases into our study by measuring survival from the date of study entry [as described by Azzato et al. (2009)] but we found the proportional hazards assumption was violated in this model (Schoenfeld global test for proportional hazards in the model containing *MC1R* score, P = 0.002). It is important that the proportional hazards assumption holds when including prevalent cases in this way (Azzato et al., 2009) so we discounted them from further analyses and focused efforts on cases recruited within 2 yrs of diagnosis. A breakdown of how cases were excluded can be found in Table S4.

The hypothesis that *MC1R* variants might be associated with survival was first tested by evaluating hair color and survival. Hair color is determined by a number of pigment genes, but red hair is predominantly (but not exclusively) determined by *MC1R*. Support for the hypothesis was seen, in that those with red hair had better survival (see Figure 2, and Table 2), and therefore the association between *MC1R* status and OS was also investigated in each of the 10 data sets. We created a combined estimate for the nine smaller data sets by including study as a stratification variable in the model.

The agouti signaling protein (coded by *ASIP*, a melanoma susceptibility locus) is a competitive agonist of melanocyte-stimulating hormone (MSH) which binds to the melanocortin receptor, so that inheritance of the risk allele at *ASIP* results in reversion to the null (pheomelanin) phenotype. Hence, it is postulated that inheritance of risk alleles at *ASIP* has a similar effect to variant *MC1R* on MITF signaling. Therefore, in the Leeds data set, where we had both *MC1R* sequence data and *ASIP* SNP genotyping (for the single nucleotide polymorphism rs4911442), we included the *ASIP* and *MC1R* data together in a separate model to investigate the combined effect of the two on survival.

Forest plots were used to compare the hazard ratio (HR) estimates across studies; HR estimates for *MC1R* score and for no consensus *MC1R* alleles versus one or more consensus *MC1R* alleles were plotted for each center, alongside a pooled estimate.

The relationship between the age variable and the log hazard for survival was suspected to be nonlinear. Details of how we tested this are provided in Supporting information.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Table S1. *MC1R* calls including and excluding rare variants determined using the bioinformatic analysis.

Table S2. *MC1R* status and hair colour in the combined dataset.

Table S3. MC1R Genotyping method, patient accrual and follow up in each group.

Table S4. Breakdown of the exclusion of cases ineligible for analysis by cohort.

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3.6. Original paper VI. The lack of E318K *MITF* germline mutation in Latvian melanoma patients

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Cancer Genetics

LETTER TO THE EDITOR

The lack of E318K *MITF* germline mutation in Latvian melanoma patients

MITF as a transcription factor is one of the main regulators of melanin synthesis, melanosome biogenesis, and the overall life cycle of melanocytes. Recently, the association between a rare *MITF* variant E318K (rs149617956) and melanoma was discovered simultaneously by two independent studies. Yokoyama et al., first, using whole genome sequencing followed by linkage analysis, found E318K to be an intermediate risk variant for melanoma. Consistent with this finding, the association was also found in a large Australian case-control sample and an independent case-control sample from the United Kingdom with a joint variant carrier frequency of 0.017 (68/3988 cases), corresponding to a 2.2-fold higher melanoma risk (1). Similar results were also obtained in the French population by Bertolotto et al., who found that the E318K variant predisposes carriers to familial and sporadic melanoma, with a minor allele frequency (MAF) of 0.014 (17/603 cases) and a 4.8-fold higher melanoma risk, as well as to renal cell carcinoma (RCC), with a MAF of 0.015 (5/164 cases) and a 5.2-fold higher RCC risk (2). Later, Ghiorzo et al., in a study from Italy, found an association between the E318K variant and melanoma, with a MAF of 0.009 (12/667 cases) and a 2.85-fold higher melanoma risk, and highlighted a potential association with not only RCC but also pancreatic cancer (3). Finally, a large Genes, Environment, and Melanoma (GEM) study from four countries (Australia, Italy, Canada, and the United States) supports E318K as a medium-penetrance melanoma susceptibility variant, with a MAF of 0.014 (44/1194 cases) and 1.7-fold melanoma risk (4). In contrast to the previously mentioned studies, a study by Gromowski et al. analyzing a Polish population found no association between the *MITF* E318K variant and melanoma risk. Among 748 melanoma patients, there were only two heterozygous E318K carriers, resulting in a MAF of only 0.001. In addition, no association was found between E318K and other types of cancer (kidney, colon, lung, breast, and prostate) (5).

Here, we examined the prevalence of the *MITF* variant E318K in Latvian melanoma patients and controls. The study was based on DNA samples and data from the Genome Database of Latvian Population (LGDB), a government-funded biobank (briefly described in Ciganoka et al. (6)).

Altogether, 477 melanoma patients (mean age 56.2 $\pm$ 14.4 y) comprising 327 women (mean age 55.8 $\pm$ 14.4 y) and 150 men (mean age 57.2 $\pm$ 14.4 y) with histopathologically confirmed cutaneous melanoma who were recruited to the LGDB between 2001 and 2011 at the Riga Eastern Clinical University Hospital Latvian Oncological Centre were included in the study. Of these patients, 18 (3.8%) had a family history of melanoma, 11 (2.3%) had relatives with pancreatic cancer, 7 (1.5%) had multiple primary melanomas, and 49 (10.3%) were early-onset (age $\leq$ 35 y) melanoma patients. All of these patients tested negative for the presence of mutations in two main melanoma susceptibility genes, *CDKN2A* and *CDK4*. The control group consisted of 225 unrelated healthy volunteers (mean age 47.3 $\pm$ 17.2 y), comprising 168 women (mean age 48.9 $\pm$ 16.8 y) and 57 men (mean age 42.6 $\pm$ 17.5 y), who were also participants in LGDB. The *MITF* sequence region that included variant E318K was amplified using the forward primer 5′-CAGGCTCGAGCTCATGGA-3′ and the reverse primer 5′-TGGGGACACTATAGGCTTGG-3′ (1), sequenced with the same primers by conventional Sanger sequencing using an Applied Biosystems ABI PRISM BigDye Terminator cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA), and analyzed on an Applied Biosystems ABI PRISM 3100 genetic analyzer (Thermo Fisher Scientific).

Because of the relatively low incidence of melanoma in Latvia, approximately 7.6 cases per 100,000 persons per annum, and the absence of mutations in well-known melanoma susceptibility genes in most families analyzed, we hypothesized that there might be other germline mutations responsible for melanoma risk in our population and that the *MITF* E318K variant could be a good candidate. However, we found the *MITF* variant E318K neither in melanoma patients (either familial, including pancreatic cancer, or sporadic) nor in controls, in a total of 702 individuals genotyped.

Admittedly, the current study is underpowered to confirm whether the absence of the *MITF* E318K variant is due to a true lack of association or rather due to insufficient sample size. A much larger study is needed to reveal the association between *MITF* E318K and risk of developing melanoma. The discrepancies between four different studies can be explained by different characteristics of the cases studied—we, similarly to Gromowski et al. (5), analyzed unselected cases—whereas in Australian (1), French (2), and Italian (3) studies, genetically enriched cases were analyzed, and by population stratification. Similar population stratification has been observed with the *CDKN2A* polymorphism A148T, which has an association with melanoma in Latvian and Polish populations, unlike in several other populations (7). Therefore, we suggest that *MITF* E318K might not be considered a contributor to melanoma risk in this geographical region combined.

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3.7. Original paper VII. Low-penetrance melanoma risk gene polymorphisms in Latvian population

Low-penetrance melanoma risk gene polymorphisms in Latvian population

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Key words: melanoma; *MC1R*; *MDM2*; *MITF*; *TP53*.

Cutaneous melanoma is cancer that develops from melanocytes and arises through the interaction of environmental, individual's pigmentation phenotype and genotype factors. The aim of this study was to analyze association between polymorphisms in medium (*MITF*) and low (*TP53, MDM2, MC1R*) penetrance melanoma risk genes and melanoma in Latvian population.

The microphthalmia-associated transcription factor gene (*MITF*) polymorphism Glu318Lys (rs149617956) has shown association with melanoma both in melanoma families and general population therefore is classified as a medium penetrance melanoma risk polymorphism (Yokoyama et al. 2011).

TP53 is tumor suppressor that is negatively regulated by mouse double minute 2 homolog (MDM2). TP53 among the other targets activates melanocortin 1 receptor (MC1R) signaling pathway subsequently leading to the synthesis of UV-protective pigment. Many studies demonstrate association between *MC1R* gene polymorphisms and melanoma (Williams et al. 2011). *MC1R* polymorphisms with the highest melanoma risk are designated as RHC (red hair color) polymorphisms. There are also several studies with regard to association between TP53 polymorphism Pro72Arg c.215C>G (rs1042522), *MDM2* gene promoter polymorphism c.14+309T>G (rs2279744) and melanoma risk, however results are conflicting (Cotignola et al. 2012; Ye et al. 2013).

MITF gene region with polymorphism Glu318Lys was sequenced in 490 melanoma patients and 377 healthy controls however Glu318Lys was found in none of them.

In the analysis of *TP53* and *MDM2* polymorphisms altogether 490 melanoma patients and 356 controls were included. *TP53* Pro72Arg analysis was performed using RFLP method with endonuclease Bsh1236I and *MDM2* gene promoter region with c.14+309T>G was sequenced. When allele and genotype frequencies of polymorphisms Pro72Arg and c.14+309T>G were examined individually, none of them was associated with melanoma (OR 1.07, 95% CI 0.87–1.32, P = 0.517 and OR 1.18, 95% CI 0.95–1.45, P = 0.131, for allele frequencies respectively). None of Pro72Arg and c.14+309T>G genotype combinations were associated with melanoma risk. When the presence of *MC1R* gene RHC polymorphisms were taken into consideration, there were more melanoma patients than controls with *TP53* Pro72Arg GG genotype and *MC1R* polymorphisms (OR 2.76, 95% CI 1.02–7.52, P = 0.040) (Table 1). There was no

Table 1. Association between *TP53* polymorphism Pro72Arg and melanoma depending on the presence of MC1R polymorphisms. WT, wild type (including synonymous polymorphisms); RHC, red hair color polymorphisms; NRHC, non-red hair color (all other nonsynonymous) polymorphisms; OR, odds ratio; CI, confidence interval; *P*, *P* value

TP53 Pro72Arg	MC1R polymorphisms	Patients (*n*) 279	%	Controls (*n*) 217	%	OR	95% CI	*P*
CC	WT	7	2.5	11	5.1	1	–	
	RHC	13	4.7	6	2.8	3.40	0.88 – 13.19	0.072
	RHC/NRHC	24	8.6	15	6.9	2.51	0.80 – 7.91	0.111
CG	WT	33	11.8	36	16.6	1.44	0.50 – 4.15	0.498
	RHC	38	13.6	25	11.5	2.39	0.82 – 6.99	0.107
	RHC/NRHC	72	25.8	61	28.1	1.85	0.68 – 5.08	0.224
GG	WT	41	14.7	36	16.6	1.79	0.63 – 5.10	0.273
	RHC	52	18.6	29	13.4	2.82	0.99 – 8.06	0.048
	RHC/NRHC	102	36.6	58	26.7	2.76	1.02 – 7.52	0.040

such an association in the presence of *TP53* Pro72Arg CC or CG genotype leading to the conclusion that Pro72Arg GG genotype in combination with *MC1R* polymorphisms has additional impact on melanoma risk.

