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Familial Melanoma and Pancreatic Cancer: studies on genotype, phenotype and surveillance

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Familial Melanoma and Pancreatic Cancer studies on genotype, phenotype and surveillance

Thomas P. Potjer

Colophon

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Familial Melanoma and Pancreatic Cancer studies on genotype, phenotype and surveillance

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We know nothing very certainly
but everything only probably

(Christiaan Huygens, 1629-1695)

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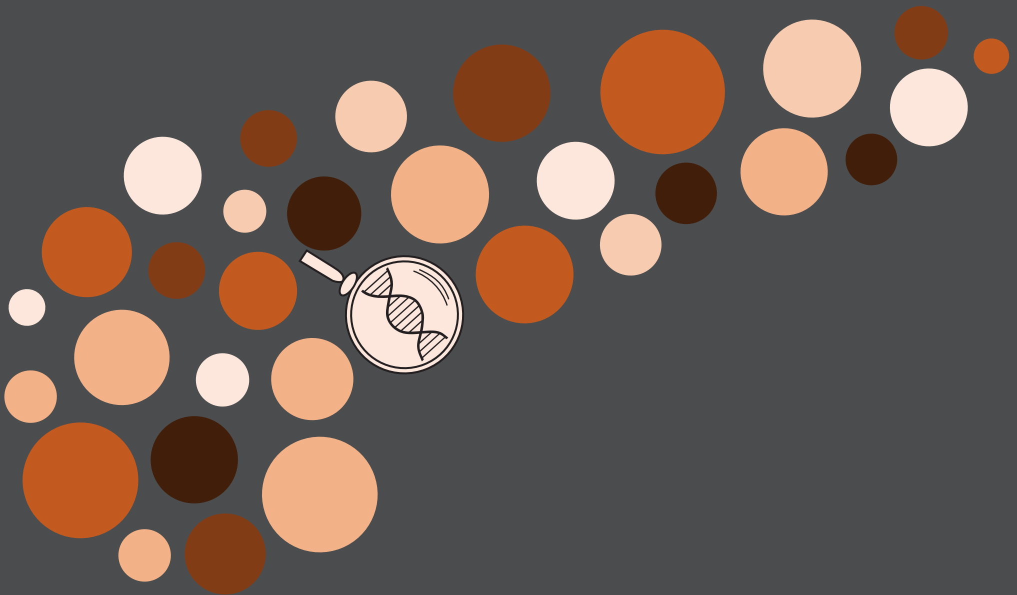

1

General Introduction

CDKN2A, P16-*LEIDEN* AND FAMILIAL MELANOMA-PANCREATIC CANCER SYNDROME

Familial clustering of cutaneous melanoma has increasingly been documented since the 1970s, and one of the first studies that reported an excess of pancreatic ductal adenocarcinoma (henceforth referred to as pancreatic cancer; PC) in unbiased melanoma families was published in 1990 by Bergman and colleagues.[1] The families in this study originated from two genetically isolated towns in the vicinity of Leiden, the Netherlands. Shortly after the identification of the first melanoma predisposition gene *CDKN2A* (MIM #600160*) in 1994,[2,3] a specific Dutch founder mutation† in the *CDKN2A* gene was described in these melanoma-pancreatic cancer prone families, a 19-base-pair deletion in exon 2 known as p16-*Leiden* (c.225_243del).[4,5] An excess of PC in *CDKN2A*-mutated melanoma families was subsequently observed in other populations as well.[6,7]

To date, the *CDKN2A* gene has remained the major high-risk predisposition gene for familial melanoma and germline mutations are identified in 10-40% of melanoma families.[8,9] The *CDKN2A* gene encodes two distinct proteins by using different first exons (1α and 1β) that are translated in alternate reading frames (*figure 1*). The proteins, p16INK4a and p14ARF, are both tumour-suppressors that act in two different pathways. The p16-retinoblastoma(Rb)-pathway controls cell-cycle G1-phase exit, and the p14ARF-p53 pathway induces cell cycle arrest or apoptosis.[10] Germline mutations associated with familial melanoma occur across the entire coding region of the *CDKN2A* gene, including both exon 1α and exon 1β. Heterozygous carriers of a germline mutation have a 70% lifetime risk for developing one or more cutaneous melanomas, and the first melanoma generally occurs at a young age (mean <45 years).[11-15] In a study that included 182 p16-*Leiden* mutation carriers, the mean age at melanoma diagnosis was 39 years and the risk of multiple primary melanomas was approximately 40%. Moreover, p16-*Leiden* mutation carriers that had a melanoma before age 40 had a twice as high risk to develop a second primary melanoma than carriers with a first melanoma after age 40.[15]

An increased risk for PC has been reported for various mutations in *CDKN2A* that affect the p16INK4a protein (exon 1α and exon 2, see *figure 1*).[16,17] The PC risk is particularly high for p16-*Leiden* mutation carriers, approximately 15-20% with a mean age at diagnosis of 58 years.[18-20] In addition to melanoma and PC, several other cancers have been described in *CDKN2A* mutation carriers, including upper and lower respiratory tract cancers [21-24], digestive tract cancers [21,25] and breast cancer [26,27]. De Snoo *et al* specifically evaluated the non-melanoma cancer risks in a large cohort of 221 p16-*Leiden* mutation carriers and

* *Mendelian Inheritance in Man*; Catalog of Human Genes and Genetic Disorders (http://www.omim.org)

† In this thesis, the word *mutation* is used as a synonym for *pathogenic variant*

668 first-degree relatives. They confirmed that these (proven or implied) carriers have a high risk for PC (RR 46.6) and additionally found an increased risk for particularly cancers of the lip, mouth and pharynx (RR 10.8), cancers of the respiratory system (RR 5.7, including laryngeal cancer), eye/brain tumours (RR 11.4) and non-melanoma skin cancers (RR 22.3).[21] Germline mutations in the *CDKN2A* gene, including p16-*Leiden*, thus seem to cause a broad cancer predisposition syndrome.

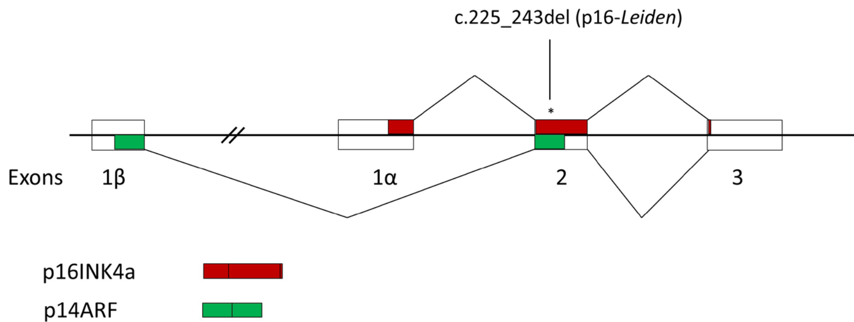

FIGURE 1. The *CDKN2A* gene and its two products, p16INK4a and p14ARF. The p16-*Leiden* mutation is located in exon 2 and affects both p16INK4a and p14ARF.
Adapted with permission from Pigment Cell Melanoma Research, 28, Aoude LG, Wadt KA, Pritchard AL, Hayward NK, Genetics of familial melanoma: 20 years after CDKN2A, 148-60 (2015)

In the first part of this thesis (chapters 2-6), we use the term Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome when referring to familial melanoma with or without a known germline *CDKN2A* mutation. However, use of this term is avoided nowadays because the correlation between atypical multiple moles (nevi) and melanoma is more complex and the atypical nevi phenotype is often absent or shows incomplete co-segregation with the melanoma phenotype in many *CDKN2A*-mutated families.[28-30] Therefore, in the second part of this thesis (chapters 7-9) we solely use the term familial melanoma, or hereditary melanoma when an underlying germline mutation has been identified.

CANCER SURVEILLANCE OF P16-*LEIDEN* MUTATION CARRIERS

MELANOMA SURVEILLANCE

Since the early 1980s, Dutch individuals from melanoma-prone families are offered yearly dermatologic surveillance at the specialized Pigmented Lesion Clinic of Leiden University

Medical Center (LUMC). A study from 1989 showed that melanomas that were detected during this surveillance (screen-detected) were at an earlier stage, i.e. lower Breslow thickness, and therefore had a more favorable prognosis than melanomas occurring in patients not participating in the surveillance program.[31] Comparable studies in other high-risk cohorts confirmed this beneficial effect of regular surveillance on prognosis.[32,33] When the p16-*Leiden* founder mutation was identified in the mid-1990s, many families participating in the Dutch surveillance program were found to carry this mutation. Van der Rhee *et al* subsequently studied the surveillance program in specifically p16-*Leiden* mutation carriers and again concluded that surveillance melanomas were significantly thinner than non-surveillance melanomas (Breslow thickness 0.50 mm and 0.98 mm, respectively).[34] The majority of melanomas in this study were detected within six months after the last surveillance and a considerable proportion were interval-melanomas (detected between regular screens; 20%). Carriers of the p16-*Leiden* mutation are therefore currently under more intensified, semi-annual, dermatologic surveillance.

PANCREATIC CANCER SURVEILLANCE – BACKGROUND

PC surveillance programs were first initiated in the United States two decades ago for families with a condition called Familial PC (FPC).[35,36] Families with at least two first-degree relatives with a diagnosis of PC without an identifiable genetic cause are, by definition, referred to as FPC.[37] Although several cancer predisposition genes are currently known that confer an increased risk for PC, germline mutations are identified in only a small minority (<10%) of families predisposed to PC.[38-41] Therefore, most PC surveillance programs to date have focused on FPC families and generally have included only few individuals with a known underlying germline mutation.[42-44]

The 2013 guideline of the International Cancer of the Pancreas Screening (CAPS) Consortium defines the resection of potentially curable lesions, that is early-stage cancer or its high-grade precursor lesions, as a general goal of surveillance.[45] The dismal prognosis of PC (5-year survival rate <5%) is generally a consequence of late diagnosis, but when a tumour is resected at an early stage, the 5-year survival rate could improve drastically.[46,47] Moreover, timely resection of high-grade precursor lesions of PC might prevent the development of PC at all. Intraductal papillary mucinous neoplasms (IPMN) and the more common pancreatic intraepithelial neoplasms (PanIN) are the most important precursor lesions that can be targeted by surveillance.[45] IPMNs are macroscopic cystic lesions, usually ≥5 mm, that have a high malignant potential when located in the main pancreatic duct (MD-IPMN) (*figure 2*).[48] A longitudinal study showed that approximately 60% of MD-IPMN displays high-grade dysplasia within 5 years, compared to 15% when the IPMN is located in one of the branch ducts (BD-IPMN).[49] PanINs are smaller, microscopic

lesions divided in grade 1 to 3 according to the degree of dysplasia and are located in the smaller pancreatic ducts (*figure 3*).[50] Low-grade PanINs (PanIN1-2) are found in a substantial proportion (28%) of non-PC specimens and can be indolent for many years or not progress to invasive cancer at all, whereas PanIN3 lesions are present in 58% of PC specimens and are considered carcinoma in situ.[51] Precursor lesions, in particular IPMNs, can be detected with imaging of the pancreas because they manifest as small cystic lesions of the pancreatic ducts, i.e. ductectasias. Abdominal MRI combined with magnetic resonance cholangiopancreatography (MRCP) is considered the most sensitive imaging modality to detect these cystic lesions.[52] Endoscopic ultrasonography (EUS) is better in detecting small solid pancreatic lesions, i.e. early-stage PC, compared to MRI/MRCP [52] and it is able to detect secondary parenchymal changes caused by PanIN and IPMN lesions.[53] Current surveillance programs for PC generally use one of these modalities or a combination of both.[42-45] PC surveillance programs have not (yet) implemented non-invasive (serum) biomarkers for PC in their protocols, since the only clinically approved biomarker carbohydrate antigen 19-9 (CA 19-9) has very limited diagnostic accuracy.[54] However, this is a subject of widespread investigation and various other biomarkers have shown promising results in detecting early-stage PC.[55,56]

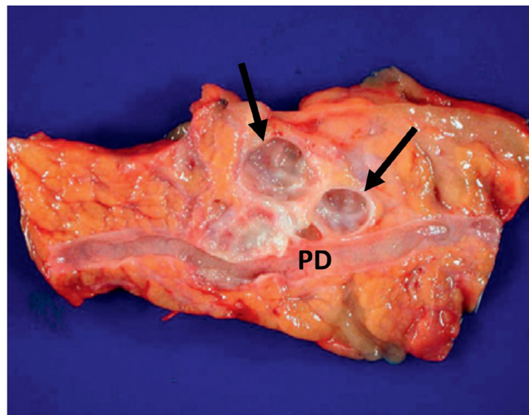

FIGURE 2. Surgical pathology specimen of resected pancreas that includes a branch-duct IPMN (arrows)
PD = main pancreatic duct

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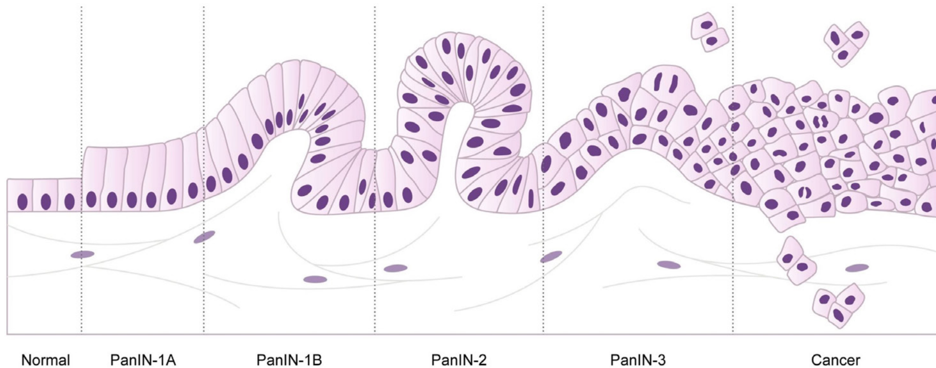

FIGURE 3. Progression model of pancreatic cancer from PanIN lesions. Normal ductal epithelial cells are short and cuboidal, while PanIN-1A lesions are flat and columnar. PanIN-1B lesions are identical to PanIN-1A, although papillary architecture can be observed in these lesions. PanIN-2 lesions can be flat or papillary and show moderate nuclear and architectural abnormalities. PanIN-3 lesions are papillary and show significant nuclear and cytological abnormalities, without the invasion of basement membrane. Pancreatic cancer (ductal adenocarcinoma) shows significant architecture and cytological abnormalities followed by basement membrane invasion.

Reprinted with permission from Susanto, J.M., 2017, Investigating the use of retinoids and epigenetic modification agents as new therapeutic strategies for the treatment of pancreatic cancer, PhD thesis, University of New South Wales, Sydney, available at https://sites.google.com/site/josus123/pancreaticcancer (accessed on December 2018).

Originally adapted from Modern Pathology, 16, Maitra A, Adsay NV, Argani P, Iacobuzio-Donahue C, De Marzo A, Cameron JL, Yeo CJ, Hruban RH, Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray, 902-12 (2003), with permission.

PANCREATIC CANCER SURVEILLANCE PROGRAM IN LEIDEN

At the LUMC, a PC surveillance program for high-risk individuals was started in the year 2000. The program is distinctive from other PC surveillance programs worldwide since it specifically focuses on the large and unique cohort of p16-*Leiden* mutation carriers that historically live in or originated from the vicinity of Leiden. All p16-*Leiden* mutation carriers, regardless of family history for PC, are eligible from age 45 and are offered annual surveillance by MRI/MRCP and, optionally, EUS. In the first evaluation by Vasen *et al* in 2011, PC was diagnosed in seven of 79 included individuals (9%) at a mean age of 59 years.[57] All patients had a resectable tumour with a size ranging 5-40 mm, although it was also shown that these tumours were aggressively growing since three of five tumours

increased in size by 10 mm or more in six months. Cystic duct lesions were detected in 11% of individuals, but 'prophylactic' surgery was performed in only one of these individuals, which revealed PanIN2 lesions on histologic examination. The authors concluded that small solid pancreatic tumours as well as small possible precursor lesions can be detected with MRI/MRCP-based surveillance of p16-*Leiden* mutation carriers, but the role of these precursor lesions in the development of PC and the timing and extent of (prophylactic) surgery remained to be determined.

GENETIC TESTING IN FAMILIAL MELANOMA

INDICATIONS FOR GERMLINE *CDKN2A* ANALYSIS

Criteria for performing germline *CDKN2A* mutation analysis in a melanoma family have been proposed in an international guideline published in 2009.[58] These criteria are based on the patient's personal and family history for melanoma and PC and the geographic location of the family. In countries with a moderate to high incidence of melanoma such as the Netherlands and other Northern European countries, the guideline recommends *CDKN2A* mutation analysis to patients with melanoma if they have at least three primary melanomas, or when there are at least two additional diagnoses of melanoma and/or PC among close (first or second-degree) family members ("rule of threes"). For lower incidence countries such as those in Southern Europe, a comparable "rule of twos" was proposed. These patients/families have a presumed 10% or greater mutation probability. Current Dutch referral guidelines generally adhere to this international guideline, although patients with a juvenile melanoma (<18 years) and patients with both melanoma and PC are also eligible for *CDKN2A* diagnostics regardless of family history (*table 1*).

OTHER GENES ASSOCIATED WITH FAMILIAL MELANOMA

Several melanoma predisposition genes other than *CDKN2A* are currently known, but mutations in these genes are much rarer compared to mutations in *CDKN2A* (*table 2*).[8,9] The *CDK4* gene, which functions in the same cell-cycle pathway as *CDKN2A*, i.e. the p16-retinoblastoma(Rb) pathway, was identified shortly after *CDKN2A* by using a candidate gene sequencing approach. *CDK4* mutations found in melanoma families are all located in codon 24 (p.R24H and p.R24C), leading to reduced p16INK4a inhibition of CDK4 and therefore an increase in CDK4 kinase activity and thus cell cycle progression. Melanoma families with a *CDK4* mutation are phenotypically comparable to *CDKN2A*-mutated families, although other cancers such as PC are not frequently seen in the very few families identified thus far.[59]

TABLE 1. Dutch referral criteria for germline *CDKN2A* diagnostics

Familial melanoma (diagnostic criteria)	▪ family with three relatives with melanoma, of which two are first-degree relatives (all first- and second-degree relatives) ▪ family with two first-degree relatives with melanoma, of which one has multiple primary melanomas
Other families	▪ family with two first-degree relatives with melanoma ▪ family with two first- or second-degree relatives with melanoma and one first- or second-degree relative with pancreatic cancer ▪ person with three or more primary melanomas ▪ person with a juvenile melanoma (<18 years) ▪ person with both melanoma and pancreatic cancer

Reference: Vasen HFA, Hes FJ and de Jong MM. Erfelijke en familiaire tumoren: Richtlijnen voor diagnostiek en preventie. Leiden: Stichting Opsporing Erfelijke Tumoren/Vereniging Klinische Genetica Nederland/Werkgroep Klinische Oncogenetica, 2017. Available from https://www.stoet.nl/wp-content/uploads/2017/02/Richtlijnen-2017.jpg

TABLE 2. Established melanoma predisposition genes other than *CDKN2A*

Gene	Pathway/Function	Non-melanoma cancers	Ref.
CDK4	Cell-cycle control	-	59
TERT	Telomere integrity	-	60
POT1	Telomere integrity	Glioma, leukaemia, possibly other cancers	61-64
ACD	Telomere integrity	Leukaemia	64,65
TERF2IP	Telomere integrity	Leukaemia	64,65
BAP1	DNA damage response	Uveal melanoma, malignant mesothelioma, renal cell carcinoma, basal cell carcinoma	66,67
MITF	Melanocyte homeostasis	Renal cell carcinoma, pancreatic cancer	68,69

The *CDKN2A* and *CDK4* genes were for many years the only known high-penetrance melanoma predisposition genes. The rise of new sequencing technologies in the last decade resulted however in the recent identification of several new predisposition genes and key pathways. One of these pathways controls telomere integrity and germline mutations have been reported in multiple genes involved in the regulation of telomere length (*TERT*) and telomere maintenance (*POT1*, *ACD*, *TERF2IP*) (*figure 4*). A specific mutation in the promotor region of *TERT* (c.-57T>G) causes an increased transcription of *TERT* and is found in only a few, although heavily affected, melanoma families.[60,70] It is hypothesized that overexpression of *TERT* results in longer telomeres and therefore enhanced survival of cancerous cells, although this has not been proven for the c.-57T>G variant.[70] The shelterin complex protects the telomeres from DNA repair mechanisms and regulates TERT activity. Germline mutations have been identified in three of its six components, *POT1*, *ACD* and

TERF2IP, and it has been demonstrated that germline *POT1* mutations do indeed result in increased telomere length.[61,62,65] Mutations in these genes are also found in families with a predisposition for glioma or leukaemia [63,64] and these cancers are reported in some of the melanoma pedigrees as well. *POT1* germline mutations are also increasingly being reported in patients and families with a wide range of other cancers, including thyroid cancer [71], colorectal cancer [72], Hodgkin's lymphoma [73] and cancers in the Li-Fraumeni (*TP53*) spectrum, in particular (cardiac) angiosarcoma [74,75]. The *POT1* gene might thus be associated with many different types of cancer other than melanoma. The *BAP1* (BRCA1-associated protein) gene is involved in several tumour suppressor pathways including the DNA damage response.

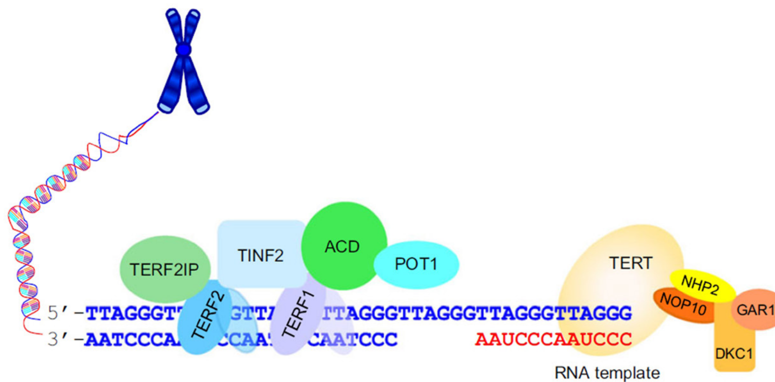

FIGURE 4 Schematic view of the telomere. The shelterin complex (TERF1, TERF2, TERF2IP, TINF2, ACD, POT1) is depicted on the left and the telomerase complex (TERT and other associated proteins) is depicted on the right. The telomerase complex adds telomere repeat sequences to the 3' end of the telomere. The shelterin complex is anchored to the double stranded TTAGGG region of the telomere by the subunits TERF1 and TERF2 and protects the telomeres from DNA repair mechanisms and regulates TERT activity.

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Germline mutations in *BAP1* cause a specific cancer predisposition syndrome with a high penetrance for uveal melanoma (28%), malignant mesothelioma (22%), cutaneous melanoma (18%), renal cell carcinoma (9%) and basal cell carcinoma (6.5%). Also, specific benign skin lesions called atypical Spitz tumours (AST) or melanocytic *BAP1*–mutated atypical intradermal tumours (MBAIT) are typically found in *BAP1* mutation carriers.[66,67] *MITF* is a lower (medium) penetrance melanoma predisposition gene and is involved in melanocyte homeostasis. Only one specific gain-of-function mutation in codon 318 (p.E318K), which causes an increase of *MITF* transcriptional activity, is associated with both

sporadic and familial melanoma.[76] *MITF* p.E318K carriers more frequently develop multiple primary melanomas and there is possibly an increased risk for renal cell carcinoma and pancreatic cancer as well.[68,69] In *figure 5*, all these currently known melanoma predisposition genes are plotted relative to their frequency and effect size. More genes with a possible association with familial melanoma are presented in chapter 8.

In addition to these high- and medium-penetrance melanoma predisposition genes, several common risk variants (single nucleotide polymorphisms; SNPs) derived from large population-based genome wide association studies (GWAS) have been associated with (sporadic) melanoma (*figure 5*).[77-79] These individual SNPs only marginally or moderately influence melanoma risk, but an aggregation of risk variants might substantially increase risk. One of the best established of these risk factors is the *MC1R* gene. The *MC1R* gene plays an important role in skin pigmentation and specific variants that are most strongly associated with a red hair colour phenotype (RHC variants) increase melanoma risk approximately twofold.[80] Other variants that are less strongly associated with red hair colour confer a much smaller melanoma risk and are called non-RHC variants. Studies have shown that both RHC and non-RHC variants also modify melanoma penetrance in *CDKN2A*-mutated families.[81,82] Common susceptibility SNPs are typical candidates to be incorporated in a polygenic risk score (PRS) model, and such models have already shown to improve risk stratification in familial breast cancer.[83,84]

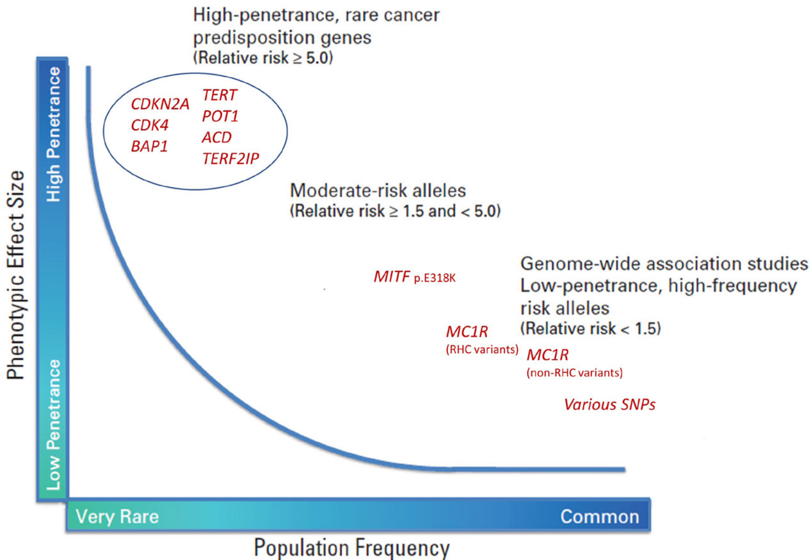

FIGURE 5. *[Legend on the next page]*

FIGURE 5. Graphic display of the phenotypic effect size of currently known genes involved in melanoma susceptibility, plotted against their frequency of occurrence. Note: the high-penetrance genes are randomly plotted within the blue circle. SNP = Single Nucleotide Polymorphism
Adapted with permission from Journal of Clinical Oncology, 28, Stadler ZK, Thom P, Robson ME, Weitzel JN, Kauff ND, Hurley KE, Devlin V, Gold B, Klein RJ, Offit K, Genome-wide association studies of cancer, 4255-67 (2010)

AIMS AND OUTLINE OF THIS THESIS

This thesis has three general aims.

- Our first aim is to investigate the full cancer phenotype of p16-*Leiden* mutation carriers and to study potential modifiers of cancer risk in these carriers (*PART I*).
- Our second aim is to evaluate and improve the p16-*Leiden* pancreatic cancer (PC) surveillance program.
- Our third and final aim is to evaluate and improve genetic testing for hereditary melanoma (*PART II*).

PART I Cancer phenotype and pancreatic cancer surveillance of p16-*Leiden* mutation carriers

In **chapter 2**, we prospectively evaluate a cohort of p16-*Leiden* mutation carriers for the occurrence of any cancer and we investigate the influence of tobacco use on cancer risk. In **chapter 3**, we genotype seven PC-associated SNPs in a nation-wide cohort of p16-*Leiden* mutation carriers and we investigate if these SNPs modify PC risk and could explain the interfamilial variability in the occurrence of PC among these families. In **chapter 4**, we compare the frequency, features and natural history of precursor lesions of PC and PC itself between two different high-risk groups (p16-*Leiden* vs. FPC surveillance cohorts). In **chapter 5**, we report two high-risk patients who developed a second primary PC after a limited resection of their first PC and we discuss the possible implications of these findings for the surgical management of patients with an early-stage screen-detected PC. In **chapter 6**, we investigate if a serum protein signature can differentiate between PC and non-PC in the p16-*Leiden* PC surveillance cohort and we discuss if this biomarker test has the potential to be implemented in the surveillance program.

PART II Genetic testing in familial melanoma; *CDKN2A* and beyond

In **chapter 7**, we study the association between germline *CDKN2A* mutations and several clinical features present in a melanoma family, and we develop a clinical scoring system (*CM-Score*) that can predict the presence of a germline *CDKN2A* mutation in melanoma

families. In **chapter 8**, we investigate the role of other (candidate) melanoma predisposition genes in a large cohort of Dutch non-*CDKN2A* melanoma families through comprehensive multi-gene panel testing.

In the final **chapter 9**, we discuss the main findings of these studies in the context of the most recent literature.

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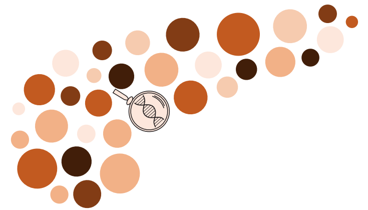
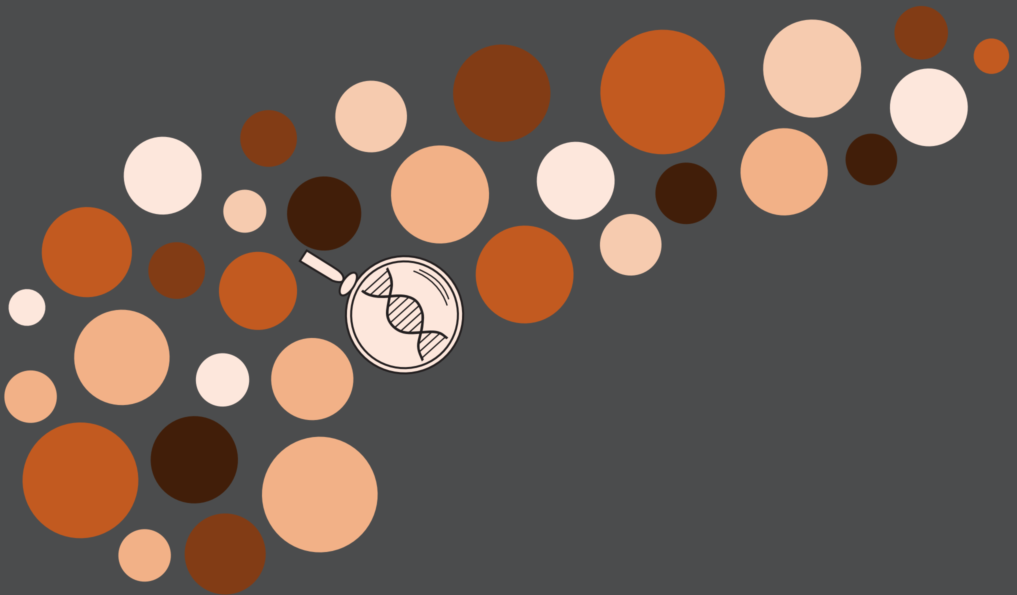
PART I

Cancer phenotype and pancreatic cancer surveillance of p16-*Leiden* mutation carriers





2

Prospective risk of cancer and the influence of tobacco use in carriers of the p16-*Leiden* germline variant

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ABSTRACT

The p16-*Leiden* germline variant in the *CDKN2A* gene is associated with a high risk of melanoma and pancreatic cancer. The aims of this study were to assess the risk of developing other cancers and to determine whether tobacco use would alter cancer risk in carriers of such a variant. We therefore prospectively evaluated individuals with a p16-*Leiden* germline variant, participating in a pancreatic surveillance program, for the occurrence of cancer (n=150). Tobacco use was assessed at the start of the surveillance program. We found a significantly increased risk for melanoma (RR 41.3; 95% CI 22.9-74.6) and pancreatic cancer (RR 80.8; 95% CI 44.7-146). In addition, increased risks were found for cancers of the lip, mouth and pharynx (RR 18.8; 95% CI 6.05-58.2) and respiratory tumours (RR 4.56; 95% CI 1.71-12.1). Current smokers developed significantly more cancers of lip, mouth and pharynx, respiratory system and pancreas compared to former and never-smokers. In conclusion, this study shows that carriers of a p16-*Leiden* variant have an increased risk of developing various types of cancer and smoking significantly increases the risk of frequently occurring cancers. Smoking cessation should be an integral part of the management of p16-*Leiden* variant carriers.

INTRODUCTION

Familial atypical multiple mole melanoma (FAMMM) syndrome is an autosomal dominant tumour syndrome characterized by the development of melanoma and dysplastic naevi of the skin. Up to 40% of FAMMM families harbour a germline variant in the *CDKN2A* gene, making it the most frequently involved gene in FAMMM syndrome.[1] More than 65 different variants in the *CDKN2A* gene have been identified worldwide.[2] In the Netherlands, the p16-*Leiden* variant, a 19-base pair deletion (c.225_243del19; RefSeq NM_000077.4), is the most common *CDKN2A* germline variant.[3] In a previous study,[4] we demonstrated that carriers of such a variant have an increased risk of developing pancreatic cancer (15-20% lifetime risk). Since then a large cohort of patients is under pancreatic surveillance.[5]

Several studies reported an increased risk of tumours other than melanoma and pancreatic cancer for various *CDKN2A* germline variants.[6-10] However, these studies have used a variety of methodological approaches and some have been limited by inclusion of heterogeneous groups or by failure to determine individual mutation status. In addition, the influence of environmental factors (e.g. smoking) on the phenotypic variability in FAMMM syndrome is yet to be elucidated.

In the present study, we analysed the prospective risk of cancer in a unique cohort of individuals with the same p16 germline variant (p16-*Leiden)*. Additionally, we examined the association between a personal history of smoking and the development of cancer.

PATIENTS AND METHODS

PATIENT COHORT

Individuals were included in this study on the basis of carrier status for the p16-*Leiden* germline variant and participation in a pancreatic surveillance program, which consisted of a yearly abdominal MRI combined with magnetic resonance cholangiopancreatography (MRCP) from age 45.[5]

A complete medical history was obtained at the start of the surveillance study. Following this first visit, patients revisited the gastroenterologist annually, at which point the occurrence of new cancers or other diseases was assessed. For the current study, all medical records (with pathological confirmation) were obtained for each individual from the electronic hospital information system. Only cancers that occurred after the first contact were included in the analysis. The study inclusion and follow-up period was from January

2000 to April 2013. The follow-up time for each individual started from inclusion until the last documented appointment with a medical specialist at the Leiden University Medical Center, or the date of death.

CANCER RISK ESTIMATES AND STATISTICAL ANALYSIS

The prospectively observed cancers were classified by International Classification of Diseases code 10 (ICD-10). To calculate the expected number of cancers, five-year cancer incidence rates of matching ICD codes, specific for sex and age, were obtained from the Netherlands Cancer Registry (NCR) for the province of South-Holland in the Netherlands.[11] To calculate the expected number of neuroendocrine tumours, national incidence rates were used for the period 2001-2010.[12] The relative risks were computed by dividing the observed cancer numbers in each group by the expected cancer numbers. Confidence intervals for the relative risks were calculated with the use of Poisson probabilities. To compute the impact of tobacco use on cancer development, individuals were classified as either ever-smokers (current or former) or never-smokers at inclusion in the study; χ^2 analysis was used for comparison. Acquired data was submitted to a public *CDKN2A* gene variant database (http://chromium.liacs.nl/LOVD2/home.php; submission ID #0014954)

RESULTS

PATIENT CHARACTERISTICS

A total of 150 proven or implied carriers of the p16-*Leiden* germline variant were included (64 males, median age at inclusion 51 years (range, 36-72 years)). One hundred and forty-four individuals had a proven p16-*Leiden* germline variant, including a homozygote for the p16-*Leiden* variant. The remaining 6 individuals had at least one melanoma in their medical history and a close relative with the p16-*Leiden* germline variant, which makes them highly likely of being a carrier (>97% according to Bayesian probabilities). The median time of follow-up was 43 months (range, 1-144 months; 1st-3rd quartile, 17-89 months). The total observation period was 682 person years.