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3.8. Original paper VIII. Inherited variation in the *PARP1* gene and survival from melanoma

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Inherited variation in the PARP1 gene and survival from melanoma

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Key words: melanoma, survival, *PARP1*, genetic determinants of survival, random effects meta-analysis, bioinformatics
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We report the association of an inherited variant located upstream of the poly(adenosine diphosphate-ribose) polymerase 1 (*PARP1*) gene (rs2249844), with survival in 11 BioGenoMEL melanoma cohorts. The gene encodes a protein involved in a number of cellular processes including single-strand DNA repair. Survival analysis was conducted for each cohort using proportional hazards regression adjusting for factors known to be associated with survival. Survival was measured as overall survival (OS) and, where available, melanoma-specific survival (MSS). Results were combined using random effects meta-analysis. Evidence for a role of the PARP1 protein in melanoma ulceration and survival was investigated by testing gene expression levels taken from formalin-fixed paraffin-embedded tumors. A significant association was seen for inheritance of the rarer variant of *PARP1*, rs2249844 with OS (hazard ratio (HR) = 1.16 per allele, 95% confidence interval (CI) 1.04–1.28, $p = 0.005$, eleven cohorts) and MSS (HR = 1.20 per allele, 95% CI 1.01–1.39, $p = 0.03$, eight cohorts). We report bioinformatic data supportive of a functional effect for rs2249844. Higher levels of *PARP1* gene expression in tumors were shown to be associated with tumor ulceration and poorer OS.

What's new?

Although staging systems predict outcome fairly well for melanoma, survival still varies among individual patients. In this meta-analysis, the authors found that inheritance of a rare genetic variant of PARP1 was associated with improved survival of melanoma patients. Increased expression of PARP1 has been associated with poorer outcome, and depletion of PARP1 may reduce both melanoma growth and angiogenesis. The identification of this and other germline variants that affect survival may help to identify key biological pathways active in host/tumor interactions, which may lead to the discovery of new therapeutic targets for treating advanced melanoma.

The aim of the research consortium BioGenoMEL (www.biogenomel.eu) is to investigate the role of germline (inherited) genetic variation in melanoma survival. Identifying genes that might have an effect on survival by moderation of host/tumor interaction would help us to understand the biology of that interaction, potentially giving rise to novel adjuvant therapies. Previous studies have reported evidence that inherited variation in genes in drug metabolism pathways may affect survival from lung cancer[1,2] and that ancestry-related polymorphisms were associated with acute lymphoblastic leukemia relapse.[3] We have previously reported evidence that melanocortin receptor 1 (*MC1R)* variants that influence melanin synthesis and are associated with increased melanoma risk were associated with reduced risk of death from melanoma.[4]

In our study, we look at the association of a germline variant in the *PARP1* gene with outcome. PARP1 is a member of the family of the poly(adenosine diphosphate-ribose) polymerase (PARP) proteins, which are DNA damage sensors, signaling to downstream effectors[5] and therefore directly involved in genomic stability, DNA repair and apoptosis. It has been suggested previously that cancer cells can become "addicted" to DNA repair pathways that protect them from lethal levels of DNA damage,[6,7] and it has been postulated that PARP1 may play a role in this "addiction." Increased expression of PARP1 protein has been reported in a number of cancer types, reviewed by Yelamos *et al.*,[5] when increased expression of PARP1 was frequently reported to be associated with a poorer outcome. The role of PARP1 in cancer is said, however, to be pleomorphic, having also been linked to inflammation *via* its role in upregulation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells),[8] which has been described as promoting the avoidance of programmed cell death.[9] Depletion of PARP1 has been reported to reduce both melanoma growth and angiogenesis, while increasing chemosensitivity in melanoma cells.[10] This effect is postulated to be *via* inhibition of a senescence-induced secretome.[11]

We report evidence of an association of a single nucleotide polymorphism (SNP) previously shown to be associated with a reduced risk of melanoma[12] with outcome. There is, as yet, no direct evidence of a functional effect of this SNP on PARP1 protein levels, so we report an investigation of relevant published bioinformatics data. Although increased expression of PARP1 in melanoma compared to benign melanocytic nevi has been reported using immunohistochemistry,[13] we thought it is important to look at melanoma tumors for an association between *PARP1* expression and survival as additional evidence that PARP1 might be important in melanoma.

Methods

Data collection

The SNP data are derived from a large Leeds dataset (the Leeds Melanoma Cohort) and from ten smaller datasets within the BioGenoMEL consortium. These cohorts have been described previously[4] but details are also provided in Supporting Information (Supporting Information Table S1).

SNP selection process

The *PARP1* SNP rs2249844 (A>G) was selected for investigation because of its association with melanoma susceptibility,

the minor allele showing a reduced risk of melanoma development. It also has a comparatively high minor allele frequency (MAF), and is in close proximity to the coding sequence of the *PARP1* gene. Rs2249844 is in linkage disequilibrium (LD; $R^2 = 0.96$) with the intronic *PARP1* SNP rs3219090, which has been shown to be associated with susceptibility to melanoma[12] and is itself associated with susceptibility (unpublished data, odds ratio, OR = 0.87; 95% confidence interval, CI 0.81–0.93, $p = 0.00005$, 2,804 cases, 7,618 controls). Both *PARP1* SNPs were typed in the Leeds cohort as part of an initial panel of 23 independent SNPs in selected candidate genes.

SNP genotyping

Genotyping for the datasets from Leeds, both Vienna cohorts, Stockholm, Lund, Athens and Riga was performed in Leeds using Taqman technology (Applied Biosystems, Foster City, CA). A total of 2 μl polymerase chain reactions (PCRs) were performed in 384-well plates using 10 ng of DNA (dried), 0.05 μl assay mix and 1 μl Universal Master Mix (Applied Biosystems) according to the manufacturer's instructions. End point reading of the genotypes was performed using an ABI 7900HT Real-time PCR system (Applied Biosystems). All genotypes were double scored by an independent analyst. The *PARP1* SNP rs2249844 was genotyped using the Taqman assay C__34511379_10 (Applied Biosystems).

Genotyping in the additional datasets

Genotyping for the Barcelona, Valencia and Essen datasets was performed in Heidelberg using a PCR-based allelic discrimination method (KBiosciences, UK) in 384-well plate format. In each plate 8% of wells were assigned for quality control. Genotypes in amplified products were determined by differences in VIC and FAM fluorescent level in plate read operation on ABI PRISM 7900HT (Applied Biosystems) using SDS 1.2 Software. Postoperation data were transferred as Microsoft Excel files and converted into genotype information. Genotypes in random samples were validated by DNA sequencing.

DNA samples for genotyping for the Tampa and Vienna FFPE cohorts were extracted from healthy skin removed during wide local excision procedures.

Gene expression analysis

Complementary analyses were run examining the association of gene expression levels with outcome in primary and metastatic formalin-fixed paraffin-embedded tumor samples. Tumor blocks were obtained from patients recruited to a Leeds retrospective study (Predicting Response to Chemotherapy) allowing identification of gene expression profiles associated with primary tumor features and overall survival (OS). Patients recruited to our study had stage IV melanoma and were treated with palliative dacarbazine or temozolomide as part of clinical trials or routine clinical care. Clinical data relating to survival and characteristics of the primary tumor were obtained from treating clinicians and diagnostic histopathology reports.

PARP1 gene expression in the tumors was explored in relation to survival but, as the intent was to study the putative effects of *PARP1* on the tumor microenvironment, we also examined the relationship of *PARP1* gene expression with tumor ulceration and angiolymphatic invasion. Further details can be found in Supporting Information and Supporting Information Table S2.

Statistical analysis for the SNP data

Link-anonymized data on date of diagnosis and date of death, and prognostic indicators such as Breslow thickness, tumor site, age at diagnosis and sex were additionally centralized in Leeds from the nine European BioGenoMEL cohorts. Analyses were carried out independently using the same procedure by the Tampa group on their dataset as this approach was compliant with their IRB approval.

Survival time was defined as the period between the date of surgical excision of the primary and date of death or last date of follow-up (at which point records were censored). Multivariable survival analyses were performed using Cox's proportional hazards model in R 2.10.1. Hazard ratio estimates were calculated for the effect of each of the SNPs on OS adjusted for sex, site (head/neck, trunk, limbs or other) and age of diagnosis and then additionally for Breslow thickness. Melanoma-specific survival (MSS) data were not available from all groups. Therefore, to reduce the number of nonmelanoma-related deaths reported, we truncated OS time at 8 years of follow-up. An additive genetic model was assumed.

Relevant per-allele effect estimates and standard errors for each study were taken from the fitted Cox's proportional hazards models. These data were then used to carry out a random-effects meta-analysis in R. Models are reported adjusted for age of diagnosis, sex and tumor site, with and without Breslow thickness. We also compared the association of the SNP for OS and MSS in the seven cohorts where cause of death was available. The proportional hazards assumption was tested by fitting Schoenfeld residuals and testing for a significant association with time. Sensitivity analyses were conducted to ensure that no single cohort skewed the results. Additional details regarding the statistical analysis, sensitivity analysis and testing of the proportional hazards assumption can be found in Supporting Information, Supporting Information Table S3, and Figures S1 and S2.

Bioinformatic analysis

The rs2242844 SNP is in close proximity to the 5′ end of the *PARP1* gene but is not known to have a functional effect on the gene itself or to be in strong LD with a known functional variant. We undertook several bioinformatic analyses to determine whether rs2249844 or a linked genetic variant is associated with a putative functional effect that may affect outcome in melanoma cases.

Identification of noncoding regulatory elements. To identify potentially important noncoding regulatory regions we queried experimentally derived data on sites of DNAse

hypersensitivity deposited in public databases; these regions are of particular interest because they indicate open stretches of DNA available to bind transcription factors that are therefore potentially transcriptionally active. We then attempted to further characterize these regions by looking at additional experimentally derived data such as observed CpG and histone methylation patterns, observed transcription factor binding and sequence conservation. We used pattern recognition algorithms and software such as ChromHMM,[14] which predicts functional elements (such as enhancers) based on the chromatin state of a region (derived from patterns learnt from experimental data), and JASPAR,[15] which predicts potential transcription factor-binding sites by matching the sequence to a database of transcription factor motif patterns. We investigated the region in LD with rs2249844 ($r^2 \geq 0.6$) for putative regulatory elements using HaploReg v2 (http://www.broadinstitute.org/mammals/haploreg/haploreg.php),[16] the Roadmap Epigenomics project (http://www.roadmapepigenomics.org/) and the ENCclopedia Of DNA Elements (ENCODE; http://genome.ucsc.edu/ENCODE/).[17,18]

Identification of functional elements using SNPinfo. We used the SNPinfo (http://snpinfo.niehs.nih.gov/)[19] function prediction tool to predict the function of SNPs in *PARP1* listed in HapMap (CEU population) up to 10 kb downstream and 150 kb upstream. SNPinfo uses several computational algorithms to predict potential transcription factor-binding sites, splice sites, miRNA-binding sites, nonsynonymous SNPs, potential stop codon mutations, sequence conservation, regulatory potential and severity of the effect of a mutation (using Polyphen, http://genetics.bwh.harvard.edu/pph2/[20] or SNPs3D, http://www.snps3d.org/[21]).

Investigation of expression quantitative trait loci associated with PARP1. We investigated the association of *PARP1* expression with SNPs in close proximity (within 1 Mb to rs2249844) using the expression quantitative trait loci (eQTL) data (http://www.sanger.ac.uk/resources/software/genevar/)[22] and the *cis*-eQTL—Gene analysis option in the GeneVar software package. We subsequently investigated the association of rs2249844 with expression of genes in close proximity to *PARP1* using the *cis*-eQTL—SNP analysis option in GeneVar. We would have preferred to use tissue-specific expression data (MuTHER resource NCBI36/ReMOAT 1.0.0, TwinsUK-S skin data, $n = 856$[23]); however, we found no data for *PARP1* expression in this dataset at the time of writing so we subsequently ran the same analysis using the MuTHER pilot study data (Twin1-S, Twin2-S, $n = 98$).[24] We also investigated data deposited in the Chicago eQTL browser for evidence of eQTLs in the *PARP1* region. Further details can be found in Supporting Information.

Analysis of the gene expression data from melanoma tumors

Differences in gene expression associated with tumor ulceration and presence of angiolymphatic invasion were tested using Mann–Whitney *U*-tests. Fold change differences in median gene expression were calculated between ulcerated and nonulcerated tumors and tumors with or without angiolymphatic invasion. Analysis of the putative association between ulceration and *PARP1* expression was then adjusted for age, sex and Breslow thickness.

Analysis was performed to identify gene expression patterns associated with OS. The Cox proportional hazards model was used to calculate HRs and 95% CIs for each gene using log-transformed data ($\log_2$). Survival analysis was conducted in the same manner as described above for the SNP survival analysis.

Results

Descriptive statistics

A total of 8,599 cases were recruited across the 11 cohorts; 3,965 of these were included in the analysis after dropping ineligible cases (Table 1). As has been reported previously,[4] there were no major differences between cohorts in age distribution (Fig. 1). Breslow thicknesses were similar across most of the cohorts with the exception of the Riga and Essen cohorts, where cases have been recruited with thicker tumors on average, or were recruited from a clinic serving as a referral center, respectively. As all cases in the Vienna FFPE cohort (recruited between 1997 and 2002) had a sentinel node biopsy this cohort also had a bias toward tumors thicker than 1 mm. There was considerable variation in the time period in which cases were recruited across the cohorts though the majority of cases had been recruited within the last 10 years.

Supporting Information Figure S1 shows Kaplan–Meier estimates for survival in each cohort. The curves for most cohorts are similar, except for Essen and Riga for which we see worse prognosis, consistent with the recruitment, on average, of cases with thicker tumors in both of these cohorts.

Analysis of the association of the PARP1 rs2249844 SNP with survival

Figures 2*a* and 2*b* and Table 2 show the association of rs2249844 with OS in the 11 BioGenoMEL cohorts. Overall, in a random effects meta-analysis there was a significant association seen across the 11 cohorts (HR = 1.17, 95% CI 1.06–1.30, $p = 0.003$, adjusted for age, sex and tumor site); the significant association persisted when the large Leeds cohort was omitted (HR = 1.20, 95% CI 1.06–1.35, $p = 0.003$). Additional adjustment for Breslow thickness did not appear to attenuate this association (HR = 1.17, 95% CI 1.04–1.33, $p = 0.01$). There was no evidence of significant heterogeneity among the cohorts ($I^2 = 0\%$, Cochran's Q, $p = 0.7$).

Observed MAF of rs2249844 ranged from 0.26 to 0.40 in the 11 cohorts. To determine whether the observed frequencies were significantly different from the frequency of the SNP recorded for the CEU population in HapMap (MAF = 0.36) we compared the counts of the SNP in each

Table 1. Cases eligible for analysis in the 11 cohorts that comprise the meta-analysis of the association of the rs2249844 SNP (A>G) with outcome

Center	Whole cohort size	Incident case (recruited <2 years after diagnosis)	Cases genotyped for the SNP	Number of cases with a single melanoma	Cases with complete data on adjusting covariates				Number with Breslow >0.75 mm	Cases with complete follow-up
					Age	Sex	Site	Breslow		
Leeds	2,180	2,131	1,696	1,648	1,648	1,648	1,648	1,608	1,422	1,419
Vienna	1,085	389	173	173	164	164	152	151	140	140
Vienna FFPE	302	302	295	287	284	284	284	280	268	268
Stockholm	870	605	264	264	264	264	264	264	240	240
Lund	355	355	342	342	342	342	321	321	166	166
Athens	200	200	197	197	197	197	197	197	191	190
Riga	243	242	226	224	224	224	224	194	175	175
Barcelona	398	358	345	304	290	289	287	278	269	269
Valencia	1,440	1,248	647	647	649	649	633	579	394	393
Essen	941	643	615	599	563	563	482	384	298	298
Tampa	585	585	422	422	422	422	422	420	407	407
Total	8,599	7,058	5,222	5,107	5,047	5,046	4,914	4,676	3,970	3,965

Cases in each column also meet the criteria of all conditions to the left of it.

cohort with those in the CEU population. No significant difference was seen for any cohort at the 5% level though a borderline difference was observed for the Austrian cohort (MAF = 0.26, Fisher's exact test $p = 0.06$) (data not shown).

By using OS as the outcome we could potentially be reporting an association of the *PARP1* SNP with a nonmelanoma-related outcome. Therefore, we investigated the association of rs2249844 with MSS in the eight cohorts for which we had data on cause of death (Fig. 2*c*, Supporting Information Table S4). Overall there was little difference in the association of the variant for MSS (HR for melanoma-specific death 1.19, 95% CI 1.01–1.39, $p = 0.03$) compared to OS for the same cohorts (HR for death 1.15, 95% CI 1.03–1.29, $p = 0.01$). This suggests that our OS data are a reasonable proxy for MSS in the cohorts for which these data are unavailable.

Determination of potential functional elements associated with rs2242844 using bioinformatics tools

Identification of noncoding regulatory elements. We found evidence of three DNAse-sensitive regions in foreskin melanocyte primary cells, one of which corresponds to the transcription initiation region (suggesting that in melanocytes the *PARP1* gene is actively expressed), one located in the first intron and one located 12 kb upstream of the gene (Supporting Information Fig. S3).

The upstream peak is predicted by the ChromHMM algorithm to contain a weak enhancer region, which suggests it could potentially have a role in increasing *PARP1* transcription levels, whereas the intron peak is predicted to contain an insulator region, which could potentially protect the gene by acting as a "barrier" that prevents silencing of the gene through advance of heterochromatin formation.[25,26] To find clues as to what kind of transcription factors may bind to these regions we queried the JASPAR database of regulatory motifs with the sequence of each peak and found that JASPAR predicted the best scoring motif to be RREB1 (ras-responsive element binding protein 1) and RUNX1 (Runt-related transcription factor 1) for the upstream peak (Supporting Information Table S5). The best scoring motifs for the intronic peak were for NF-κB and TATA-binding proteins (Supporting Information Table S6). It should be stressed that JASPAR has poor sensitivity and these predictions require confirmation through experimental data. We did, however, see some experimental evidence of NF-κB binding in the region of the intron peak from ENCODE ChiP-seq data (Supporting Information Fig. S4).

We found experimental evidence in other cell lines for the existence of a Maf transcription factor-binding site in close proximity to the SNP (details are given in Supporting Information and Supporting Information Figs. S5–S10). There is no evidence to show that this region is active in normal foreskin melanocyte primary cells but it could potentially play an active role in melanoma cells.

Identification of functional elements using SNPinfo. We used the SNPinfo prediction tool to predict the function of SNPs in *PARP1* 10 kb downstream and 150 kb upstream listed for the CEU population in HapMap. We saw no evidence that the SNP itself was associated with any transcription factor-binding sites, miRNA-binding sites or that it is particularly well conserved (see Supporting Information). There was no evidence of a common coding SNP within the gene that would have a large effect on PARP1 function. There was some evidence of miRNA binding seen in the 3′ tip of the gene, which may have functional relevance. Two

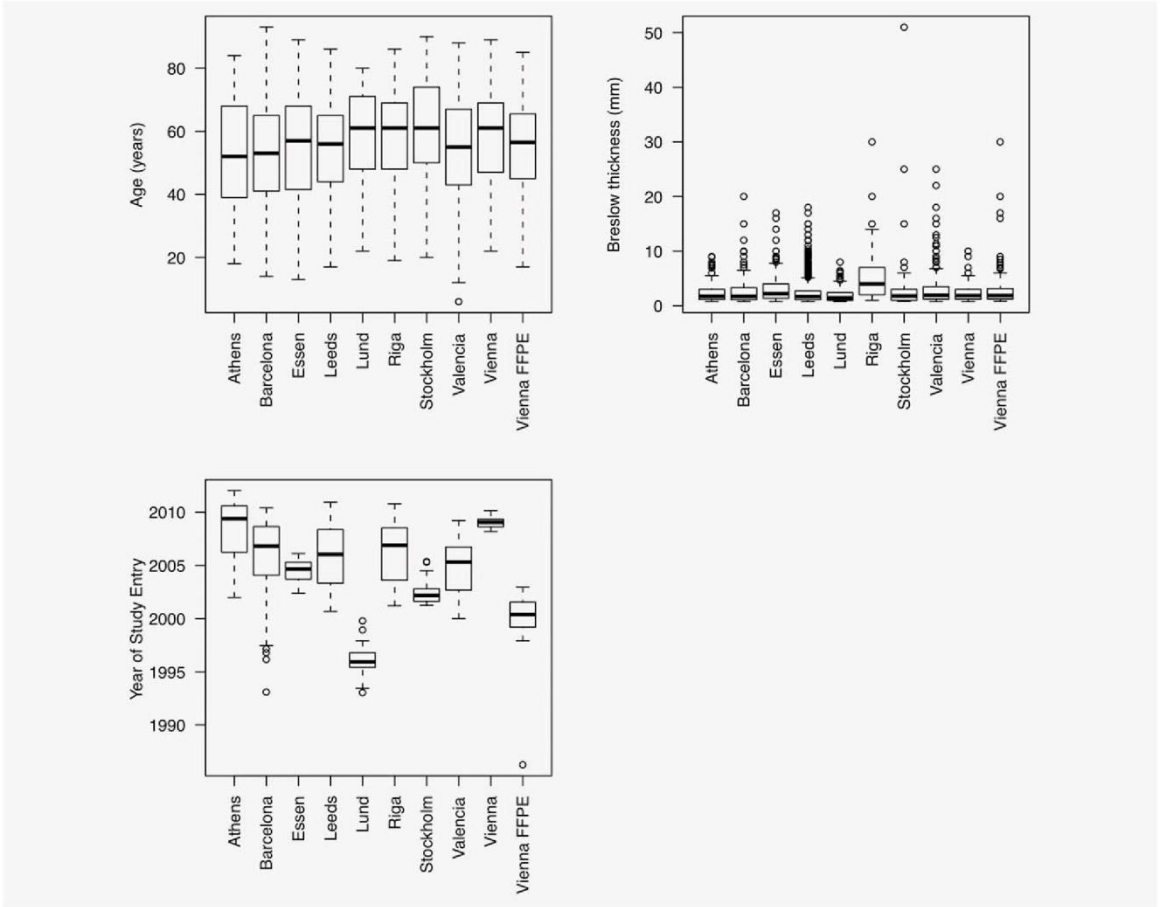

Figure 1. Box plots showing variation of Breslow thickness (>0.75mm), age of diagnosis and date of study entry in each of the ten cohorts. Individual patient data were not available for the Tampa cohort. For the Lund and Athens cohorts date of study entry was not available so date of diagnosis is presented; date of study entry was within 2 years of diagnosis.