PROSPECTIVE TUMOURS

A total of 47 prospective tumours were diagnosed in 36 (24%) of the 150 individuals. Due to the relatively small numbers of observed cancers, classification was based on organ system rather than individual site, with the exceptions of melanoma and pancreatic cancer. *Table 1* shows the relative risks for developing various types of cancer. Melanoma and pancreatic cancer were the most frequently occurring cancers (n=11 each, RR 41.3 (95% CI 22.9-74.6) and 80.8 (95% CI 44.7-146), respectively). When these tumours were excluded

from the analysis, the risk of developing any type of cancer remained significantly increased (RR 4.31; 95% CI 2.91-6.37). The highest risks were found for cancers of the lip, mouth and pharynx (RR 18.8; 95% CI 6.05-58.2), respiratory tumours (RR 4.56; 95% CI 1.71-12.1) and digestive tract tumours (RR 3.71; 95% CI 1.39-9.90). The relatively small numbers of observed cancers, however, resulted in broad confidence intervals, which is especially true for cancers of bone and soft tissue.

TABLE 1. Relative risk of developing cancer in a prospective series of p16-*Leiden* variant carriers (n=150)

Site/organ system	ICD-10 code	Observed (95% CI)	Expected	RR (95% CI)
Bone	c40-c41	1 (0.141-7.10)	0.0149	66.9 (9.43-475)*
Digestive	c15-c24, c26	4 (1.50-10.7)	1.08	3.71 (1.39-9.90)*
Female Breast	c50	3 (0.967-9.30)	1.15	2.61 (0.840-8.08)
Haematological	c81-c96	1 (0.141-7.10)	0.462	2.16 (0.305-15.3)
Lip, mouth, pharynx	c00-c14	3 (0.968-9.30)	0.160	18.8 (6.05-58.2)*
Male genital	c60-c63	1 (0.141-7.10)	0.689	1.45 (0.204-10.3)
Melanoma[§]	c43	11 (6.09-19.9)	0.266	41.3 (22.9-74.6)*
Nonmelanoma skin[#]	c44	4 (1.50-10.7)	0.327	12.3 (4.60-32.6)*
Pancreas	c25	11 (6.09-19.9)	0.136	80.8 (44.7-146)*
Respiratory	c32-c34	4 (1.50-10.7)	0.877	4.56 (1.71-12.1)*
Soft tissue	c38, c47-c49	2 (0.500-8.00)	0.0336	59.5 (14.9-238)*
Unknown primary site	c80	1 (0.141-7.10)	0.138	7.22 (1.02-51.3)
Urinary	c64-c68	1 (0.141-7.10)	0.333	3.00 (0.423-21.3)
All cancers		47 (35.3-62.6)	6.20	7.58 (5.69-10.1)*
All cancers except melanoma and pancreas		25 (16.9-37.0)	5.80	4.31 (2.91-6.37)*

** Significant*

[§] First as well as subsequent melanomas are registered In the Netherlands Cancer Registry (NCR)

[#] Basal cell carcinoma is not registered in the NCR and therefore not included in the calculation

Details of 21 prospective cancers (all cancers except those of skin and pancreas) are shown in *table 2*. Notably, the observed number of carcinoid tumours was higher than expected (0.0168; RR 119; 95% CI 29.7-475). When excluding carcinoid tumours from the risk calculation for digestive tract tumours, the increased risk for a digestive tract tumour no longer reached significance (RR 1.86; 95% CI 0.465-7.43).

Seven individuals developed a total of 11 melanomas during the follow-up period. However, a much larger number of individuals (91 out of 150) had a diagnosed melanoma prior to

starting surveillance for pancreatic cancer (median age at diagnosis of first melanoma 40 years). *Table 3* shows tumours diagnosed before inclusion, of which melanoma forms by far the major part. Only one individual developed a first melanoma during the follow-up period. Melanoma therefore remains the most frequently occurring cancer in this p16-*Leiden* study cohort and first melanomas mostly occur prior to the age of inclusion (45 years). A more exhaustive description of the melanoma phenotype in carriers of the p16-*Leiden* germline variant is given by van der Rhee *et al*.[13]

TABLE 2. Characteristics of prospective cancers (excluding skin cancer and pancreatic cancer)

Subject number	Sex	Tumour type/organ	Histopathology	Age at diagnosis
1	F	Caecum	Carcinoid	72
2	M	Appendix	Carcinoid	58
		Bone	Papillary squamous cell carcinoma of mandible	62
3	M	Stomach	Adenocarcinoma of cardia	64
4	M	Haematopoietic	Multiple myeloma	67
		Stomach	Adenocarcinoma	67
5	F	Breast	Ductal adenocarcinoma	48
6	F	Breast	Ductal adenocarcinoma	53
7	F	Breast	Ductal adenocarcinoma	49
8	M	Hypopharynx	Squamous cell carcinoma	51
		Lung	Squamous cell carcinoma	52
9*	M	Floor of mouth	Squamous cell carcinoma	58
		Larynx	Squamous cell carcinoma	58
10	F	Tongue	Carcinoma not specified	51
11	F	Lung	Non-small cell carcinoma	60
12	M	Larynx	Squamous cell carcinoma	55
13	F	Bladder	Small cell carcinoma	58
14	M	Prostate	Adenocarcinoma	69
15	F	Knee	Myxofibrosarcoma	48
16	M	Neck	Leiomyosarcoma	66
17	F	Unknown	Metastatic adenocarcinoma	67

**This patient had two primary tumours detected concurrently.*

TOBACCO USE

With regard to a personal history of smoking, information was complete for 147 (98%) out of 150 individuals. At inclusion, 92 individuals were ever-smokers (of which 26 were current smokers). Eleven of 92 ever-smokers (12%) and four of 55 never-smokers (7%) developed

pancreatic cancer, respiratory cancer or cancer of the lip, mouth and pharynx (p=0.364). Four of 11 patients with pancreatic cancer were never-smokers. When only current smokers were considered, seven of 26 (27%) developed above mentioned cancers, versus only eight of 121 (7%) of the former and never-smokers. Therefore, current smokers in our cohort have a fourfold increased risk of developing these types of cancer when compared to former and never-smokers (p=0.002).

CHRONIC DISEASES

We also evaluated the occurrence of other (chronic) diseases. We found that six out of 150 individuals (4%) had a medical history of sarcoidosis, which is much higher than expected (estimated prevalence in Europe approximately 15-20 per 100,000 individuals).[14] There was no kinship between these individuals.

CAUSES OF DEATH

Eighteen of the 150 individuals died during follow-up (median age of death 62 years (range, 49-78 years)). Seventeen individuals died from cancer; seven from pancreatic cancer (median age 59 years) and four from melanoma (median age 61 years).

TABLE 3. Tumours diagnosed before inclusion in the surveillance program

Site/organ system	Observed cancer	Individual(s)
Digestive	1	1
Female Breast	4	4
Female Genital	1	1
Lip, mouth, pharynx	3	2
Melanoma	194	91
Nonmelanoma skin	2	2
Respiratory	4	4
Urinary	1	1
All cancers	210	98

DISCUSSION

This prospective study analysed the risk of cancers in a cohort of homogeneous *CDKN2A* variant carriers (p16-*Leiden*). A significantly increased risk of both melanoma and pancreatic cancer was found. However, when excluding these cancers from the risk calculation, a marked increased risk for developing any cancer (RR 4.31; 95% CI 2.91-6.37) remained. Most notable were the increased risk of respiratory and lip, mouth and pharynx cancer,

and the relatively frequent occurrence of carcinoid tumours. Being a current smoker at the start of surveillance was significantly associated with the development of tumours of the pancreas, respiratory tract and head and neck region. In addition, we found an association between the p16-*Leiden* variant and sarcoidosis.

Without considering melanoma and pancreatic cancer, tumours of the respiratory tract (including laryngeal tumours) and of the lip, mouth and pharynx were the most frequently occurring tumours in our cohort. A previous retrospective study by de Snoo *et al* also found significantly increased risks for these tumours in a cohort of p16-*Leiden* variant carriers.[9] Oldenburg *et al* described a p16-*Leiden* variant positive family in which many relatives had developed lung cancer and head and neck tumours.[15] Several other case reports have also described the occurrence of head and neck tumours in *CDKN2A* variant positive families.[16,17] In sum, it seems that tumours of the head and neck and respiratory tract are part of the spectrum of cancers occurring in *CDKN2A* variant-positive FAMMM families.

Two interesting observations were the relatively frequent occurrence of carcinoid tumours and sarcoidosis in unrelated variant carriers in our cohort. Both have not been previously reported in carriers of a *CDKN2A* variant. Although only two individuals developed a carcinoid tumour during follow up, another individual had a medical history of carcinoid. It has been shown that p16 inactivation plays a role in the pathogenesis of sporadic neuro-endocrine tumours, as a substantial amount of these tumours show loss of p16 expression,[18] and also promoter methylation of the p16 gene is frequently found [19]. Further studies are needed to confirm the possible association between a *CDKN2A* germline variant and carcinoid tumours or sarcoidosis.

Our current study has several strengths. Due to its prospective design, patient participation was not influenced by the occurrence of tumours. In addition, due to the yearly follow-up at the outpatient Department of Gastroenterology, it is unlikely that cancers and other important medical information were missed. Another strength is the homogeneity of the cohort; all individuals have the same *CDKN2A* germline variant. An important limitation was, however, the relatively high age of inclusion of individuals (median age 51 years), which was due to the threshold of 45 years of age for inclusion in the pancreatic surveillance program. Tumours generally occurring before this age were therefore not included in the calculations, which is reflected by the observation of a high incidence of melanoma prior to start of the surveillance program. Because the number of participants and observed cancers was relatively small, risk factor analysis for each cancer separately could not be carried out.

Pancreatic cancer is the leading cause of death in our cohort. Pancreatic cancer surveillance may improve survival, as most tumours are detected in a resectable stage.[5] In view of the increased risk of head and neck tumours (including tumours of the larynx), patients should be advised to contact their doctor if they have complaints of hoarseness, dysphagia or ulcers in mouth or throat. A low threshold for reference to an otolaryngologist should be advocated. A surveillance program for tumours of the head and neck region should possibly be considered in the future, which could simply consist of yearly inspection of the mouth and throat. The clear relation of many of the frequently occurring cancers in our cohort to smoking indicates that active intervention to quit smoking is of the utmost importance in this group.

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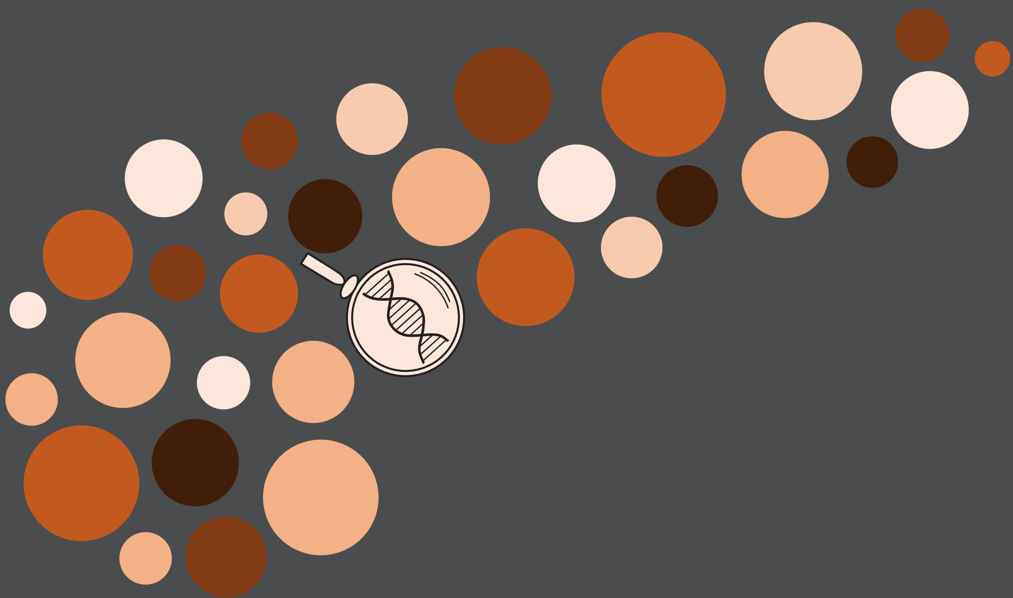

3

Pancreatic cancer-associated gene polymorphisms in a nation-wide cohort of p16-*Leiden* germline mutation carriers; a case-control study

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ABSTRACT

BACKGROUND

The p16-*Leiden* founder mutation in the *CDKN2A* gene is the most common cause of Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome in the Netherlands. Individuals with this mutation are at increased risk for developing melanoma of the skin, as well as pancreatic cancer. However, there is a notable interfamilial variability in the occurrence of pancreatic cancer among p16-*Leiden* families. We aimed to test whether previously identified genetic risk factors for pancreatic cancer modify the risk for pancreatic cancer in p16-*Leiden* germline mutation carriers.

METHODS

Seven pancreatic cancer-associated SNPs were selected from the literature and were genotyped in a cohort of 185 p16-*Leiden* germline mutation carriers from 88 families, including 50 cases (median age 55 years) with pancreatic cancer and 135 controls (median age 64 years) without pancreatic cancer. Allelic odds ratios per SNP were calculated.

RESULTS

No significant association with pancreatic cancer was found for any of the seven SNPs.

CONCLUSIONS

Since genetic modifiers for developing melanoma have already been identified in *CDKN2A* mutation carriers, this study does not exclude that genetic modifiers do not play a role in the individual pancreatic cancer risk in this cohort of p16-*Leiden* germline mutation carriers. The search for these modifiers should therefore continue, because they can potentially facilitate more targeted pancreatic surveillance programs.

BACKGROUND

The melanoma gene *CDKN2A* produces two important proteins: p16^{INK4a}, which is a cyclin-dependent kinase inhibitor, and p14^{ARF}, which binds the p53-stabilizing protein MDM2.[1] In the Netherlands, a founder mutation in the *CDKN2A* gene, a 19-base pair deletion called p16-*Leiden* (c.225_243del19; RefSeq NM_000077.4), is the most common cause of Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome.[2] In addition to a marked increased risk of developing melanoma of the skin (70% lifetime risk), these mutation carriers also have a 15-20% lifetime risk of developing pancreatic cancer with a mean age of 58 years at diagnosis.[3] Interestingly, there is a notable interfamilial variability in the occurrence of pancreatic cancer among p16-*Leiden* families.[3] Therefore, the p16-*Leiden* mutation might not be the only genetic risk factor in these individuals causing an increased susceptibility for pancreatic cancer. Since pancreatic cancer has a very poor prognosis due to late occurrence of symptoms and therefore late detection, surveillance for pancreatic cancer is currently offered to p16-*Leiden* germline mutation carriers in a research setting to investigate whether pancreatic cancer, or, even more preferable, high-grade precursor lesions can be detected earlier in a potentially still curable stage.[4] By identifying additional genetic risk factors (genetic modifiers) in these individuals, surveillance could possibly be more individualized.

In recent years, genome-wide association studies (GWAS) have identified several common risk variants associated with pancreatic cancer.[5-7] In this study, we genotyped a selected number of these variants (SNPs) in a unique cohort of p16-*Leiden* mutation carriers with and without pancreatic cancer. We hypothesized that these SNPs might modify the risk of pancreatic cancer in these p16-*Leiden* mutation carriers.

METHODS

STUDY POPULATION AND DNA SAMPLE COLLECTION

For this case-control study, only proven carriers of the p16-*Leiden* germline mutation were included. From all Dutch p16-*Leiden* mutation carriers, DNA is stored in the Laboratory for Diagnostic Genome Analysis (LDGA) of the Leiden University Medical Center. All p16-*Leiden* germline mutation carriers diagnosed at the LDGA between the initiation of *CDKN2A* gene diagnostics at the LDGA in 1998 and January 1st 2014 were eligible for inclusion. Cases were defined as having been diagnosed with exocrine pancreatic cancer at the time of data collection; controls were at least 55 years old on January 1st 2014 or died beyond that age, and were not diagnosed with pancreatic cancer. Individuals who

were younger than 55 years or died before this age were excluded from the control group. Medical records were obtained for each individual from the electronic hospital information system of the medical center where this individual initially received genetic counselling by a clinical geneticist for *CDKN2A* gene diagnostics. Access to these medical records was granted for the (co)authors since they are clinical geneticists working in these medical centers. Additional follow up data was acquired from two ongoing pancreatic surveillance studies (a single-center study at Leiden University Medical Center and a multi-center study at Erasmus MC University Medical Center Rotterdam) and from the Netherlands Foundation for the Detection of Hereditary Tumours, a central registration institute for hereditary tumours (FAMMM, amongst others) in the Netherlands. This study was approved by the Ethics Committee of the Leiden University Medical Center, by issuing a *declaration of no objection* (#P14.148). This is an assessment of the study protocol on due diligence, that is, if it serves the codes of *good practice* and *good conduct*. It is not a formal ethical assessment, because the study does not fall within the scope of the Dutch law for medical research on human subjects; the medical records and the DNA were already available and the involved human subjects were not specifically recruited for the study and were not subjected to any actions. Therefore, no separate ethical assessment or approval was needed for the collection of data in the participating medical centers.

SNP selection and genotyping

SNPs for genotyping in this cohort were selected from recent GWAS studies with large cohorts of sporadic pancreatic cancer patients.[5-7] Selection was based on significance of association with pancreatic cancer and reported odds ratios, as well as expected allele frequencies. In the first place, SNPs with the largest odds ratios and smallest p-values were selected. Subsequently, only those SNPs with a relatively high minor allele frequency (MAF) were considered for genotyping, because of the limited sample size of the cohort. This would optimize the number of carriers of the minor allele and thereby augment the potential of reaching significance between subgroups. In order to test a relatively wide variety of genes, a maximum of two different SNPs per gene was maintained. All included individuals were genotyped for the selected SNPs using high-resolution DNA melting curve analysis.[8] Melting assays were performed with Lightscanner® (Biofire Defense Inc, Salt Lake City, UT).

STATISTICAL ANALYSIS

Given our relatively small sample size, we performed a power calculation with Bonferroni correction for multiple testing prior to the study. Despite a calculated power of approximately 15%, we wanted to pursuit this small but tangible chance of finding a trend of association.

The frequencies of the risk alleles were computed and compared between cases and

controls by calculating a SNP-specific allelic odds ratio, including the 95% confidence interval. Additional p-values were calculated using a basic χ^2 test and the Bonferroni correction for multiple testing was applied. Therefore, a p-value of less than 0.007 was considered significant. All statistical analyses were performed using SPSS 20.0.0 (IBM Inc, Armonk, NY).

RESULTS

PATIENT CHARACTERISTICS *(TABLE 1)*

In total, 422 p16-*Leiden* germline mutation carriers were eligible for inclusion. Of these, 50 individuals (18 males, 36%) were diagnosed with pancreatic cancer, with a median age at diagnosis of 55 years (range 21-76 years), and could all be included in the case group. The remaining 372 individuals were not diagnosed with pancreatic cancer, but 237 individuals were excluded from the control group because of not having reached the age of 55 years. The remaining control group consisted of 135 individuals (50 males, 37%) with a median age of 64 years (range 55-88 years). Thus, for this study a total of 185 p16-*Leiden* germline mutation carriers (from 88 families) were included. A considerable number of individuals (98 of 185 [53%]) developed melanoma, and 37 of 98 individuals with melanoma (38%) had multiple melanoma. In the case group, 24 of 50 individuals (48%) had a medical history of melanoma, and in the control group 74 of 135 individuals (55%) had a medical history of melanoma.

TABLE 1. Patient characteristics

	p16-*Leiden* mutation carriers (n=185)	
	Cases with pancreatic cancer (n=50)	**Controls without pancreatic cancer (n=135)**
Median age in years (range)	55 (21-76)	64 (55-88)
Gender (m:f)	18:32	50:88
Medical history of melanoma	24 (48%)	74 (55%)
Of which multiple	8/24 (33%)	29/74 (39%)

SNP GENOTYPING AND ASSOCIATION WITH PANCREATIC CANCER

A total of seven SNPs in five different genes were selected for genotyping. *Table 2* shows the minor allele frequencies of these SNPs in cases and controls, and the calculated association (allelic odds ratio and 95% confidence interval) with pancreatic cancer in our cohort of p16-*Leiden* germline mutation carriers. No significant association with pancreatic cancer was found for any of the seven SNPs.

TABLE 2. Association of seven selected SNPs with pancreatic cancer in this study

Marker*	Minor Allele Frequency		Allelic OR (95% CI)	Sign. (p-value)
	Cases	Controls		
rs2821367 (T, C) 1q32.1 *NR5A2*	0.37	0.38	1.0 (0.62-1.62)	1.00
rs7310409 (G, A) 12q24.31 *HNF1A*	0.35	0.39	0.87 (0.54-1.41)	0.57
rs735396 (A, G) 12q23.31 *HNF1A*	0.33	0.36	0.90 (0.55-1.47)	0.67
rs1805100 (G, A) 8q21.11 *HNF4G*	0.49	0.56	0.76 (0.48-1.21)	0.24
rs505922 (T, C) 9q34.2 *ABO*	0.30	0.33	0.87 (0.53-1.44)	0.59
rs657152 (G, T) 9q34.2 *ABO*	0.30	0.37	0.73 (0.44-1.20)	0.22
rs172310 (C, A) 7q36.3 *SHH*	0.43	0.39	1.18 (0.74-1.89)	0.49

** SNP reference (major / minor allele), chromosome location, gene*

DISCUSSION

In this case-control study, we analysed seven pancreatic cancer-associated SNPs in a distinctively homogeneous cohort of 185 *CDKN2A* germline mutation carriers. That is, all cases with (n=50) and all controls without (n=135) pancreatic cancer carry the same p16-*Leiden* germline mutation. We hypothesized that (a subset of) these SNPs would be associated with an increased pancreatic cancer risk in these individuals, and that genetic modifiers would explain, at least partially, the variability in the occurrence of pancreatic cancer in p16-*Leiden* families. However, in our cohort, no significant association was found between the occurrence of pancreatic cancer and any of the seven SNPs.

Currently, research on genetic modifiers in *CDKN2A* mutation carriers is mainly focused on identifying low-risk variants that influence melanoma risk.[9,10] To date, the *MC1R* gene, which is known to have a role in the skin pigmentation process, is the most important modifier gene identified so far.[11,12] Penetrance of *CDKN2A* mutations regarding melanoma risk is thus proven to be subject to low-risk genetic variants in other genes. Because of these

encouraging results from previous studies, it can be expected that genetic variants could also be identified in *CDKN2A* mutation carriers which influence pancreatic cancer risk. It is however unlikely that these variants will be the same as those which influence melanoma risk, as Wu *et al* demonstrated.[13] Other genetic variants should thus be considered when studying the risk of pancreatic cancer in *CDKN2A* mutation carriers. Yang *et al* made a first attempt at this by sequencing the *PALB2* gene in a small cohort of *CDKN2A* mutation carrying families with pancreatic cancer.[14] The *PALB2* gene (OMIM #610355) is one of the relatively few known high-risk pancreatic cancer susceptibility genes associated with familial pancreatic cancer to date.[15] However, no pathogenic mutations were identified in their study and also no association was found between SNPs in the *PALB2* gene and the occurrence of pancreatic cancer.

The strength of this study is that our cohort of p16-*Leiden* mutation carriers is homogeneous regarding the type of mutation, and therefore relatively large in its kind. Yet, in order to study modifier effects in a cohort of individuals with a genetically inherited predisposition to cancer, a large sample size is important.[16] Because the p16-*Leiden* mutation is a rare mutation, an important limitation of this study is that sample size cannot easily be increased. Therefore, the lower age limit of the control group was set at 55 years (the mean age of pancreatic cancer in the case group). It is possible that individuals in the control group will develop pancreatic cancer in the future and this could have influenced the results. Possibly, recalculations in the future, taking into account new clinical follow-up data of these individuals, could change the results significantly.

For this study, we selected seven promising pancreatic cancer-associated SNPs from the literature, based on reported odds ratios, p-values and reproducibility. The sample size limitation allowed us to test only a restricted number of SNPs and because of the limited sample size we chose for those with a relatively high minor allele frequency. Our selection of seven SNPs is however only a subset of a much larger set of SNPs associated with pancreatic cancer and it is therefore still possible that these other SNPs do play a role in the pancreatic cancer risk in p16-*Leiden* germline mutation carriers (see Lin *et al* [17] for an overview of associated SNPs). It is also possible that other, non-genetic, risk factors play a role in the variable pancreatic cancer phenotype. However, we do not have sufficient data available of these external risk factors in our studied mutation carriers. One of the most important non-genetic risk factors for developing pancreatic cancer is tobacco use, and even in *CDKN2A* germline mutation carriers it increases the risk for pancreatic cancer significantly.[18,19]

CONCLUSIONS

This study aimed at identifying genetic modifiers that influence pancreatic cancer risk in a homogeneous cohort of Dutch p16-*Leiden* germline mutation carriers. Despite the fact that no significant association could be found for the seven tested pancreatic cancer-associated SNPs, it is still possible that these or other genetic modifiers play a significant role in the individual pancreatic cancer risk in these individuals. The search for genetic modifiers for pancreatic cancer in p16-*Leiden* germline mutation carriers should therefore continue.

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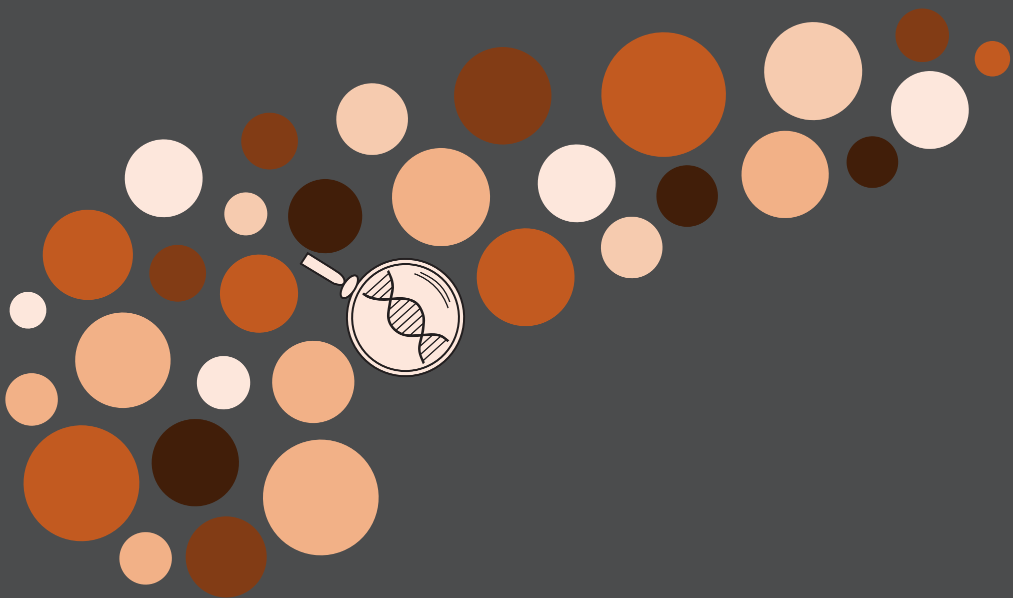

4

Variation in precursor lesions of pancreatic cancer among high-risk groups

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ABSTRACT

PURPOSE

Pancreatic ductal adenocarcinoma (PDAC) surveillance programs are currently offered to high-risk individuals aiming to detect precursor lesions or PDAC at an early stage. We assessed differences in frequency and behaviour of precursor lesions and PDAC between two high-risk groups.

EXPERIMENTAL DESIGN

Individuals with a p16-*Leiden* germline mutation (n=116; median age 54 years) and individuals from familial pancreatic cancer (FPC) families (n=125; median age 47 years) were offered annual surveillance by magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP) with or without endoscopic ultrasound (EUS) for a median surveillance period of 34 months (0-127 months) or 36 months (0-110 months), respectively. Detailed information was collected on pancreatic cystic lesions detected on MRCP and precursor lesions in surgical specimens of patients who underwent pancreatic surgery.

RESULTS

Cystic lesions were more common in the FPC cohort (42% versus 16% in p16-*Leiden* cohort), while PDAC was more common in the p16-*Leiden* cohort (7% versus 0.8% in FPC cohort). Intraductal papillary mucinous neoplasm (IPMN) was a common finding in surgical specimens of FPC-individuals, and was only found in two patients of the p16-*Leiden* cohort. In the p16-*Leiden* cohort, a substantial proportion of cystic lesions showed growth or malignant transformation during follow-up whereas in FPC-individuals most cystic lesions remain stable.

CONCLUSION

In p16-*Leiden* mutation carriers, cystic lesions have a higher malignant potential than in FPC-individuals. Based on these findings, a more intensive surveillance program may be considered in this high-risk group.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the western world. It is one of the most lethal cancers with an incidence rate almost equaling the mortality rate and an overall 5-year survival of approximately 5%.[1,2] There has been no improvement in prognosis in the last decades. However, longer survival has been reported for patients with early stage tumors.[3] Probably, the only way to detect PDAC at an early stage and to improve the prognosis is by surveillance of asymptomatic individuals. Such a surveillance program should ideally focus on the detection of known precursor lesions, that is, intraductal papillary mucinous neoplasms (IPMNs) and pancreatic intraepithelial neoplasias (PanINs).[4,5] Because of the low incidence rate of PDAC, surveillance for this cancer would not be appropriate in the general population. However, in high-risk groups, i.e. individuals with an inherited predisposition to PDAC, screening could be valuable in improving the prognosis.

Approximately 3-5% of PDAC cases are associated with an inherited predisposition.[6,7] Individuals with certain tumor syndromes, such as familial atypical multiple mole melanoma (FAMMM), Peutz-Jeghers syndrome (PJS) and hereditary breast cancer (*BRCA2* mutation carriers), have a marked increase in risk of developing PDAC.[8] In FAMMM syndrome, which is associated with a mutation in the *CDKN2A* (or p16) gene, individuals are at increased risk of developing melanoma of the skin. FAMMM members with the Dutch founder mutation, a 19-base pair deletion of exon 2 of the *CDKN2A* gene (p16-*Leiden*), have a 15-20% lifetime risk of developing PDAC.[9]

When there is no proven tumor syndrome, but apparent familial clustering of PDAC, the condition is referred to as familial pancreatic cancer (FPC), which represents the largest proportion of hereditary PDAC. By definition, there should be at least two first degree relatives with PDAC to fulfill the criteria for FPC. The risk of developing PDAC increases with the number of family members affected. Individuals with two affected first degree relatives have a 6.4-fold increased risk, and the risk increases to 32-fold in case of three or more first degree relatives affected.[10]

Several studies on screening for PDAC in high-risk individuals, predominantly FPC, have been published during the last decade.[11-22] Various screening modalities have been used in these studies, but the optimal strategy for surveillance in high-risk groups remains undetermined. Endoscopic ultrasonography (EUS) is able to detect small solid tumors, but it is an invasive procedure. Magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP) are appropriate for detecting small cystic lesions, but

are less sensitive in detecting small solid tumors.[23,24]

In studies focusing on FPC, a high frequency of precursor lesions have been described, but an overall low rate of PDAC. [11-14,17-21] On the other hand, screened individuals with the p16-*Leiden* mutation are reported to have a much lower frequency of precursor lesions but a high rate of PDAC.[22] Therefore, the question arises whether there is a different role of precursor lesions in the development of PDAC in the various high-risk groups.

In the present study, we evaluated screening data from a large p16-*Leiden* cohort and a large FPC cohort from the Leiden University Medical Center and the German FaPaCa registry, respectively. The aims were to compare the frequency of precursor lesions and PDAC between these two cohorts, to compare the features and natural course of precursor lesions, and to discuss possible implications for the surveillance protocol.

PATIENTS AND METHODS

SURVEILLANCE GROUP

Individuals at risk (IAR) from two different registries were included in this study. The current study is a retrospective analysis of two ongoing prospective surveillance studies. A subset of these have been published earlier and were updated for this study.[17,22] Individuals with a p16-*Leiden* germline mutation were referred from the Clinical Genetics Department to the Department of Gastroenterology and Hepatology of the Leiden University Medical Center in The Netherlands to participate in a surveillance program. Individuals from FPC families were recruited via the FaPaCa registry, a German national case collection for FPC families which is coordinated by the Philipps-University of Marburg in Germany. The diagnosis of FPC was based on the presence of two or more first degree relatives with a confirmed diagnosis of PDAC. Also, individuals with a *BRCA2* or a *PALB2* mutation and familial clustering of PDAC (primary tumor burden in family) were included in the FPC cohort. Individuals with two first degree relatives with PDAC were classified as moderate risk (5- to 10-fold), individuals with three or more first degree relatives with PDAC or with a *BRCA2* or *PALB2* mutation were classified as high risk (>10-fold). Both inclusion procedures and criteria were previously described for the two cohorts.[17,22] The ongoing surveillance studies in Leiden and Marburg were approved by the Ethics Committee of the Leiden University Medical Center and the Phillips-University of Marburg, respectively. For the current study, evaluation was from January 2000 to August 2011 at Leiden University Medical Center and from June 2002 to December 2011 at the FaPaCa registry.

SCREENING MODALITY

The surveillance program that was used for the FaPaCa FPC-families consisted of both MRI/MRCP and EUS. In the p16-*Leiden* families, MRI/MRCP and optionally EUS was performed. However, for this study, only the results of the MRI/MRCP were used for comparison. IARs without any MRI/MRCP accomplished were excluded. MRI/MRCP was performed yearly in both centers. In case of an abnormal finding, either close follow-up with MRI/MRCP and EUS or surgery was advised by a multidisciplinary team. Detailed information regarding follow-up and MRI-technique were previously described for both groups.[17,22] MRIs were evaluated by specialized radiologists at the centers in Marburg and Leiden. All abnormal MRIs from the p16-*Leiden* cohort were revised by the radiologist from Marburg (J.T.H.).

CYSTIC LESIONS

Cystic lesions were defined as radiologically detected cystic lesions including those originating from the pancreatic ducts. For the current study, cystic lesions were subdivided into (1) main duct type (MD) lesions, (2) branch duct type (BD) lesions with a clear connection to the main duct on imaging and (3) other cystic lesions with uncertain connection to pancreatic ducts. Cystic lesions were further classified as multicystic single lesions consisting of multiple small cysts, single or multiple unicystic lesions.

INDICATION FOR SURGERY

In the event of a pathological finding in the pancreas by the imaging modalities, the findings were reviewed by an interdisciplinary board consisting of geneticists, psychooncologists, surgeons and gastroenterologists at both sides. Criteria to recommend surgery included cystic lesions >3 cm, cystic lesions of any size with a substantial solid component, cystic lesions with irregular boundaries in IAR with a strong family history (e.g. three or more affected first degree relatives), significant change in size and morphology during follow up, positive or highly-suspicious EUS fine needle aspiration cytology or patients preference.