SNPs (rs11541664 and rs1059040) were predicted by the PolyPhen feature of SNPinfo to be potentially damaging to PARP1 functionality. Both of these occur in regions that SNPinfo predicts are exonic splicing silencers, which could disrupt splicing activity. These are, therefore, both theoretically good functional candidates; however, these variants appear to be very rare (no variant alleles were seen for rs11541664 in the HapMap dataset and no information on allele frequency is available for rs1059040). In practice it is unlikely that these two variants alone could explain the association of rs2242844 with outcome.

Investigation of eQTL associated with PARP1. We used Genevar to investigate whether rs2242844 was associated with expression of genes in the region around *PARP1*. We found no evidence to support a significant association of this SNP with expression of any expression probe in the region, in the full published MuTHER Twins UK Skin data (Supporting Information Figs. S11 and S12), although we could not test *PARP1* directly as no probe data for it were present. No significant association of the SNP was seen in the smaller MuTHER Twins pilot data in which, curiously, *PARP1* probes were present (Supporting Information Figs. S13–S15). From the Chicago eQTL browser one significant QTL was identified in the region around *PARP1*: a variant 4 kb downstream of *PARP1* was found to be significantly associated with DNAseI sensitivity.[27] DNAse sensitivity is a marker of regions of open chromatin, but there is no complimentary evidence of an association of an eQTL with the tagged variant (Supporting Information Fig. S16).

Expression data

To investigate the association of tumor gene expression levels with outcome, RNA was extracted from tumor blocks from the Leeds Predicting Response to Chemotherapy Study. Higher levels of *PARP1* expression were seen where the primary tumors had been ulcerated (Supporting Information

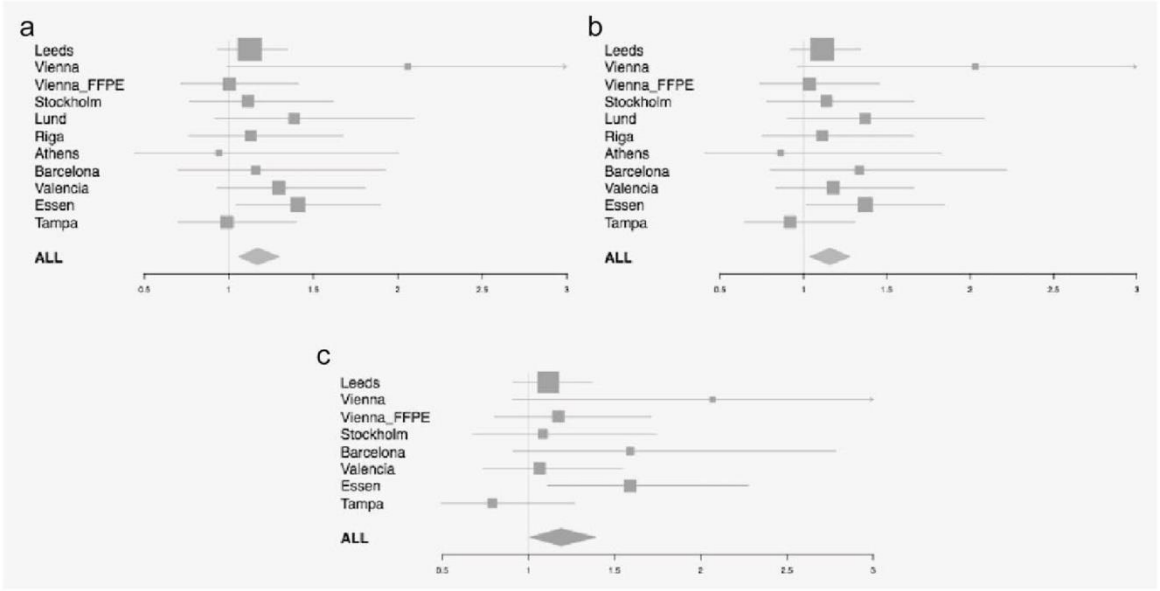

Figure 2. Forest plot of the association of the rs2249844 SNP (A>G) with overall survival in 11 cohorts. Adjusted for (*a*) age, sex, site, (*b*) additionally for Breslow thickness and (*c*) using melanoma-specific survival as the end point.

Table 2. Association of the rs2249844 SNP (A>G) with overall survival (truncated at 8 years of follow-up) in each of the eight melanoma cohorts

Center	Cases	Minor allele frequency[1]	No. of deaths	HR (95% CI)[2]	*p*-Value	HR (95% CI)[3]	*p*-Value
Leeds	1,419	0.32	258	1.12 (0.93–1.35)	0.2	1.11 (0.92–1.34)	0.3
Vienna	140	0.26	15	2.06 (0.99–4.28)	0.05	2.03 (0.97–4.27)	0.06
Vienna FFPE	268	0.33	77	1.00 (0.72–1.41)	1	1.04 (0.74–1.46)	0.8
Stockholm	240	0.40	65	1.12 (0.77–1.62)	0.6	1.14 (0.78–1.66)	0.5
Lund	166	0.33	45	1.39 (0.92–2.10)	0.1	1.37 (0.90–2.08)	0.1
Athens	190	0.31	17	0.94 (0.44–2.00)	0.9	0.86 (0.41–1.83)	0.7
Riga	175	0.33	56	1.13 (0.76–1.68)	0.5	1.11 (0.75–1.66)	0.6
Barcelona	269	0.29	41	1.16 (0.70–1.93)	0.6	1.34 (0.80–2.22)	0.3
Valencia	393	0.30	77	1.30 (0.93–1.81)	0.1	1.18 (0.83–1.66)	0.4
Essen	298	0.32	96	1.41 (1.04–1.90)	0.03	1.37 (1.02–1.85)	0.04
Tampa	407	0.33	82	0.99 (0.70–1.40)	0.97	0.92 (0.65–1.31)	0.7
Combined− Leeds[4]	2,546		571	1.20 (1.06–1.35)	0.003	1.17 (1.04–1.33)	0.01
Combined+ Leeds[4]	3,965		829	1.17 (1.06–1.30)	0.003	1.16 (1.04–1.28)	0.005

Cox proportional hazard models were fitted assuming an additive effect.
[1]Frequency in CEU population in HapMap = 0.36.
[2]Cases adjusted for age, sex, site of primary and a single primary melanoma recruited no more than 2 years after diagnosis.
[3]Cases additionally adjusted for Breslow thickness data > 0.75 mm.
[4]Meta-analysis results assume a random effects model.

Table S7, $n = 152$, fold change 1.31, $p = 0.007$), although this effect was restricted to primary ($n = 51$) rather than metastatic tumors ($n = 58$). The association between ulceration and *PARP1* expression remained significant in logistic regression analysis when adjusted for age, sex and Breslow thickness (Table 3, $n = 109$, OR = 1.99 per $\log_2$ expression, 95% CI 1.02–3.88, $p = 0.04$; unadjusted values, Supporting Information Table S8). In multivariable survival analysis (Table 4; unadjusted values, Supporting Information Table S9 and Fig. S17), increased *PARP1* expression levels were associated with

Table 3. The association of *PARP1* gene expression taken from formalin-fixed paraffin-embedded tissue samples with ulceration

		PARP1 gene expression (per log2 expression unit)	
	n	OR (95% CI)	*p*-Value
Overall	109	1.99 (1.02–3.88)	0.04
Primary tumors	51	7.25 (1.69–31.08)	0.008
Metastatic tumors	58	1.00 (0.46–2.35)	1

A significant association of greater ulceration with greater *PARP1* expression is seen for these data in both primary tumors and metastatic tumors. Analyses are adjusted for age, sex and Breslow thickness.

Table 4. The association of *PARP1* gene expression taken from formalin-fixed paraffin-embedded tissue samples with overall survival

		PARP1 gene expression (per log2 expression unit)	
	n	HR (95% CI)	*p*-Value
Overall	149	1.61 (1.20–2.15)	0.002
Primary tumors	67	1.95 (1.20–3.15)	0.007
Metastatic tumors	82	1.73 (1.15–2.58)	0.008

A significant association of poorer outcome with greater *PARP1* expression is seen for these data in both primary tumors and metastatic tumors. Analyses are adjusted for age, sex and Breslow thickness.

poorer outcome (HR for death = 1.61 per $\log_2$ unit of expression, 95% CI 1.20–2.15, $p = 0.002$).

Discussion

We have investigated the association of rs2249844 with survival across 11 melanoma cohorts. We have demonstrated a significant association with increased risk of death for individuals with the minor allele. Rs2249844 is located 5.3 kb 5′ of *PARP1* and is in LD ($R^2 = 0.96$) with the intronic *PARP1* SNP rs3219090, which has been shown to be associated with reduced risk of melanoma.[12] We have also presented additional evidence for a role of PARP1 in melanoma, as elevated *PARP1* gene expression in tumors was associated with poorer outcome in primary tumors of individuals who ultimately developed stage IV disease and were associated with ulceration in primary tumors. The observed association was not demonstrated to be strong enough to reach genome-wide significance. However, prior knowledge of the functional roles of *PARP1* and its previous association with melanoma risk increases the confidence we have that the association observed is a true positive.

The strengths of our study are the size of the Leeds cohort, combined with ten melanoma cohorts from Europe and North America. Although some of these cohorts are small, we have demonstrated that combination in a meta-analysis gives sufficient power to identify significant associations with survival.

A weakness of the study was that we were only able to use OS as the end point across all cohorts. However, MSS was available for the majority of cohorts, and in a direct comparison of cohorts that have both MSS and OS data there was no overall difference in the estimated association of the variant with both outcome measures. We applied sensitivity analysis, but showed no evidence that any one cohort unduly influenced the observed association. A second weakness of the study is that there are no published data demonstrating whether the *PARP1* SNP results in lower or higher expression of PARP1. PARP1 fulfills a plethora of cellular roles, including the regulation of inflammation,[28] differentiation[29] and control of the secretome,[11] which might have effects on the tumor or the normal tissue responses to the tumor, and we cannot postulate which of those effects is most biologically relevant.