HISTOLOGY

For both cohorts, pancreatic surgical specimens were investigated by pathologists at each centre and reassessed by a single experienced pathologist (G. K), with a special expertise in pancreatic pathology. All available sections were reviewed and particular attention was given to the slides showing tumorous/cystic alterations and duct changes (average number per specimen/case: 4 (range 3–6). In the sections (range 3–4) containing nontumorous/noncystic tissue all PanINs were recorded and their numbers listed in *tables 4-6*. PanINs were classified by their grade of dysplasia in low (1) moderate (2) or high (3). IPMNs were subtyped as gastric, intestinal, oncocytic or pancreatobiliary type with low grade, moderate or high grade dysplasia.[4,25,26]

STATISTICAL ANALYSIS

Descriptive statistics were compiled for both groups. Categorical features were compared using χ^2 analysis. Continues variables were compared using the independent samples *t* test or, when indicated, the Mann-Whitney test. A *P* value of <0.05 was considered significant. Statistical analyses were performed using SPSS 17.0 (SPSS Inc, Chicago, IL).

RESULTS

PATIENT CHARACTERISTICS

4

A total of 116 IAR with a p16-*Leiden* germline mutation and 125 IAR from FPC families were available for evaluation and included in this study (*table 1*). In the FPC cohort, 66 individuals were classified as moderate risk and 59 individuals as high risk. In the high-risk group, 9 individuals (7%) had a known mutation (6x *PALB2*, 3x *BRCA2*). Median age at start screening was 54 years for the p16-*Leiden* cohort (range 38-72 years) and 47 years for the FPC cohort (range 27-73 years). The median time under surveillance was 34 months for the p16-*Leiden* cohort (range 0-127 months) and 36 months for the FPC cohort (range 0-110 months). A total of 507 MRIs were performed in the p16-*Leiden* cohort (mean 4.4 per individual) and 457 in the FPC cohort (mean 3.7 per individual). All abnormal MRI's from both cohorts were confirmed by one experienced radiologist (J.T.H.).

TABLE 1. Patient characteristics of the two cohorts

	Median age at start screening (range)	Gender m:f	Median time under surveillance [mo] (range)	Total MRI (pp)
◆ **FPC (n=125)**	47 (27-73)	54:71	36 (0-110)	457 (3.7)
◆ **p16-*Leiden* (n=116)**	54 (38-72)	50:66	34 (0-127)	507 (4.4)

pp = per person (mean), n = number

CYSTIC LESIONS AND PDAC DETECTED BY MRI

Cystic lesions were present in 18 of 116 individuals with the p16-*Leiden* germline mutation (16%). In the FPC cohort, 52 of 125 individuals had cystic lesions (42%, p<0.001) (*table* 2). In the p16-*Leiden* cohort, PDAC was diagnosed in 8 of 116 individuals (7%). In the FPC cohort, only 1 of 125 individuals was diagnosed with PDAC (0.8%, p=0.013).

Four of the eight PDAC cases (50%) in the p16-*Leiden* cohort were prevalent cases (detected at the first screening round) and the other 4 were incident cases (detected

during follow-up). The patient with PDAC in the FPC cohort was a high-risk FPC-patient and PDAC was detected during follow-up.

TABLE 2. Frequency of radiologically detected cystic lesions and of PDAC

	Cystic lesions (%)*	PDAC (%)	Operation (%)
◆ **FPC (n=125)**	52 (42)	1 (0.8)	12 (10)
◆ **p16-*Leiden* (n=116)**	18 (16)	8 (7)	7 (6)

n = number

** Numbers represent the number of individuals with one or more radiologically detected cystic lesions of the pancreas*

FEATURES AND NATURAL COURSE OF CYSTIC LESIONS

IAR with cystic lesions in the FPC cohort were significantly younger than in the p16-*Leiden* cohort (54 vs. 60 years, p=0.026) (*table 3*). In both cohorts, most IAR had cystic lesions not located in the main duct (89% in p16-*Leiden*, 98% in FPC), but in the p16-*Leiden* cohort, significantly more cystic lesions were located in the main duct compared to the FPC cohort (p=0.020). In both cohorts, most individuals had single unicystic or multiple unicystic lesions, only a few had multicystic lesions. All lesions were comparable in size between the two cohorts. Unicystic lesions were mostly small (mean size 3-6 mm). In the FPC cohort, one high-risk individual had a relatively large unicystic lesion (31 mm) at baseline screening, which was located in the main duct (the only main duct ectasia in the FPC cohort). This patient is scheduled for resection as recommended by the consensus guidelines due to the high risk of malignancy inherent to main duct lesions.[27] The distribution of cystic lesions over the pancreas in the two cohorts is shown in *table 3*. Cystic lesions were significantly more often located in the corpus of the pancreas in the p16-*Leiden* mutation carriers than in the FPC-cohort. In the FPC cohort, only three of 52 (6%) individuals had a cystic lesion detected after the first screening round (incident), which was significantly less than in the p16-*Leiden* cohort (56%, p<0.001).

In the p16-*Leiden* cohort, thirteen of 18 (72%) individuals had follow-up of their cystic lesions (mean duration of follow-up: 2.5 years). Three individuals (23%) with follow-up MRIs showed progression, i.e. growth of a cystic lesion or PDAC-development. The individual with growth of the cystic lesion had a multicystic lesion with a diameter of 15 mm. During six years of follow-up there was no change in size, but one year later the diameter of the lesion increased to 17 mm. The two other individuals with progression at follow-up developed PDAC at the site of the cystic lesion. One of these individuals had two multicystic lesions (14.2 mm and 12 mm) and developed a 20 mm cancer detected by MRI one year later. The second patient had a small solitary lesion and irregular duct and developed a 10 mm

cancer detected by MRI five months later. The two other incident cases of PDAC in the p16-*Leiden* cohort did not have a cystic lesion detected on previous MRI. One individual developed a 15 mm cancer 12 months after a normal

TABLE 3. Features and course of cystic lesions on radiology

		p16-*Leiden*	FPC	p-value
No. of patients (%)		18 (16)	52 (42)	
Mean age at detection (range)		60 (50-72)	54 (31-71)	0.026
Localization	Main duct	3	1	0.020
	Other than main duct*	16	51	ns
Detection	Prevalent	9	49	<0.001
	Incident	10	3	<0.001
Appearance	Multicystic	5	7	ns
	Multiple unicystic	9	21	ns
	Single unicystic	7	26	ns
Mean size (range)	Multicystic	14 mm (11-18)	11 mm (6-18)	ns
	Multiple unicystic	4 mm (2-14)	5 mm (1-10)	ns
	Single unicystic	3 mm (2-4)	6 mm (2-31)	ns
Site of pancreas	Head	10	18	ns
	Corpus	13	23	0.041
	Tail	9	27	ns
	Proc. Uncinatus	1	-	ns
Follow-up	No. of patients	13	33	ns
	Mean follow-up (range)	2.5 years (0.25-8)	3.8 years (1-7)	0.027
	Growth of lesion	1	3	-
	Development of PDAC[†]	2	1	-

Numbers represent the number of individuals. Since an individual is able to have more than one lesion, overlap may exist
ns = not significant
** includes branch duct cystic lesions with clear connection to the main duct and cystic lesion with uncertain connection to pancreatic ducts*
[†] at the same site of the cystic lesion(s)

MRI; the other individual developed a 40 mm cancer 28 months after a normal MRI. Thus, of the four incident PDAC cases, two had one or more cystic lesions detected on previous MRI.

A comparable number of individuals in the FPC cohort had follow-up of their cystic lesions (33/52=63%, p=0.500). Mean follow-up of these lesions was however significantly longer (mean duration of follow-up: 3.8 years, Mann-Whitney test: p=0.027). Only four individuals had progression of their cystic lesions (12%). The MRIs of three individuals showed growth

of a lesion, of which one was a multicystic lesion and two were unicystic lesions. Growth was slow in all three cases. One individual developed PDAC in the pancreatic head two years after the first and only MRI. This MRI showed multiple tiny unicystic lesions in the whole pancreas, the largest located in the head with a diameter of 5 mm. The proportion of individuals with progression of their cystic lesions was higher in the p16-*Leiden* cohort (23%) than in the FPC cohort (12%) .

HISTOLOGIC FINDINGS IN SURGICAL SPECIMENs

In the p16-*Leiden* cohort, seven cases underwent surgery, of which six had PDACs (*table 4*). Three of these cases had single low grade PanIN lesions (PanIN1 and 2) adjacent to the carcinoma. One case (*table 4*, case A), with the smallest PDAC of the series, showed a small gastric type BD-IPMN with low- to high-grade dysplasia, and another case (*table 4*, case G) showed multifocal PanIN1 and 2 disease combined with peripheral foci of lobular fibrosis and small gastric-type BD-IPMNs in the subtotal pancreatectomy specimen. The surgical specimens of four additional PDACs from symptomatic patients with a p16-*Leiden* germline mutation diagnosed in the same time period at the Leiden University Medical Center, were histologically reviewed (*table 5*). In two cases, the PDAC was accompanied by few low grade PanIN lesions. One of the two cases had in addition a PanIN3 lesion. IPMNs were not found in these cases. In total, five of the 10 operated PDAC cases (50%) (*table 4* and *table 5*) revealed PanIN lesions and 1 of 10 had IPMNs in the surrounding tissue. Only one case (*table 4*, case G) in the screened p16-*Leiden* cohort was operated because of growth of a cystic lesion on MRI. This patient who was already previously mentioned showed multifocal PanIN-disease but no infiltrating PDAC (as discussed earlier).

TABLE 4. p16-*Leiden* cohort: histologic findings in surgical specimens

		Histologic characteristics	
	Age	**Tumor diagnosis**	**Precursor lesions in the peritumorous tissue (n)**
A	62	Ductal adenocarcinoma G1	BD-IPMN; PanIN1 (1)
B	49	Ductal adenocarcinoma G1	-
C	47	Ductal adenocarcinoma G3	Few PanIN1-2 (2)
D	72	Ductal adenocarcinoma G1	-
E	58	Ductal adenocarcinoma G1	-
F	57	Ductal adenocarcinoma G1	Few PanIN1 (3)
G	62	No PDAC. Multifocal PanIN1-2; BD-IPMN	n/a

G = grade, n/a = not applicable, n = number of lesions

TABLE 5. p16-*Leiden*: histologic findings in surgical specimens of additional (symptomatic) PDAC cases, not screened

		Histologic characteristics	
	Age	**Tumor diagnosis**	**Precursor lesions in the peritumorous tissue (n)**
A	38	Ductal adenocarcinoma G2	Few PanIN1 (3)
B	58	Ductal adenocarcinoma G2	-
C	40	Ductal adenocarcinoma G1	-
D	47	Ductal adenocarcinoma G3	PanIN1, 3 (2)

G = grade, n = number of lesions

In the FPC cohort, one of the twelve cases that underwent pancreatic resection had PDAC (*table 6*). Five cases had small BD-IPMN lesions. Three cases had one or more PanIN3 lesions as highest grade, of which two were found in combination with a BD-IPMN. Two cases had one or more PanIN2 lesions as highest grade, of which again one occurred in association with a BD-IPMN. One case had only PanIN1 lesions, one case had in addition to a PanIN1 lesion a serous cystadenoma (SCA) and two cases only had a SCA.

DISCUSSION

In this study we compared a FPC cohort with a p16-*Leiden* cohort to evaluate the role of precursor lesions in the early detection of PDAC in these two high-risk groups. We demonstrated a significant difference in recognition of precursor lesions and PDAC between the two groups. Cystic lesions were more common in the FPC cohort (42% vs. 16%), while the incidence of PDAC was ten times higher in the p16-*Leiden* cohort (7% vs. 0.8%). Interestingly, on histologic examination of resected pancreas specimens, the FPC cohort showed both PanIN lesions as well as IPMN lesions, whereas patients in the p16-*Leiden* cohort revealed mainly a few low-grade PanIN lesions. In the p16-*Leiden* cohort, a substantial proportion of cystic lesions showed growth or malignant transformation during follow-up whereas in the FPC cohort most cystic lesions were stable. These findings suggest a high malignant potential of cystic lesions occurring in p16-*Leiden* mutation carriers.

TABLE 6. FPC cohort: histologic findings in surgical specimens

			Histologic characteristics			
	Age	**Risk group**[†]	**PDAC**	**PanIN**	**IPMN**	**other**
1	42	Moderate				SCA
2	58	Moderate		PanIN1-2 (multifocal*)	BD-IPMN, gastric type	
3	61	Moderate				SCA
4	64	Moderate		PanIN1-3 (multifocal)	BD-IPMN, gastric type (multiple)	
5	54	Moderate		PanIN1-2		
6	51	High		PanIN1-3 (multifocal)		
7	53	High		PanIN1	BD-IPMN, gastric type microscopic)	
8	54	High		PanIN1		
9	52	High	yes			
10	61	High		PanIN1		SCA
11	69	High		PanIN1-3 (multifocal)	BD-IPMN, gastric type (multiple)	
12	70	High		PanIN1-2 (multifocal)	BD-IPMN, gastric type	

† Moderate risk = two first degree relatives with PDAC, High risk = three or more first degree relatives with PDAC, or with a BRCA2 or PALB2 germline mutation
** multifocal indicates more than 3 PanIN lesions*

To date, a number of studies focused on screening for PDAC has been published, predominantly concerning individuals from FPC families.[11-14,17-21] Overall, in these studies both PanIN lesions and IPMN lesions were detected in FPC-individuals, but there was an overall low incidence of PDAC (<1%). To date, there is only one screening study that solely looked at a large FAMMM/p16-*Leiden* cohort.[22] It showed a high incidence of PDAC (9%) and revealed no confirmed IPMN lesions. Other studies that included FAMMM patients in their screening program also did not report confirmed IPMN lesions.[15,16,20] IPMNs were lacking in the pancreas of genetically engineered mice with K-RAS and p16 germline mutations.[28] Taken together these data show that the results of the current study are in line with previous screening investigations on FPC and p16-*Leiden*.

What is the role of cystic lesions in the development of PDAC? De Jong *et al* studied the prevalence of cystic lesions in the pancreas in the general population and demonstrated that 2.4% of almost 3000 asymptomatic individuals who had a screening abdominal MRI had a pancreatic cyst of any kind, but only 8% of these cysts (0.2% of total) communicated with the pancreatic duct, which can be considered a cystic duct lesion.[29] Our current study demonstrated a frequency of cystic lesions in the FPC and p16-*Leiden* cohort of 42% and 16%, respectively, of which the majority probably originate from pancreatic ducts. Thus, the rate of cystic lesions in high-risk groups compared to the general population is much higher, which suggests an association between these lesions and the development of

PDAC. However, in the study by De Jong *et al*, no MRCP was performed and the MRI was not directed to imaging of the pancreas, so the difference could be overestimated.

In the development of PDAC, usually only PanIN2-3 or IPMN are considered relevant lesions. Andea *et al* compared tumor free pancreatic tissue from pancreas specimens with PDAC with that of entirely nonneoplastic pancreatic tissue.[30] A substantial proportion (28%) of normal pancreas specimens harbored low-grade PanIN (PanIN1 and 2) lesions but no PanIN3 lesions whereas the latter lesions were detected in more than half (58%) of pancreas specimens with PDAC, an observation which suggests the pathological significance of these lesions. Shi *et al* found, in their comparison of specimens from FPC associated PDACs with sporadic PDACs, that IPMNs are common lesions in FPC-individuals. In the FPC series, 33% of the individuals had IPMNs (20% high-grade), whereas the surrounding tissue of sporadic PDACs only harbored IPMNs in 6% of cases (none high-grade).[31]

In our p16-*Leiden* cohort, including the four PDAC cases not under surveillance, three of 10 PDAC cases (30%) had a few associated PanIN1 lesions, whereas in the FPC cohort, six of 12 patients (50%) had PanIN2-3 lesions that were not associated with a PDAC. In the FPC cohort, five of the 12 patients (42%) had BD-IPMNs of gastric type. These lesions were only seen twice in our p16-*Leiden* cohort, but in both patients the findings resembled the precursor pattern observed in FPC cohort. These results suggest that PanINs and BD-IPMNs of gastric type play an important role in the FPC phenotype, but have much less significance for the p16-*Leiden* phenotype. Our study also showed that in the p16-*Leiden* cohort some PDACs developed without evidence for the presence of precursor lesions.
A common finding in our FPC cohort was serous cystadenoma (SCA), confirmed in three cases. SCAs were not observed in p16-families. The screening studies in FPC families by Canto *et al* [13] and Ludwig *et al* [19] also reported serous cystadenomas and a serous microcystic adenoma, all variants of serous cystic neoplasms (SCN), which are considered rare benign lesions [32]. The relatively high frequency of SCAs in FPC might be explained by selection bias as FPC patients underwent surgery because of suspicion of an IPMN.

Overall, our findings and the findings reported in the literature suggest an important role of precursor lesions in the carcinogenesis of PDAC in different high-risk groups which justifies the goal of screening, i.e. to identify these precursor lesions.

The current study has some limitations. First of all it is a *retrospective* analysis of the presence of precursor lesions and PDAC in two high-risk groups. However, the data were retrieved from two ongoing *prospective* surveillance studies. Secondly, there are some differences between the two cohorts that might have influenced the results. The mean age

of the FPC group at the start of surveillance is seven years younger than the age of the p16-*Leiden* group. Because the frequency of cystic lesions was higher in the FPC group, we would expect that the differences would be even larger if the age distribution in the two groups was similar. However, because the mean age at diagnosis of PDAC in FPC is in the mid-60s and the mean age at the start of the surveillance of the FPC cohort was only 47 years, it is likely that the incidence of PDAC will increase over the coming decades. The difference in frequency of PDACs might thus become smaller, although the incidence of PDAC in other cohorts consisting of participants that enrolled in their mid-50s was also low (<1%). In the present study we compared only the outcome of the MRI/MRCP between the two cohorts. A possible source of bias is the fact that in the FPC cohort also EUS was used in the surveillance protocol whereas only MRI/MRCP was applied in the p16-*Leiden* cohort. The use of EUS in the FPC-cohort could have increased the detection of cystic lesions. However, because the sensitivity of MRCP for detection of such lesions is higher compared to EUS, we don't think that adding EUS to the FPC-protocol had a major effect on the results.

The results of our current study could have implications for the current screening protocol. In FPC, the incidence of PDAC is low (0.8%) and almost all lesions (88%) detected by screening are stable at follow-up (or only slowly growing). This would suggest a relatively low malignant potential of precursor lesions in the setting of FPC. Because of these findings, it could be argued that it is safe to screen young FPC-individuals (e.g. <55 years) without evidence of precursor lesions with larger intervals between examinations, for instance once every two years and those with lesions at shorter intervals. In p16-*Leiden*, however, we demonstrated a high incidence of PDAC and a probably high malignant potential of precursor lesions. A more intensive surveillance program with MRI/MRCP as well as EUS is probably needed for the timely detection of early stage tumors or precursor lesions._

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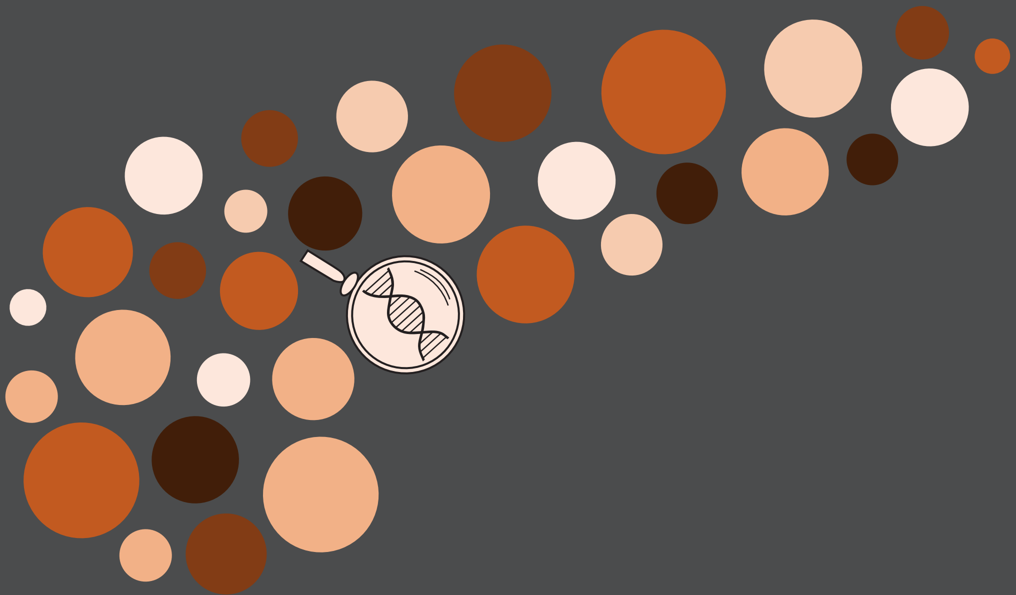

5

Limited resection of pancreatic cancer in high-risk patients can result in a second primary

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SUMMARY

Up to 10% of patients with pancreatic ductal adenocarcinoma (PDAC) have either a positive family history for pancreatic cancer (Familial Pancreatic Cancer), or an underlying germline mutation in specific genes (e.g. *CDKN2A*, *BRCA2*) associated with hereditary tumour syndromes. Guidelines have recently been established for the surveillance and management of individuals with a high risk for PDAC, but no recommendations were provided regarding the extent of surgery, that is partial or total pancreatectomy, in cases with a small screen-detected PDAC. This is an important issue because it seems very likely that a hereditary background increases the risk for a second primary cancer of the pancreas. Here we describe two high-risk individuals who developed a second primary cancer after a partial pancreatectomy of an early-stage cancer. Based on these cases, we discuss the pros and cons of total pancreatectomy in high-risk individuals with an early-stage PDAC.

BACKGROUND

Despite medical progress and improved diagnostic and surgical procedures, pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal cancers and is currently the fourth leading cause of cancer-related death in the western world. Only a minority of patients are diagnosed at an early stage of the disease.[1] Pancreatic surveillance of asymptomatic high-risk individuals could potentially increase the proportion of patients with early-stage PDAC and thus improve overall survival. A well-established group of individuals at high risk are those with an inherited predisposition for the disease. About 5-10% of PDAC cases have either a positive family history for pancreatic cancer, a condition referred to as Familial Pancreatic Cancer (FPC), or an underlying germline mutation in specific genes associated with certain tumour syndromes that also predispose to PDAC.[2] Tumour syndromes that are relatively frequently associated with PDAC include Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome caused by a mutation in the *CDKN2A* gene and hereditary breast cancer caused by a mutation in the *BRCA2* gene in particular.[2]

A number of studies have described pancreatic surveillance in high-risk individuals, using screening tools such as endoscopic ultrasonography (EUS) and magnetic resonance imaging (MRI) combined with magnetic resonance cholangiopancreatography (MRCP).[3] At an international multidisciplinary consensus meeting in 2011 on the surveillance and management of individuals at high risk for PDAC, indications for surgery of these individuals were addressed, but no recommendations were given regarding the extent of surgery, that is, partial or total pancreatectomy, in patients with a screen-detected PDAC.[3] This is an important issue because it seems very likely that a hereditary background increases the risk for a second primary cancer of the pancreas.

Here we describe two high-risk individuals who developed a second primary cancer after a partial pancreatectomy of an early-stage cancer. Based on these cases we discuss the pros and cons of a total pancreatectomy.

CASES

Patient 1 is a 62-year-old female with the Dutch '*p16-Leiden*' founder mutation in the *CDKN2A* gene (c.225_243del19; RefSeq NM_000077.4) and a medical history of melanoma at age 56. This patient was enrolled in the pancreatic surveillance program at Leiden University Medical Center in 2008. The first MRI showed a lesion in the head-corpus region of the pancreas, suspicious for an adenocarcinoma. The lesion was confirmed by CT scanning,

with no signs of distant metastases. A partial pancreaticoduodenectomy was performed. Histopathologic examination showed a well-differentiated (grade 1) adenocarcinoma of 5 mm, surrounded by PanIN1 lesions and an IPMN lesion. The resection margins were free of tumour and seven lymph nodes were unaffected (T1N0M0, UICC stage IA). A *KRAS* hotspot mutation in codon 12 was detected in the tumour (c.35G>T). This patient continued pancreatic surveillance. After 4 years and 6 months, a solitary lesion of 7 mm was found in the corpus-tail region with EUS. Cytological examination of an EUS-guided fine-needle aspirate showed atypical cells compatible with adenocarcinoma. Of note, no *KRAS* mutation was detected in these cells. CT scanning confirmed the presence of the lesion without evidence for distant metastases. A completion pancreatectomy with splenectomy was performed and histopathologic examination showed one small duct suspicious for adenocarcinoma surrounded by multifocal PanIN1-3 lesions. The resection margins of the specimen were free of tumour and 13 lymph nodes were unaffected (T1N0M0, stage IA). Fifteen months after completion pancreatectomy, the patient is alive with no evidence of disease.

Patient 2 is a 46-year-old female with a germline mutation in the *BRCA2* gene and three affected relatives with PDAC. In 1984, she developed a painless icterus; CT scanning and endoscopic retrograde cholangiopancreatography (ERCP) revealed a tumour in the pancreatic head. A partial pancreaticoduodenectomy was performed. Histopathologic examination showed a moderately differentiated (grade 2) adenocarcinoma of 22 mm. The resection margins were free of tumour and none of 14 lymph nodes were affected (T2N0M0, stage IB). In 1987, 2 years and 9 months later, the tumour marker CA 19.9 increased to 190 U/mL (normal <39 U/ml) and CT scanning revealed a tumour in the tail of the pancreas. A resection of the remnant pancreas was performed and histopathologic examination showed a poorly differentiated (grade 3) adenocarcinoma of 20 mm. The resection margins and eight lymph nodes were free of tumour (T2N0M0, stage IB). At the last follow-up, 28 years after completion pancreatectomy, the (currently 76-year-old) patient is alive with no evidence of PDAC.

DISCUSSION

This is the first report of the development of a second primary PDAC after partial resection of a first pancreatic tumour in patients with a genetically increased risk for the development of PDAC. The first patient carried a *CDKN2A* mutation and the second patient had a mutation in *BRCA2*; both gene defects are associated with the development of PDAC. Development of a metachronous second primary tumour in a remnant pancreas in

apparently *sporadic* PDAC cases has been previously described [4], but this is a very rare event, which is probably due to the poor survival of these patients.

The cases presented here raise a number of questions, the *first* of which is: did these patients actually develop a second primary or was the second tumour simply a local recurrence of the original tumour? Arguments that would support a second primary tumour rather than a local recurrence include: (1) a long interval between diagnoses of the tumours, (2) a location of the second tumour in another part of the pancreas (distant from resection lines), (3) differences in the pathology of the tumours, (4) different *KRAS* mutations in the tumours, and (5) both first and second tumours are early-stage without evidence for metastatic disease. The two cases comply with most of these criteria: (1) intervals between diagnoses were 4 years and 6 months in case 1 and 2 years and 9 months in case 2; (2) the location of the second tumour was distant from the resection lines in both cases; (3) in patient 2, the first tumour was a moderately differentiated (grade 2) adenocarcinoma, whereas the second tumour was a poorly differentiated (grade 3) adenocarcinoma; (4) in patient 1, the *KRAS* hotspot mutation detected in the first tumour was not detected in the cells obtained by cytology from the second tumour, suggesting a different aetiology; (5) in both cases, the first and second cancer were early-stage cancers (T1-2N0M0). Taken together, these findings suggest that these tumours are most likely second primary tumours.

A *second* important question is: what is the risk, in a patient with a genetic predisposition, of developing a second primary cancer after resection of a first PDAC? A previous study on surveillance outcomes for Dutch carriers of a *p16-Leiden* mutation reported that, while the program substantially increased the proportion of patients with resectable tumours, very few patients had a long survival.[5] This was echoed in the German surveillance program, where patients with a longer survival following resection of PDAC were also very rare.[6] The observation of a second tumour in these 'rare' (n=2) patients therefore suggests that a genetic predisposition contributes a substantial risk of developing a second primary tumour if the patient survives the first tumour. Moreover, the development of a second cancer within a relatively short follow-up time (2 to 4 years after the first tumour) also indicates substantial risk.

What are the implications of our findings for the surgical management of high-risk patients? Should we offer total pancreatectomy (TP) to all patients with a genetic predisposition and an early-stage cancer? A well-known disadvantage of TP is the development of 'brittle' diabetes which is associated with substantial morbidity. However, recent studies all concluded that TP is safe, with acceptable mortality and morbidity.[7,8] Studies that compared the perioperative mortality and morbidity of TP with partial pancreatectomy (PP, mostly

pancreaticoduodenectomy [PD]) produced more conflicting results (see *table*).[9-15] Some studies reported no significant difference in mortality and morbidity between TP and PD, whereas others reported a 1.5-3 fold increased risk of mortality and a (lesser) increased morbidity risk. Interestingly, two recent studies assessed quality of life (QoL) in TP cases compared with matched PD cases.[8,10] These studies demonstrated that QoL following TP is acceptable and similar to that reported for PD. Moreover, while brittle diabetes has a negative impact on QoL after TP, the level of impact is comparable to that of diabetes following PP or due to other causes. This conclusion was supported by another study that assessed QoL (without comparisons) in TP cases.[7] In light of these recent studies, the best approach may be to openly discuss the various advantages and disadvantages of TP with high-risk patients with early-stage PDAC and come to a decision together.

In conclusion, we describe two high-risk patients who developed a second primary PDAC two to four years after a partial pancreatic resection of an early-stage PDAC. In view of the acceptable perioperative mortality and morbidity risk of TP and an improvement of quality of life after TP in recent years, this type of surgery should be seriously considered in high-risk patients with an early-stage (screen-detected) tumour.

TABLE. Perioperative outcome of total pancreatectomy (TP) versus partial pancreatectomy (PP)

Reference	Cohort	Mortality (%)	Sign. (p-value)	Morbidity (%)	Sign. (p-value)
Schmidt *et al* [9]	TP n=33	6	n.s.	36	n.s.
	PD n=28	7		54	
Muller *et al* [10]	TP n=87	6	n.s.	31	n.s.
	PD n=87	3		23	
McPhee *et al* [11]	TP n=1,399	8.3	0.0002	n/a	n/a
	PD n=27,289	6.6		n/a	
Reddy *et al* [12]	*Period 1970-2007*				
	TP n=100	8	0.0007	69	<0.0001
	PD n=1,286	1.5		38.6	
	Subanalysis of period 2000-2007				
	TP n=53	*1.9*	*0.17*	*No significant change*	
	PD n=?	*1.2*			
Nathan *et al* [13]	TP n=376	8.6	0.09	n/a	n/a
	PP n=3,645	6.3		n/a	
Simons *et al* [14]	TP n=5,966	OR 2.90	<0.0001	OR 1.29	0.0025
	PD n=56,207	*Ref*		*Ref*	
Bhayani *et al* [15]	TP n=198	6.1	0.02	38	0.02
	PD n=6,314	3.1		30	

PD = Pancreaticoduodenectomy, OR = odds ratio, n.s. = not significant, n/a = not available

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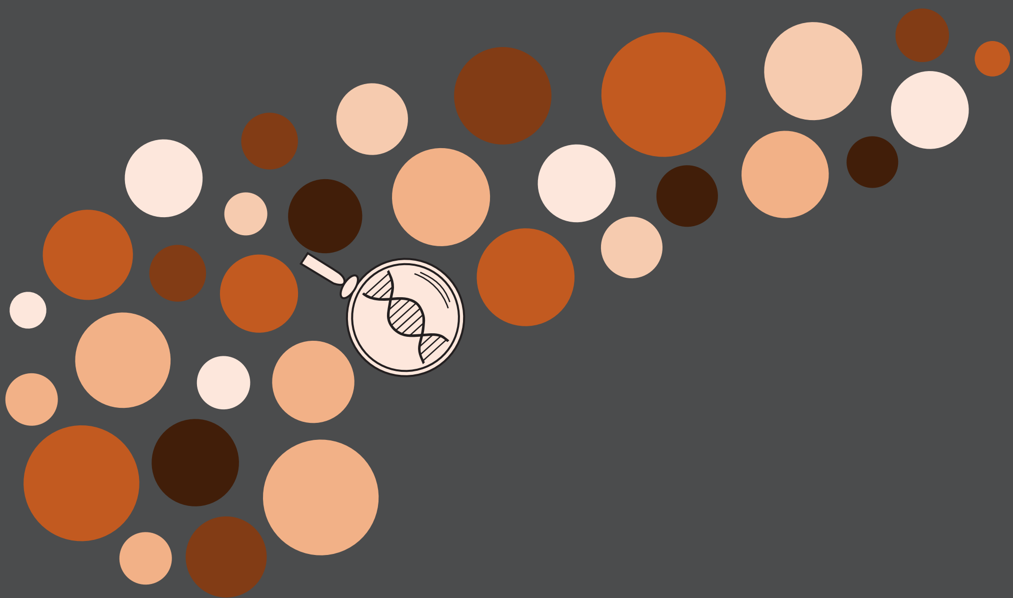

6

Application of a serum protein signature for pancreatic cancer to separate cases from controls in a pancreatic surveillance cohort

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ABSTRACT

BACKGROUND

Pancreatic cancer (PC) surveillance is currently offered to individuals with a genetic predisposition to PC, but routinely used radiological screening modalities are not entirely reliable in detecting early-stage PC or its precursor lesions. We recently identified a discriminating PC biomarker signature in a sporadic patient cohort. In this study, we investigated if protein profiling can accurately distinguish PC from non-PC in a pancreatic surveillance cohort of genetically predisposed individuals.

METHODS

Serum samples of 66 individuals with a *CDKN2A* germline mutation who participated in the pancreatic surveillance program (5 cases, 61 controls) were obtained following a standardized protocol. After sample clean-up, peptide and protein profiles were obtained on an ultrahigh resolution MALDI-FTICR mass spectrometry (MS) platform. A discriminant score for each sample was calculated with a previously designed prediction rule, and the median discriminant scores of cases and controls were compared. Individuals with precursor lesions of PC (n=4) and individuals with a recent diagnosis of melanoma (n=4) were also separately considered.

RESULTS

Cases had a higher median discriminant score than controls (0.26 vs 0.016; p=0.001). The only individual with pathologically confirmed precursor lesions of PC could also be clearly distinguished from controls, and having a (recent) medical history of melanoma did not influence the protein signatures.

CONCLUSIONS

Peptide and protein signatures are able to accurately distinguish PC cases from controls in a pancreatic surveillance setting. MS-based protein profiling therefore seems to be a promising candidate for implementation in the pancreatic surveillance program as an additional screening modality.

INTRODUCTION

Pancreatic cancer (PC) is one of the most lethal cancers with a 5-year survival rate of only 5%.[1] The first clinical symptoms generally appear relatively late when the tumour is already in an advanced stage. To improve prognosis, PC has to be detected at an earlier stage in which curative surgical resection is still possible. Therefore, in the last decade, pancreatic surveillance programs for high-risk individuals have been set up, aimed at detecting early-stage PC or relevant precursor lesions in individuals with a genetic predisposition to PC.[2]

At the Leiden University Medical Center (LUMC), such a pancreatic surveillance program was initiated in the year 2000 for individuals with a *CDKN2A* germline mutation.[3] These individuals have a familial predisposition for developing cutaneous melanoma, a condition known as Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome, but also a 15-20% lifetime risk for developing PC.[4] Because many individuals with a specific Dutch founder mutation in the *CDKN2A* gene (a 19bp deletion known as p16-*Leiden*) are living in the vicinity of Leiden, a relatively large cohort of these patients is under pancreatic surveillance in the LUMC. The surveillance program consists of annual abdominal magnetic resonance imaging (MRI and MRCP) and optionally endoscopic ultrasound (EUS). Although these screening modalities are generally able to detect early-stage PC or relevant precursor lesions of PC, the diagnostic yield of surveillance programs using these modalities varies greatly and only a subset of patients with a screen-detected PC have an early-stage cancer.[2,5] Therefore, there is a need to improve the current pancreatic surveillance program.

One way to improve PC surveillance programs is to use serum biomarkers as an additional non-invasive screening modality.[6-9] These biomarkers have to discriminate cancer patients from non-cancer patients or even patients with precursor lesions of PC. Currently, only the mucin-associated carbohydrate antigen CA 19-9 is routinely used, but has not proven to be an adequate biomarker for detecting early-stage PC.[10] Many studies have been published on novel individual biomarkers for the early detection of PC, but none of them have been implemented in daily practice so far.[11,12]

In our center, a discriminating PC biomarker signature was recently identified by following a serum peptide and protein profiling strategy based on a combination of automated single-step sample clean-up and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[13,14] The most detailed protein signatures were obtained using an ultrahigh resolution MALDI Fourier transform ion cyclotron resonance (FTICR) MS

platform that provided case-control classifications with a sensitivity and specificity both well above 85%.[15] A discriminating prediction rule was validated for this classification. The methodology used in our previous studies is graphically displayed in *figure 1* (left-hand side). Based on these encouraging results it was concluded that such protein signatures are a promising candidate for implementation in the current pancreatic surveillance program as an additional screening modality. The aim of the current study is therefore to determine whether ultrahigh resolution protein profiling (using MALDI-FTICR-MS) in serum can accurately distinguish individuals with PC from non-PC in a novel cohort of *CDKN2A* mutation carriers enrolled in the pancreatic surveillance program, using the previously designed and validated prediction rule for the classification of individual samples (*figure 1*, right-hand side).

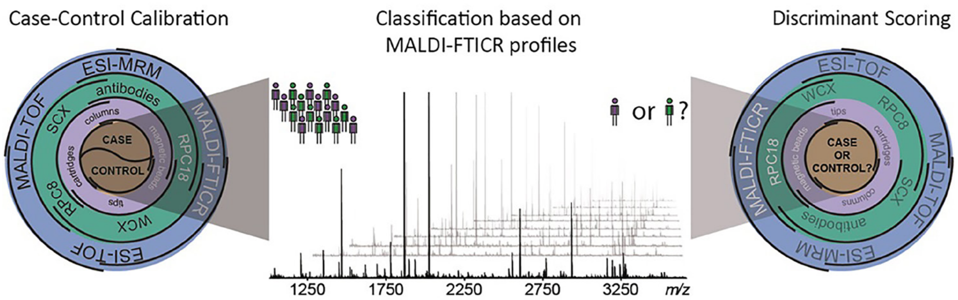


FIGURE 1: Serum peptide and protein profiling strategy, aiming for patient classification based on matrix-assisted laser desorption/ionization (MALDI) Fourier transform ion cyclotron resonance (FTICR) mass spectrometry.

Various peptide and protein signatures have been reported based on a single-step sample clean-up procedure using a combination of a carrier (depicted in the inner shell) with capture material (depicted in the middle shell), and a mass spectrometer (depicted in the outer shell). Previously, our group has reported signatures for PC based on weak-cation exchange (WCX) with MALDI time-of-flight (TOF) [Velstra et al. 2013], and reversed-phase (RP) C18 with MALDI-TOF [Velstra et al. 2015]. In the current study an ultrahigh resolution RPC18-MALDI-FTICR signature is used that was obtained in a case-control calibration and validation design (left-hand side) [Nicolardi et al. 2015]. Serum samples from *CDKN2A* mutation carriers are analysed in an identical way to obtain a discriminant score (right-hand side).

PATIENTS AND METHODS

PATIENT COHORT AND BLOOD SAMPLING

Individuals with a *CDKN2A* germline mutation who participate in the pancreatic surveillance program at the Leiden University Medical Center were eligible for inclusion. A complete medical history was obtained at the start of surveillance, including a medical history of melanoma or other cancers. Subsequently, annual MRI and MRCP with optionally EUS was performed and in case of an abnormal finding, either close follow-up with MRI/MRCP and EUS or surgery was advised by a multidisciplinary team, as previously described.[3] Any cancer occurring in follow up was registered. Cases were defined as having a pathologically confirmed diagnosis of PC. Controls were not diagnosed with PC, and included individuals with relevant precursor lesions of PC. These were defined as either pathologically proven precursor lesions (intraductal papillary mucinous neoplasm (IPMN) and pancreatic intraepithelial neoplasia (PanIN) [16]), or radiological cystic lesions ≥ 5 mm suspicious for IPMN.

Serum samples from the cases with PC were obtained prior to surgery. Serum samples from the controls without PC were obtained during their annual surveillance visit at the outpatient gastroenterology clinic. Only one sample was collected per individual. Samples were collected over a time period ranging from April 2008 until January 2015. Additional serum samples of *CDKN2A* mutation carriers with PC who did not participate in the surveillance program were available through an on-going research project of the Department of Surgery, in which serum samples of all patients with PC are obtained prior to surgery. Samples were collected and processed following a standardized high-throughput clean-up protocol as previously described.[17,18] Informed consent was obtained from all individuals, and the study was approved by the Ethics Committee of the Leiden University Medical Center (#P03.147).

SAMPLE PROCESSING AND MALDI-FTICR MASS SPECTROMETRY PEPTIDE PROFILING

The isolation of peptides and protein from serum was performed using a fully automated, high-throughput protocol based on solid-phase extraction (SPE) with RPC18-funtionalized magnetic beads, as previously described.[15,18] Subsequently, MALDI-profiles were obtained on a MALDI-FTICR platform that allows mass analysis of serum peptides and proteins with isotopic resolution up to 15,000 Da. A detailed description of this approach and workflow, as well as the subsequent data processing, was previously described by Nicolardi *et al*.[15] For this study, only so-called low-mass (LM) data (i.e., up to *m/z*-value 4000) was used for statistical analysis. The serum samples were blindly analysed.

STATISTICAL ANALYSIS

Our group previously designed a prediction rule to classify a serum sample as either case or control using logistic regression ridge shrinkage (LRRS) analysis.[15,19] By applying the same prediction rule to the LM data acquired in this study, a “discriminant score” was calculated for each sample. Samples were grouped according to their known disease status and the median discriminant scores per group were compared using a Mann-Whitney-Wilcoxon test. Individuals with precursor lesions and individuals with a recent diagnosis of melanoma were also separately considered.

RESULTS

PATIENTS

A total of 66 individuals (42 females, 64%) were included in the study. Sixty-one individuals had a molecularly proven *CDKN2A* germline mutation, of which 60 had the p16-*Leiden* mutation (c.225_243del19; RefSeq NM_000077.4). One individual carried the c.67G>C mutation, which is also associated with PC [not published data]. The remaining 5 individuals had a medical history of melanoma (or PC, #4 *table 2*), and a close relative with a proven *CDKN2A* germline mutation, which makes them highly likely of being a carrier. Patient characteristics are shown in *table 1*. Five individuals (all female) had PC, with a mean age of 54 years (range 39-62 years). Two of five cases had a medical history of melanoma, but no other cancers occurred in the case group. The remaining 61 individuals (37 females, 61%) had no PC. The mean age of the control group was 53 years (range 42-72 years). Thirty-eight controls had a medical history of melanoma, and a few other cancers occurred in the control group (see *table 1*). One individual in the control group had a melanoma 1 month prior to serum sampling (#2 *table 3*), and one individual had a melanoma 1 month after serum sampling. Two other individuals had cancer ≤12 months before or after serum sampling (both melanoma; 12 months prior and 9 months after). These melanomas were non-metastatic.

Detailed information about the case group is shown in *table 2*. Three cases were participating in the surveillance program, of which two were diagnosed with PC at the first screening round (prevalent) and one was diagnosed on a subsequent screening round (incident). This latter individual (#1, *table 2*) had a normal MRI two years earlier but missed her MRI a year later. She was diagnosed with a 3.6 cm tumour in the subsequent year. Two of five cases were not participating in the surveillance program, and had their serum drawn prior to surgery as part of standard (research) procedure at the Department of Surgery.

TABLE 1. Patient characteristics

Diagnosis	No. of Patients	Age (range)	M:F	Medical History of Melanoma (of which multiple)	Medical History of Other Cancers (No. of Individuals) *
PC	5	54 (39-62)	0:5	2 (1)	None
No PC	61	53 (42-74)	24:37	38 (12)	SCC of larynx (1) † SCC of mouth (1) † SCC of skin (1) BCC of skin (3) Phyllodes sarcoma of breast (1)
With precursor lesions	*4/61*	*54 (45-63)*	*2:2*	*3 (1)*	None
Total	66	53 (39-74)	24:42	40 (13)	As above

SCC = Squamous Cell Carcinoma, BCC = Basal Cell Carcinoma
** None of these cancers occurred within a year prior to serum sampling*
† These cancers occurred synchronously in one individual

TABLE 2. Tumour Characteristics of Cases with PC

	Age	M/F	Medical History of Cancer	Mode of Diagnosis	Loc.	Tumour Size (cm)	Tumour Stage (TNM)	Tumour Grade
1	57	F	-	Surveillance, incident	Tail	3.6	T2N0M0 (Stage IB)	2
2	62	F	Me 56 yrs	Surveillance, prevalent	Head-corpus	0.5	T1N0M0 (Stage IA)	1
3	62	F	Me 31 yrs (2x)	Symptomatic	Head	5.0	T3N1M0 (Stage IIB)	2
4	39	F	-	Symptomatic	Proc. uncinatus	1.5	T3N1M0 (Stage IIB)	n/a
5	47	F	-	Surveillance, prevalent	Corpus	5.7	T3N1M0 (Stage IIB)	3

Me = Melanoma

Four individuals in the control group had relevant precursor lesions of PC, of which detailed information is shown in *table 3*. All four individuals had cystic lesions ≥ 5 mm suspicious for IPMN, but only one individual had a surgical resection due to growth of the lesion. Pathological examination of the resected pancreas of this patient confirmed the presence of an IPMN lesion, as well as multifocal PanIN1-2 lesions.

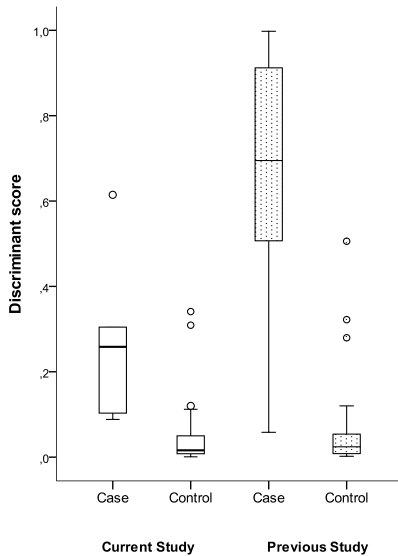
TABLE 3. Precursor Lesions of PC in the Control Group

	Age	M/F	Medical History of Cancer	Findings Pancreatic Surveillance	Surgical Intervention	Pathology
1	63	F	-	Multicystic lesion of 15 mm in head-corpus region, stable for 6 years and growth to 17 mm in the 7th year. Suspicious for BD-IPMN. Two cystic lesions (8 mm, head and 5 mm, tail), stable for 2 years. Suspicious for BD-IPMN	Subtotal pancreatectomy	BD-IPMN; Multifocal PanIN1–2
2	59	M	>15 Me from age 27, most recent at age 59	Multicystic lesion of 7 mm in proc. uncinatus, suspicious for BD-IPMN, stable for 2 years	Not performed	n/a
3	45	F	Me 42 yrs	Cystic lesion of 7 mm and multicystic lesion of 7 mm in head region, both suspicious for BD-IPMN, stable for 2 years	Not performed	n/a
4	49	M	Me 44 yrs	Cystic lesion of 13 mm in corpus-tail region, suspicious for BD-IPMN, stable for 2 years	Not performed	n/a

Me = Melanoma, BD-IPMN = Branch duct intraductal papillary mucinous neoplasm, PanIN = Pancreatic intraepithelial neoplasia

Statistical classification of serum profiles

High-quality MALDI-FTICR data was obtained from all samples and therefore all samples were suitable for further statistical analysis. In *figure 2*, boxplots of the calculated discriminant scores for cases (n=5) and controls (n=61) are shown. Boxplots of the data from our previous study are displayed in *figure 2* as well. Cases from our previous study had a noticeable higher score than cases from the current study, as can be seen in *figure 2*. This can probably be explained by the fact that more cases in our previous study had metastatic (lymph nodes positive or distant) disease, i.e. stage IIB or higher (83% compared to 60% in the current study). As was shown in our previous study, a more advanced tumour stage is associated with a higher discriminant score. The difference could further be caused by a systematic re-calibration effect. Nonetheless, the boxplots show that cases with PC are accurately distinguished from controls without PC in the new surveillance data. The median discriminant score for cases is 0.26 and for controls 0.016, which differs significantly (p value 0.001 using the Mann-Whitney-Wilcoxon test).



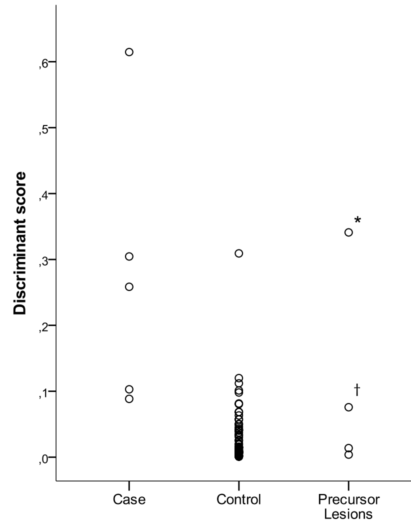


FIGURE 2.

FIGURE 3.

FIGURE 2: Boxplots of the discriminant scores for cases and controls of the current study and of our previous study

The boxplots on the left represent the data of the current study. For comparison, boxplots of the data from our previous study [Nicolardi et al. 2015] are displayed on the right. The generally higher discriminant scores of cases in the previous study compared to cases in the current study can probably be explained by the fact that more cases in our previous study had metastatic (lymph nodes positive or distant) disease, i.e. stage IIB or higher (83% compared to 60% in the current study). A more advanced tumour stage is associated with a higher discriminant score. A systematic re-calibration effect could further explain the difference.

O = Outliers

FIGURE 3: Scatter plot of the discriminant scores of the current study; individuals with precursor lesions are separated from controls

This figure shows all the individual discriminant scores of the 66 included individuals, subdivided in cases (n=5), controls (n=57) and individuals with precursor lesions (n=4).

** Individual #1 (table 3); discriminant score of 0.34, † Individual #2 (table 3); discriminant score of 0.08*

6

Scores of individuals with precursor lesions of PC are separately shown in *figure* 3. The only individual with pathologically proven precursor lesions of PC (#1 in *table 3*, * in *figure 3*) had a relatively high score of 0.34, well above the median score of controls and comparable with the scores of the cases. The individual with precursor lesions as well as a melanoma 1 month prior to serum sampling (#2 in *table 3*, † in *figure 3*) had a score of 0.08 and scored above the 75th percentile of the median score of the control group. The other two individuals with (radiological) precursor lesions had a score below the median of the control group. Apart from individual #2 (*table 3*), there were three other individuals with a melanoma diagnosed shorty before or after serum sampling. These individuals had a score near or well below the median score of the control group.

DISCUSSION

In this study, we analysed biomarker profiles in a pancreatic surveillance cohort of *CDKN2A* mutation carriers with and without PC using the same methodology as in our earlier work. By applying the previously designed prediction rule for the classification of serum samples, cases with PC could be accurately distinguished from controls without PC. Also, individuals with suspicious precursor lesions of PC might be distinguished from controls, and having a (medical history of) melanoma probably does not influence the protein signatures. Protein profiling therefore has potential to be included in the pancreatic surveillance program, where it, as an addition to current screening methods, can aid in the decision whether a patient will need surgery or not.

Different biomarkers have been extensively studied in sporadic patient cohorts over the last decades,[12,20,21] but this is the first study to investigate the role of biomarkers in a pancreatic surveillance cohort of genetically predisposed individuals. Recent studies from the University of Marburg did however investigate biomarkers in familial PC (FPC) individuals with PC or relevant precursor lesions of PC in a non-surveillance setting.[22,23] Interestingly, the (few) individuals with pathologically confirmed high-grade precursor lesions (PanIN2-3) in their studies had significantly elevated serum biomarker levels prior to surgery and the levels dropped to the normal range after surgery. FPC individuals having relevant precursor lesions of PC could thus accurately be distinguished from healthy controls using their proposed biomarker sets, and the authors argued that biomarkers may be suitable for the early detection of precursor lesions of PC in high-risk individuals.

Indeed, a major goal of screening is the detection of precursor lesions of PC,[2] and their prevalence in *CDKN2A* mutation carriers is evident. Vasen *et al* reported that 11% of *CDKN2A*

carriers in the surveillance program had possible precursor lesions (*ductectasias*) on radiology.[3] Potjer *et al* reported an even higher number (16%), and concluded that precursor lesions might have a high malignant potential in *CDKN2A* carriers, compared to precursor lesions in FPC individuals.[24] In order to be implemented in a pancreatic surveillance cohort, it is therefore important that potential serum biomarkers not only distinguish non-cancer patients from cancer patients, but also from patients with relevant precursor lesions of PC. In this study, there was only one patient with histologically confirmed precursor lesions (IPMN and PanIN1-2), and as mentioned those precursor lesions, especially the IPMN, might have a relatively high malignant potential because the patient was a *CDKN2A* mutation carrier. This patient had a protein signature comparable to those with PC. The other three patients with less suspicious precursor lesions on radiology had a normal to near-normal protein profile. Therefore, it seems likely that patients with substantial precursor lesions might be accurately distinguished from healthy *CDKN2A* carriers using serum protein profiling, although numbers are too small to make definite conclusions.

A second requirement for biomarkers to be implemented in a pancreatic surveillance cohort of high-risk individuals, especially *CDKN2A* carriers, is that the signatures are not disturbed by the occurrence of other types of cancer. The FAMMM syndrome (due to a *CDKN2A* germline mutation) is mainly characterized by a very high risk (70%) of developing cutaneous melanoma, and 62% of the carriers in this study indeed had a medical history of melanoma. Having a medical history of melanoma did not influence the protein signatures in general, as cases could still accurately be distinguished from controls in this cohort. Also, the four controls with a recent diagnosis of melanoma did not evidently diverge from the other control patients. Only the individual with both a recent diagnosis of melanoma and radiological precursor lesions had a slightly higher discriminant score than the other controls, but that could be caused by the presence of precursor lesions as argued above. In addition to the high risk of developing melanoma and PC, *CDKN2A* mutation carriers also have a higher risk of developing head and neck squamous cell carcinoma, which emphasises that FAMMM syndrome is a true tumour syndrome.[25,26] It is therefore also important to know if these cancers influence the protein signatures, but that could not be investigated in the current study due to the fact that there was no recent diagnosis of this type of cancer in the study group. There was only one individual in this cohort with two synchronous tumours of the larynx and mouth 4 years prior to serum sampling, without recurrence after treatment and a very low discriminant score.

The most important limitation of this study is sample size. More individuals with PC and, preferably, histologically confirmed high-grade precursor lesions are needed to investigate if these individuals definitely can be distinguished from healthy *CDKN2A* individuals. These

patients are however very rare and it would take years to collect only a few more patients. Also, more patients with other tumours than PC at or around the time of serum sampling are needed in order to investigate if those tumours intervene with the protein signatures. A second limitation is that we did not collect samples after surgical treatment, and therefore we could not investigate if the high discriminant scores declined after surgery. Future implementation of protein profiling in the surveillance program, with standardized yearly serum sampling, including post-surgery sampling, will ensure more patients with different types of cancer or precursor lesions of PC.

Since current screening strategies for PC are not entirely reliable for detecting early-stage PC or its (high-grade) precursor lesions, there is a strong need to improve the pancreatic surveillance program. As is shown in this preliminary study, protein profiling seems a very promising method to be included as an additional non-invasive screening modality. Previously, similar MS-based profiling studies in our group provided promising results with regard to peptide and protein signatures for the early detection of breast cancer and colorectal cancer,[18,27] and thus protein profiling seems suitable for cancer surveillance in general.

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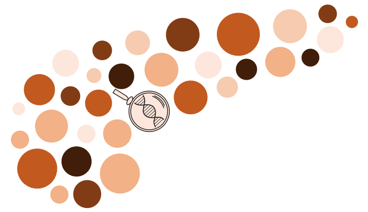
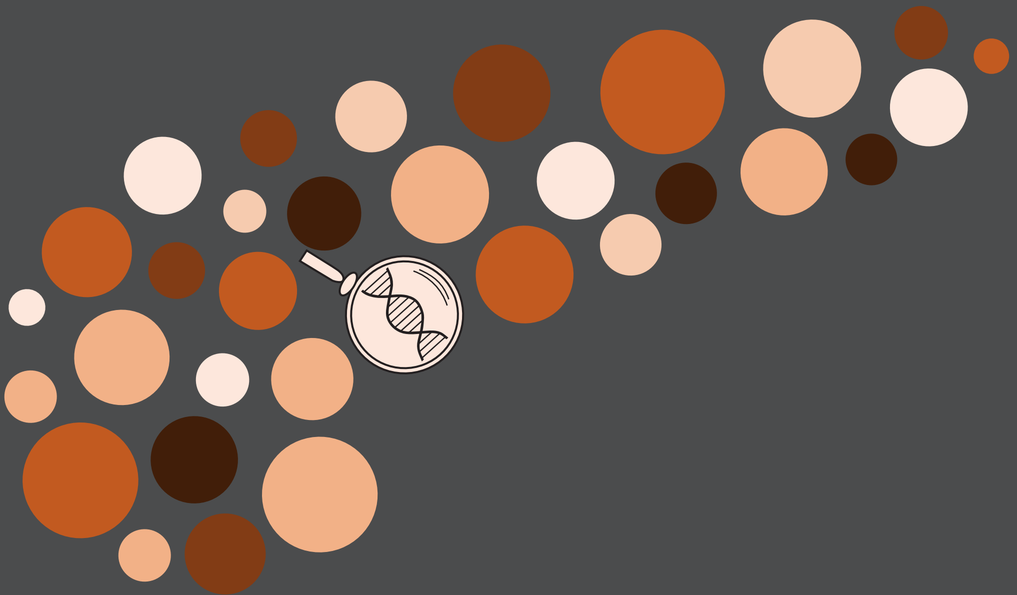
PART II



Genetic testing in familial melanoma; *CDKN2A* and beyond



7

CM-Score: A validated scoring system to predict *CDKN2A* germline mutations in melanoma families from Northern Europe

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ABSTRACT

BACKGROUND

Several factors have been reported that influence the probability of a germline *CDKN2A* mutation in a melanoma family. Our goal was to create a scoring system to estimate this probability, based on a set of clinical features present in the patient and his or her family.

METHODS

Five clinical features and their association with *CDKN2A* mutations were investigated in a training cohort of 1227 Dutch melanoma families (13.7% with *CDKN2A* mutation) using multivariate logistic regression. Predefined features included number of family members with melanoma and with multiple primary melanomas, median age at diagnosis and presence of pancreatic cancer or upper airway cancer in a family member. Based on these five features, a scoring system (*CDKN2A Mutation (CM)-Score*) was developed and subsequently validated in a combined Swedish and Dutch familial melanoma cohort (n=421 families; 9.0% with *CDKN2A* mutation).

RESULTS

All five features were significantly associated ($p<0.05$) with a *CDKN2A* mutation. At a CM-Score of 16 out of 49 possible points, the threshold of 10% mutation probability is approximated (9.9%; 95% CI 9.8-10.1). This probability further increased to >90% for families with ≥36 points. A CM-Score under 16 points was associated with a low mutation probability (≤4%). CM-Score performed well in both the training cohort (AUC 0.89; 95% CI 0.86-0.92) and the external validation cohort (AUC 0.94; 95% CI 0.90-0.98).

CONCLUSION

We developed a practical scoring system to predict *CDKN2A* mutation status among melanoma-prone families. We suggest that *CDKN2A* analysis should be recommended to families with a CM-Score of ≥16 points.

INTRODUCTION

Since its identification in 1994,[1] the *CDKN2A* gene (MIM 600160) has remained the major high-risk susceptibility gene for cutaneous melanoma. Germline mutations are present in approximately 10-40% of familial cases.[2] Carriers of a germline mutation in the *CDKN2A* gene have an increased risk for developing melanoma, with a penetrance of up to 70% at 80 years of age, and 40% of carriers develop multiple primary cutaneous melanomas.[3] Furthermore, mutation carriers have an increased risk for other types of malignancies, the most important of which is pancreatic cancer.[4] Due to the high risk of melanomas and other types of cancer and the advantages of regular surveillance in improving prognosis and survival,[5,6] it is important to identify families that carry a *CDKN2A* germline mutation. However, the probability of a *CDKN2A* mutation strongly depends both on the clinical characteristics of a family and personal (dermatological) and environmental factors such as skin type and the amount of sun exposure. Thus, *CDKN2A* mutation analysis might not be indicated in some lower-risk melanoma families.

The Netherlands and Sweden both have a high incidence of melanoma (age-standardized rate 19.4 and 18.0 per 100.000, respectively[7]) and specific founder mutations in the *CDKN2A* gene are the predominant cause of *familial* melanoma in these countries. In the Netherlands, the 19-base pair deletion termed p16-*Leiden* (c.225_243del, p.Ala76Cysfs*64; RefSeq NM_000077.4) not only confers an increased risk for melanoma but also for tumors of the pancreas and upper airway tract (larynx, pharynx, oral cavity), and to a lesser extent tumors of the lungs and digestive tract.[8-10] Carriers of the Swedish founder mutation (c.335_337dup, p.Arg112dup; RefSeq NM_000077.4) also show an increased risk for these tumors.[11,12] Although it is recognized that the risk-spectrum for non-melanoma cancers differs among carriers of different mutations in the *CDKN2A* gene, pancreatic and upper airway tract cancers have repeatedly been reported in a variety of carrier populations.[4,13-17]

Over the past decade, research groups from Europe, the United States and Australia have attempted to identify clinical features that are associated with germline *CDKN2A* mutations in melanoma families.[18-24] Studied features included (1) number of melanoma patients in a family, (2) number of patients with multiple primary melanomas in a family, (3) median age at diagnosis of melanoma and (4) presence of pancreatic cancer in a family. The most significant associations reported in these studies were the presence of more than two melanoma cases in a family, an early age of onset, and having at least one family member with multiple primary melanomas and/or pancreatic cancer. Based on a literature review from 2009, it was suggested that melanoma patients from areas with a moderate to high incidence of melanoma are candidates for genetic testing of *CDKN2A* if they have at

least three primary melanomas, or when there are at least two additional diagnoses of melanoma and/or pancreatic cancer among close (first or second-degree) family members ("rule of threes").[25] The authors argued that these families have an estimated 10% or greater probability of carrying a germline *CDKN2A* mutation, which is a commonly used threshold in clinical practice for gene sequencing in hereditary cancer.[26-28]

The goal of this study was to create a scoring system for clinicians to estimate the probability of a germline *CDKN2A* mutation based on a set of clinical features present in the patient and his or her family. Using a training cohort of Dutch melanoma families, we therefore analysed the association of four previously reported clinical features that are associated with a *CDKN2A* mutation, and investigated the association with upper airway cancer as an additional feature. A combined cohort of Swedish and Dutch melanoma families was used for external validation of the scoring system.

PATIENTS AND METHODS

TRAINING COHORT

The training cohort included all cutaneous melanoma index patients and their families in the Netherlands referred for *CDKN2A* mutation analysis between 1998 and 2015. According to current Dutch referral guidelines, *CDKN2A* mutation analysis is indicated if one of the following criteria is met: a family with 1) two first-degree relatives with melanoma, 2) two first or second-degree relatives with melanoma and one first or second-degree relative with pancreatic cancer, 3) three or more primary melanomas in one individual, 4) an individual with juvenile melanoma (< 18 years), or 5) an individual with a history of both melanoma and pancreatic cancer. At the Department of Clinical Genetics at Leiden University Medical Centre, the Laboratory for Diagnostic Genome Analysis (LDGA) has been the primary sequencing facility for *CDKN2A* in the Netherlands since 1998, and receives diagnostic requests from across the Netherlands. Essential pedigree information was gathered for the families and added to the Leiden Familial Melanoma Database. These data included the number of first and second-degree family members (of each other) with cutaneous melanoma (invasive or in situ), whether these patients had single or multiple primary melanomas (MPM), the age of each melanoma patient at first diagnosis and the number of family members with pancreatic cancer (PC) and upper airway cancer (UAC), i.e. cancer of larynx, pharynx or oral cavity. We restricted our analysis of these latter tumours to the first and second-degree relatives of the index patient and the first-degree relatives of melanoma patients. We relied on the referring clinical geneticists for complete pedigree information and, if necessary, histologic confirmation of cancer diagnoses (melanoma

and others). We included all information on cancer diagnoses, also those unconfirmed by the clinical geneticist, since index patient reports of melanomas in family members have a high known level of accuracy (true positive predictive value 77-87%).[29] We imputed the age of melanoma diagnosis for family members where the age at diagnosis was not reported in the pedigree (n=320 individuals from 212 families [61 with *CDKN2A* mutation]). Imputation was based on median age at diagnosis in *CDKN2A* mutation families (40 years) and sporadic (non-*CDKN2A*) patients (55 years), as reported by van der Rhee *et al*.[30] When the patient was younger than this age or was deceased prior to this age at time of *CDKN2A* analysis in the family, that specific age was used for imputation. Families without a *CDKN2A* mutation were excluded from the study if *CDKN2A* analysis was only performed in a non-affected family member (n=84). Families in which *CDKN2A* sequencing was unsuccessful were also excluded (n=4). The Leiden University Medical Centre Ethics Committee issued a declaration of no objection (#C14.064) regarding the creation of the Leiden Familial Melanoma Database.

VALIDATION COHORT

The greater portion of the validation cohort in this study consisted of members of melanoma-prone families from Sweden.[31] Families were identified by questioning newly-diagnosed melanoma patients about their familial melanoma history. Melanoma families were defined as kindreds with at least two relatives (first, second or third-degree) with histologically or clinically verified melanoma. Since 1995, germline *CDKN2A* mutation analysis is offered to members of these families after informed consent is obtained. The study was approved by Research Ethical Review Boards at Lund University and Karolinska Institute in Stockholm, the sites where the genetic tests were performed. In Stockholm, patients with multiple primary melanomas (regardless of family history) are also invited to undergo germline *CDKN2A* mutation analysis. In 2012, a study was performed to broaden understanding of the identified familial melanoma kindreds and of multiple primary melanoma patients through linkage to Swedish national registries.[11,12,32,33] Further linkage to the Swedish Cancer Registry (established in 1958 with register completeness estimated to be 96%)[34] provided data on all registered cancers in the *CDKN2A* genotyped individuals and their first and second-degree relatives.

Additional Dutch melanoma families were recruited at the Department of Dermatology, Leiden University Medical Centre, according to the inclusion criteria of the GenoMEL study (http://www.genomel.org/). After providing written informed consent, melanoma patients were asked about their familial melanoma history. A melanoma family was defined by the presence of three or more cases with histologically-confirmed melanoma, or two cases with histologically-confirmed melanoma in first-degree relatives.

DNA ANALYSIS

In the Dutch cohorts (both training and validation), DNA was extracted from whole blood samples of index patients and was used for sequencing of all coding exons of *CDKN2A* (1α, 1β, 2 and 3), including exon/intron boundaries. To detect larger deletions or duplications, multiplex ligation-dependent probe amplification (MPLA) was performed. In the early years of *CDKN2A* diagnostics, analysis was limited to a mutation-specific PCR for the detection of the p16-*Leiden* mutation. However, only a very small subset of *CDKN2A* wild type families in the training cohort were analysed in this manner (n=32). In an additional 89 families from the training cohort, exon 1β was not sequenced. For the Swedish cohort, procedures used for PCR of all *CDKN2A* exons and direct sequencing of PCR products has been described previously.[11] Presence of a *CDKN2A* mutation was defined as having either a pathogenic or likely pathogenic variant in the *CDKN2A* gene (class 4 or 5 variant)[35] or an unclassified variant (class 3) shown to be located on a pathogenic *CDKN2A* haplotype. Classification of these variants was based on (previously reported) co-segregation with disease, strong evidence of impaired protein function, and in some families, shared pathogenic haplotypes.

STATISTICAL ANALYSIS

Five clinical features were predefined and used for analysis: the number of first and second-degree family members (including the index patient) with (1) melanoma and (2) multiple primary melanomas (MPM), (3) the median age at diagnosis of (first) melanoma in the family and the presence of (4) pancreatic cancer (PC) and (5) upper airway cancer (UAC) in a family. Median age at diagnosis was divided into three age groups (<30 years, 30-50 years and ≥50 years). A univariate analysis was performed to independently evaluate these features and a multivariate logistic regression model was used to assess the association between all five features and the presence of a germline *CDKN2A* mutation. The formula of the logistic regression model is P(robability)=$e^L/(1+e^L)$ where L= constant + $\beta_1 {*} C_1$ (number of family members with melanoma [1=0, 2=1, 3=2, ≥4=3]) + $\beta_2 {*} C_2$ (number of family members with MPM [0=0, 1=1, ≥2=2]) + $\beta_3 {*} C_3$ (median age at primary diagnosis [≥50=0, <50=1]) + $\beta_4 {*} C_4$ (presence of PC [No=0, Yes=1]) + $\beta_5 {*} C_5$ (presence of UAC [No=0, Yes=1]), and where β is the feature-specific β-coefficient. All statistical analyses were carried out in SPSS (version 23.0).

DEVELOPMENT OF A SCORING SYSTEM: CM-SCORE

The β-coefficients derived from the multivariate analysis were converted to points for each feature using the formula Points=$(C_x {*} \beta_C)/B$ (as described by Sullivan *et al*,[36] where C_x is the feature-specific numeral from the logistic regression formula, β_C is the β-coefficient and B is the fixed multiplier or constant [defined 0.22]). The total number of points was calculated for each family in the training cohort. Since there were often considerable differences in

the number of families with successive point totals (for instance, there were 6 families with 21 points (33% mutation) and 37 families with 22 points (16% mutation)), the cohort was subsequently split into eight point-groups. This grouping would ensure a more accurate calculation of the observed mutation frequencies per group with narrower confidence intervals. For each of these groups, the observed mutation frequencies, the mean of the predicted probabilities and their 95% confidence intervals were calculated. The scoring system, CM- (*CDKN2A* Mutation) Score, was subsequently applied to the validation cohort, with the families split into the same point-groups as in the training cohort. The observed mutation frequencies and their 95% confidence intervals were again calculated for each group. The performance of the scoring system was assessed for both the training cohort and the validation cohort with the Hosmer-Lemeshow goodness of fit test (calibration) and Receiver Operator Characteristic (ROC) curve analysis with calculation of the Area Under the Curve (AUC) (discrimination). The slope of the calibration line was estimated with linear regression. The proposed cut-off value in CM-Score for performing *CDKN2A* analysis was determined as the score that corresponds to a predicted mutation probability of ~10%.[26-28]

RESULTS

TRAINING COHORT

A total of 1227 families were included in the study, 168 of which had a (likely) pathogenic variant in the *CDKN2A* gene (13.7%). The p16-*Leiden* founder mutation was present in 77% of these families (n=130) (*supplementary table S1)*. Most of the families had two or more members with melanoma (853 families; 70%) and included 503 two-case families, 233 three-case families and 117 families with four or more melanoma cases. In 654 (77%) of these multiple-case families, at least one additional clinical feature was present (i.e. median age <50 years or presence of MPM, PC or UAC in the family, see *supplementary table S2*). In the 374 single-case families, 207 families (55%) had at least two other clinical features and 150 families (40%) had one other clinical feature. The majority of melanomas in the training cohort were confirmed by histology reports (76%). Pancreatic cancer and upper airway cancer diagnoses were less frequently confirmed by the referring clinical geneticist (both 43%).