A third weakness was that we did not exchange samples for quality control; however, samples were screened in three centers only (Leeds, Heidelberg and Tampa) and we have exchanged samples in the past within the consortium and demonstrated very high concordance for *CDKN2A* mutation detection[30] and *MC1R* variant detection.[4] Finally, we may have introduced a small amount of survival bias into the study by allowing recruitment for up to 2 years after diagnosis. However, SEER 13 data (generated from white melanoma cases, 1992–2009) show that overall less than 5% of cases die within the first 2 years.

Our findings have potential clinical significance. The tumor gene expression data (see below) suggest that higher levels of *PARP1* are associated with poorer outcome. A recent article by Rodríguez *et al.* supports the notion that *PARP1* plays a role in melanoma progression; PARP inhibition interfered with the endothelial to mesenchymal transition, suppressed vasculogenic mimicry and protected against lung metastasis in mice models.[31]

Where cells have become overly reliant or "addicted" to a DNA repair pathway, therapeutic efficacy may result from targeted disruption of the pathway. Our observations of increased expression of *PARP1* in poor prognosis tumors do lend support to the view that PARP inhibitors might play a role in combination therapies for melanoma patients.

We report an association between *PARP1* expression in tumors and ulceration, which was independent of Breslow thickness. Ulceration is a powerful prognostic indicator and is recognized as such in the AJCC staging system.[32] Its presence rather paradoxically also appears to predict benefit from adjuvant interferon therapy, and therefore it is possible that it is a biomarker of a specific biological difference between melanomas.[33] We have previously reported that ulceration is associated with evidence of macrophage-driven tumor inflammation and lymphatic invasion[34] and the data reported here suggest that *PARP1* may play a role in determining this phenotype.

Inheritance of the minor allele in the *PARP1* SNP rs2249844 was associated with a reduced risk of melanoma in

a genome-wide association study[12] but increased risk of death from melanoma. Intuitively, we might expect that in general alleles associated with greater risk would be associated with poorer survival, but examples of the opposite relationship have been previously documented in the literature.[4,35] To understand the biological implications of this observation, the functional correlates of the inherited variant SNP would require correlation between germline genotyping and expression data from normal tissues of the same individual, which is beyond the scope of this article. Bioinformatic analyses may offer tentative indicators of mechanisms by which a gene's cellular activity may be altered but are greatly limited by a paucity of existing data. Our bioinformatic analyses show some evidence that rs2249844 lies in melanocytes within a transcriptionally active region, which contains putative binding sites for transcription factors such as RREB1 and NF-κB that are involved in signaling pathways known to be important in melanoma.

The study has provided further support for the view that inherited variation may moderate survival expectations for cancer patients and reveal biological pathways of importance in host/tumor interaction.

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4. DISCUSSION

This thesis reports associations between various germline variants and melanoma risk using data from the Latvian population. Several previous studies have reported the role of genetic variants in *CDKN2A* and *CDK4* on melanoma in the Latvian population mainly in a family setting (Pjanova *et al.* 2009; Pjanova *et al.* 2007; Pjanova *et al.* 2006a; Pjanova *et al.* 2006b; Pjanova *et al.* 2003). This work has continued and expanded research of melanoma genetics in the Latvian population.

Since the very beginning of the research of germline genetic factors underlying melanoma risk and development in 1990-ies, the field has largely expanded. The first studies were mostly dedicated for variations in two high melanoma risk genes *CDKN2A* and *CDK4* in melanoma families, however, soon enough it was established that changes in these genes are found only in about half of melanoma families leading to the conclusion that there must be other still unknown genes involved in disease development in these families.

4.1. High melanoma risk genes in the Latvian population

4.1.1. *CDKN2A*

CDKN2A is known as the main high melanoma risk gene all around the world, and many studies have demonstrated its role in melanoma development in families all over the world (Potrony *et al.* 2015). Previous studies in the Latvian population have identified several variants in *CDKN2A*, however, none of them showed a convincing association with melanoma (Pjanova *et al.* 2009; Pjanova *et al.* 2007; Pjanova *et al.* 2003). These studies examined protein coding, 5' and 3' untranslatable regions of *CDKN2A*. In the study presented here analysis also included deeper *CDKN2A* intron sequences as well as both promoter regions.

Using Multiplex Ligation-dependent Probe Amplification (MLPA) approach, a novel deletion c.-20677_ 20682delGTACGC in the promoter region of *CDKN2A* alternative product p14ARF was detected in a patient who had developed melanoma at a relatively early age of 40. His father was diagnosed with melanoma at the age of 78. Unfortunately, DNA material from the father or other family members was not available for analysis therefore it was not possible to estimate deletion segregation with the disease within this family. Genotyping of 211 sporadic melanoma patients and 326 control persons demonstrates a statistically significant association between the deletion and melanoma with OR=6.35 and p=0.02, however, data should be interpreted cautiously due to the limited study power. Previous studies have detected up to several kbp large melanoma-associated deletions affecting more than one exon of *CDKN2A* (Helsing *et al.* 2008; Lesueur *et al.* 2008; Knappskog *et al.* 2006; Mistry *et al.* 2005). The deletion found in this study is much smaller and located rather far from the coding region. However, several single nucleotide variants within promoter region of p16INK4A—the second product of the *CDKN2A*—have previously been demonstrated to have an association with familial melanoma and some of them are also proven to be functionally relevant (Andreotti *et al.* 2016; Bisio *et al.* 2010; Harland *et al.* 2000; Liu *et al.* 1999). Deletion c.-20677_ 20682delGTACGC could also potentially affect p14ARF transcriptional regulation through an autoregulatory feedback loop that involves TP53 and MDM2 (Robertson & Jones 1998).

Besides 6bp p14ARF promoter deletion, a novel intronic *CDKN2A* variant IVS2+82C>T was discovered in a melanoma patient with one case of melanoma and several cases of other cancer types in the family. Variant IVS1+1569T>A (c.150+1569T>A; rs138967562) was found in two melanoma patients with a family history of melanoma as well

as other types of cancer. This variant has not previously been described in relation to melanoma. Genotyping in the case-control sample did not reveal a statistically significant association within the sporadic melanoma patient group. Previous studies have described other variants located within *CDKN2A* introns associated with melanoma in families. Some of them are located in splice sites and cause aberrant splicing, for example, IVS1-1G>C (Sargen *et al.* 2016; Hocevar *et al.* 2006; Petronzelli *et al.* 2001) or IVS2+1G>T (Loo *et al.* 2003; Rutter *et al.* 2003; Hussussian *et al.* 1994). Intronic variants discovered in this study are located far from canonical splice acceptor and donor sites, however, other studies describe several deep intron variants such as IVS1+1104C>A, IVS1-1104C>G and IVS2-105A>G that are associated with melanoma development within families and the latter also creates a false splice donor site (Goldstein *et al.* 2006; Harland *et al.* 2005a; Harland *et al.* 2001). Thus, variants described in the current study could also potentially be functionally important, however, further studies are required.

A plausible explanation for the absence of *CDKN2A* disease causing variants in Latvian melanoma families might be the fact that these two recurrent cases within the family are actually sporadic melanomas and segregation is a simple coincidence or there might be shared environmental factors. Given the fact that several other high risk genes have been discovered in recent years, and there is a wide spectrum of known medium and low melanoma risk genes, alternatively, disease in these families might be caused by some currently unknown underlying genetic factor or combination of them.

4.1.2. *CDK4*

The second high melanoma risk gene analysed within the framework of the thesis study was *CDK4*. Previously two Latvian melanoma families carrying variant in *CDK4* hot-spot position p.Arg24His have been described (Pjanova *et al.* 2009; Pjanova *et al.* 2007). This study reports the third *CDK4* melanoma family in Latvia with the same missense change p.Arg24His. The patient was 40 years old at the time of diagnosis and his father, grandmother and two of the grandmother's siblings also had melanoma. Besides melanoma, several other types of cancer were observed in the family history. Most of the known Latvian melanoma families described so far comprise two melanoma cases, however, all three currently known Latvian *CDK4* families including the third family reported here have five confirmed melanoma cases. Haplotype analysis of the third Latvian *CDK4* melanoma family showed that this haplotype is similar to that of the two previously described families indicating a founder effect (Pjanova *et al.* 2009).

Besides p.Arg24His, another missense change p.Arg24Cys has been found in several melanoma families around the world. Haplotype analysis shows that these variants have independently arisen multiple times in different populations indicating that codon 24 is a mutational hot-spot (Molven *et al.* 2005). During the thesis study, a larger international collaborative study was carried out where information and data about all *CDK4* melanoma families known at that time were analysed. As a part of the thesis information about all three Latvian melanoma families, including phenotype data, was collected and summarized as well as additional genotyping of *MC1R* gene within these three families was performed. Data was sent to the collaborative partners and included in the overall analysis. Altogether information was collected about 17 melanoma families from 8 countries. Given the fact that at the time of the study execution, only such a small number of *CDK4* melanoma families had been reported, three families from Latvia is a relatively large number. Knowing that so far only one change in *CDKN2A* promoter has been associated with melanoma, *CDK4* remains the main high melanoma risk in Latvia and, given the small population (less than two million

inhabitants), it exhibits a strong founder effect resulting in relatively high occurrence of *CDK4* variant in the Latvian population. The overall study results demonstrated that *CDK4* melanoma families are phenotypically characterized by an early disease onset, development of MPM and increased number of atypical naevi—features that are all also characteristic to *CDKN2A* melanoma families (Taylor *et al.* 2017; Taylor *et al.* 2016; Pedace *et al.* 2011; Goldstein *et al.* 2007). Thus *CDK4* and *CDKN2A* families phenotypically cannot be distinguished. Therefore *CDK4* exon 2 should be tested whenever a melanoma family is negative for *CDKN2A* disease causing variants.

4.2. Medium and low melanoma risk genes in the Latvian population

Currently three genes have been titled as medium melanoma risk genes (*MC1R*, *MITF*, *CLC45A2*). Within the framework of this study two of these genes (*MC1R*, *MITF*) were analysed in the Latvian population.

4.2.1. *MC1R*

After the discovery and initial studies of *CDKN2A* and *CDK4*, *MC1R* was one of the next major melanoma risk gene candidates as it functionally is highly involved in pigmentation synthesis regulation. In the fair-skinned population, this gene is highly polymorphic in the general population (Pérez Oliva *et al.* 2009) with overall variant frequency in Europe ranging from about 20-40% in Mediterranean countries and increasing according to geographical latitude to 60% in the British Isles and Nordic countries (Höiom *et al.* 2009; Gerstenblith *et al.* 2007). The study presented here is the first study of *MC1R* gene variants and their association with melanoma risk in the Latvian populations. According to the results of this study, frequency of *MC1R* variants in the Latvian population is 58% that is close to the British Isles and Sweden (Höiom *et al.* 2009; Gerstenblith *et al.* 2007).