UNIVARIATE AND MULTIVARIATE ANALYSIS

Having at least three family members with melanoma was significantly associated with the presence of a *CDKN2A* mutation in the univariate analysis (*table 1*). A median age of under 50 years and one or more cases with multiple melanomas in a family were also significantly associated with a *CDKN2A* mutation. Age under 30 years at time of diagnosis did not result in a higher odds ratio than age 30 to 50 years (OR 5.1 [95% CI 2.5-10.4] versus OR 7.1 [95%

CI 4.1-12.3], respectively). A significantly increased risk for a *CDKN2A* mutation was seen in families in which pancreatic cancer and upper airway cancer co-occurred with melanoma; a mutation was present in 33% of the families with one or more pancreatic cancer patients and 46% of the families with one or more upper airway cancer patients.

TABLE 1. Univariate analysis showing the independent association between each clinical feature and a germline *CDKN2A* mutation

Features	Total (n=1227)	*CDKN2A* wild type (n=1059)	*CDKN2A* mutation (n=168)	OR*	95% CI	P-Value
No. of family members with melanoma†						
1	374	346	28 (7.5%)	1.0	-	-
2	503	461	42 (8.3%)	1.1	0.7-1.9	0.641
3	233	194	39 (16.7%)	2.5	1.5-4.2	<0.001
≥4	117	58	59 (50.4%)	12.6	7.4-21.3	<0.001
No. of family members with MPM†						
0	749	697	52 (6.9%)	1.0	-	-
1	406	329	77 (19.0%)	3.1	2.2-4.6	<0.001
≥2	72	33	39 (54.2%)	15.8	9.2-27.3	<0.001
Median age at primary diagnosis						
≥50 years	437	422	15 (3.4%)	1.0	-	-
30-50 years	666	532	134 (20.1%)	7.1	4.1-12.3	<0.001
<30 years	124	105	19 (15.3%)	5.1	2.5-10.4	<0.001
Presence of pancreatic cancer¥						
No	956	877	79 (8.3%)	1.0	-	-
Yes	271	182	89 (32.8%)	5.4	3.9-7.6	<0.001
Presence of upper airway cancer¥						
No	1117	999	118 (10.6%)	1.0	-	-
Yes	110	60	50 (45.5%)	7.1	4.6-10.7	<0.001

MPM = multiple primary melanomas, OR = odds ratio, CI = confidence interval

* The variable with the smallest risk was defined as baseline with an odds ratio of 1.0, and odds ratios for the other variables were calculated against this baseline value.

† First and second-degree relatives of each other; including the index patient

¥ First and second-degree relatives of the index patient and first-degree relatives of melanoma patients

In a multivariate logistic regression model, the five features investigated in the univariate model remained significantly associated with a mutation (*table 2*). Since in the univariate analysis age under 30 years was not a stronger predictor than age 30 to 50 years, these age groups were combined into one group (age <50 years) for the multivariate analysis. The highest odds ratios were found for median age under 50 years (OR 8.5 [95% CI 4.5-

16.0]) and for presence of pancreatic or upper airway cancer in a family (OR 7.5; [95% CI 4.8-11.7] and OR 6.0 [95% CI 3.4-10.5], respectively), but these features had only two possible outcomes (<50 or ≥50 years, Yes or No), whereas the other melanoma-specific features had three or four possible outcomes and increasing odds ratios for each step.

TABLE 2. Multivariate logistic regression model showing the association between all five clinical features combined and a germline *CDKN2A* mutation

Clinical feature	β-coefficient	OR	95% CI	P-Value
No. of family members with melanoma (1, 2, 3, ≥4)	0.871	2.4	1.9-3.0	<0.001
No. of family members with MPM (0, 1, ≥2)	1.096	3.0	2.2-4.1	<0.001
Median age at primary diagnosis (≥50, <50)	2.142	8.5	4.5-16.0	<0.001
Presence of pancreatic cancer (No, Yes)	2.013	7.5	4.8-11.7	<0.001
Presence of upper airway cancer (No, Yes)	1.790	6.0	3.4-10.5	<0.001

MPM = multiple primary melanomas, OR = odds ratio, CI = confidence interval

The formula of the logistic regression model:

$P= e^L / (1+ e^L)$ where L= -6.220 + 0.871 x C_1 (no. of family members with melanoma [1=0, 2=1, 3=2, ≥4=3]) + 1.096 x C_2 (no. of family members with MPM [0=0, 1=1, ≥2=2]) + 2.142 x C_3 (median age at primary diagnosis [≥50=0, <50=1]) + 2.013 x C_4 (presence of pancreatic cancer [No=0, Yes=1]) + 1.790 x C_5 (presence of upper airway cancer [No=0, Yes=1])

CM-SCORE

The points assigned to each clinical feature are shown in *table 3*. The predicted mutation probabilities and observed mutation frequencies per point-group are shown in *table 4*. Below a total of 16 of 49 possible points, the predicted mutation probability is low (≤4.0%). Between 16 and 19 points, the predicted mutation probability is 9.9% and substantially increases in subsequent point-groups (20-23 points: 20.9%, 24-27 points: 34.7%, 28-31 points: 52.1%, 32-35 points: 71.4%, ≥36 points: 90.7%).

The concordance between observed and predicted mutation probabilities (calibration) is graphically displayed in *figure 1A*. The slope of the calibration line (1.03) indicates a good calibration, and the Hosmer-Lemeshow test (p=0.925) provided no evidence of a poor fit. *Figure 2A* shows the ROC curve analysis. The AUC is 0.89 (95% CI 0.86-0.92, p< 0.001), which indicates that the model has a good ability to discriminate between families with and without a *CDKN2A* mutation. The threshold of 10% predicted probability is approximated at the cut-off value of 16 points in CM-Score, with a sensitivity of 90.5% (95% CI 84.7-94.2) and a specificity of 68.0% (95% CI 65.1-70.8). The majority of families (n=736; 60%) had a CM-Score of less than 16 points.

TABLE 3. Scoring system (CM-Score) based on the multivariate logistic regression model

Features	Points
No. of family members with melanoma†	
1	0
2	4
3	8
≥4	12
No. of family members with MPM†	
0	0
1	5
≥2	10
Median age at primary diagnosis	
≥50 years	0
<50 years	10
Presence of pancreatic cancer¥	
No	0
Yes	9
Presence of upper airway cancer¥	
No	0
Yes	8

MPM = multiple primary melanomas

† First and second-degree relatives of each other; including the index patient

¥ First and second-degree relatives of the index patient and first-degree relatives of melanoma patients

TABLE 4. Point totals from CM-Score with the corresponding mean predicted mutation probabilities and the observed mutation frequencies in the training and validation cohorts.

CM-Score	Predicted mutation probability		Observed mutation frequency					
			Training cohort (n=1227)			Validation cohort (n=421)		
Points	Prob. (%)	95% CI	Freq.	%	95% CI	Freq.	%	95% CI
≤11	1.0	0.9-1.0	*4 / 383*	*1.0*	*0.4-2.7*	*0 / 159*	*0*	*0.0-2.4*
12 – 15	4.0	3.9-4.1	*12 / 353*	*3.4*	*2.0-5.9*	*4 / 166*	*2.4*	*0.9-6.0*
16 – 19	9.9	9.8-10.1	*26 / 203*	*12.8*	*8.9-18.1*	*4 / 38*	*10.5*	*4.2-24.1*
20 – 23	20.9	20.4-21.4	*18 / 99*	*18.2*	*11.8-26.9*	*1 / 17*	*5.9*	*1.1-27.0*
24 – 27	34.7	33.1-36.3	*23 / 75*	*30.7*	*21.4-41.8*	*4 / 12*	*33.3*	*13.8-60.9*
28 – 31	52.1	49.4-54.7	*16 / 32*	*50.0*	*33.6-66.4*	*4 / 6*	*66.7*	*30.0-90.3*
32 – 35	71.4	69.6-73.1	*30 / 40*	*75.0*	*59.8-85.8*	*5 / 7*	*71.4*	*35.9-91.8*
≥36	90.7	89.0-92.4	*39 / 42*	*92.9*	*81.0-97.5*	*16 / 16*	*100*	*80.6-100.0*

The predicted mutation probability for each point-group is the mean of the predicted probabilities of the point totals in that group in the training cohort. The corresponding 95% confidence interval is estimated using the standard error of the mean.

Prob = probability, Freq = frequency, CI = confidence interval

EXTERNAL VALIDATION OF THE SCORING SYSTEM

The validation cohort consisted of a total of 421 families (403 from Sweden; 18 from the Netherlands), of which 38 had a (likely) pathogenic variant in the *CDKN2A* gene (9.0%). Most of these families (n=30; 79%) carried the Swedish founder mutation p.Arg112dup and two Dutch families carried the p16-*Leiden* founder mutation (*supplementary table S3*). The majority were multiple-case families (294 families; 70%) and included 232 two-case families, 37 three-case families and 25 families with four or more cases. All melanomas in the validation cohort were histologically confirmed. Pancreatic cancer was present in 29 families (28 histologically confirmed; 72% *CDKN2A* mutation) and upper airway cancer in 24 families (23 histologically confirmed; 63% *CDKN2A* mutation).

The observed mutation frequencies per point-group in the validation cohort are shown in *table 4*. The performance of CM-Score in the validation cohort is displayed in *figures 1B* and *2B*. The slope of the calibration line is 1.14 with a non-significant Hosmer-Lemeshow test (p=0.615). The AUC is 0.94 (95% CI 0.90-0.98, p<0.001), indicating good performance of CM-Score in the validation cohort. The sensitivity and specificity at the cut-off value of 16 points is 89.5% (95% CI 74.3-96.6) and 83.8% (95% CI 79.6-87.3), respectively. Similar to the training cohort, the majority of families in the validation cohort (n=325; 77%) had a CM-Score of less than 16 points.

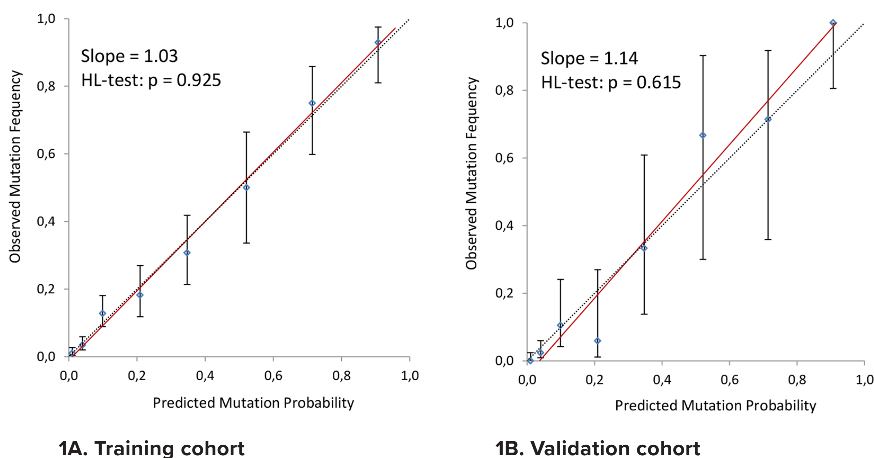
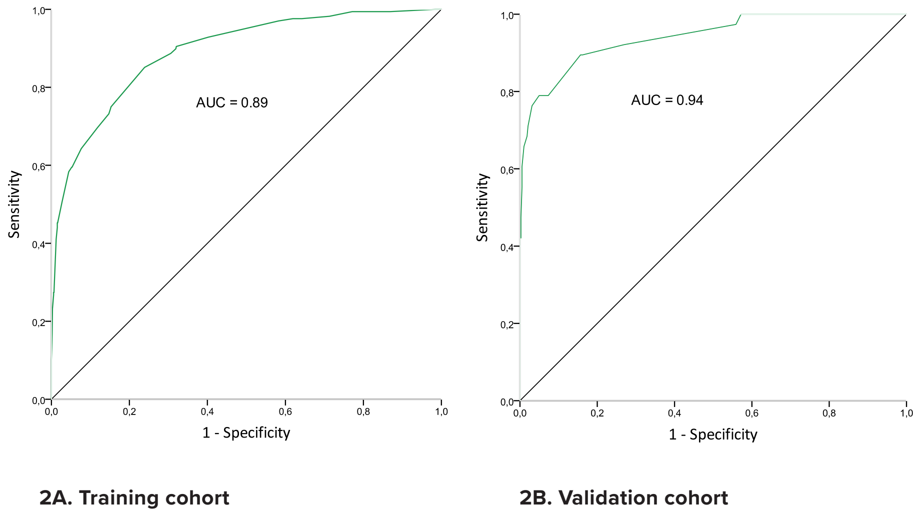


FIGURE 1. Calibration of CM-Score

The calibration line (red) is a linear regression line that shows the relation between observed mutation frequency and predicted mutation probability in the training cohort (A) and the validation cohort (B). The dashed line is the reference line of perfect calibration. The 95% confidence intervals of the observed mutation frequencies per point-group are displayed by the vertical lines.

HL-test = Hosmer-Lemeshow test



2A. Training cohort **2B. Validation cohort**

FIGURE 2. Discriminative ability of CM-Score

Receiver Operator Characteristic (ROC) curve analysis of the training cohort (A) and the validation cohort (B).Point total was used as the test variable and mutation status was used as the state variable. Comparable results were obtained when the calculated predicted probability was used as test variable.

AUC = Area Under the Curve

DISCUSSION

This study in a large Dutch training cohort of 1227 melanoma families confirmed the importance of four previously established clinical features that are associated with the presence of a germline *CDKN2A* mutation in a melanoma patient. Furthermore, a fifth feature, the presence of upper airway cancer in the family, could be validated. Based on these clinical features and their odds ratios in our multivariate logistic regression model, we developed the CM-Score system to predict *CDKN2A* mutation probability, which performed very well in a combined Swedish and Dutch external validation cohort (AUC 0.94). At a cut-off value of 16 out of 49 points, the predicted probability approximates the commonly used 10% predicted probability threshold for germline gene sequencing in hereditary cancer, with a sensitivity of 89% and a specificity of 84% in the validation cohort. This cut-off value is also clinically relevant, since the majority of families in the training and validation cohorts scored less than 16 points (60% and 77%, respectively), a threshold below which the probability of a mutation decreases substantially (≤4%). Use of CM-Score could potentially spare many families (extensive) genetic testing, which may be particularly

relevant in countries where resources for genetic testing are limited. Conversely, in families with a high CM-Score and therefore high mutation probability, genetic testing is even more urgent. A scoring system should, however, always only complement the clinical judgment of the clinical geneticist requesting DNA diagnostics (for instance taking into account family size, age of family members, whether a patient has a certain combination of different malignancies and the availability of reliable medical information).

Risk models involving melanoma[37] and *CDKN2A* mutation probability[23,24] have been described previously. Niendorf *et al* incorporated the features (1) number of primary proband melanomas, (2) number of primary melanomas in the family and (3) age in a logistic regression model they named MELPREDICT.[23] The AUC was 0.881 in the training set (n=116 families) and 0.803 in the external validation set (n=143 families). A computerized optimization of this model, renamed MelaPRO, was published in 2010, and outperformed the former model with an AUC of 0.86 in a validation set of 167 families.[24] MelaPRO includes the same clinical (familial) features as MELPREDICT, but also takes into account regional melanoma incidence rates and the geographical penetrance of *CDKN2A*. In contrast, while our CM-Score was trained and validated using families of Northern European descent, its strength lies in its simple, non-computerized scoring system that incorporates five features (including the presence of pancreatic cancer and upper airway cancer in a family), and despite this simplicity shows a superior performance in very large sets of melanoma-prone families.

The guidelines for *CDKN2A* mutation testing proposed by Leachman *et al* in 2009[25] were recently updated.[38] In view of the recent reports of non-*CDKN2A* melanoma syndromes, such as those related to germline mutations in *BAP1*[39] (MIM 603089), *POT1*[40] (MIM 606478) and *MITF*[41] (MIM 156845), the authors propose tailored multi-gene panel testing in melanoma families instead of *CDKN2A* mutation testing alone. The 2009 criteria for genetic testing were converted into a points system, with points awarded for cancers that occur in so-called melanoma-dominant syndromes and melanoma-subordinate syndromes (where melanoma is not the predominant cancer type, such as in hereditary breast and ovarian cancer). Based on these points, the clinical geneticist can subsequently select the appropriate gene panel(s) to be tested in a family. In the selection and genetic assessment of melanoma families, this is a rather different approach to the one we propose in the current study. Firstly, CM-Score is designed for families where melanoma is the predominant cancer type. Secondly, since *CDKN2A* is still by far the major susceptibility gene in familial melanoma, we based the selection of families for genetic assessment on the probability of specifically detecting a *CDKN2A* mutation in these families. Because other melanoma-dominant syndromes (such as those related to *BAP1*, *POT1*, *CDK4* and *MITF*) are very

rare compared to *CDKN2A*-related familial melanoma (each gene contributing <1%),[42] we hypothesize that the calculated mutation probability from CM-Score largely reflects the joint probability of detecting a germline *CDKN2A* mutation and other rare melanoma-dominant mutations. However, it should be noted that some tumors that are not part of CM-Score are highly specific to non-*CDKN2A* melanoma syndromes, especially *BAP1*-related tumors such as uveal melanoma and mesothelioma.[43,44] *BAP1* germline analysis should therefore be specifically offered when these tumors co-occur with cutaneous melanoma in a family, either together with *CDKN2A* or as part of a multi-gene panel test. It is not within the scope of this study to elaborate on the choice between multi-gene panel testing and *CDKN2A* mutation testing alone in melanoma-prone families. Although multi-gene panel testing increases the detection rate of cancer-predisposing germline mutations, there is also an elevated risk of identifying a variant of unknown significance in one of the genes and therefore increasing the uncertainty for a family regarding their genetic risk. The chance of this happening increases as more genes are included in a panel or when multiple panels are considered. Pros and cons of multi-gene panel testing should therefore always be carefully discussed with the patient.

Strengths of our study include relatively large and homogeneous cohorts and the broad analysis of five clinical features, including one more recently described feature (i.e. upper airway cancer). However, because the scoring system is based on populations with a high melanoma incidence, it is possible that it will underestimate the probability of finding a *CDKN2A* mutation in lower melanoma incidence areas such as Southern (Mediterranean) Europe or overestimate the probability in extreme incidence areas such as Australia. Additional validation in other geographical areas would therefore be valuable. Another limitation of our study is information bias. In the training cohort we had to rely on information supplied by the referring clinical geneticists and not all melanoma diagnoses were therefore histologically confirmed (76%). However, since the reporting of additional melanomas in family members by the index patient is known to be highly accurate, this factor is unlikely to have influenced the results.[29] Unfortunately, only 43% of all pancreatic tumors and 43% of all upper airway tumors were confirmed. Nevertheless, all melanomas and other cancers in the validation cohort were verified since the majority of diagnoses were derived from the Swedish Cancer registry.

In conclusion, we have developed and validated a non-computerized and clinically easy-to-use scoring system that shows high utility in predicting the probability of a germline *CDKN2A* mutation in melanoma-prone families from Northern Europe. The scoring system is based on clinical information on melanoma diagnoses in the patient´s family, and additionally includes diagnoses of pancreatic and upper airway cancer. As CM-Score was

trained and validated in large sets of Northern European families, we suggest that the system should be further validated in other regions as well. In view of the 10% mutation probability threshold, we suggest that *CDKN2A* analysis should be recommended to families with a CM-Score of ≥16 points.

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COLLABORATORS

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Spectrum of (likely) pathogenic *CDKN2A* variants and variants of uncertain significance located on a pathogenic haplotype in Dutch melanoma families (training cohort)

	CDKN2A **nucleotide change[¥]**	**p16INK4a amino acid change**	**p14ARF amino acid change**	**No. of families**
5’ UTR	c.-34 G>T	p.?	None	4
Exon 1β[‡]	c.193+1G>A	None	p.?	5
	c.193G>C	None	p.Gly65Arg	4
Exon 1α	c.47T>G	p.Leu16Arg	None	2[§]
	c.67G>C	p.Gly23Arg	None	8[§]
	c.71G>C	p.Arg24Pro	None	2
	c.131_132insAA	p.Tyr44*	None	1
	c.143C>A	p.Pro48Gln	None	1
Exon 2	c.151-2A>G	p.?	p.?	1
	c.159G>A	p.Met53Ile	p.Asp68Asn	2
	c.203C>T	p.Ala68Val	p.Arg82Arg	2[§]
	c.225_243del[†]	p.Ala76Cysfs*64	p.Arg90Valfs*76	130
	c.301G>T	p.Gly101Trp	p.Arg115Leu	2
	c.352G>A	p.Ala118Thr	p.Gly132Asp	3[§]
Exon 1+2+3	Deletion 155 kb of *CDKN2A*, *CDKN2B* and *MTAP*	Whole gene deletion	Whole gene deletion	1
Total				168

¥ RefSeq NM_000077.4 isoform p16INK4a

‡ RefSeq NM_058195.3 isoform p14ARF

† p16-*Leiden*. Dutch founder mutation

§ located on pathogenic haplotype

SUPPLEMENTARY TABLE S2. Clinical characteristics of families in the training cohort

No. of features	≥2 members with melanoma	Presence of MPM	Median age at primary diagnosis <50 years	Presence of PC	Presence of UAC	No. of families
5	+	+	+	+	+	18 **18**
4	+	+	+	+	-	42
	+	+	+	-	+	15
	+	+	-	+	+	1
	+	-	+	+	+	5
	-	+	+	+	+	4 **67**
3	+	+	+	-	-	129
	+	+	-	+	-	17
	+	+	-	-	+	4
	+	-	+	+	-	30
	+	-	+	-	+	22
	+	-	-	+	+	4
	-	+	+	+	-	23
	-	+	+	-	+	6
	-	+	-	+	+	0
	-	-	+	+	+	8 **243**
2	+	+	-	-	-	69
	+	-	+	-	-	260
	+	-	-	+	-	32
	+	-	-	-	+	6
	-	+	+	-	-	92
	-	+	-	+	-	9
	-	+	-	-	+	3
	-	-	+	+	-	51
	-	-	+	-	+	9
	-	-	-	+	+	2 **533**
1	+	-	-	-	-	199
	-	+	-	-	-	46
	-	-	+	-	-	76
	-	-	-	+	-	25
	-	-	-	-	+	3 **349**
0	-	-	-	-	-	17 **17**
					Total	**1227**

MPM = multiple primary melanomas, PC = pancreatic cancer, UAC = upper airway cancer

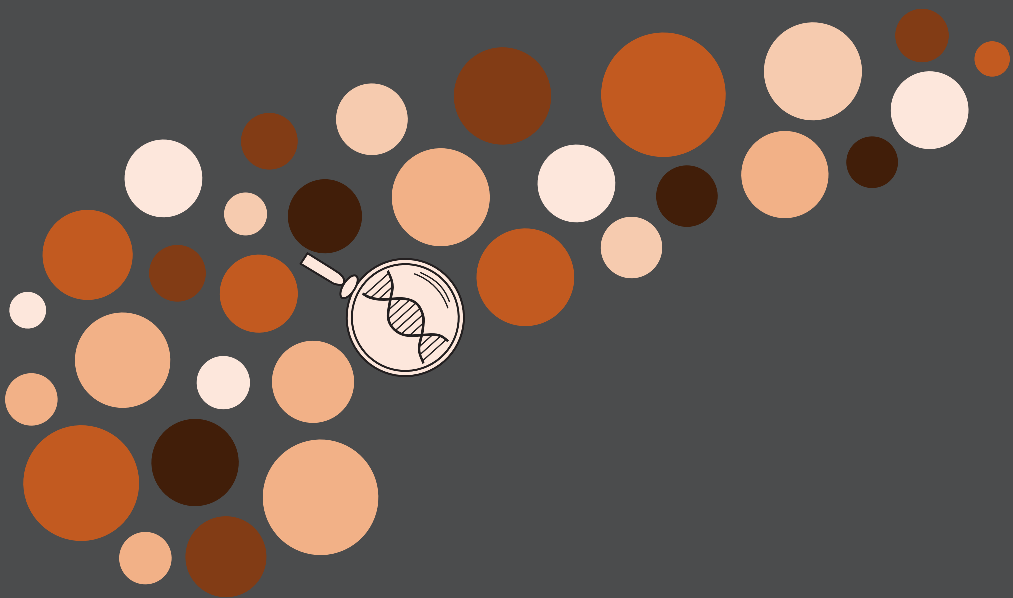
SUPPLEMENTARY TABLE S3. Spectrum of (likely) pathogenic *CDKN2A* variants in Swedish and Dutch melanoma families (validation cohort)

	***CDKN2A* nucleotide change**[¥]	**p16INK4a amino acid change**	**p14ARF amino acid change**	**No. of families**
Exon 1α	c.83dup	p.Arg29Alafs*15	None	1
	c.134G>T	p.Gly45Val	None	1
	c.143C>T	p.Pro48Leu	None	1
Exon 2	c.179_202del	p.Ala60_Gly67del	p.Gly75_Arg82del	1
	c.225_243del[†]	p.Ala76Cysfs*64	p.Arg90Valfs*76	2
	c.241C>T	p.Pro81Ser	p.Thr95Ile	1
	c.335_337dup[‡]	p.Arg112dup	p.Ser127dup	30
	c.353C>T	p.Ala118Val	None (p.Gly132Gly)	1
Total				38

¥ RefSeq NM_000077.4 isoform p16INK4a

† p16-*Leiden*. Dutch founder mutation

‡ Swedish founder mutation



8

Multi-gene panel sequencing of established and candidate melanoma susceptibility genes in a large cohort of Dutch non-*CDKN2A/CDK4* melanoma families

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Thomas P. Potjer, Sander Bollen, Anneliese J.E.M. Grimbergen, Remco van Doorn, Nelleke A. Gruis, Christi J. van Asperen, Frederik J. Hes, Nienke van der Stoep, on behalf of the Dutch Working Group for Clinical Oncogenetics

ABSTRACT

Germline mutations in the major melanoma susceptibility gene *CDKN2A* explain genetic predisposition in only 10-40% of melanoma-prone families. In this study we comprehensively characterized 488 melanoma cases from 451 non-*CDKN2A/CDK4* families for mutations in 30 established and candidate melanoma susceptibility genes using a custom-designed targeted gene panel approach. We identified (likely) pathogenic variants in established melanoma susceptibility genes in 18 families (n=3 *BAP1*, n=15 *MITF* p.E318K; diagnostic yield 4.0%). Among the three identified *BAP1*-families, there were no reported diagnoses of uveal melanoma or malignant mesothelioma. We additionally identified two potentially deleterious missense variants in the telomere maintenance genes *ACD* and *TERF2IP*, but none in the *POT1* gene. *MC1R* risk variants were strongly enriched in our familial melanoma cohort compared to healthy controls (R variants: OR 3.67, 95% CI 2.88-4.68, $p<0.001$). Several variants of interest were also identified in candidate melanoma susceptibility genes, in particular rare (pathogenic) variants in the albinism gene *OCA2* were repeatedly found. We conclude that multi-gene panel testing for familial melanoma is appropriate considering the additional 4% diagnostic yield in non-*CDKN2A/CDK4* families. Our study shows that *BAP1* and *MITF* are important genes to be included in such a diagnostic test.

INTRODUCTION

Cutaneous melanoma is the most aggressive type of common skin cancers and incidence has been increasing worldwide over the past decades.[1] With an age-standardized rate of 19.4 per 100.000, the Netherlands is among the countries with the highest incidence rates in the world, comparable to incidence rates in the northernmost European (Scandinavian) countries.[2] Well-established personal and environmental risk factors for melanoma include a fair skin type, having (many) atypical nevi, a high level of ultraviolet radiation exposure, and a history of sunburns in childhood.[3] A family history for the disease is also a significant risk factor and suggests a shared genetic predisposition among family members. This familial clustering occurs in approximately 5-10% of melanoma cases, and is referred to as familial melanoma.[4]

The major high-risk susceptibility gene for familial melanoma is *CDKN2A* and germline mutations are identified in 10-40% of familial cases.[5,6] In the Netherlands, a specific founder mutation in *CDKN2A*, known as p16-*Leiden* (c.225_243del, p.A76Cfs*64; RefSeq NM_000077.4), is the most frequent cause of familial melanoma (~80% of *CDKNA* mutations). Carriers of this mutation show not only a markedly increased risk for (multiple) cutaneous melanomas, but also for other cancers, especially pancreatic cancer and cancers of the upper respiratory tract (larynx, pharynx, oral cavity).[7,8] *CDKN2A* is an unusual gene in that it encodes two distinct proteins, p16INK4a and the alternatively spliced p14ARF, both of which are tumour-suppressors that act in two distinct pathways. The p16-retinoblastoma(Rb)-pathway controls cell-cycle G1-phase exit, while the p14ARF-p53 pathway induces cell cycle arrest or apoptosis.[9] Despite the major role of these pathways in melanoma susceptibility, only one other gene in the p16-retinoblastoma(Rb)-pathway, the *CDK4* gene, has been shown to be associated with familial melanoma, and only a small number of families with germline mutations in this gene have been identified to date.[10]

However, new melanoma susceptibility pathways have emerged in recent years.[5,6] Several high-penetrance genes involved in telomere lengthening (*TERT*) or telomere maintenance (Shelterin complex: *POT1*, *ACD*, *TERF2IP*) have been identified, and mutations in these genes each account for approximately 1% of familial melanoma predisposition.[11-13] Furthermore, germline mutations in the BRCA1-associated protein (*BAP1*) gene cause a specific cancer predisposition syndrome mainly characterized by an increased susceptibility for uveal melanoma and malignant mesothelioma, but also including cutaneous melanoma, renal cancer, basal cell carcinoma and characteristic skin lesions called atypical Spitz tumours (AST) or melanocytic *BAP1*–mutated atypical intradermal tumours (MBAIT).[14] The *MITF* gene is a medium-penetrance melanoma susceptibility gene and shows incomplete co-segregation with the phenotype. MITF is a basic-helix-loop-helix-leucine zipper transcription

factor that has a key function in melanocyte homeostasis. Loss-of-function mutations in this gene cause auditory-pigmentary syndromes, such as Waardenburg syndrome type 2A (MIM #193510). However, a specific missense variant (c.952G>A, p.E318K; RefSeq NM_000248.3) located in a small-ubiquitin-like modifier (SUMO) consensus site impairs the SUMOylation of MITF, which results in a gain-of-function increase in *MITF* transcriptional activity. Carriers of this variant have an approximately three- to fourfold increased risk for melanoma and are more likely to develop multiple primary melanomas.[15] Several other cancers (renal cancer, pancreatic cancer) have also been reported in carriers of this variant.[16,17] In addition to these known high- and medium-penetrance melanoma susceptibility genes, there are several well-established (common) variants in the lower-penetrance *MC1R* gene that are associated with an increased risk for melanoma in the general population. *MC1R* encodes the receptor for α-melanocyte stimulating hormone (α-MSH), which plays an important role in skin pigmentation. Variants in *MC1R* that are most strongly associated with red hair color (RHC) confer an approximately twofold increased risk for melanoma (R variants), while other variants (r variants) show a weaker association with RHC (non-RHC) and confer a much smaller increase in risk for melanoma.[18] It has also been shown that both R and r variants in *MC1R* act as modifiers of melanoma risk in families with a *CDKN2A* germline mutation.[19] Furthermore, mutations in other cancer susceptibility genes have been recently reported in melanoma families in studies using mainly Whole Exome Sequencing (WES) technologies,[20-22] but the exact role of these and other candidate melanoma susceptibility genes in the familial setting remains unclear and requires further evaluation.

Although Dutch melanoma families are well characterized for *CDKN2A* and *CDK4* mutations,[23] no large scale investigation has yet been performed to identify (potential) deleterious variants in other established or candidate melanoma susceptibility genes. In the current study, we therefore sequenced a comprehensive panel of 30 (candidate) melanoma susceptibility genes in a large cohort of Dutch melanoma-prone families without a known *CDKN2A* or *CDK4* mutation. Our goal was to determine the frequency of pathogenic variants in established melanoma susceptibility genes and to investigate the role of a broad range of candidate susceptibility genes in familial melanoma.

PATIENTS AND METHODS

PATIENT COHORT

Both cutaneous melanoma (CM) and uveal melanoma (UM) patients were eligible for inclusion in the study if they had at least one other relative (up to third-degree) with CM and/or UM, and no previously identified pathogenic germline variant in the melanoma core genes *CDKN2A*

or *CDK4*. Diagnostic sequencing of these two genes was performed at the Laboratory for Diagnostic Genome Analysis (LDGA) at the Department of Clinical Genetics of the Leiden University Medical Centre (LUMC), which has served as the primary sequencing facility for *CDKN2A* and *CDK4* in the Netherlands since 1998. In a small minority of referred families, the *CDKN2A* gene was only partly sequenced and/or the *CDK4* gene was not sequenced. Both genes were included in our research gene panel in order to exclude the presence of pathogenic variants in these genes. The study was approved by the LUMC Ethics Committee (#P15.341) and informed consent was obtained from all included individuals.

We initially selected 500 patients from 460 families for inclusion in the study. After critical re-evaluation of these families, 11 samples were excluded from the analysis based on failure to meet above mentioned inclusion criteria. In one of these samples, a pathogenic variant in the 5'UTR region of *CDKN2A* (c.-34G>T) was identified. Another sample was excluded because sequencing was unsuccessful. In total, 488 samples from 451 families remained for analysis *(table 1)*. Most families had a proband with CM (n=446) and the majority of these probands had at least one other relative with CM (n=442 families; n=478 samples). This 'familial CM' subgroup included 208 two-case families (83% of which consisted of first-degree relatives), 182 three-case families and 52 families with four or more melanoma cases. An additional four probands with CM had one or more relatives with UM, but no CM. The remaining five families had a proband with UM and one or more relatives with UM and/or CM. A control cohort consisted of a total of 449 adult individuals sequenced at the LUMC for a non-melanoma, non-oncogenic indication (MODY; MIM #606391). MODY is an autosomal dominant form of diabetes mellitus which manifests in young adults.

TABLE 1. Characteristics of the cohort

Proband history	Family history	No. of families	No. of samples
Cutaneous melanoma (CM)	*Total no. of CM cases in family*[a]		
	1	*4*	5
	2	*208*	218
	3	*182*	198
	4+	*52*	62
	Total	**446**	**483**
Uveal melanoma (UM)	*Total no. of UM cases in family*[b]		
	1	*2*	2
	2	*3*	3
	Total	**5**	**5**
Total		**451**	**488**

[a] Uveal melanoma was present in all four single-case families (one additional sample included), six two-case families, one three-case family and six families with four or more cases

[b] Cutaneous melanoma was present in both single-case families and in one two-case family

GENE SELECTION AND SEQUENCING

A total of 30 genes were selected by a multidisciplinary expert team (TP, RvD, NG, FH, NvdS; July 2016) and incorporated into a custom-designed targeted gene panel. This included nine established melanoma susceptibility genes and an additional 21 candidate genes identified in previous studies (*table 2*). Sequencing of all coding exons, including exon-intron boundaries, was performed on the Illumina HiSeq4000 platform to yield 150 basepair, paired-end reads. Targets were captured using a custom-designed, gene panel-specific Agilent SureSelect ^{XT} Clearseq enrichment kit and sequenced using the 200 ng XT protocol. Capture, enrichment and sequencing were performed at the GenomeScan sequencing facility in Leiden (https://www.genomescan.nl/). Subsequent data analysis was performed using our in-house developed set-up for diagnostic next generation sequence (NGS) analysis. In brief, FastQ sequence data was analyzed using an in-house developed and stringent post-sequencing annotation pipeline (using BWA-GATK-VEP).