Numerous association studies in different populations all over the world have investigated the influence of *MC1R* variants on genetic predisposition to melanoma and many of these studies have demonstrated associations between *MC1R* variants and the risk of melanoma development, with an OR up to seven (Table 2 in the Literature). The magnitude of risk also depends on the number and type of the *MC1R* variants present (Pasquali *et al.* 2015; Williams *et al.* 2011; Kanetsky *et al.* 2010). Gene dosage effect was replicated in the study presented here where presence of two or more *MC1R* variants is associated with twice higher risk than the presence of only one *MC1R* variant. Individually four *MC1R* variants display a statistically significant association with an increased melanoma risk in the Latvian population, namely, p.Val60Leu, p.Val92Met, p.Arg151Cys, and p.Arg160Trp, with the strongest association being for p.Arg151Cys (OR 4.47; 95% CI=2.19–9.14, $p<0.001$). Variants p.Arg151Cys and p.Arg160Trp demonstrate very strong association in most of the association studies as well as meta-analyses (Table 2 in the Literature) (Antonopoulou *et al.* 2015; Pasquali *et al.* 2015; Williams *et al.* 2011) and this study replicates these results in the Latvian population. Furthermore, functional consequences of both of these variants on receptor activity have been well described (Table 1 in the Literature).

The association between the remaining two *MC1R* variants p.Val60Leu and p.Val92Met and an increased melanoma risk varies more among different populations. About half of the studies show no association between p.Val60Leu and the risk of melanoma, however, a positive association has been demonstrated in studies from Swedish (Höiom *et al.* 2009), Polish (Debniak *et al.* 2006), Dutch (Kennedy *et al.* 2001), French (Matichard *et al.* 2004), Greek (Stratigos *et al.* 2006) and Spanish populations (Scherer *et al.* 2009; Gudbjartsson *et al.* 2008; Fernandez *et al.* 2007) and it is also reflected in meta-analyses with

OR ranging from 1.14 to 1.47 (Antonopoulou *et al.* 2015; Pasquali *et al.* 2015; Williams *et al.* 2011). Variant p.Val92Met shows no association with melanoma in the majority of the studies, still an association has been found in Dutch (Kennedy *et al.* 2001), Spanish (Fernandez *et al.* 2007) and US populations (Han *et al.* 2006a) as well as in meta-analyses with ORs 1.08–1.55 (Antonopoulou *et al.* 2015; Pasquali *et al.* 2015; Williams *et al.* 2011). While several other variants are associated with melanoma risk in different populations, none of them are associated with melanoma risk in the Latvian population; however, it could be explained by relatively low variant frequency and insufficient sample size. For example, variant p.Asp84Glu is associated with melanoma in Norwegian (Helsing *et al.* 2012), Swedish (Gudbjartsson *et al.* 2008), German (Scherer *et al.* 2009; Mossner *et al.* 2007), Dutch (Kennedy *et al.* 2001), French (Guedj *et al.* 2008) and Australian (Duffy *et al.* 2010a) populations and another variant p.Asp294His is associated with melanoma risk in Swedish (Gudbjartsson *et al.* 2008), French (Guedj *et al.* 2008), Spanish (Ibarrola-Villava *et al.* 2010; Scherer *et al.* 2009; Gudbjartsson *et al.* 2008; Fernandez *et al.* 2007), Italian (Fargnoli *et al.* 2006), Australian (Duffy *et al.* 2010a) and US (Guan *et al.* 2013; Council *et al.* 2009; Han *et al.* 2006a) populations, and both of these variants are associated with melanoma risk in meta-analyses (Antonopoulou *et al.* 2015; Pasquali *et al.* 2015; Williams *et al.* 2011). However, in the Latvian population variants p.Asp84Glu and p.Asp294His were found only in one patient each and were not found in controls, therefore association with melanoma risk could not be properly evaluated. A couple of other variants are associated with melanoma in several populations—p.Arg142His in German (Scherer *et al.* 2009) and Greek (Stratigos *et al.* 2006) populations, variant p.Ile155Thr in German (Scherer *et al.* 2009) and Spanish populations (Fernandez *et al.* 2007) and Ashkenazi Jews (Galore-Haskel *et al.* 2009), and variant p.Arg163Gln in Polish population (Debniak *et al.* 2006) as well as all three of these variants in meta-analyses (Antonopoulou *et al.* 2015; Pasquali *et al.* 2015; Williams *et al.* 2011), however, in our population they were found with similar frequencies in patients and controls. As sample size is the main limitation of this association study, a larger sample possibly would reveal effects of both rare variants and those with similar frequencies within these patient and control groups. The same should be mentioned with regards to subgroup analyses as some of the subgroups were small with limited ability to detect associations. It also should be noted that due to the sample size multiple testing for the association analysis was not performed, which might have led to the overestimation of some p-values. The results also could possibly not reflect the complete truth as the controls enrolled in the study on a volunteer basis, which may have caused a selection bias.

Besides *MC1R*, some other variants within the chromosomal region encompassing *MC1R* have demonstrated association with melanoma risk in GWAS studies—rs258322 (located within an intron of the cyclin-dependent kinase 10 gene, *CDK10*), rs4785763 (located within the AFG3-like matrix AAA peptidase subunit 1 of the pseudogene *AFG3L1P*) and rs8059973 (located within flanking 5'UTR of dysbindin domain containing 1 gene, *DBNDD1*) (Barrett *et al*. 2011; Bishop *et al*. 2009). Moreover, meta-analyses indicate that rs4785763 and rs258322 are associated with melanoma risk (Antonopoulou *et al*. 2015; Gerstenblith *et al*. 2010) and recently the associations between both variants and melanoma were replicated in a two-stage GWAS (Ransohoff *et al*. 2017). However, none of the genotyping arrays used in these GWAS contained *MC1R* variants. Therefore a question existed, whether the risk conveyed by variants in the 16q24.3 region is independent or is it accounted for by *MC1R* variants. Variants rs258322 and rs4785763 have also demonstrated association with hair colour, skin pigmentation, and tanning in several pigmentation GWASs, and in these studies after the adjustment for *MC1R* variants p.Arg151Cys, p.Arg160Trp and

p.Asp294His these associations mostly disappeared (Gerstenblith *et al*. 2010; Nan *et al*. 2009b; Han *et al*. 2008; Sulem *et al*. 2007). In order to characterize these interactions in conjunction with melanoma, variants in the *MC1R* were juxtaposed with the above mentioned non-coding variants in the 16q24.3 region as well as detailed haplotype analysis was performed. Results confirmed previous observation that variant p.Arg151Cys among all *MC1R* variants have the strongest association with melanoma in our population. Further analysis also showed that p.Arg151Cys retained its significance in multivariate models. Besides p.Arg151Cys, small nominal association with melanoma was also detected for variant rs258322, however, it did not enter any multivariate model after the inclusion of p.Arg151Cys. In addition, the LD analysis suggested a rather high correlation between p.Arg151Cys and rs258322. Altogether these results demonstrate that the observed influence on melanoma risk by rs258322 can most likely be accounted by p.Arg151Cys. Previously one study presented in a conference (Demenais *et al*. 2009) has also explored whether the association signals in the 16q24.3 region might be accounted for by *MC1R* variants using dataset in which the associations of chromosome 16 variants with melanoma were first discovered (Bishop *et al*. 2009). Both their stepwise regression models and haplotype analyses showed that the rs258322's association was accounted for by *MC1R* variants p.Arg151Cys and p.Arg160Trp.

Another variant nominally associated with an increased risk of melanoma was rs4785763. Unlike rs258322, variant rs4785763 was selected by stepwise regression, with and without cofactors, alongside the *MC1R* variant p.Arg151Cys thus suggesting some degree of independence between these variants. It was also supported by the respective haplotype analyses and by the rather small LD between these two variants ($r^2 = 0.160$). Interestingly, rs4785763 demonstrates some interaction with age and sex, as the inclusion of age and sex in both univariate and multivariate regression models enhanced the power of detecting rs4785763 as significant. Variant rs4785763 is located ~80 kb away from *MC1R* and resides within the pseudogene *AFG3L1P* that is orthologous to the murine gene Afg3l1. Murine Afg3l1 encodes a subunit of the ATP-dependent proteolytic complex localized in the inner membranes of mitochondria, while the human orthologue only produces noncoding mRNA (Kremmidiotis *et al*. 2001). While rs4785763 is located only in a pseudogene, evidence suggests that pseudogene products play a variety of roles in cancer biology (Poliseno *et al*. 2015) and one could speculate that genetic variants in these transcripts also might have some influence on the activity of pseudogene products. In the previous study (Demenais *et al*. 2009) rs4785763 effect similarly to rs258322 was explained by the presence of *MC1R* variants, however, results from study presented here suggests that rs4785763 operate quite independently from p.Arg151Cys.

Several studies have noted that the association of *MC1R* variants with melanoma risk was stronger or limited to persons with protective cutaneous phenotypes, that is, persons with darker hair and darker skin (Pasquali *et al.* 2015; Kanetsky *et al.* 2010; Raimondi *et al.* 2008; Matichard *et al.* 2004; Kennedy *et al.* 2001; Palmer *et al.* 2000). The study presented here also shows that after stratification for the different pigmentation characteristics, *MC1R* still contributes to melanoma risk, demonstrating that *MC1R* variants and pigmentation are independent melanoma risk factors and the *MC1R* genotype provides information about melanoma risk beyond that of the cutaneous phenotype. Thus the combination of *MC1R* genotype and phenotype data might be important to the prediction of melanoma risk in persons with otherwise protective cutaneous phenotypes. It should be noted that this study used self-reported pigmentation characteristics that, due to individual subjectivity, might be inaccurate.

Some evidence shows that *MC1R* variants are associated with one of the major measures of tumour progression—tumour thickness (Landi *et al.* 2005) especially in sun-sensitive individuals (Taylor *et al.* 2015), however, similarly to the study of the Greek population (Stratigos *et al.* 2006) data from the study presented here does not support this finding. Low percentage of thin tumours in Latvian melanoma patients hampers the likelihood to find the difference between *MC1R* variant carriers versus non-carriers stratified by tumour thickness.

This study, however, found an association between the presence of *MC1R* variants and age of melanoma onset. Carriers of *MC1R* variants were older than non-carriers (55 vs 49 years) thus suggesting a possible protective effect of *MC1R* variants as indicated by the belated age of onset. Previous studies had not detected any association between *MC1R* variants and age of disease onset (Stratigos *et al.* 2006; Landi *et al.* 2005). Interestingly, a large collaborative study that explored effects of *MC1R* variants on the survival of melanoma in altogether 3060 patients from 10 cohorts found association between the presence of *MC1R* variants and improved disease survival (HR=0.78, 95% CI=0.65–0.94) thus supporting the observation from the Latvian population. A sample from the Latvian population was also included in this large survival study and while the sample size was relatively small (n=137), results from the Latvian population are in concordance with the combined results (HR=0.87). While initially these results seem counterintuitive as it is well known that *MC1R* variants increase melanoma risk and therefore it is very tempting to think that they might also be associated with a worse disease prognosis, however, underlying mechanisms of disease development are more complex. In this case the protective effect of *MC1R* variants could be explained by non-pigmentary effects of one of its downstream transcription factors *MITF*.

MC1R is known to have a modifying effect in families with *CDKN2A* variants (Demenais *et al.* 2010; Fargnoli *et al.* 2010; Chaudru *et al.* 2005; Box *et al.* 2001a; van der Velden *et al.* 2001); however; it could not be tested in the Latvian sample due to the absence of disease causing *CDKN2A* variants in melanoma families. The comprehensive study of *CDK4* families presented above revealed that there might also be some modifying effect of *MC1R* variants in *CDK4* families. Summarized data showed that *CDK4* positive unaffected family members had significantly fewer *MC1R* RHC variants compared to either *CDK4* positive affected individuals or a *CDK4* negative control group. In addition, patients with MPM had more *MC1R* RHC variants than patients with single primary melanoma. This suggests some enhancing effect of *MC1R* variants on melanoma development.

The study of the *MC1R* variants shown here also explored functional effects of the novel and rare variants found in the Latvian population. Previous studies have mostly explored functional effects of the nine most common and strong *MC1R* non-synonymous variants p.Val60Leu, p.Asp84Glu, p.Val92Met, p.Arg142His, p.Arg151Cys, p.Ile155Thr, p.Arg160Trp, p.Arg163Gln and p.Asp294His. These variants code partially 'loss-of-function' receptors with diminished plasma membrane trafficking and ability to activate the cAMP pathway (Table 1 in the Literature). However, there are some studies that have also investigated functional consequences of some not so common *MC1R* variants and demonstrate that 'loss-of-function' variants are found among them as well (Garcia-Borron *et al.* 2005; Sánchez-Más *et al.* 2002; Jiménez-Cervantes *et al.* 2001a; Jiménez-Cervantes *et al.* 2001b). As a part of the *MC1R* study, an analysis of eight previously functionally uncharacterized rare *MC1R* variants found in the Latvian population was carried out. Four of the rare variants identified in the study p.Phe45Leu, p.Ser83Leu, p.Gly89Arg, and p.Thr95Met are located in the receptor region that is involved in the formation of a ligand-binding pocket (Prusis *et al.* 1997) and thus potentially could alter receptor ligand binding

properties. One more variant potentially involved in ligand binding was p.Asp121Glu as previously p.Asp121Lys, another variant causing a different amino acid substitution in the same position, has been shown to be directly involved in the receptor ligand binding process (Yang *et al.* 1997). Using i*n silico* tools all five variants p.Phe45Leu, p.Ser83Leu, p.Gly89Arg, p.Thr95Met and p.Asp121Lys participating in ligand binding were predicted to be damaging. Three other variants p.Val165Ile, p.Val188Ile, and p.Arg213Trp *in silico* were predicted to be benign, however, results from *in vitro* analyses were not completely consistent with *in silico* predictions. Variants p.Phe45Leu and p.Thr95Met, that *in silico* were predicted to be damaging, resembled wild type receptors in terms of both plasma membrane trafficking and signalling via the cAMP pathway (variant p.Phe45Leu even showed an increased level of cAMP response compared with wild type). Variants p.Val165Ile and p.Val188Ile that were predicted to be benign, indeed had normal cell surface density and normal or only slightly reduced signalling via the cAMP pathway therefore they most likely are benign as predicted *in silico* and their functional activity are comparable to the wild type receptor. The rest of the *MC1R* non-synonymous variants p.Ser83Leu, p.Gly89Arg, p.Asp121Lys and p.Arg213Trp *in vitro* displayed various degrees of diminished cAMP signalling, most likely due to the reduction in cell surface expression, as shown by confocal microscopy. Thus four of eight rare *MC1R* variants p.Ser83Leu, p.Gly89Arg, p.Asp121Glu, and p.Arg213Trp are comparable to 'loss-of-function' RHC alleles that have been widely described before (Table 1 in the Literature). One of the limitations of this study regarding the *in vitro* analyses of the *MC1R* variants was that the model system consisted of cells without melanocytic origin (BHK—baby hamster kidney cells). However, previous studies on other *MC1R* variants were performed using different cell lines and the overall result tendencies remained the same (Beaumont *et al.* 2005). In addition, this study also successfully replicated effects of the well-characterized variant p.Arg151Cys and therefore the overall results from this study should be comparable with data from similar studies.

To sum up, results from the functional analyses demonstrate that a significant subset of rare *MC1R* variants is functionally relevant. Different association studies have used various approaches to categorising less common and novel *MC1R* variants and according to these results many of them should be treated similarly to commonly known RHC variants.

Practically *MC1R* variants might influence risk of melanoma through various pathways. Firstly, diminished receptor activity leads to insufficient production of protective pigments in the skin that might lead to increased somatic mutation rate (Robles-Espinoza *et al.* 2016). There are also a couple of more direct observations of the effect of *MC1R* variants on the risk of melanoma. MC1R with variants might have an advantage in early melanoma development due to better proliferation rates and more effective binding of melanoma cells to the extracellular matrix (Robinson & Healy 2002). MC1R is also expressed in a variety of immune cells, which suggests potential roles in immune-related functions (Maaser *et al.* 2006).

4.2.2. *MITF*

The second intermediate melanoma risk gene investigated within the thesis was a wide profile transcription factor coding gene *MITF* that functionally belongs to the same signalling pathway as the above described medium melanoma risk gene *MC1R*. Melanoma risk has been associated with a particular non-synonymous *MITF* variant p.Glu318Lys that has been demonstrated to have an association with familial as well as sporadic melanoma in several studies from different populations—Australian and the United Kingdom (Yokoyama *et al.* 2011), French (Bertolotto *et al.* 2011), Italian (Ghiorzo *et al.* 2013) as well as a large

Genes, Environment, and Melanoma (GEM) study from Australia, Italy, Canada, and the United States (Berwick *et al.* 2014). These studies showed that variant p.Glu318Lys is rare (MAF<0.01), it segregates with the disease in several melanoma families, has an association with sporadic melanoma with ORs ranging from 2 to 5 and also has an association with RCC and pancreatic cancer. However, a study from Poland found no association between p.Glu318Lys and melanoma risk. Among 748 melanoma patients analysed in the Polish study, there were only two heterozygous carriers of p.Glu318Lys, resulting in a MAF of only 0.001. In addition, no association was found between p.Glu318Lys and other types of cancer (kidney, lung, breast, and prostate) (Gromowski *et al.* 2014). In the study presented here a screening of the *MITF* variant p.Glu318Lys was carried out in the Latvian population. Altogether 702 persons (477 melanoma patients and 225 healthy controls) were included in the study with a particular focus on patients with a family history of melanoma, pancreatic cancer, MPM and early onset patients who all were negative for high melanoma risk variants in *CDKN2A* and *CDK4* genes. Therefore at the time of the study *MITF* variant p.Glu318Lys was a good melanoma risk gene candidate in the Latvian population. However, variant p.Glu318Lys was not found in any of the 702 persons analysed. A possible explanation is that this study simply was underpowered due to an insufficient sample size and a much larger sample is needed to detect variant alleles and carry out statistical analysis. These discrepancies between studies can also be explained by different characteristics of the cases that were included in the analyses. This study in the Latvian population similarly to the Polish study analysed unselected cases, whereas in studies that detected association between p.Glu318Lys and melanoma risk, genetically enriched cases were analysed. Another explanation is genetic differences between populations. Similar population stratification affecting relatively geographically close Polish and Latvian populations has been observed with the *CDKN2A* variant p.Ala148Thr, which has an association with melanoma in Latvian and Polish populations, unlike in several other populations (Pjanova *et al.* 2007). These results suggest that *MITF* variant p.Glu318Lys might not be considered a contributor to melanoma risk in this particular geographical region.

4.2.3. *TP53* and *MDM2*

Besides high and medium melanoma risk genes, so far at least 30 low melanoma risk loci have been identified (Table 3 in the Literature). In this study closer attention was paid to *TP53* and *MDM2* that functionally are interconnected with *MC1R* in pigmentation synthesis signalling pathway. MC1R is activated by α-MSH that initiates an intracellular signal cascade leading to the production of the photoprotective pigment melanin (Hunt *et al.* 1995). Hormone α-MSH in turn is produced in posttranslational processing of pro-opiomelanocortin (POMC). It has been demonstrated that after UV irradiation POMC promoter is activated by one of the major tumour suppressor proteins TP53 (Cui *et al.* 2007). Almost half of human cancers harbour somatic *TP53* mutations (Kandoth *et al.* 2013) and the impact of *TP53* on cancer is not limited only to somatic changes but may also manifest itself through germline variants (Stracquadanio *et al.* 2016).

The most studied *TP53* germline variant with regard to melanoma is p.Pro72Arg (c.215C>G; rs1042522). It is located in a proline rich domain of TP53 that has an important role in TP53-mediated apoptosis (Sakamuro *et al.* 1997; Walker & Levine 1996). Interestingly, Arg and Pro display different functional activity and properties. Arg allele induces apoptosis better and faster than Pro (Pim & Banks 2004; Sullivan *et al.* 2004; Dumont *et al.* 2003; Thomas *et al.* 1999), while Pro allele induces cell-cycle arrest better than Arg (Pim & Banks 2004), more efficiently activates transcription (Thomas *et al.* 1999) and

induces TP53-dependent DNA-repair genes (Siddique & Sabapathy 2006). Both alleles also differ in their geographical distribution—Arg allele is more prevalent in individuals having lighter skin and living in higher latitudes while Pro allele is more widespread in populations with darker skin that live closer to equator, most probably due to evolutionary selection (Beckman *et al.* 1994). Results from the Latvian population are in concordance with this observation—Arg allele is more common in the Latvian population than Pro allele. In previous studies from other populations a positive association with melanoma has been found for both alleles—Arg in Brasilian and US populations (Oliveira *et al.* 2013; Li *et al.* 2008b; Shen *et al.* 2003) and Pro in Greek and German populations (Stefanaki *et al.* 2007; Gwosdz *et al.* 2006). In the Latvian population the common allele Arg is also more common in melanoma patients than controls, however, results from this study did not find a positive association between Arg allele and melanoma risk. Similarly, studies of several other populations also have not found an association between melanoma and any of p.Pro72Arg alleles—the Dutch population (Bastiaens *et al.* 2001c), US Nurses' Health Study (Han *et al.* 2006b), Scottish (Povey *et al.* 2007) and Italian population studies (Capasso *et al.* 2010). However, in a recent meta-analyses study a small effect attributable to Arg allele was identified when specific genotype subgroups (Arg/Pro vs Pro/Pro) were analysed (Geng *et al.* 2015).

Previously only one study has analysed relationships between p.Pro72Arg and *MC1R* gene variants where authors found that the association of Pro allele with melanoma risk was stronger for patients carrying Pro/Pro homozygote who simultaneously did not carry *MC1R* RHC variants p.Arg151Cys, p.Arg160Trp and p.Asp294His (OR=2.99, 95% CI=1.02–8.78) (Stefanaki *et al.* 2007). In the study presented here, when the presence of *MC1R* gene RHC variants were taken into consideration, there were more melanoma patients than controls with Arg/Arg genotype and *MC1R* variants (OR=2.76; 95% CI 1.02–7.52, p=0.040). No such association was found in the presence of p.Pro72Arg Pro/Pro or Pro/Arg genotype leading to the conclusion that Arg/Arg genotype of variant p.Pro72Arg in combination with *MC1R* variants has an additional impact on melanoma risk.