Only variants that occurred with a minor allele frequency (MAF) of less than 5% in the 1000 Genomes variant database were collected and annotated. Subsequent variant filtering and analysis was performed using a second in-house developed variant analysis tool called LOVDplus. Only variants that had an optimal Genotype Quality (GQ) score of 99 (range 0-99) were considered for further interpretation. The obtained sequencing data had an average depth of >1000 (>99% at least 30x) with horizontal coverage >99%, and were aligned to human reference genome build GRCh37. Variants with an alternate read ratio of <0.2 were excluded.

VARIANT SELECTION AND INTERPRETATION

We used Alamut® Visual (V.2.9.0, Interactive Biosoftware, Rouen, France) as an in silico tool for interpretation of the variants. In the primary filtering step, we selected exonic variants and intronic variants up to 10 nucleotides from the exon-intron junction with a MAF of less than 0.01 in the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/) and Genome of the Netherlands (GoNL; http://nlgenome.nl) public variant databases. Synonymous variants without a possible effect on splicing were excluded. The functional effect of missense variants was predicted by the in silico tools SIFT (http://sift.jcvi.org/), Align GVGD (http://agvgd.hci.utah.edu/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and the CADD score (http://cadd.gs.washington.edu/). A further selection of variants of interest (secondary filtering) was based on the following criteria: 1) known pathogenic variants in literature, 2) truncating variants, 3) missense variants with a CADD score >15 and at least two out of three in silico protein prediction tools predicting a possible functional effect, 4) in-frame indels, and 5) variants that likely affect splicing (predicted by SpliceSiteFinder-like, MaXEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder, incorporated in Alamut®).

TABLE 2. List of genes included in the panel

Gene	Full Name	Alt. Name	MIM no.	Refs.
Established melanoma susceptibility genes				
High to medium penetrance:				
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A		600160	
CDK4	Cyclin-Dependent Kinase 4		123829	
BAP1	BRCA1-Associated Protein 1		603089	
POT1	Protection of Telomeres 1		606478	*Reviewed in: Aoude et al.,*[5] *Read et al.*[6]
ACD	Adrenocortical Dysplasia Homolog	*TPP1*	609377	
TERF2IP	TERF2-Interacting Protein	*RAP1*	605061	
TERT	Telomerase Reverse Transcriptase		187270	
MITF	Microphthalmia-Associated Transcription Factor		156845	
Low to medium penetrance:				
MC1R	Melanocortin 1 receptor		155555	
Shelterin complex candidate genes				
TERF1	Telomeric Repeat-Binding Factor 1	*TRF1*	600951	
TERF2	Telomeric Repeat-Binding Factor 2	*TRF2*	602027	*Aoude et al.*[12]
TINF2	TERF1-Interacting Nuclear Factor 2	*TIN2*	604319	
Candidate genes from WES/WGS and GWA studies				
BRIP1	BRCA1-Interacting Protein 1		605882	*Tuominen et al.*[22]
RAD51B	RAD51 Paralog B	*RAD51L1*	602948	*Wadt et al.*[21]
POLE	DNA Polymerase Epsilon		174762	*Aoude et al.*[20]
NEK2	NIMA-Related Kinase 2		604043	-
NEK4	NIMA-Related Kinase 4		601959	-
NEK10	NIMA-Related Kinase 10		-	-
NEK11	NIMA-Related Kinase 11		609779	-
DOT1L	DOT1-Like Histone Lysine Methyltransferase		607375	-
PARP1	Poly (ADP-Ribose) Polymerase 1		173870	-
CENPS	Centromere Protein S	*APITD1*	609130	-
CREB3L1	CAMP Responsive Element Binding Protein 3 Like 1		616215	-
MLLT6	Mixed-Lineage Leukemia, Translocated to, 6		600328	-
ERCC3	ERCC Excision Repair 3		133510	-
CBLB	Cbl Proto-Oncogene B		604491	-
Other candidate genes				
PTEN	Phosphatase and Tensin Homolog		601728	*Bubien et al.*[48]
RASEF	RAS and EF-Hand Domains-Containing Protein		611344	*Maat et al.*[49]
POLH	DNA Polymerase Eta		603968	*Di Lucca et al.*[50]
OCA2	OCA2 Melanosomal Transmembrane Protein		611409	*Hawkes et al.*[45]

MIM = Mendelian Inheritance in Man (http://www.omim.org)

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Analysis of the *POLE* gene was confined to variants in the exonuclease domain (exon 9-14),[20] while analysis of *CDK4*, *TERT*, *MITF* and *MC1R* was restricted to specific variants known to be associated with an increased melanoma risk. This included the p.R24H and p.R24C variants in *CDK4*,[10] the c.-57T>G promoter variant in *TERT*,[13] the p.E318K variant in *MITF*,[15] and the R and r variants in *MC1R*.[18] Co-segregation analysis of the detected variants was possible for families in which more than one case was included in the study. Finally, all variants of interest were evaluated using a recently published in silico prediction tool, UMD-predictor (http://umd-predictor.eu/). This tool uses a combinatorial approach to predict pathogenicity of coding single nucleotide variants by pooling information at the nucleotide level, the protein level and at the mRNA level, and has an exceptionally good reported performance.[24]

RESULTS

In our cohort of 488 samples (451 families), a total of 171 variants passed our primary filtering criteria (see *supplementary table S1*). These included 151 exonic variants, of which eight were truncating (four frameshift, four nonsense), 138 missense, three in-frame indels, and two synonymous variants with a possible effect on splicing. The remaining 20 variants were intronic. Of the 171 variants, 44 were novel (not reported in the reference databases ExAC and GoNL), 41 were extremely rare (MAF<0.0001), 29 were very rare (MAF<0.001), and the remaining 57 variants were rare (MAF<0.01). Subsequent filtering resulted in 60 variants of interest in 20 genes (*tables 3-5*). These selected variants were only detected in probands with CM and in none of the probands with UM. The *MC1R* risk variants were separately analyzed (*table 6*).

VARIANTS OF INTEREST IN ESTABLISHED MELANOMA SUSCEPTIBILITY GENES AND SHELTERIN COMPLEX GENES

We detected two novel splice variants and one novel truncating variant in the *BAP1* gene in three probands (0.7% of families) (*table 3*). The c.122+1G>T, p.? and c.1730-1G>A, p.? variants are both located in a canonical splice site and are predicted to inactivate the splice donor site of intron 3 and splice acceptor site of intron 13, respectively, likely resulting in a prematurely truncated protein. The c.1936_1937insTT, p.(Y646Ffs*10) frameshift variant is also predicted to cause a truncated protein due to a premature stop codon. All three families had multiple members with CM (see *supplementary figure S1*). In two families, possible *BAP1*-associated nevi (Spitz nevi) were reported in first-degree relatives, and in one of these families, multiple relatives were also diagnosed with (one or several) basal cell carcinomas.

No other *BAP1*-specific tumours, such as UM, malignant mesothelioma or renal cell carcinoma, were reported in these families. Interestingly, in the proband who carried the *BAP1* c.122+1G>T, p.? variant we also identified a novel nonsense variant in the *BRIP1* gene (c.894C>A, p.(C298*)). Ovarian cancer was not reported in this family.

The *MITF* p.E318K risk variant was detected in a total of fifteen probands (3.3%), a frequency more than twice that of the Dutch reference population (MAF 0.015; GoNL: 0.007) (*table 3*). All *MITF* p.E318K families had at least two members with CM ('familial CM'; seven two-case families, six three-case families, and two families with four or more cases). The median age of probands at melanoma diagnosis was 41 years (range 27-74). One proband had multiple primary melanomas, a feature also present in two additional families. Renal cancer and pancreatic cancer were present in two families and in one family, respectively.

In the three shelterin complex subunits that have been reported as high-penetrance melanoma susceptibility genes (*POT1*, *ACD*, *TERF2IP*), we identified two potentially deleterious variants (*table 3*). A rare missense variant in the *ACD* gene (c.871A>G, p.(T291A)), detected in a proband from a two-case family, is located in the POT1 binding domain in which previously reported pathogenic variants seem to cluster.[12] A very rare missense variant in the *TERF2IP* gene (c.398G>A, p.(R133Q)), located in the MyB DNA binding domain, was detected in a proband of another two-case family. These variants had a CADD score >20 and were predicted to be damaging by at least two in silico tools, although UMD-predictor classified both variants as polymorphisms. Remarkably, we did not detect any potentially deleterious variants in the *POT1* gene. In the other shelterin complex subunit genes *TERF1*, *TERF2* and *TINF2*, we identified eight potentially deleterious variants (six missense, two in-frame dups) (*table 3*). These included a novel variant in the ACD/TERF2 binding motif domain of the *TINF2* gene (c.38G>T, p.(R13L)) and two extremely rare variants in the *TERF1* gene (c.1193A>G, p.(Y398C); MyB DNA binding domain) and the *TERF2* gene (c.794G>A, p.(R265H)). An in-frame duplication in the *TERF1* gene (c.186_188dup, p.(E62dup); telomeric repeat binding factor homology domain) was shared among two third-degree relatives with CM in one family, but as this is a common variant in Asian and African populations (MAF ~2% in ExAC) it is unlikely to be pathogenic. None of the patients in our cohort carried the known melanoma susceptibly variant in the *TERT* promoter region (c.-57T>G).

TABLE 3. Selected variants of interest in established melanoma susceptibility genes and shelterin complex candidate genes

Gene	Variant	Type	Allele count	MAF (AN=976)	MAF in ExAC[a] / GoNL
Established melanoma susceptibility genes					
ACD	c.871A>G, p.(Thr291Ala)	missense	1	0.0010025	0.0012/0.001
BAP1	c.122+1G>T, p.?	splicing	1	0.0010025	-/-
BAP1	c.1730-1G>A, p.?	splicing	1	0.0010025	-/-
BAP1	c.1936_1937insTT, p.(Tyr646Phefs*10)	frameshift	1	0.0010025	-/-
MITF	c.952G>A, p.(Glu318Lys)	missense	15	0.015369	0.0025/0.007
TERF2IP	c.398G>A, p.(Arg133Gln)	missense	1	0.0010025	0.00022/-
Shelterin complex candidate genes					
TERF1	c.186_188dup, p.(Glu62dup)	in-frame duplication	2	0.002049	0.0005/-[d]
TERF1	c.212_217dup, p.(Glu71_Ala72dup)	in-frame duplication	1	0.0010025	0.00014/-
TERF1	c.1193A>G, p.(Tyr398Cys)	missense	1	0.0010025	0.000009/-
TERF2	c.56A>G, p.(Asp19Gly)	missense	1	0.0010025	0.00012/-
TERF2	c.794G>A, p.(Arg265His)	missense	1	0.0010025	0.000027/-
TERF2	c.1492G>A, p.(Glu498Lys)	missense	4	0.004098	0.0022/0.003
TINF2	c.38G>T, p.(Arg13Leu)	missense	1	0.0010025	-/-
TINF2	c.734C>A, p.(Ser245Tyr)	missense	3	0.003074	0.00073/-

Gene reference sequences: *ACD*: NM_001082486.1, *BAP1*: NM_004656.3, *MITF*: NM_000248.3, *TERF2IP*: NM_018975.3, *TERF1*: NM_017489.2, *TERF2*: NM_005652.4, *TINF2*: NM_001099274.1
AN = allele number, MAF = minor allele frequency, CADD = Combined Annotation Dependent Depletion, FD = in known functional domain, CoS = co-segregation with melanoma in one or more families, Y = yes, N = no, delet = deleterious, pos = possibly, prob = probably
[a] In European (Non-Finnish) population
[b] Possible classifications in Align GVGD are C0, C15, C25, C35, C45, C55 and C65. Variants in class C0 have the least probability of being pathogenic, variants in class C65 have the highest probability of being pathogenic. See also http://agvgd.hci.utah.edu/classifiers.php
[c] HumVar trained PolyPhen-2 model used for prediction
[d] Common variant (MAF>1%) in one or more non-European populations
[e] Co-segregation analyses of variants with melanoma phenotype: *TERF1* p.E62dup: 2/2

CADD	SIFT	Align GVGD[b]	PolyPhen-2[c]	UMD-Predictor	FD	CoS[e]
23.2	delet.	C55	prob. damaging	polymorphism	Y	
					Y	
					Y	
					Y	
27.9	tol.	C0	prob. damaging	prob. polymorphism	Y	
23.4	delet.	C35	benign	polymorphism	Y	
					Y	Y
					Y	
24.7	delet.	C25	prob. damaging	pathogenic	Y	
16.35	delet.	C0	pos. damaging	n.a.	N	
28.3	delet.	C0	pos. damaging	prob. polymorphism	N	
34	delet.	C55	pos. damaging	prob. polymorphism	Y	
27	delet.	C0	prob. damaging	pathogenic	Y	
22.7	delet.	C15	benign	polymorphism	N	

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TABLE 4. Selected variants of interest in candidate melanoma susceptibility genes *BRIP1, POLE* and *OCA2*

Gene	Variant	Type	Allele count	MAF (AN=976)	MAF in ExAC[a] / GoNL
BRIP1	c.517C>T, p.(Arg173Cys)	missense	9	0.009221	0.0047/0.004
BRIP1	c.790C>T, p.(Arg264Trp)	missense	1	0.0010025	0.0012/0.003
BRIP1	c.894C>A, p.(Cys298*)	nonsense	1	0.0010025	-/-
BRIP1	c.1198G>T, p.(Asp400Tyr)	missense	2	0.002049	0.000027/-
BRIP1	c.1255C>T, p.(Arg419Trp)	missense	1	0.0010025	0.00046/0.001
BRIP1	c.2069G>A, p.(Gly690Glu)	missense	1	0.0010025	-/-
BRIP1	c.2582C>G, p.(Ser861Cys)	missense	1	0.0010025	0.000027/-
BRIP1	c.2593C>T, p.(Arg865Trp)	missense	1	0.0010025	0.000027/-
POLE	c.861T>A, p.(Asp287Glu)	missense	9	0.009221	0.0017/0.004
POLE	c.893A>G, p.(Tyr298Cys)	missense	1	0.0010025	-/-
POLE	c.1230G>A, p.(Trp410*)	nonsense	1	0.0010025	-/-
OCA2	c.163del, p.(Ala55Leufs*47)[f]	frameshift	1	0.0010025	0.000019/-
OCA2	c.796C>T, p.(Arg266Trp)	missense	1	0.0010025	0.0018/0.003[d]
OCA2	c.1255C>T, p.(Arg419Trp)[f]	missense	1	0.0010025	0.00011/-
OCA2	c.1261C>T, p.(Arg421Trp)	missense	1	0.0010025	0.000065/-
OCA2	c.1327G>A, p.(Val443Ile)[f]	missense	18	0.018443	0.0051/0.008
OCA2	c.1441G>A, p.(Ala481Thr)[f]	missense	1	0.0010025	0.0026/0.001[d]
OCA2	c.1465A>G, p.(Asn489Asp)[f]	missense	7	0.007172	0.0007/0.003
OCA2	c.1592A>G, p.(Tyr531Cys)	missense	1	0.0010025	0.00011/0.001
OCA2	c.2037G>C, p.(Trp679Cys)[f]	missense	1	0.0010025	0.00015/-

Gene reference sequences: *BRIP1*: NM_032043.2, *POLE*: NM_006231.2, *OCA2*: NM_000275.2
AN = allele number, MAF = minor allele frequency, CADD = Combined Annotation Dependent Depletion, FD = in known functional domain, CoS = co-segregation with melanoma in one or more families, Y = yes, N = no, delet = deleterious, pos = possibly, prob = probably
[a] In European (Non-Finnish) population
[b] Possible classifications in Align GVGD are C0, C15, C25, C35, C45, C55 and C65. Variants in class C0 have the least probability of being pathogenic, variants in class C65 have the highest probability of being pathogenic. See also http://agvgd.hci.utah.edu/classifiers.php
[c] HumVar trained PolyPhen-2 model used for prediction
[d] Common variant (MAF>1%) in one or more non-European populations
[e] Co-segregation analyses of variants with melanoma phenotype: *BRIP1* p.R419W: 1/2, *BRIP1* p.R865W: 1/2, *OCA2* p.R421W: 1/2, *OCA2* p.V443I: 1/2 (two families), *OCA2* p.N489D: 3/3 (one family), *POLE* p.W410*: 1/2
[f] Variants reported in patients with oculocutaneous albinism type 2

CADD	SIFT	Align GVGD[b]	PolyPhen-2[c]	UMD-Predictor	FD	CoS[e]
27.6	delet.	C55	prob. damaging	pathogenic	Y	
32	delet.	C0	prob. damaging	pathogenic	Y	
36				pathogenic	Y	
33	delet.	C35	prob. damaging	pathogenic	Y	
33	delet.	C35	prob. damaging	pathogenic	Y	N
32	delet.	C65	prob. damaging	pathogenic	Y	
28.5	delet.	C65	prob. damaging	pathogenic	Y	
34	delet.	C25	prob. damaging	pathogenic	Y	N
25.7	delet.	C35	prob. damaging	pathogenic	Y	
28.3	delet.	C65	prob. damaging	pathogenic	Y	
38				pathogenic	Y	N
					N	
18.24	delet.	C0	pos. damaging	prob. polymorphism	N	
32	delet.	C0	prob. damaging	pathogenic	Y	
28	delet.	C0	prob. damaging	pathogenic	Y	N
34	tol.	C0	prob. damaging	polymorphism	Y	N
27.6	tol.	C0	pos. damaging	prob. polymorphism	Y	
28.2	delet.	C0	prob. damaging	pathogenic	Y	Y
25.3	delet.	C0	prob. damaging	pathogenic	Y	
34	delet.	C0	prob. damaging	pathogenic	Y	

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TABLE 5. Selected variants of interest in candidate melanoma susceptibility genes (excluding *BRIP1*, *POLE* and *OCA2*)

Gene	Variant	Type	Allele count	MAF (AN=976)	MAF in ExAC[a] / GoNL
CBLB	c.770A>T, p.(His257Leu)	missense	1	0.0010025	-/-
CBLB	c.1402C>G, p.(Arg468Gly)	missense	1	0.0010025	0.000018/-
ERCC3	c.496G>A, p.(Val166Ile)	missense	1	0.0010025	-/-
ERCC3	c.847C>T, p.(Arg283Cys)	missense	5	0.005123	0.0014/0.002
ERCC3	c.1421dup, p.(Asp474Glufs*2)	frameshift	1	0.0010025	0.00014/-
ERCC3	c.1776T>G, p.(Ile592Met)	missense	1	0.0010025	-/-
ERCC3	c.2111C>T, p.(Ser704Leu)	missense	2	0.002049	0.0022/0.001
MLLT6	c.655C>T, p.(Arg219Trp)	missense	1	0.0010025	0.000064/-
MLLT6	c.2195A>C, p.(Glu732Ala)	missense	1	0.0010025	-/-
MLLT6	c.2755G>A, p.(Gly919Arg)	missense	1	0.0010025	-/-
NEK2	c.97-2A>G	splicing	1	0.0010025	0.00029/-
NEK2	c.137A>G, p.(Glu46Gly)	missense	1	0.0010025	-/-
NEK2	c.952C>T, p.(Arg318*)	nonsense	1	0.0010025	0.000018/-
NEK4	c.500T>C, p.(Ile167Thr)	missense	1	0.0010025	0.000009/-
NEK4	c.1953_1955del, p.(Glu651del)	in-frame deletion	1	0.0010025	0.0011/0.002
NEK4	c.2093+1G>C	splicing	1	0.0010025	-/-
NEK10	c.1094G>A, p.(Arg365Gln)	missense	7	0.007172	0.0094/0.009
NEK11	c.127G>C, p.(Val43Leu)	missense	2	0.002049	0.00016/-[d]
PARP1	c.1814C>T, p.(Pro605Leu)	missense	1	0.0010025	0.000036/-
PARP1	c.2656G>A, p.(Val886Met)	missense	1	0.0010025	0.000027/-
POLH	c.626G>T, p.(Gly209Val)	missense	2	0.002049	0.0032/0.003[d]
POLH	c.890G>A, p.(Trp297*)	nonsense	1	0.0010025	-/-
RASEF	c.157C>T, p.(Arg53Trp)	missense	1	0.0010025	0.000049/-
RASEF	c.1049_1050del, p.(His350Argfs*3)	frameshift	1	0.0010025	0.000063/-
RASEF	c.2078A>G, p.(Asp693Gly)	missense	1	0.0010025	0.000018/-
RASEF	c.2207A>T, p.(Asn736Ile)	missense	1	0.0010025	0.000027/-

Gene reference sequences: *CBLB*: NM_170662.4, *ERCC3*: NM_000122.1, *MLLT6*: NM_005937.3, *NEK2*: NM_002497.3, *NEK4*: NM_003157.5, *NEK10*: NM_152534.4, *NEK11*: NM_024800.4, *PARP1*: NM_001618.3, *POLH*: NM_006502.2, *RASEF*: NM_152573.3

AN = allele number, MAF = minor allele frequency, CADD = Combined Annotation Dependent Depletion, FD = in known functional domain, CoS = co-segregation with melanoma in one or more families, Y = yes, N = no, delet = deleterious, pos = possibly, prob = probably

[a] In European (Non-Finnish) population

[b] Possible classifications in Align GVGD are C0, C15, C25, C35, C45, C55 and C65. Variants in class C0 have the least probability of being pathogenic, variants in class C65 have the highest probability of being pathogenic. See also http://agvgd.hci.utah.edu/classifiers.php

[c] HumVar trained PolyPhen-2 model used for prediction

[d] Common variant (MAF>1%) in one or more non-European populations

CADD	SIFT	Align GVGD[b]	PolyPhen-2[c]	UMD-Predictor	FD	CoS[e]
33	delet.	C0	prob. damaging	pathogenic	Y	
23.6	delet.	C0	pos. damaging	pathogenic	Y	
24.6	delet.	C25	benign	prob. polymorphism	Y	
34	delet.	C65	benign	pathogenic	Y	N
					Y	
24.9	delet.	C0	prob. damaging	prob. pathogenic	Y	
24	delet.	C15	benign	pathogenic	N	N
25.2	delet.	C15	pos. damaging	pathogenic	N	
24.6	delet.	C0	prob. damaging	pathogenic	Y	
26.2	delet.	C0	pos. damaging	pathogenic	N	
					Y	
28	delet.	C0	prob. damaging	pathogenic	Y	N
39				pathogenic	Y	
26.9	delet.	C25	prob. damaging	pathogenic	Y	
					N	
					N	
25.2	delet.	C0	pos. damaging	polymorphism	N	Y
27.5	delet.	C25	pos. damaging	prob. polymorphism	Y	
22.6	delet.	C15	benign	pathogenic	Y	
32	delet.	C0	prob. damaging	pathogenic	Y	
28.1	delet.	C15	prob. damaging	prob. polymorphism	Y	
40				pathogenic	Y	Y[f]
28.4	delet.	C0	prob. damaging	prob. pathogenic	Y	
					Y	
32	delet.	C0	pos. damaging	pathogenic	Y	
27.4	delet.	C0	pos. damaging	pathogenic	Y	

[e] Co-segregation analyses of variants with melanoma phenotype: *ERCC3* p.R283C: 1/2 (one family), *ERCC3* p.S704L: 1/2 (one family), *NEK2* p.E46G: 1/2, *NEK10* p.R365Q: 2/2 (one family)

[f] The proband with the *POLH* p.W297* variant had a father with the recessively inherited disease xeroderma pigmentosum (MIM #278750) and he is therefore highly likely to have carried this variant as well

Since we were particularly interested in the frequency of *MC1R* risk variants in *familial* CM cases, we only analyzed the *MC1R* gene in the 'familial CM' subgroup (n=478 individuals). In this cohort, we observed a substantial enrichment of R variants compared to controls (OR 3.67, 95% CI 2.88-4.68, p<0.001) (*table 6*). The frequency of p.D84E was most strikingly increased in our cohort (OR 5.66, 95% CI 1.88-17.06, p=0.001), followed by p.R160W (OR 3.82, 95% CI 2.72-5.37, p<0.001) and p.R151C (OR 3.78, 95% CI 2.68-5.34, p<0.001). Although less prominent, r variants were also enriched in familial CM cases (any r variant: OR 1.53, 95% CI 1.22-1.91, p<0.001).

TABLE 6. Association of *MC1R* risk variants with familial cutaneous melanoma

	familial CM cohort[a] (AN=956)	control cohort[a] (AN=898)	OR	95% CI	p value[b]
No. of individuals	478	449			
Reference sequence[c]	388	549	*Ref.*	*Ref.*	*Ref.*
All R variants	**0.342**	**0.140**	**3.67**	**2.88 – 4.68**	**<0.001**
c.252C>A, p.D84E	0.017	0.004	5.66	1.88 – 17.06	0.001
c.425G>A, p.R142H	0.008	0.008	1.62	0.58 – 4.50	0.431
c.451C>T, p.R151C	0.145	0.058	3.78	2.68 – 5.34	<0.001
c.478C>T, p.R160W	0.150	0.059	3.82	2.72 – 5.37	<0.001
c.880G>C, p.D294H	0.022	0.011	2.79	1.38 – 6.38	0.005
All r variants	**0.252**	**0.248**	**1.53**	**1.22 – 1.91**	**<0.001**
c.178G>T, p.V60L	0.105	0.104	1.52	1.12 – 2.08	0.008
c.274G>A, p.V92M	0.082	0.081	1.51	1.07 – 2.13	0.021
c.464T>C, p.I155T	0.006	0.006	1.70	0.52 – 5.60	0.540
c.488G>A, p.R163Q	0.060	0.058	1.55	1.04 – 2.31	0.032

MC1R reference sequence: NM_002386.3
AN = allele number
[a] minor allele frequency (MAF)
[b] using Fisher's exact test (two-sided)
[c] number of alleles without any R or r variant

VARIANTS OF INTEREST IN CANDIDATE MELANOMA SUSCEPTIBILITY GENES

In addition to the novel, truncating variant in the *BRIP1* gene (c.894C>A, p.(C298*)) found in one of the *BAP1*-families, an additional seven potentially deleterious missense variants were identified in *BRIP1* (*table 4*). This included one novel variant (c.2069G>A, p.(G690E)) and two extremely rare variants (c.2582C>G, p.(S861C) and c.2593C>T, p.(R865W)) located in the DNA helicase domain and predicted to be damaging by all in silico tools including UMD-predictor. However, the latter variant did not co-segregate with the phenotype in a two-case family. In this same domain, a different missense variant was previously

reported to co-segregate in a three-case melanoma family.[22] The remaining four variants were located in the ATPase/helicase core domain, and included an extremely rare variant (c.1198G>T, p.(D400Y)) in two probands and a very rare variant (c.1255C>T, p.(R419W)) in one proband. Currently, little is known from literature about the effect of these missense variants and no functional testing has been performed.

We further identified two missense variants in the exonuclease domain of the *POLE* gene: one novel variant (c.893A>G, p.(Y298C)) in a single proband and a rare variant (c.861T>A, p.(D287E)) in nine other probands *(table 4*). Both variants were predicted to be damaging by all in silico tools including UMD-predictor. In another proband, we identified a novel truncating variant in *POLE* (c.1230G>A, p.(W410*)), but this variant did not co-segregate with the phenotype in a two-case family.

In the *OCA2* gene, we identified nine (potentially) deleterious variants, of which six were previously reported in patients with the recessively inherited condition oculocutaneous albinism type 2 (MIM #203200) (*table 4*). Two of these established pathogenic variants, c.1327G>A, p.(V443I) and c.1465A>G, p.(N489D), were detected in multiple individuals (n=17 and 7, respectively) and the frequency of these variants was more than twice that found in the Dutch GoNL reference database (MAF: 0.018 and 0.0071; GoNL: 0.008 and 0.003, respectively). Co-segregation analysis was, however, ambiguous: the c.1465A>G, p.(N489D) variant co-segregated with the phenotype in a three-case family (all first-degree relatives), but the c.1327G>A, p.(V443I) variant did not co-segregate in two two-case families. Interestingly, one proband was homozygous for the c.1327G>A, p.(V443I) variant. This proband had a medical history of three primary melanomas from age 57 and a first-degree relative (sibling) with melanoma. Although the proband was reported to have a fair skin type and reddish hair, no other physical signs of albinism were reported.

Another proband, with a medical history of three primary melanomas from age 48 and a first-degree relative (child) with melanoma at age 32, carried two pathogenic variants in the *OCA2* gene (c.1327G>A, p.(V443I) and c.2037G>C, p.(W679C)). Since physical signs of albinism were not reported in the proband, it is possible that these variants are located on the same allele, but this could not be confirmed because co-segregation data was unavailable.

In the other included candidate melanoma susceptibility genes, largely derived from whole exome/genome sequencing studies by both our own research group and other research groups, we detected four truncating variants (in *ERCC3*, *NEK2*, *POLH*, *RASEF*), two canonical splice site variants (in *NEK2*, *NEK4*) and several potentially deleterious missense

variants (in *CBLB*, *ERCC3*, *MLLT6*, *NEK2*, *NEK4*, *NEK10*, *NEK11*, *PARP1*, *POLH*, *RASEF*) (*table 5*). All of these variants occurred in only one proband and co-segregation data was only occasionally available. UMD-predictor classified the majority of these variants as (probably) pathogenic.

DISCUSSION

In this study, we performed multi-gene panel testing of 30 (candidate) melanoma susceptibility genes in 451 Dutch melanoma-prone families without a *CDKN2A* or *CDK4* mutation. We identified (likely) pathogenic variants in established high- and medium-penetrance melanoma susceptibility genes in 4.0% of these families (18/451; n=3 *BAP1*, n=15 *MITF*). In addition, two potentially deleterious missense variants were detected in important functional domains of the *ACD* and *TERF2IP* genes (0.4%) and, surprisingly, none of the 451 families carried a variant of interest in the *POT1* gene.

The frequency of *BAP1* mutations in our cohort (n=3; 0.7%) is in line with a reported frequency of ~1% among melanoma-prone families worldwide.[25] *BAP1* is a deubiquitinating hydrolase that acts as a tumour suppressor and is involved in the regulation of key pathways including cell proliferation, cell differentiation, cell survival and the DNA damage response. Germline *BAP1* mutations have been reported in patients with several types of tumours, but particularly in UM and malignant mesothelioma.[14] Interestingly, these two major cancers were not present in our three families. Although CM itself is relatively common in *BAP1* mutation carriers (13-18%),[14,26] *BAP1* mutations are rarely reported in CM families without these other cancers: a study by Njauw *et al*[27] detected only one *BAP1* mutation in 193 CM families (0.5%), and a study by Wadt *et al*[28] found no *BAP1* mutations in 133 high-risk CM patients (of which 94 CM *families*). By contrast, Gerami *et al*[29] found a *BAP1* mutation in a single case with multiple primary cutaneous melanomas and a dysplastic nevus phenotype, with no family history for either CM or UM or any other *BAP1*-associated cancers. A recent population-based study reported only three loss-of-function *BAP1* mutations in CM cases (<0.2%), and all these cases had relatives with *BAP1*-associated cancers, although none had UM.[30] Our study demonstrates that *BAP1* mutations can indeed be detected in some CM families without UM or malignant mesothelioma and it is therefore important to incorporate the *BAP1* gene in a diagnostic (cutaneous) melanoma gene panel test. However, it should be noted that basal cell carcinoma and (atypical) Spitz nevi, features also associated with *BAP1* mutations, were reported in two of the families.

Fifteen probands in our familial CM cohort (15/442; 3.4%) carried the *MITF* p.E318K risk variant,

which is amongst the highest frequencies reported in familial non-*CDKN2A* cases. Only one small study from Switzerland reported a higher frequency, 7.7% (2/26), in melanoma-prone families.[31] A similar frequency, 3.4% (19/558) in familial cases, was found in a study from the United States, although it is unclear if these patients were all pre-screened for *CDKN2A* mutations.[32] Frequencies in various other cohorts range from 0-3% [16,28,33-35], with the lowest frequency (<1%) reported in familial cases from Italy.[17,36] In the Netherlands, diagnostic testing for the *MITF* p.E318K risk variant is now included in the default genetic work-up for familial CM and all carriers are offered regular dermatologic surveillance (regardless of the familial burden for CM). This regular surveillance is recommended because carriers are at increased risk for developing subsequent (multiple primary) melanomas [15] that might also be fast-growing [35] and/or amelanotic [37], a subtype less easily recognized by the patient and/or the dermatologist. Hence, knowledge about *MITF* p.E318K mutation status can be relevant for both the patient and the dermatologist. Surveillance for other cancers such as renal- or pancreatic cancer is not (yet) offered because the actual risk for these cancers is insufficiently established and surveillance methods are more challenging.

Germline mutations in the telomere maintenance pathway genes in melanoma families have been described in several studies.[11-13] The present study demonstrates that mutations in these genes are probably very rare in the Dutch familial melanoma population. We identified only two potentially deleterious missense variants in *ACD* and *TERF2IP* (0.4%) and none in *POT1* or the promoter region of *TERT*. In the *ACD* and *TERF2IP* genes, both nonsense and pathogenic missense variants have been previously reported in familial melanoma kindreds.[12] Interestingly, the *TERF2IP* p.(R133Q) variant that we detected in a two-case melanoma family was previously reported in a three-case chronic lymphocytic leukemia (CLL) family (without melanoma).[38] Because the variant co-segregated with only two of the cases, the authors concluded that this is a medium-penetrance variant for CLL. Leukemia was not reported in relatives of the proband in our cohort. Of the eight potentially deleterious missense variants detected in the *TERF1*, *TERF2* and *TINF2* genes, co-segregation analysis was only possible for one of these variants. There is no additional evidence for pathogenicity of these missense variants, and as yet no protein truncating variants have been reported in these latter genes. Therefore, their role in melanoma susceptibility remains uncertain.

We identified several variants of interest in the known cancer susceptibility genes *BRIP1* and *POLE*, including a nonsense variant in *BRIP1*. BRIP1 (BRCA1-interacting protein C-terminal helicase 1) is a Fanconi anemia group protein and is required for the double-strand break repair function of BRCA1. Heterozygous protein truncating variants in *BRIP1* have mainly been associated with an increased susceptibility for ovarian cancer,[39] but there were

no diagnoses of ovarian cancer in family members of the proband with the nonsense *BRIP1* variant in this study. Interestingly, this variant co-occurred with a canonical splice site variant in *BAP1* in the same proband, the latter presumably being the predominant melanoma susceptibility factor in this family. We additionally identified several potentially deleterious missense variants in *BRIP1,* some novel or extremely rare, and most of which were predicted to be damaging by all in silico tools used. In a recent study from Sweden, an extremely rare missense variant in the DNA helicase domain of *BRIP1* was found to co-segregate in a three-case melanoma family.[22] Three missense variants in our cohort were located in this same functional domain. Based on these findings, the *BRIP1* gene might be involved in melanoma susceptibility, but more research is needed to clarify this, in particular replication studies in other melanoma cohorts and functional studies to address the pathogenicity of missense variants. The *POLE* gene is a polymerase gene involved in DNA repair and replication and is primarily associated with colorectal cancer. It appears that only missense variants in the exonuclease domain confer an increased susceptibility for cancer through impaired proofreading, which results in tumours with a high mutation burden.[40] Therefore, we restricted our analysis of variants to this specific exonuclease domain and, consequently, all reported variants in *POLE* are located within this domain. Recently, a novel missense variant in the exonuclease domain of *POLE* was reported in a seven-case melanoma family and showed near-complete co-segregation.[20] Although we were not able to perform co-segregation analysis for the novel missense variant (c.893A>G, p.(Y298C)) detected in our cohort, functional analysis of melanoma tissue (mutation burden test) might provide more insight. Of note, colorectal cancer was not reported in this family.

Biallelic germline mutations in *OCA2* cause oculocutaneous albinism type 2 (MIM #203200). *OCA2* encodes the P-protein which has multiple functions in the biosynthesis of melanin. Loss-of-function of the P-protein results in hypopigmentation of the skin, hair and iris and an increased risk for sun-induced skin cancers, in particular basal cell carcinoma and squamous cell carcinoma.[41] Although melanoma is not known to be a common cancer type in patients with *OCA2*-related albinism, families with multiple members with albinism and melanoma have been reported.[42] In our cohort, one proband with a possible subclinical phenotype of albinism carried a homozygous pathogenic *OCA2* variant. Additionally, we observed an increased frequency of rare heterozygous variants in the *OCA2* gene, in particular the known pathogenic variants c.1327G>A, p.(V443I) and c.1465A>G, p.(N489D).[43,44] The association with melanoma predisposition of the c.1327G>A, p.(V443I) variant in combination with another *OCA2* variant was also studied by Hawkes *et al*[45] in one albinism-melanoma family. They concluded that these variants might be high-penetrance loci for melanoma in this family (OR 6.5). In a recent study by Goldstein *et al*,[46] the *OCA2* gene was included in a multi-gene panel test of 42 (candidate) melanoma

susceptibly genes that were sequenced in 144 melanoma cases from 76 American families. Comparable to our study, numerous rare variants in *OCA2* were found. The frequency of rare variants in other albinism genes (*TYR, TYRP1*) was also significantly increased in the Goldstein study. Interestingly, a nonsense variant in *TYR* showed near-complete co-segregation in a large family with six melanoma cases. The precise role of *OCA2* (and other albinism genes) in melanoma predisposition remains to be determined, but based on these findings a medium-penetrance or modifier effect can be hypothesized. The albinism genes are therefore good candidates for further investigation.

There is extensive literature on the association between *MC1R* R and r variants and sporadic melanoma in population-based cohorts.[18] In our 'familial CM' cases, we observed a high frequency of *MC1R* R variants in particular, a finding comparable to the results of a Danish high-risk melanoma cohort.[28] This suggests that these common risk variants also play a significant role in the familial setting. Since some of the familial occurrence of melanoma might be explained by the aggregation of common risk variants in a family, we are currently incorporating all *MC1R* R and r variants in a polygenic risk score (PRS) model that also includes approximately 40 other common risk variants derived from large melanoma GWAS. PRS models have already been shown to improve risk stratification in other familial cancer cohorts, in particular familial breast cancer.[47]

A major strength of our study is cohort size. With the inclusion of 451 families lacking a mutation in the *CDKN2A* or *CDK4* genes, of which 442 families had at least two cases of CM, to our knowledge this is the largest melanoma gene panel study to date. Although our inclusion criteria were not highly stringent, most families had at least two close relatives with melanoma (for instance, 83% of the two-case families consisted of first-degree relatives). Furthermore, our panel included all eight currently known high- and medium-penetrance melanoma susceptibility genes and therefore our reported 4% diagnostic yield for these genes (excluding *CDKN2A* and *CDK4*) is probably very accurate. As a custom-designed targeted gene panel was used, filtering of variants was less strict compared to most reported WES studies. It is therefore very unlikely that potential pathogenic variants in the selected genes were missed in our study. A limitation is that co-segregation analysis of variants was not possible in many families. This was primarily due to Ethics Committee restrictions that prohibited us from re-contacting patients when variants of uncertain significance (VUS) or variants in non-established genes were detected. However, co-segregation analysis of (likely) pathogenic variants in known cancer susceptibility genes (*BAP1, MITF, BRIP1*) is currently being initiated.

To conclude, we demonstrate that multi-gene panel testing for familial melanoma results in

an additional 4% diagnostic yield in non-*CDKN2A/CDK4* families. The identification of several families with pathogenic variants in the *BAP1* and *MITF* genes suggests a significant role of these genes in melanoma predisposition and it is therefore important to include these in a diagnostic test. Conversely, variants in the telomere maintenance genes, especially *POT1*, seem to be (very) rare in the Dutch population. When including these genes in a panel test, one should be aware of identifying variants of uncertain significance, as we did in the current study. In view of the relatively high frequency of (potential) pathogenic variants in the *OCA2* gene in both our own and in a recently published American familial melanoma cohort, further elucidation of the role of heterozygous *OCA2* variants in melanoma predisposition appears to be of particular interest. In the future, candidate susceptibility genes such as *OCA2* could potentially be added to routine germline diagnostics, given sufficient evidence for their pathogenicity in melanoma predisposition. This will in turn enhance the diagnostic yield of the panel and improve tumour risk assessment in melanoma families.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. All variants with a MAF <1% (after primary filtering)

Gene	mRNA refSeq	Chr. Position (GRCh37)	cDNA change	Exon	Protein change	Variant type	dbSNP
ACD	NM_001082486.1	chr16: 67694102	c.280G>A	1	p.(Val94Ile)	ms	rs149365469
ACD	NM_001082486.1	chr16: 67694044	c.338G>A	1	p.(Arg113Gln)	ms	rs142507451
ACD	NM_001082486.1	chr16: 67692863	c.871A>G	7	p.(Thr291Ala)	ms	rs139438549
ACD	NM_001082486.1	chr16: 67691917	c.1436G>A	10	p.(Arg479Lys)	ms	rs531580930
BAP1	NM_004656.3	chr3: 52443569	c.122+1G>T	-	p.?	intron	-
BAP1	NM_004656.3	chr3: 52439834	c.878C>T	10	p.(Pro293Leu)	ms	rs777664260
BAP1	NM_004656.3	chr3: 52437424	c.1729+8T>C	-	p.?	intron	rs150945583
BAP1	NM_004656.3	chr3: 52437315	c.1730-1G>A	-	p.?	intron	-
BAP1	NM_004656.3	chr3: 52436841	c.1936_1937in-sTT	15	p.(Tyr-646Phefs*10)	fs	-
BRIP1	NM_032043.2	chr17: 59924572	c.517C>T	6	p.(Arg173Cys)	ms	rs4988345
BRIP1	NM_032043.2	chr17: 59924512	c.577G>A	6	p.(Val193Ile)	ms	rs4988346
BRIP1	NM_032043.2	chr17: 59924505	c.584T>C	6	p.(Leu195Pro)	ms	rs4988347
BRIP1	NM_032043.2	chr17: 59924502	c.587A>G	6	p.(Asn196Ser)	ms	rs550707862
BRIP1	NM_032043.2	chr17: 59885956	c.790C>T	7	p.(Arg264Trp)	ms	rs28997569
BRIP1	NM_032043.2	chr17: 59885852	c.894C>A	7	p.(Cys298*)	ns	-
BRIP1	NM_032043.2	chr17: 59876603	c.1198G>T	9	p.(Asp400Tyr)	ms	rs764711572
BRIP1	NM_032043.2	chr17: 59876546	c.1255C>T	9	p.(Arg419Trp)	ms	rs150624408
BRIP1	NM_032043.2	chr17: 59853928	c.1936-5T>A	-	p.?	intron	-
BRIP1	NM_032043.2	chr17: 59853790	c.2069G>A	14	p.(Gly690Glu)	ms	-
BRIP1	NM_032043.2	chr17: 59821955	c.2098-3T>C	-	p.?	intron	-
BRIP1	NM_032043.2	chr17: 59821830	c.2220G>T	15	p.(Gln740His)	ms	rs45589637
BRIP1	NM_032043.2	chr17: 59763520	c.2582C>G	19	p.(Ser861Cys)	ms	rs774415723
BRIP1	NM_032043.2	chr17: 59763509	c.2593C>T	19	p.(Arg865Trp)	ms	rs578022079
CBLB	NM_170662.4	chr3: 105572408	c.269G>A	3	p.(Ser90Asn)	ms	-

Minor Allele Frequencies				Evolutionary Conservation / Distance			In-Silico Prediction				
Allele count	MAF (AN=976)	MAF ExAC[a]	MAF GoNL	NT	AA	GD	CADD Phred	SIFT	Align GVGD[b]	Poly Phen-2[c]	Spl
1	0.001025	0.00076	-	W	M	S	12.74	T	C0	B	
3	0.003074	0.0022	0.002	N	W	S	18.17	T	C0	B	
1	0.001025	0.0012	0.001	M	H	S	23.2	D	C55	D	
1	0.001025	0.000099	0.001	N	W	S	0.006	T	C0	B	
1	0.001025	-	-	-	-	-	-	-	-	-	+
1	0.001025	0	-	M	M	M	23.8	D	C0	B	
5	0.005123	0.006	0.007	-	-	-	-	-	-	-	
1	0.001025	-	-	-	-	-	-	-	-	-	+
1	0.001025	-	-	-	-	-	-	-	-	-	
9	0.009221	0.0047	0.004	M	H	L	27.6	D	C55	D	
4	0.004098	0.0054	0.007	N	W	S	0.002	T	C0	B	
1	0.001025	0.002	0.002	W	W	M	4.462	T	C0	B	
1	0.001025	0.000018	-	W	W	S	0.003	T	C0	B	
1	0.001025	0.0012	0.003	M	M	M	32	D	C0	D	
1	0.001025	-	-	-	-	-	36	-	-	-	
2	0.002049	0.000027	-	H	H	L	33	D	C35	D	
1	0.001025	0.00046	0.001	W	H	M	33	D	C35	D	
1	0.001025	-	-	-	-	-	-	-	-	-	
1	0.001025	-	-	H	H	M	32	D	C65	D	
2	0.002049	-	-	-	-	-	-	-	-	-	
1	0.001025	0.00065	0.001	W	M	S	25.7	T	C0	P	
1	0.001025	0.000027	-	M	H	M	28.5	D	C65	D	
1	0.001025	0.000027	-	W	H	M	34	D	C25	D	
1	0.001025	0	-	M	M	S	21.2	T	C0	B	

SUPPLEMENTARY TABLE S1 CONTINUED.

Gene	mRNA refSeq	Chr. Position (GRCh37)	cDNA change	Exon	Protein change	Variant type	dbSNP
CBLB	NM_170662.4	chr3: 105572313	c.364A>G	3	p.(Ile122Val)	ms	rs748358316
CBLB	NM_170662.4	chr3: 105464836	c.770A>T	6	p.(His257Leu)	ms	-
CBLB	NM_170662.4	chr3: 105438947	c.1351G>A	10	p.(Asp451Asn)	ms	rs377118360
CBLB	NM_170662.4	chr3: 105438896	c.1402C>G	10	p.(Arg468Gly)	ms	-
CBLB	NM_170662.4	chr3: 105421032	c.1865G>C	12	p.(Ser622Thr)	ms	rs41302192
CBLB	NM_170662.4	chr3: 105421025	c.1872T>G	12	p.(Asn624Lys)	ms	-
CBLB	NM_170662.4	chr3: 105400624	c.2240A>T	15	p.(His747Leu)	ms	rs149189614
CBLB	NM_170662.4	chr3: 105397298	c.2546A>T	17	p.(Gln849Leu)	ms	-
CENPS	NM_199294.2	chr1: 10494754	c.209+7A>G	-	p.?	intron	rs760512781
CENPS	NM_199294.2	chr1: 10502454	c.409G>A	5	p.(Glu137Lys)	ms	rs146240548
CREB3L1	NM_052854.3	chr11: 46329489	c.454G>A	3	p.(Ala152Thr)	ms	rs199951144
CREB3L1	NM_052854.3	chr11: 46332586	c.599A>T	5	p.(Asp200Val)	ms	rs187725533
CREB3L1	NM_052854.3	chr11: 46337910	c.1105G>A	9	p.(Ala369Thr)	ms	rs201046043
DOT1L	NM_032482.2	chr19: 2191003	c.265-8G>A	-	p.?	intron	rs374436091
DOT1L	NM_032482.2	chr19: 2191226	c.480G>A	5	p.(=)	syn	-
DOT1L	NM_032482.2	chr19: 2210451	c.1058C>G	13	p.(Ala353Gly)	ms	rs138206172
DOT1L	NM_032482.2	chr19: 2211098	c.1352A>G	15	p.(Asp451Gly)	ms	rs377185393
DOT1L	NM_032482.2	chr19: 2214564	c.1892C>T	19	p.(Ser631Leu)	ms	rs200661860
DOT1L	NM_032482.2	chr19: 2216470	c.2114G>C	20	p.(Ser705Thr)	ms	-
DOT1L	NM_032482.2	chr19: 2216545	c.2189C>T	20	p.(Ser730Leu)	ms	rs750873331
DOT1L	NM_032482.2	chr19: 2216610	c.2254C>T	20	p.(Pro752Ser)	ms	rs370203392
DOT1L	NM_032482.2	chr19: 2216658	c.2302G>C	20	p.(Ala768Pro)	ms	rs758184437
DOT1L	NM_032482.2	chr19: 2217034	c.2489C>T	21	p.(Pro830Leu)	ms	rs368118931
DOT1L	NM_032482.2	chr19: 2217094	c.2544+5G>A	-	p.?	intron	rs202211033
DOT1L	NM_032482.2	chr19: 2217786	c.2560G>A	22	p.(Ala854Thr)	ms	rs201843576
DOT1L	NM_032482.2	chr19: 2217801	c.2575G>A	22	p.(Gly859Arg)	ms	rs753001418
DOT1L	NM_032482.2	chr19: 2222325	c.3157G>A	24	p.(Ala1053Thr)	ms	rs144165419

Minor Allele Frequencies				Evolutionary Conservation / Distance			In-Silico Prediction				
Allele count	MAF (AN=976)	MAF ExAC[a]	MAF GoNL	NT	AA	GD	CADD Phred	SIFT	Align GVGD[b]	Poly Phen-2[c]	Spl
1	0.001025	0	-	M	H	S	13.69	T	C0	B	±
1	0.001025	-	-	H	H	M	33	D	C0	D	
1	0.001025	0	-	H	M	S	27.7	T	C0	D	
1	0.001025	0.000018	-	W	M	M	23.6	D	C0	P	
9	0.009221	0.0083	0.007	W	W	S	5.998	T	C0	B	
1	0.001025	-	-	W	W	M	0.007	T	C0	B	
2	0.002049	0.000045	-	M	M	M	19.56	T	C0	B	
1	0.001025	0.000018	-	M	W	M	20.7	T	C0	B	
1	0.001025	0.000046	-	-	-	-	-	-	-	-	
1	0.001025	0.0014	0.002	M	M	S	10.71	T	C0	B	
1	0.001025	0.0065[d]	0.006	N	M	S	3.186	T	C0	B	
7	0.007172	0.0059	0.006	M	H	L	26.9	D	C0	B	
1	0.001025	0.00033	0.001	W	M	S	22.7	D	C0	B	
4	0.004098	0.00034	0.001	-	-	-	-	-	-	-	
1	0.001025	0.000018	-	N	-	-	-	-	-	-	±
1	0.001025	0.0024	0.004	M	W	S	19.18	T	C0	B	
1	0.001025	0.00025	0.001	M	M	M	23.3	T	C0	D	±
1	0.001025	0.00055	0.001	H	H	L	34	T	C0	D	
1	0.001025	-	-	M	M	S	23	T	C0	B	
1	0.001025	0.000027	-	M	W	L	25.9	T	C0	B	
1	0.001025	0.000027	-	M	M	M	25.8	T	C0	B	
1	0.001025	0.0001	-	N	W	S	22.2	T	C0	B	
3	0.003074	0.000018	-	W	M	M	26.4	T	C0	B	
2	0.002049	0.0015	0.003	-	-	-	-	-	-	-	±
2	0.002049	0.0017	0.004	N	W	S	3.499	T	C0	B	
1	0.001025	0.000009973	-	M	M	M	25.8	T	C0	P	
1	0.001025	0.0018	-	N	W	S	0.136	T	C0	B	

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SUPPLEMENTARY TABLE S1 CONTINUED.

Gene	mRNA refSeq	Chr. Position (GRCh37)	cDNA change	Exon	Protein change	Variant type	dbSNP
DOT1L	NM_032482.2	chr19: 2223364	c.3475G>C	25	p.(Asp1159His)	ms	rs377512955
DOT1L	NM_032482.2	chr19: 2226219	c.3699C>T	27	p.(=)	syn	rs771189396
DOT1L	NM_032482.2	chr19: 2226539	c.4019A>G	27	p.(Lys1340Arg)	ms	-
DOT1L	NM_032482.2	chr19: 2226592	c.4072G>A	27	p.(Gly1358Ser)	ms	rs376766280
DOT1L	NM_032482.2	chr19: 2226694	c.4174G>A	27	p.(Gly1392Ser)	ms	rs375002753
DOT1L	NM_032482.2	chr19: 2226728	c.4208C>A	27	p.(Thr1403Asn)	ms	rs200561588
DOT1L	NM_032482.2	chr19: 2226833	c.4313T>G	27	p.(Leu1438Arg)	ms	rs371610616
DOT1L	NM_032482.2	chr19: 2226929	c.4409C>T	27	p.(Pro1470Leu)	ms	-
DOT1L	NM_032482.2	chr19: 2226935	c.4415C>G	27	p.(Pro1472Arg)	ms	-
DOT1L	NM_032482.2	chr19: 2227081	c.4561C>T	27	p.(His1521Tyr)	ms	-
ERCC3	NM_000122.1	chr2: 128047825	c.496G>A	4	p.(Val166Ile)	ms	-
ERCC3	NM_000122.1	chr2: 128047311	c.611G>A	5	p.(Gly204Glu)	ms	rs751705179
ERCC3	NM_000122.1	chr2: 128046416	c.847C>T	7	p.(Arg283Cys)	ms	rs145201970
ERCC3	NM_000122.1	chr2: 128044468	c.1153G>A	8	p.(Asp385Asn)	ms	-
ERCC3	NM_000122.1	chr2: 128038129	c.1421dup	9	p.(Asp474Glufs*2)	fs	rs587778281
ERCC3	NM_000122.1	chr2: 128030492	c.1776T>G	11	p.(Ile592Met)	ms	-
ERCC3	NM_000122.1	chr2: 128016978	c.2111C>T	14	p.(Ser704Leu)	ms	rs4150521
MITF	NM_000248.3	chr3: 70014091	c.952G>A	9	p.(Glu318Lys)	ms	rs149617956
MLLT6	NM_005937.3	chr17: 36864133	c.354+8G>A	-	p.?	intron	rs113618401
MLLT6	NM_005937.3	chr17: 36868139	c.592G>A	7	p.(Ala198Thr)	ms	rs2241012
MLLT6	NM_005937.3	chr17: 36868202	c.655C>T	7	p.(Arg219Trp)	ms	rs369771793
MLLT6	NM_005937.3	chr17: 36869017	c.794C>T	8	p.(Pro265Leu)	ms	rs754493479
MLLT6	NM_005937.3	chr17: 36872024	c.979G>A	9	p.(Ala327Thr)	ms	rs146278240
MLLT6	NM_005937.3	chr17: 36872922	c.1339G>T	10	p.(Ala447Ser)	ms	rs145966494
MLLT6	NM_005937.3	chr17: 36876664	c.2195A>C	15	p.(Glu732Ala)	ms	-
MLLT6	NM_005937.3	chr17: 36878443	c.2755G>A	17	p.(Gly919Arg)	ms	-

Minor Allele Frequencies				Evolutionary Conservation / Distance			In-Silico Prediction				
Allele count	**MAF (AN=976)**	**MAF ExAC[a]**	**MAF GoNL**	**NT**	**AA**	**GD**	**CADD Phred**	**SIFT**	**Align GVGD[b]**	**Poly Phen-2[c]**	**Spl**
2	0.002049	0.00022	0.001	H	M	M	28.9	T	C0	D	
1	0.001025	0.00018	-	W	-	-	-	-	-	-	±
1	0.001025	-	-	W	W	S	25.2	T	C0	D	
1	0.001025	0.000059	-	W	W	S	8.724	T	C0	B	
1	0.001025	0.00052	0.002	N	W	S	2.832	T	C0	B	
1	0.001025	0.00024	-	N	W	S	12.39	T	C0	B	
1	0.001025	0.00011	-	W	W	M	11.78	T	C0	D	
1	0.001025	-	-	M	W	M	25.3	D	C0	B	
1	0.001025	-	-	M	W	M	23.3	T	C0	B	
1	0.001025	-	-	M	W	M	25.4	T	C0	D	
1	0.001025	-	-	H	H	S	24.6	D	C25	B	
1	0.001025	0.000009	-	M	H	M	18.35	T	C0	B	
5	0.005123	0.0014	0.002	H	H	L	34	D	C65	B	
2	0.002049	-	-	H	H	S	21.8	T	C0	B	
1	0.001025	0.00014	-	-	-	-	-	-	-	-	
1	0.001025	-	-	W	H	S	24.9	D	C0	D	
2	0.002049	0.0022	0.001	H	M	L	24	D	C15	B	
15	0.015369	0.002527	0.007	H	H	S	27.9	T	C0	D	
1	0.001025	0.00031[d]	-	-	-	-	-	-	-	-	
1	0.001025	0.00001[d]	-	N	W	S	0.011	T	C0	B	
1	0.001025	0.000064	-	W	H	M	25.2	D	C15	P	
1	0.001025	0.000063	-	M	M	M	25	D	C0	B	
9	0.009221	0.0067	0.008	W	M	S	18.24	D	C0	B	
9	0.009221	0.0069	0.008	N	W	M	6.782	T	C0	B	
1	0.001025	-	-	M	H	M	24.6	D	C0	D	
1	0.001025	-	-	M	H	M	26.2	D	C0	P	

SUPPLEMENTARY TABLE S1 CONTINUED.

Gene	mRNA refSeq	Chr. Position (GRCh37)	cDNA change	Exon	Protein change	Variant type	dbSNP
MLLT6	NM_005937.3	chr17: 36881009	c.3020C>T	19	p.(Ala1007Val)	ms	rs150198262
NEK2	NM_002497.3	chr1: 211847857	c.97-2A>G	-	p.?	intron	rs201869074
NEK2	NM_002497.3	chr1: 211847815	c.137A>G	2	p.(Glu46Gly)	ms	-
NEK2	NM_002497.3	chr1: 211842488	c.952C>T	6	p.(Arg318*)	ns	rs146817802
NEK4	NM_003157.5	chr3: 52800252	c.500T>C	3	p.(Ile167Thr)	ms	-
NEK4	NM_003157.5	chr3: 52786252	c.1064A>G	7	p.(Asn355Ser)	ms	-
NEK4	NM_003157.5	chr3: 52783745	c.1469G>A	8	p.(Arg490Gln)	ms	rs189287859
NEK4	NM_003157.5	chr3: 52780883	c.1544G>T	9	p.(Gly515Val)	ms	-
NEK4	NM_003157.5	chr3: 52777417	c.1953_1955del	12	p.(Glu651del)	del	rs534558039
NEK4	NM_003157.5	chr3: 52775426	c.2093+1G>C	-	p.?	intron	-
NEK10	NM_152534.4	chr3: 27343261	c.1094G>A	14	p.(Arg365Gln)	ms	rs75891446
NEK10	NM_152534.4	chr3: 27233631	c.2394G>T	27	p.(Gln798His)	ms	rs766212798
NEK10	NM_152534.4	chr3: 27216236	c.2594C>A	28	p.(Pro865His)	ms	rs140958685
NEK10	NM_152534.4	chr3: 27216215	c.2615A>G	28	p.(Tyr872Cys)	ms	rs141326474
NEK10	NM_152534.4	chr3: 27203966	c.2996A>G	32	p.(Asn999Ser)	ms	-
NEK10	NM_152534.4	chr3: 27182990	c.3124A>G	34	p.(Ile1042Val)	ms	rs41487750
NEK10	NM_152534.4	chr3: 27161337	c.3275C>T	36	p.(Pro1092Leu)	ms	rs34545563
NEK11	NM_024800.4	chr3: 130748679	c.127G>C	3	p.(Val43Leu)	ms	rs140058289
NEK11	NM_024800.4	chr3: 130947497	c.1525G>C	15	p.(Glu509Gln)	ms	-
OCA2	NM_000275.2	chr15: 28326992	c.29G>A	2	p.(Arg10Gln)	ms	rs199752361
OCA2	NM_000275.2	chr15: 28326984	c.37G>A	2	p.(Gly13Ser)	ms	rs201554429
OCA2	NM_000275.2	chr15: 28326977	c.44C>G	2	p.(Pro15Arg)	ms	-
OCA2	NM_000275.2	chr15: 28326858	c.163del	2	p.(Ala55Leufs*47)	fs	-
OCA2	NM_000275.2	chr15: 28263599	c.751G>A	7	p.(Val251Met)	ms	rs147432138
OCA2	NM_000275.2	chr15: 28263554	c.796C>T	7	p.(Arg266Trp)	ms	rs33929465
OCA2	NM_000275.2	chr15: 28261316	c.824C>T	8	p.(Thr275Met)	ms	rs369750458

Minor Allele Frequencies				Evolutionary Conservation / Distance			In-Silico Prediction				
Allele count	MAF (AN=976)	MAF ExAC[a]	MAF GoNL	NT	AA	GD	CADD Phred	SIFT	Align GVGD[b]	Poly Phen-2[c]	Spl
4	0.004098	0.0021	0.002	M	M	S	33	D	C0	B	
1	0.001025	0.00029	-	-	-	-	-	-	-	-	+
1	0.001025	-	-	H	H	M	28	D	C0	D	
1	0.001025	0.000018	-	-	-	-	39	-	-	-	
1	0.001025	0.000009	-	H	H	M	26.9	D	C25	D	
1	0.001025	-	-	N	W	S	0.001	T	C0	B	
2	0.002049	0.00088	-	W	W	S	6.179	T	C0	B	
1	0.001025	-	-	N	W	M	11.36	T	C0	B	±
1	0.001025	0.0011	0.002	W	M	-	-	-	-	-	
1	0.001025	-	-	-	-	-	-	-	-	-	+
7	0.007172	0.0094	0.009	M	M	S	25.2	D	C0	P	
2	0.002049	0	-	N	W	S	8.073	T	C0	B	
2	0.002049	0.00065	-	W	W	M	21	D	C0	B	
5	0.005123	0.0016	0.006	W	W	L	1.542	T	C0	B	
1	0.001025	0.000018	-	W	W	S	2.977	T	C0	B	
3	0.003074	0.0063[d]	0.004	W	W	S	13.03	T	C0	B	
1	0.001025	0.000064	-	W	W	M	12.07	T	C0	B	
2	0.002049	0.00016[d]	-	H	H	S	27.5	D	C25	P	
1	0.001025	0	-	W	W	S	5.17	T	C0	B	
1	0.001025	0.00051	0.002	W	W	S	11.75	T	C0	B	
1	0.001025	0.000038	-	W	W	S	10.86	T	C0	B	
1	0.001025	-	-	W	W	M	11.18	T	C0	B	
1	0.001025	0.000019	-	-	-	-	-	-	-	-	
1	0.001025	0.00017[d]	-	N	W	S	4.551	T	C0	B	
1	0.001025	0.0018[d]	0.003	W	H	M	18.24	D	C0	P	
1	0.001025	0.000036	-	M	M	M	25.7	T	C0	P	

SUPPLEMENTARY TABLE S1 CONTINUED.

Gene	mRNA refSeq	Chr. Position (GRCh37)	cDNA change	Exon	Protein change	Variant type	dbSNP
OCA2	NM_000275.2	chr15: 28230319	c.1255C>T	13	p.(Arg419Trp)	ms	rs143218168
OCA2	NM_000275.2	chr15: 28230313	c.1261C>T	13	p.(Arg421Trp)	ms	rs372899234
OCA2	NM_000275.2	chr15: 28230247	c.1327G>A	13	p.(Val443Ile)	ms	rs121918166
OCA2	NM_000275.2	chr15: 28230238	c.1336A>G	13	p.(Met446Val)	ms	rs140566426
OCA2	NM_000275.2	chr15: 28228553	c.1441G>A	14	p.(Ala481Thr)	ms	rs74653330
OCA2	NM_000275.2	chr15: 28228529	c.1465A>G	14	p.(Asn489Asp)	ms	rs121918170
OCA2	NM_000275.2	chr15: 28211880	c.1592A>G	15	p.(Tyr531Cys)	ms	rs143699063
OCA2	NM_000275.2	chr15: 28202728	c.1784+6G>A	-	p.?	intron	rs779188429
OCA2	NM_000275.2	chr15: 28171315	c.2037G>C	19	p.(Trp679Cys)	ms	rs121918169
OCA2	NM_000275.2	chr15: 28116379	c.2165T>C	21	p.(Ile722Thr)	ms	rs1800417
PARP1	NM_001618.3	chr1: 226595491	c.120+9_120+20 del	-	p.?	intron	-
PARP1	NM_001618.3	chr1: 226578278	c.450G>T	4	p.(Gln150His)	ms	rs142376976
PARP1	NM_001618.3	chr1: 226573230	c.986A>G	7	p.(Asn329Ser)	ms	-
PARP1	NM_001618.3	chr1: 226570767	c.1129C>T	8	p.(Pro377Ser)	ms	rs2230484
PARP1	NM_001618.3	chr1: 226570748	c.1148C>A	8	p.(Ser383Tyr)	ms	rs3219062
PARP1	NM_001618.3	chr1: 226567661	c.1505C>T	10	p.(Ala502Val)	ms	rs183533639
PARP1	NM_001618.3	chr1: 226564936	c.1814C>T	13	p.(Pro605Leu)	ms	rs369900729
PARP1	NM_001618.3	chr1: 226564855	c.1895C>T	13	p.(Thr632Met)	ms	rs138228205
PARP1	NM_001618.3	chr1: 226552705	c.2656G>A	19	p.(Val886Met)	ms	rs776746526
PARP1	NM_001618.3	chr1: 226549169	c.3037C>A	23	p.(Leu1013Met)	ms	rs138906127
POLE	NM_006231.2	chr12: 133253180	c.861T>A	9	p.(Asp287Glu)	ms	rs139075637
POLE	NM_006231.2	chr12: 133253148	c.893A>G	9	p.(Tyr298Cys)	ms	-
POLE	NM_006231.2	chr12: 133250290	c.1230G>A	13	p.(Trp410*)	ns	-
POLH	NM_006502.2	chr6: 43550882	c.272+4A>G	-	p.?	intron	rs373430329
POLH	NM_006502.2	chr6: 43555032	c.296T>C	4	p.(Val99Ala)	ms	rs750026446
POLH	NM_006502.2	chr6: 43555151	c.415G>A	4	p.(Ala139Thr)	ms	rs554936509

	Minor Allele Frequencies			Evolutionary Conservation / Distance			In-Silico Prediction				
Allele count	MAF (AN=976)	MAF ExAC[a]	MAF GoNL	NT	AA	GD	CADD Phred	SIFT	Align GVGD[b]	Poly Phen-2[c]	Spl
1	0.001025	0.00011	-	N	H	M	32	D	C0	D	
1	0.001025	0.000065	-	W	M	M	28	D	C0	D	
18	0.018443	0.0051	0.008	H	H	S	34	T	C0	D	
2	0.002049	0.00025	0.001	W	M	S	1.045	T	C0	B	
1	0.001025	0.0026[d]	0.001	H	H	S	27.6	T	C0	P	
7	0.007172	0.0007	0.003	M	H	S	28.2	D	C0	D	
1	0.001025	0.00011	0.001	W	M	L	25.3	D	C0	D	
1	0.001025	0.000019	-	-	-	-	-	-	-	-	
1	0.001025	0.00015	-	H	H	L	34	D	C0	D	
1	0.001025	0.00091[d]	-	W	W	M	0.342	T	C0	B	
1	0.001025	-	-	-	-	-	-	-	-	-	
3	0.003074	0.00051	0.001	W	H	S	23.4	T	C0	D	
1	0.001025	-	-	W	W	S	7.255	T	C0	B	
3	0.003074	0.0068	0.004	M	H	M	13.58	T	C0	B	
9	0.009221	0.0027	0.002	H	M	L	24.6	D	C0	B	
3	0.003074	0.00082	-	W	M	S	10.01	T	C0	B	
1	0.001025	0.000036	-	M	M	M	22.6	D	C15	B	
2	0.002049	0.00034	-	M	M	M	26.2	T	C0	B	
1	0.001025	0.000027	-	H	H	S	32	D	C0	D	
2	0.002049	0.00095	-	W	H	S	18.79	T	C0	B	
9	0.009221	0.0017	0.004	W	H	S	25.7	D	C35	D	
1	0.001025	-	-	H	H	L	28.3	D	C65	D	
1	0.001025	-	-	-	-	-	38	-	-	-	
1	0.001025	0.00041	-	-	-	-	-	-	-	-	±
1	0.001025	0.000036	-	H	H	S	27.4	T	C0	D	
2	0.002049	-	0.001	M	M	S	23	T	C0	P	

SUPPLEMENTARY TABLE S1 CONTINUED.