TP53 is negatively regulated by an auto-regulatory feedback loop with E3 ubiquitin ligase MDM2. *MDM2* intron 1 comprises an alternative promoter P2 to *MDM2* that is induced by TP53 (Zauberman *et al.* 1995). *MDM2* variant c.14+309T>G (rs2279744) is located within this promoter and it has been shown that the G allele of rs2279744 increases the affinity for transcription activation factor Sp1 thus leading to increased *MDM2* expression and subsequently to TP53 inhibition that might promote tumour formation (Bond *et al.* 2004). Association studies for melanoma demonstrate an association between *MDM2* variant rs2279744's G/G genotype and melanoma risk depending on age and sex. A couple of studies have demonstrated an association between the rs2279744 minor allele homozygote G/G and melanoma risk, especially for younger women or women with hereditary melanoma (Thunell *et al.* 2014; Firoz *et al.* 2009). Contrary to the previous, another study showed that women with G/G genotype might actually be at lower risk for developing melanoma at a younger age (Cotignola *et al.* 2012). One study also gave evidence for an association between s2279744 genotype G/G and presence of tumours with Breslow thickness >0.75mm (Capasso *et al.* 2010). However, overall, studies do not tend to find a convincing association between the *MDM2* variant and melanoma risk (Oliveira *et al.* 2014; Capasso *et al.* 2010; Nan *et al.* 2009c), and meta-analysis supports this lack of association (Qin *et al.* 2015). This indicates that this variant's association with melanoma risk is present only in smaller subgroups. In the Latvian population c.14+309T>G also did not show association with melanoma risk.

4.2.4. *PARP1*

Another low risk gene briefly examined within this study is poly(ADP-ribose) polymerase 1 gene (*PARP1*), particularly variant rs2249844 that has previously been shown to be associated with a reduced risk of melanoma in GWAS (MacGregor *et al.* 2011). Altogether 243 cases from the Latvian population were included in a large collaborative study that comprised altogether 8599 cases from 11 melanoma cohorts all over the world. Results from all cohorts combined demonstrate that minor allele rs2249844 is associated with an increased risk of death (HR=1.16, 95% CI=1.04–1.28), and results from the Riga cohort were consistent with overall results (HR=1.11, 95% CI=0.75–1.66). These results intuitively contradict with results from GWAS where minor allele of rs2249844 was associated with reduced risk, but here it shows association with poor survival. However later Law *et al*. (2015b) demonstrated similar opposite effects in study of another *PARP1* variant rs3219090—variant, that is in strong linkage disequilibrium with rs2249844. Similarly paradoxical results were detected in another study of *MC1R* discussed above (Paper V) where presence of melanoma risk associated *MC1R* variants was associated with better melanoma survival. Such results illustrate complex underlying functional relations between these genes and variations within them, and other genetic and cellular factors.

4.3. Concluding remarks and future prospects

Studies of melanoma risk genes in the Latvian population so far have demonstrated that the main risk gene in Latvian melanoma families is *CDK4*. Apart from one deletion in p14ARF promoter, no other melanoma associated variants have been found in *CDKN2A*—the gene that is considered to be the main gene associated with familial melanoma development worldwide. Other medium and low risk genes examined in the Latvian population so far contribute a small effect in melanoma predisposition both in a family setting and sporadic cases, which is in concordance with overall findings in other populations worldwide. Researchers are still looking for other high risk genes in about half of the melanoma families with 'missing heritability' and several candidates have already been identified as shown in the literature review. We have also created a HaloPlex^{TM} targeted Ion Torrent sequencing panel that included three of these novel high melanoma risk genes—*BAP1*, *POT1*, *TERT* (as well as already well known high melanoma risk genes *CDKN2A* and *CDK4*) for analyses of patients with an increased likelihood to have a stronger genetic component contributing to disease development. Those were patients with a family history of melanoma and/or other types of cancers, patients with an early age of disease onset (<40 years) and patients with multiple melanoma. So far, we have found several novel or rare potentially functionally significant genetic variants in genes *BAP1*, *POT1* and *TERT*. A rare *BAP1* variant p.Pro293Leu was discovered in one of the UM patients. A novel nonsense *POT1* variant p.Leu153* was detected in a melanoma patient with multiple other cancer cases in the family. A novel *POT1* splice donor site variant c.546+1G>A was found in a young (28 year old) melanoma patient with other cancer cases in the family. In several young patients with other types of cancer in the family rare *TERT* variants p.Ala279Thr and p.Ala1062Thr that previously have been associated with esophageal, lung cancer and leukaemia were identified. Thus, these initial results show that NGS approach has led to the discovery of several novel and potentially functionally significant variants associated with melanoma in Latvia; however, these are findings that need to be further validated.

CONCLUSIONS

1. For the first time in a Latvian melanoma family disease associated 6 bp deletion (c.–20677_ – 20682delGTACGC) in the *CDKN2A* locus has been discovered and is located in the promoter of *CDKN2A* alternative product p14ARF.

2. *CDK4* gene is the main high-risk gene in Latvian melanoma families so far.

3. Melanoma families with *CDK4* germline variants in codon 24 cannot be distinguished phenotypically from *CDKN2A* positive melanoma families—both are characterised by an early onset of disease, increased occurrence of clinically atypical naevi, and development of MPM indicating that in a clinical setting, the *CDK4* gene should always be examined when a melanoma family is negative for *CDKN2A* disease associated variants.

4. The highest melanoma development risk in the Latvian population is associated with *MC1R* variant p.Arg151Cys (OR=4.47, 95% CI=2.19–9.14, p<0.001), and *MC1R* variants have a gene dosage effect—melanoma risk for carriers of two *MC1R* variants is twice as high (OR=3.98, 95% CI=2.15–7.38, p<0.001) than that of carriers of one variant (OR=1.98, 95% CI=1.26–3.11, p=0.003).

5. After *MC1R* gene variant carrier stratification according to the pigmentation phenotype, the risk of melanoma remained in groups with otherwise protective phenotypes.

6. A subset of the rare *MC1R* variants are functionally relevant and significantly hamper functional activity of the receptor, therefore they might be important in melanoma development and should be considered as the high risk variants in *MC1R* analysis.

7. The absence of any consensus *MC1R* alleles is associated with a significantly lower risk of death suggesting a survival benefit for inherited *MC1R* variants in melanoma patients.

8. In addition to *MC1R* variant p.Arg151Cys the association with melanoma in 16q24.3 region was found for variant rs4785763 in pseudogene *AFG3L1P*, and this variant acts independently from p.Arg151Cys.

9. Medium melanoma risk gene *MITF* and its variant p.Glu318Lys variant does not appear to be significant melanoma risk factor in the Latvian population.

10. Variants in low melanoma risk genes *TP53* (p.Pro72Arg) and *MDM2* (c.14+309T>G) are not associated with melanoma in the Latvian population, except for the small impact of *TP53* gene variant p.Pro72Arg Arg/Arg genotype in the presence of *MC1R* RHC variants (OR=2.76, 95% CI=1.02–7.52, p=0.04).

11. The inheritance of a genetic variant rs2249844 of *PARP1* gene in melanoma patients is associated with an increased risk of death.

MAIN THESIS FOR DEFENCE

1. Variant p.Arg24His in *CDK4* gene is the main genetic high melanoma risk factor in the Latvian population, and *CDK4* families can't be phenotypically distinguished from *CDKN2A* families.

2. The main melanoma risk variant in 16q24.3 locus is *MC1R* variant p.Arg151Cys.

3. Melanoma risk in the Latvian population increases depending on the number and presence of specific *MC1R* variants irrespectively to pigmentation phenotype.

4. *MC1R* variants are associated with positive melanoma survival rate.

5. Medium melanoma risk gene *MITF* variant p.Gly318Lys is not a significant melanoma risk factor in the Latvian population.

6. Variants in low melanoma risk genes—p.Pro72Arg in *TP53* and c.14+309T>G in *MDM2*—have a minor impact on melanoma risk, but low risk variant rs2249844 in *PARP1* gene is associated with an increased risk of death of melanoma.

LIST OF ORIGINAL PUBLICATIONS

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7. Ozola A, Pjanova D. 2015. **The lack of E318K *MITF* germline mutation in Latvian melanoma patients.** *Cancer* Genet 208: 355-356.

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APPROBATION OF THE RESEARCH

Thesis results:

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EDUCATION AND RESEARCH EXPERIENCE OF THE AUTHOR

Education

2011–2014	University of Latvia, Faculty of Biology, PhD program in biology—"Melanoma predisposition genes in the Latvian population"
2009–2011	University of Latvia, Faculty of Biology, MSc. biol. with honours—"Low melanoma risk gene polymorphisms in Latvian population"
2006–2009	University of Latvia, Faculty of Biology, BSc. biol.—"Melanocortin-1 receptor gene polymorphisms in melanoma development in Latvian population"

Additional education

2017	The training of the Leica TCS SP8 confocal microscope, Riga, Latvia
2017	Summer Short Course "Introduction to the statistical analysis of genome-wide association studies" organized by Department of Genomics of Common Disease, Imperial College, London, UK
2015	BBMRI-LPC course "Applicability of biobanks: analysis of next-generation sequencing data", Riga, Latvia
2008–2011	German language courses in Goethe-Institut Riga, Latvia

Awards and scholarships

2009–2011	Scholarship from European Social Fund project 2009/0162/1DP/1.1.2.1.1./09/IPIA/VIAA/004 "Support for realization of master study programs in University of Latvia"
2008	19th European Students' Conference (Charité Medical school of Berlin Humboldt University). Award for the best oral presentation in Dermatology session

Work experience

2011–2018	Latvian Biomedical Research and Study Centre, D. Pjanova's lab, scientific assistant
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Teaching experience

2011–2018	Supervision and reviewing of several University of Latvia, Faculty of Biology students' term papers; execution of several laboratory works for biology and medicine students of University of Latvia

Research project experience

2015–2018	TRANSCAN project GENMEL Z/15/1285 - PRL15/15 "Identification of genetic markers involved in development of metastases and second cancers in melanoma (GENMEL)"
2014–2015	European Regional Development Fund project 2014/0021/2DP/2.1.1.1.0/14/APIA/VIAA/058 "Development of novel in vitro tests for diagnostics and prognostics of individualized therapies of tumors and mitochondrial disease treatment"
2011–2013	European Regional Development Fund project 2010/0233/2DP/2.1.1.1.0/10/APIA/VIAA/076 "Prognostic testing for assessment of the effectiveness of immunotherapeutic drugs: method for personalized drug usage in oncology"
2011–2012	European Social Fund project 1DP/1.1.1.2.0/09/APIA/VIAA/150 "The investigation of molecular and genetic mechanisms of pathogenesis and the development of novel means for diagnosis and therapy"
2009–2010	European Economic Area Financial Mechanism grant EEZ09AP-38/08 "Molecular genetic changes in cutaneous malignant melanoma in Latvia"
2009–2010	Scientific project of Student Council of University of Latvia "Deletion analysis in locus 9p21 in melanoma patients in Latvian population"
2007–2011	GenoMEL Nr.018702 "Genetic and environmental determinants of melanoma: translation into behavioural change"

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