Gene	mRNA refSeq	Chr. Position (GRCh37)	cDNA change	Exon	Protein change	Variant type	dbSNP
POLH	NM_006502.2	chr6: 43565568	c.626G>T	5	p.(Gly209Val)	ms	rs2307456
POLH	NM_006502.2	chr6: 43572357	c.890G>A	8	p.(Trp297*)	ns	-
POLH	NM_006502.2	chr6: 43573062	c.1074+6A>G	-	p.?	intron	-
POLH	NM_006502.2	chr6: 43581662	c.1510C>T	11	p.(Pro504Ser)	ms	-
POLH	NM_006502.2	chr6: 43581782	c.1630C>A	11	p.(Leu544Ile)	ms	-
POLH	NM_006502.2	chr6: 43581915	c.1763C>T	11	p.(Ser588Phe)	ms	-
POT1	NM_015450.2	chr7: 124491972	c.903G>T	11	p.(Gln301His)	ms	rs116916706
POT1	NM_015450.2	chr7: 124465356	c.1742A>G	20	p.(Lys581Arg)	ms	rs201023336
PTEN	NM_000314.4	chr10: 89690796	c.210-7_210-3del	-	p.?	intron	rs587780544
RAD51B	NM_133509.3	chr14: 68301816	c.218A>G	4	p.(Gln73Arg)	ms	rs774570772
RAD51B	NM_133509.3	chr14: 68331840	c.436G>A	5	p.(Ala146Thr)	ms	rs200741476
RAD51B	NM_133509.3	chr14: 68352579	c.453-7C>T	-	p.?	intron	rs201722637
RAD51B	NM_133509.3	chr14: 68352672	c.539A>G	6	p.(Tyr180Cys)	ms	rs28910275
RAD51B	NM_133509.3	chr14: 68353784	c.619G>T	7	p.(Val207Leu)	ms	rs28908168
RAD51B	NM_133509.3	chr14: 69061228	c.1063G>A	11	p.(Ala355Thr)	ms	rs61758785
RASEF	NM_152573.3	chr9: 85677626	c.157C>T	1	p.(Arg53Trp)	ms	rs766102616
RASEF	NM_152573.3	chr9: 85640842	c.432-9_432-6dup	-	p.?	intron	-
RASEF	NM_152573.3	chr9: 85622429	c.960-9A>G	-	p.?	intron	rs375961814
RASEF	NM_152573.3	chr9: 85620394	c.1049_1050del	8	p.(His350Argfs*3)	fs	rs755447494
RASEF	NM_152573.3	chr9: 85619464	c.1151G>A	9	p.(Arg384Lys)	ms	rs138418572
RASEF	NM_152573.3	chr9: 85613340	c.1745C>A	13	p.(Thr582Asn)	ms	rs143848788
RASEF	NM_152573.3	chr9: 85605345	c.2078A>G	16	p.(Asp693Gly)	ms	-
RASEF	NM_152573.3	chr9: 85597608	c.2207A>T	17	p.(Asn736Ile)	ms	rs762067279
TERF1	NM_017489.2	chr8: 73921307	c.186_188dup	1	p.(Glu62dup)	dup	rs149294115
TERF1	NM_017489.2	chr8: 73921333	c.212_217dup	1	p.(Glu71_Ala72dup)	dup	rs755588334
TERF1	NM_017489.2	chr8: 73939257	c.857A>G	6	p.(Glu286Gly)	ms	-

Minor Allele Frequencies				Evolutionary Conservation / Distance			In-Silico Prediction				
Allele count	MAF (AN=976)	MAF ExAC[a]	MAF GoNL	NT	AA	GD	CADD Phred	SIFT	Align GVGD[b]	Poly Phen-2[c]	Spl
2	0.002049	0.0032[d]	0.003	M	H	M	28.1	D	C15	D	
1	0.001025	-	-	-	-	-	40	-	-	-	
1	0.001025	-	-	-	-	-	-	-	-	-	
1	0.001025	0.000009	-	W	M	M	0.001	T	C0	B	
1	0.001025	-	-	W	M	S	9.276	T	C0	B	
1	0.001025	-	-	W	M	L	17.46	T	C0	B	
1	0.001025	0.0042	0.003	N	H	S	15.65	T	C0	P	
3	0.003074	0.000045	-	W	W	S	10.18	T	C0	B	
1	0.001025	0.0003	-	-	-	-	-	-	-	-	±
1	0.001025	0.000018	-	W	W	S	4.787	T	C0	B	
1	0.001025	0.00027	0.002	H	H	S	25.3	T	C0	P	
1	0.001025	0.0004	0.001	-	-	-	-	-	-	-	
8	0.008197	0.0039[d]	-	W	M	L	18.52	T	C0	B	
4	0.004098	0.0029	0.004	W	H	S	15.32	D	C0	B	
9	0.009221	0.0041	0.004	W	W	S	7.995	T	C0	B	
1	0.001025	0.000049	-	W	M	M	28.4	D	C0	D	
1	0.001025	-	-	-	-	-	-	-	-	-	
3	0.003074	0.000027	-	-	-	-	-	-	-	-	±
1	0.001025	0.000063	-	-	-	-	-	-	-	-	±
1	0.001025	0.0005	0.001	H	M	S	15.71	T	C0	B	
1	0.001025	0.00017	-	M	H	S	22.6	D	C0	B	
1	0.001025	0.000018	-	H	H	M	32	D	C0	P	±
1	0.001025	0.000027	-	W	M	L	27.4	D	C0	P	
2	0.002049	0.0005[d]	-	W	W	-	-	-	-	-	
1	0.001025	0.00014	-	M	H	-	-	-	-	-	
1	0.001025	-	-	W	M	M	28.4	D	C0	B	

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SUPPLEMENTARY TABLE S1 CONTINUED.

Gene	mRNA refSeq	Chr. Position (GRCh37)	cDNA change	Exon	Protein change	Variant type	dbSNP
TERF1	NM_017489.2	chr8: 73958245	c.1193A>G	10	p.(Tyr398Cys)	ms	rs760966818
TERF2	NM_005652.4	chr16: 69419852	c.23C>G	1	p.(Ala8Gly)	ms	-
TERF2	NM_005652.4	chr16: 69419819	c.56A>G	1	p.(Asp19Gly)	ms	rs773981277
TERF2	NM_005652.4	chr16: 69419801	c.74C>T	1	p.(Pro25Leu)	ms	rs749171225
TERF2	NM_005652.4	chr16: 69406163	c.693+9G>A	-	p.?	intron	rs191776266
TERF2	NM_005652.4	chr16: 69404432	c.794G>A	5	p.(Arg265His)	ms	rs763347805
TERF2	NM_005652.4	chr16: 69401088	c.962C>T	7	p.(Pro321Leu)	ms	-
TERF2	NM_005652.4	chr16: 69395387	c.1346C>T	8	p.(Pro449Leu)	ms	-
TERF2	NM_005652.4	chr16: 69390938	c.1492G>A	10	p.(Glu498Lys)	ms	rs150757154
TERF2IP	NM_018975.3	chr16: 75682178	c.398G>A	1	p.(Arg133Gln)	ms	-
TINF2	NM_001099274.1	chr14: 24711501	c.38G>T	1	p.(Arg13Leu)	ms	-
TINF2	NM_001099274.1	chr14: 24711477	c.62A>G	1	p.(Gln21Arg)	ms	rs367835995
TINF2	NM_001099274.1	chr14: 24711465	c.74G>C	1	p.(Gly25Ala)	ms	rs202093758
TINF2	NM_001099274.1	chr14: 24709976	c.710G>A	6	p.(Gly237Asp)	ms	rs17102313
TINF2	NM_001099274.1	chr14: 24709965	c.721C>T	6	p.(Pro241Ser)	ms	rs17102311
TINF2	NM_001099274.1	chr14: 24709952	c.734C>A	6	p.(Ser245Tyr)	ms	rs142777869

AN = allele number, NT = nucleotide (PhyloP score), AA = amino acid, GD = Grantham distance, Spl = splicing effect
Variant type: ms = missense, fs = frameshift, ns = nonsense, syn = synonymous, del = in-frame deletion, dup = in-frame duplication
Evolutionary Conservation/Distance: N = not, W = weak, M = moderate, H = high, S = small, L = large
In-Silico Prediction Tools: T = tolerated, D = deleterious (SIFT), B = benign, P = possibly damaging, D = probably damaging (PolyPhen-2), + = likely affects splicing, ± = possibly affects splicing
[a] in European (Non-Finnish) population
[b] Possible classifications in Align GVGD are C0, C15, C25, C35, C45, C55 and C65. Variants in class C0 have the least probability of being pathogenic, variants in class C65 have the highest probability of being pathogenic.
See also http://agvgd.hci.utah.edu/classifiers.php
[b] HumVar trained PolyPhen-2 model used for prediction
[d] Common variant (MAF>1%) in one or more non-European populations

Minor Allele Frequencies				Evolutionary Conservation / Distance			In-Silico Prediction				
Allele count	**MAF (AN=976)**	**MAF ExAC[a]**	**MAF GoNL**	**NT**	**AA**	**GD**	**CADD Phred**	**SIFT**	**Align GVGD[b]**	**Poly Phen-2[c]**	**Spl**
1	0.001025	0.000009	-	W	H	L	24.7	D	C25	D	
1	0.001025	-	-	W	W	S	12.75	D	C0	-	
1	0.001025	0.00012	-	W	W	M	16.35	D	C0	P	
2	0.002049	0.00043	-	M	W	M	19.54	D	C0	B	
4	0.004098	0.0037	0.002	-	-	-	-	-	-	-	
1	0.001025	0.000027	-	M	M	S	28.3	D	C0	P	
1	0.001025	-	-	W	M	M	21.2	D	C0	B	
1	0.001025	-	-	W	W	M	20.5	T	C0	B	
4	0.004098	0.0022	0.003	M	H	S	34	D	C55	P	
1	0.001025	0.00022	-	W	H	S	23.4	D	C35	B	
1	0.001025	-	-	M	H	M	27	D	C0	D	
1	0.001025	0.00023	-	N	M	S	0.001	T	C0	B	
1	0.001025	0.0024	0.001	W	M	S	12.31	T	C0	B	
1	0.001025	0.00015[d]	-	W	H	M	7.836	T	C0	B	
1	0.001025	0.000063[d]	-	W	H	M	23.4	T	C0	D	
3	0.003074	0.00073	-	N	W	L	22.7	D	C15	B	

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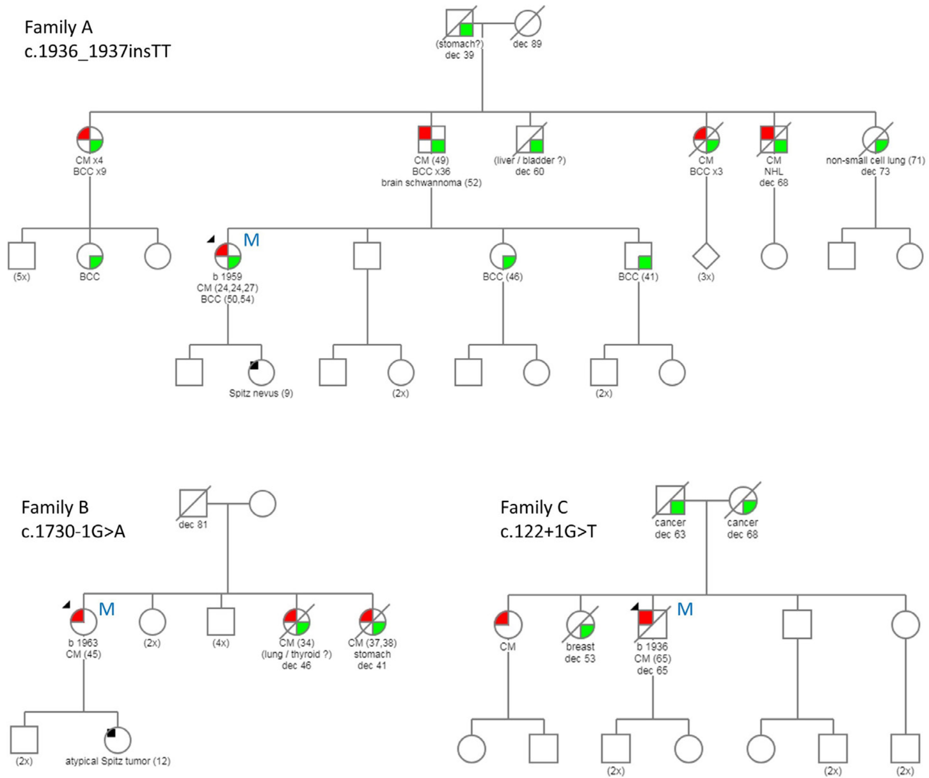


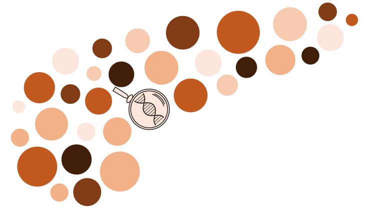
SUPPLEMENTARY FIGURE S1. Families with a (likely) pathogenic variant in *BAP1*.

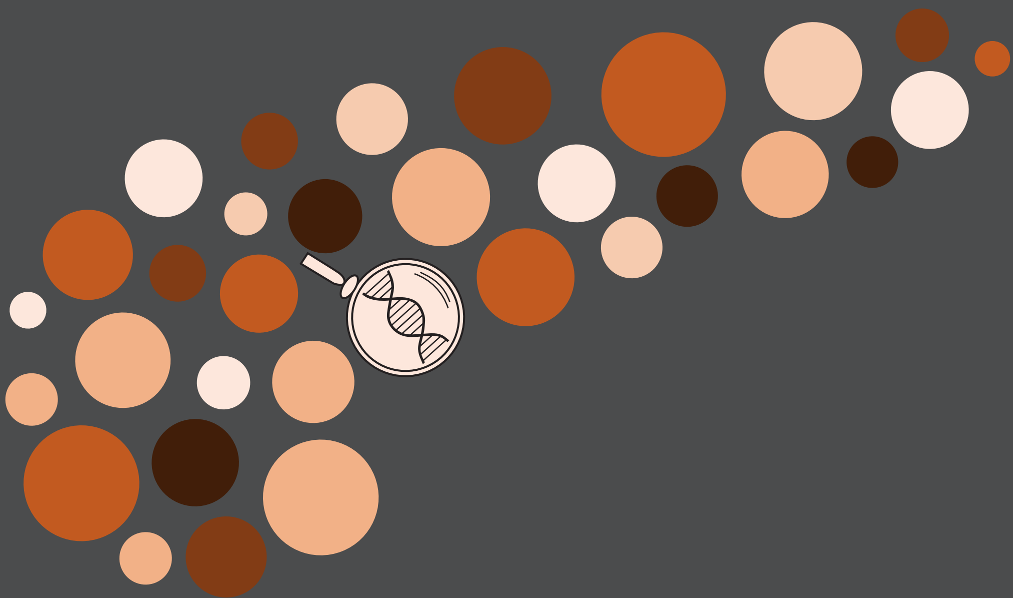
Symbols quarter-filled in upper left corner (red) represent melanoma, symbols quarter-filled in lower right corner (green) represent other cancers, symbols filled with a square in upper left corner (black) represent benign skin lesions. Age of onset in years is shown in parentheses. Unconfirmed cancer diagnoses are also shown in parentheses with "?". Probands are indicated with an arrow point and M (=mutation).

CM = cutaneous melanoma, BCC = basal cell carcinoma, NHL = Non-Hodgkin lymphoma, b = year of birth, dec = age deceased

Note: the proband in family C also carried a (likely) pathogenic variant in BRIP: c.894C>A, p.(C298)*

PART III





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General Discussion

BRIEF SYNOPSIS

In this thesis, we performed several genotype and phenotype studies in hereditary and familial melanoma patient cohorts. In the first part, our studies focused on patients with the p16-*Leiden* founder mutation in the *CDKN2A* gene. This founder mutation is the major cause of the familial melanoma-pancreatic cancer syndrome in the Dutch population. Since many p16-*Leiden* mutation carriers live in the vicinity of Leiden and are under frequent dermatologic and/or pancreatic cancer (PC) surveillance at Leiden University Medical Center (LUMC), the relatively large and homogeneous p16-*Leiden* cohort of the LUMC is unique in its kind. We studied the cancer phenotype (**chapter 2**) and modifiers of cancer risk (**chapters 2 and 3**) in these p16-*Leiden* mutation carriers. We also evaluated the p16-*Leiden* PC surveillance program by studying the role of precursor lesions in the development and early detection of PC (**chapter 4**), by reflecting on the surgical management of early-stage screen-detected PC (**chapter 5**) and by studying potential biomarkers for the early detection of PC (**chapter 6**). The LUMC has also been the primary sequencing facility for *CDKN2A* in the Netherlands since 1998 and therefore, a large collection of DNA samples and clinical information of melanoma families from across the Netherlands has been acquired in the last two decades. In the second part of this thesis, our studies focused on this familial melanoma cohort, of which ~85% do not harbour a germline *CDKN2A* mutation. We studied which clinical features are associated with the presence of a *CDKN2A* mutation in a melanoma family and created a scoring system to predict *CDKN2A* mutation probability (**chapter 7**). Furthermore, we genetically characterized melanoma families without a *CDKN2A* mutation for variants in other (candidate) melanoma predisposition genes (**chapter 8**). In this final chapter, we discuss the main findings of these studies in the context of the most recent literature.

THE EXTENDED CANCER PHENOTYPE OF *CDKN2A*

The *CDKN2A* gene is primarily a melanoma predisposition gene, and up to 40% of melanoma-prone families harbour a germline mutation in *CDKN2A*.[1,2] In many *CDKN2A*-mutated melanoma families, a clear excess of PC has also been reported,[3,4] and the lifetime risk for PC is estimated to be approximately 15-20% for carriers of the p16-*Leiden* founder mutation.[5,6] Because of the frequent co-occurrence of these two cancers in *CDKN2A*-mutated families, the condition is sometimes referred to as familial melanoma-pancreatic cancer syndrome (MIM #606719[^]). There is now increasing evidence that *CDKN2A* mutation carriers are also at risk for cancers at sites other than skin and pancreas, in particular cancer of the upper respiratory tract, i.e. head and neck region (larynx, pharynx and oral region), and possibly also cancer of the lower respiratory tract (lung). Head and neck cancers and lung cancer were initially reported in several case studies of *CDKN2A*-mutated families with various mutations.[7-9] In a large study by de Snoo *et al* in 2008, a cohort of 221 p16-*Leiden* mutation carriers and 668 first-degree relatives was retrospectively evaluated for the occurrence of any cancer and significant risks were found for cancers of the lip, mouth and pharynx (RR 10.8) and cancers of the respiratory system including laryngeal cancer (RR 5.7).[6] In **chapter 2**, we aimed to confirm the findings of the de Snoo *et al* study by prospectively evaluating a cohort of 150 p16-*Leiden* mutation carriers that were participating in the PC surveillance program and we again found a particular excess of cancers of the lip, mouth and pharynx (n=3, RR 18.8) and cancers of the respiratory system (n=4, RR 4.6). Since two of the four respiratory system cancers in our study were laryngeal cancers, a total of five cancers were located in the head and neck cancer region, making it the third most frequent cancer site in these p16-*Leiden* mutation carriers (following skin and pancreas). This number is striking considering the relatively low incidence of head and neck cancers in the general population (approximately 3000 new cases each year in the Netherlands; age standardized rate 9 per 100.000).[10] A study with very similar results was concomitantly published by a Swedish group that assessed cancer risks in a cohort of 120 individuals with a specific Swedish *CDKN2A* founder mutation (p.Arg112dup).[11] In this study, significantly increased risks other than melanoma and PC were again observed for cancers of the upper digestive tract (including oral region and pharynx; RR 17.1) and respiratory tract (including larynx; RR 15.6), and the majority of these cancers were indeed located in the head and neck region. In a subsequent collaboration study with the Swedish group (**chapter 7**), we further investigated the occurrence of cancers in the head and neck region[*] in *CDKN2A* mutation carriers by incorporating this

^ *Mendelian Inheritance in Man*; Catalog of Human Genes and Genetic Disorders (http://www.omim.org)

* In chapter 7, the term upper airway cancer (UAC) is used as a synonym for cancers of the head and neck region (larynx, pharynx, oral region).

clinical feature and four additional features in a *CDKN2A* mutation probability scoring system (*CM-Score*). We showed that the presence of these cancers in Dutch melanoma families was significantly associated with the presence of a germline *CDKN2A* mutation (OR 6.0). Since the scoring system could accurately predict *CDKN2A* mutation status in both the Dutch training cohort and the Swedish validation cohort, we demonstrated that cancers in the head and neck region are indeed an important component of the *CDKN2A* cancer phenotype in different populations with different *CDKN2A* mutations. Therefore, it might be more appropriate to use the term '*CDKN2A*-associated cancer predisposition syndrome' when referring to the familial melanoma-pancreatic cancer syndrome, because this term better reflects the broader tumour spectrum to which *CDKN2A* mutation carriers are predisposed to. It should however be noted that ~80% of both Dutch and Swedish *CDKN2A*-mutated families in chapter 7 had a *CDKN2A* founder mutation located in exon 2 (p16-*Leiden* and p.Arg112dup, respectively), which might indicate a possible genotype-phenotype correlation between specific *CDKN2A* (exon 2) mutations and head and neck cancers. Yet, case studies reporting a high incidence of head and neck cancers in *CDKN2A*-mutated families have not been confined to families with mutations in exon 2; Cabanillas *et al* reported a melanoma family with multiple cases of head and neck cancer and a truncating *CDKN2A* mutation in exon 1α.[8] Replication studies in large cohorts with different *CDKN2A* mutations are needed to further explore possible genotype-phenotype correlations. Only one such study has recently been published, in which 29 American *CDKN2A*-mutated melanoma families were evaluated over a relatively long period of four decades. No increased risk for cancers other than melanoma or PC were found in this study. Unfortunately, the specific *CDKN2A* mutations found in these families were not reported.[12]

MODIFIERS OF CANCER RISK IN *CDKN2A* MUTATION CARRIERS

NON-GENETIC MODIFIERS

Several phenotypic characteristics known to be associated with increased melanoma risk in the general population, such as the number of dysplastic nevi, poor tanning ability and sunburns in childhood, have also been shown to modify melanoma risk in carriers of a germline *CDKN2A* mutation.[13] Therefore, early patient education on sun protection is essential for carriers and their first-degree relatives. Interestingly, the most important non-melanoma cancers associated with *CDKN2A* mutations, i.e. PC, head and neck cancer and lung cancer, are strongly related to tobacco use in the general population.[14,15] Previously, it was already shown that tobacco use is an independent risk factor for

developing PC in the setting of familial PC (FPC).[16] We investigated in **chapter 2** if tobacco use is also associated with a higher risk of PC and other tobacco-related cancers in p16-*Leiden* mutation carriers. In total, 27% of current smokers in the cohort developed a tobacco-related cancer versus 7% of the former- and never-smokers. Current smokers therefore had a fourfold increased risk of developing these types of cancers (P=0.002). These findings were confirmed in the Swedish *CDKN2A* cancer risk study, where ever-smokers had an odds ratio of 9.3 for developing tobacco-related cancers compared to never-smokers.[11] Thus, tobacco use seems a significant modifier of cancer risk in *CDKN2A* mutation carriers and carriers should be strongly discouraged to smoke.

GENETIC MODIFIERS

A well-established modifier gene of melanoma risk in germline *CDKN2A* mutation carriers is *MC1R*, a gene involved in skin pigmentation.[17,18] In contrast, little is known about genetic modifiers that might influence the risk of other cancers such as PC in *CDKN2A* mutation carriers. Since there is notable interfamilial variability in the occurrence of PC among p16-*Leiden* families that might not be fully explained by differences in tobacco use or other non-genetic risk factors among mutation carriers, we investigated in **chapter 3** whether common Single Nucleotide Polymorphisms (SNPs) that are associated with PC in the general population might influence PC risk in p16-*Leiden* families. We genotyped seven PC-associated SNPs in a cohort of 185 p16-*Leiden* mutation carriers of whom 50 were diagnosed with PC, but found no significant association with PC for any of these SNPs. The study might have been underpowered for a detectable effect but it might also be possible that other PC-associated SNPs that were not genotyped in this study modify PC risk in p16-*Leiden* families. No additional studies have yet been performed that investigate SNPs as potential genetic modifiers of PC in *CDKN2A*-mutated families. However, Yang *et al* investigated if patients with PC and a germline *CDKN2A* mutation might also have one or more rare variants in 24 other (putative) PC-related genes.[19] In this study, nominally significant associations between PC and rare variants in the mismatch repair (*MMR*) genes were found, and some patients had more than one rare variant in PC-related genes. However, no loss-of-function variants were detected in these patients and only a subset of variants was classified as potentially deleterious. Therefore, the relevance of these findings remains uncertain and additional studies are needed.

PANCREATIC CANCER SURVEILLANCE AND -MANAGEMENT OF P16-*LEIDEN* MUTATION CARRIERS

P16-*Leiden* mutation carriers are offered yearly PC surveillance at the LUMC from the age of 45. Promising results of this program and several other PC surveillance programs worldwide have been published in the last two decades, however, the diagnostic yield of these programs varies greatly.[20-22] One explanation might be that there are differences in the definition of diagnostic yield among these programs. For instance, some programs included histologically confirmed lower-grade precursor lesions (PanIN1-2) in their diagnostic yield and it can be questioned if the resection of these lesions is relevant in these patients. Moreover, cystic duct lesions detected with pancreatic imaging might very well be benign or lower-grade lesions if there is no histological confirmation, and including such lesions in the diagnostic yield of a surveillance program can cause significant bias. There are also differences in which high-risk individuals are included in the surveillance programs. Most programs largely focus on individuals from FPC families, but some also include a significant number of individuals with a germline mutation in a known cancer predisposition gene such as *CDKN2A* or *BRCA1/2*. Since the PC surveillance program of the LUMC is almost entirely focused on *CDKN2A* mutation carriers, the majority of which have the p16-*Leiden* founder mutation, it is distinctive from other PC surveillance programs worldwide. To further explore possible phenotypic differences between high-risk groups that might influence diagnostic yield, we studied in **chapter 4** the frequency and behaviour of precursor lesions and PC in two different surveillance cohorts, that is, the LUMC p16-*Leiden* cohort (n=116) and a German FPC cohort (n=125). We reported substantially more diagnoses of PC in the p16-*Leiden* cohort (7% versus 0.8% in the FPC cohort), but cystic duct lesions were much more common in the FPC cohort (42% versus 16% in the p16-*Leiden* cohort). Histological examination of cystic lesions in the FPC cohort often revealed IPMN or PanIN2-3 lesions, whereas in the p16-*Leiden* cohort few such lesions were seen. Yet, cystic lesions in the p16-*Leiden* cohort frequently showed growth or malignant transformation compared to cystic lesions in the FPC cohort. Recently, our findings were confirmed in a similar study by Konings *et al* that compared the prevalence of cystic lesions and their natural behaviour between 88 FPC individuals and 98 carriers of a germline mutation that predisposes to PC (of which 64 were *CDKN2A* mutation carriers (65%)).[23] In this study, 5% of mutation carriers had a pancreatic cyst >10 mm compared to 16% in FPC individuals, but cysts in mutation carriers were more likely to grow or develop radiological features that suggest high-grade dysplasia (16% vs 2% in FPC individuals). Interestingly, the only two patients that developed PC in this study were a *CDKN2A* mutation carrier (exact mutation not reported) and a patient with the rare but highly penetrant Peutz-Jeghers syndrome. These studies show that *CDKN2A*/p16-*Leiden* mutation carriers have a much higher risk

for PC than FPC individuals. Furthermore, these studies suggest that precursor lesions in these carriers might have a higher malignant potential compared to precursor lesions in FPC individuals. However, the role of precursor lesions in the development of PC in p16-*Leiden* mutation carriers has been questioned in a recent study by Ibrahim *et al* that found a similar malignancy rate in p16-*Leiden* mutation carriers with a cystic lesion compared to those without cystic lesions.[24] Still, 50% of larger cysts (>10 mm) progressed to PC in this study. The authors also reported a particular high growth rate of PC of approximately 15 mm/year and thus confirmed previous observations of aggressively growing tumours in p16-*Leiden* mutation carriers.[20] Taken together, p16-*Leiden* mutation carriers have a particularly high risk for aggressively growing PCs, and cystic lesions, especially when >10 mm, are often instable at follow-up and might precede the development of PC in some carriers. Therefore, a more intensive PC surveillance program, for instance semi-annual surveillance with alternating MRI/MRCP and EUS, might be considered for this group if future studies show this program to be cost-effective.

The ultimate goal of PC surveillance is to improve survival of high-risk individuals, through timely resection of early-stage PC or its high-grade precursor lesions. In 2016, a large multicentre study by Vasen *et al* was the first to show a beneficial effect of regular surveillance on survival.[25] In the subset of *CDKN2A*-p16-*Leiden* mutation carriers (n=178), the resection rate of PC was high (75% compared to 15% in sporadic PC) and patients with screen-detected PC had a substantial improved 5-year survival rate (24% compared to only 4-7% in symptomatic sporadic PC). The survival benefit in the FPC subset (n=233) was less clear, since only one PC and a few high-grade precursor lesions were detected in this subset. However, Canto *et al* recently demonstrated a positive effect of surveillance on survival in FPC individuals as well.[26] In this large (n=354) and long-term follow-up (16 years) surveillance study, which mainly included FPC individuals, nine of ten PCs detected during surveillance were resectable and the 3-year survival rate of these patients was greatly improved to 85%. An additional ten individuals had a resection of one or more high-grade precursor lesions and none of these patients died during follow-up (median 7.9 years). In general, the survival rate after surgical resection of PC is largely determined by the chance of local disease recurrence or distant metastases. In the Vasen *et al* surveillance study, the resection margin was free of cancer in 78% of p16-*Leiden* mutation carriers that underwent surgery, and 56% had cancer-free lymph nodes.[25] However, in patients with a hereditary predisposition for PC such as p16-*Leiden*, the occurrence of other primary cancers (melanoma in particular) might also influence survival. For instance, one p16-*Leiden* mutation carrier with PC reported by the Vasen *et al* study died 10 months after diagnosis from melanoma metastases. Also, second primary melanomas are frequently seen in p16-*Leiden* mutation carriers, and an increased risk for developing second primary cancers in

the same tissue is characteristic for many hereditary forms of cancer. Therefore, it is very well conceivable that p16-*Leiden* mutation carriers who underwent a partial resection for PC are still at risk for a second primary PC in the remnant pancreas, and developing such a second primary PC will also influence survival. In **chapter 5**, we report the first p16-*Leiden* mutation carrier with a second primary PC after a partial resection. This patient had a small (5 mm) early-stage PC that was detected during surveillance and developed a second primary PC 4.5 years later that was also detected by continuing surveillance. Subsequently, several additional p16-*Leiden* mutation carriers with a second primary PC (synchronous and metachronous) have been reported by our group,[27,28] and more patients will probably be diagnosed in the future because of improved survival of p16-*Leiden* mutation carriers with a screen-detected PC, as reported by Vasen *et al*.[25] The development of a second primary PC in these patients can only be completely prevented when a total pancreatectomy of the first cancer is performed. Prevention of a second primary PC is especially relevant for patients that are diagnosed with an early-stage and prognostically favourable PC, and we therefore recommend to consider a total pancreatectomy as one of the surgical options in these patients. Although a total pancreatectomy is a major operation, the post-surgical outcomes and quality of life are, present-day, comparable to those who underwent a partial pancreatectomy.[29,30] The disadvantages of a total pancreatectomy, such as the development of (brittle) diabetes, should however be carefully discussed with these patients and shared-decision making is essential. Future studies that assess the actual risk for developing a second primary PC are needed and results from these studies might aid in the difficult decision of what type of surgery should be performed in p16-*Leiden* mutation carriers with an early-stage PC.

To further improve PC surveillance programs, some important limitations of the current surveillance programs need to be considered. The most challenging limitations are the suboptimal diagnostic performance of imaging modalities that are currently being used (MRI/MRCP and EUS) and the fact that extensive surgery (partial or total pancreatectomy) is the only way to remove a suspicious lesion. Pancreatic lesions might not always be clearly visible or properly interpreted by the radiologist, and patients that had surgical resection for a non-relevant lesion on histologic examination (false-positives) have repeatedly been reported.[31,32] The Vasen *et al* surveillance study showed that at least 5 out of 13 FPC individuals that had a surgical resection of a suspicious lesion had a non-relevant (precursor) lesion on histological examination.[25] In the p16-*Leiden* subset, only two patients without PC had surgery for suspicious lesions. One of these patients had multifocal PanIN1-2 and branch duct (BD-)IPMNs combined with severe multifocal fibrosis (we also reported this patient in chapter 4), but the second patient only had one low-grade IPMN. Since pancreatic resection is associated with significant morbidity and mortality,

it is very important to minimize the amount of false-positive findings and unnecessary resections in otherwise healthy individuals participating in a PC surveillance program.[32] When these individuals do develop PC, a successful surveillance program should be able to consistently detect it in the earliest possible stage (high sensitivity; few false-negatives). One way to improve the diagnostic performance of surveillance programs is to add (serum) biomarkers to the program that can differentiate between PC, relevant precursor lesions and non-relevant or no lesions. Among several types of biomarkers that are currently being investigated in this context, microRNA panels and global protein profiling (proteomics) are relatively new and results are encouraging.[33-35] In the LUMC, a specific proteomic biomarker signature for PC was previously identified in cohorts of patients with sporadic PC.[36-38] In **chapter 6**, we investigated this biomarker signature in a cohort of 66 p16-*Leiden* mutation carriers, of which 5 had developed PC. We could accurately distinguish patients with PC from patients without PC using this biomarker signature. Many included p16-*Leiden* mutation carriers had a (recent or non-recent) medical history of one or more melanomas (62%), but this did not influence the biomarker signature. The patient with histologically confirmed multifocal PanIN1-2 and BD-IPMNs previously reported in chapter 4 could also be distinguished from controls. The results of this preliminary study are very promising, but additional studies that include more patients with PC and with (relevant and non-relevant) precursor lesions will be needed before this biomarker test can be implemented in the p16-*Leiden* PC surveillance program.

The effectiveness of a cancer surveillance program does not only depend on its technical performance, but also on the motivation of participants and their adherence to the surveillance protocol. Adherence might be jeopardized when participants experience significant psychological distress, which is imaginable when there is a continuing risk for a highly lethal form of cancer such as PC that might already have occurred in (close) family members. Factors that determine or influence psychological distress should therefore be identified and recognized in an early stage.[39] Several recent studies that have investigated the psychological feasibility of PC surveillance in high-risk groups have shown that most participants have a positive attitude towards the program and find that the advantages of PC surveillance outweigh the limitations.[40-42] Approximately one third of participants worry significantly about the possibility of getting cancer, but this does not affect their mood or interfere with their daily activities.[40,41] Cancer worries decrease each following year and getting a positive surveillance result does not influence the level of cancer worries.[41,42] The only reported predictor of cancer worries is having a first degree relative with PC under the age of 50 years.[42] Importantly, anxiety and depression levels of participants are comparable with the general population and stable during follow-up.[40,41] Based on these studies, surveillance for PC seems well feasible from a psychological point of view.

GENETIC TESTING AND COUNSELLING OF MELANOMA FAMILIES: *CDKN2A* AND BEYOND

Identifying a causative germline mutation such as p16-*Leiden* in a melanoma family is relevant, since carriers can be enrolled in targeted cancer surveillance programs and their family members have the possibility to undergo presymptomatic genetic testing. Moreover, gene-specific lifestyle advices and patient education on early cancer symptoms can be given to confirmed carriers. For instance, *CDKN2A* mutation carriers are strongly advised to refrain from smoking and to seek medical advice in an early stage when there are possible signs of head and neck cancer (hoarseness, dysphagia, ulcers in mouth or throat), as we discussed in chapter 2. Knowledge about individual mutation status might also influence reproductive choices, since most cancer predisposition syndromes (*CDKN2A* included) have an autosomal dominant inheritance pattern with a 50% risk for (future) offspring to inherit the predisposition. Pre-implementation genetic diagnosis (PGD) is an assisted reproductive technique that can prevent a future child from inheriting this predisposition, but is, by definition, only available for patients with a known underlying germline mutation and has indeed been performed for *CDKN2A* mutation carriers in the Netherlands.[43] Although some patients might experience significant psychological distress from *CDKN2A* testing, several studies have shown that, in general, *CDKN2A* testing in melanoma families does not result in increased psychological distress or cancer worries among carriers and may even enhance compliance with lifestyle advices such as sun protection behaviour.[44-48]

Genetic testing for hereditary melanoma can thus be beneficial for patients and their family members and should therefore be routinely offered to melanoma families. However, the chance of identifying a causative germline mutation strongly depends on the cancer burden in a family and therefore, selection criteria for performing germline *CDKN2A* analysis were proposed in 2009.[49] These criteria are based on the total number of melanomas and PCs in a family but do not include age at melanoma diagnosis or the presence of other cancers in a family such as head and neck cancers. Recently, a French melanoma research group suggested to add age at melanoma diagnosis (<40 years) to the 2009 criteria in order to improve the detection rate of *CDKN2A* mutations.[50] In **chapter 7**, we included all the above mentioned features in a multivariate logistic regression model and found significant associations with the presence of a germline *CDKN2A* mutation for every feature in a cohort of 1227 melanoma families. For further practical purposes we developed *CM-Score*, a non-computerized scoring system that can accurately predict *CDKN2A* mutation status based on the five clinical features from the logistic regression model. Clinical geneticists, but also dermatologists and oncologists, can use this tool in

daily clinical practice to address questions on heritability of melanoma patients before genetic testing is performed. In our model, median age at melanoma diagnosis <50 years and the presence of head and neck cancer[†] in a family were both strong predictors for a germline *CDKN2A* mutation (OR 8.5 and 6.0, respectively). We therefore propose to add these features to the current Dutch referral criteria for germline *CDKN2A* diagnostics as shown in *table 1*. By adding these features, the overall mutation detection rate will likely improve and genetic testing of families with a very low probability for a *CDKN2A* mutation (<5%) will be more avoided. Of note, these criteria for *CDKN2A* diagnostics and our *CM-Score* system are only applicable to melanoma index patients and families and are not designed for families with familial pancreatic cancer (FPC). Yet, germline *CDKN2A* mutations are also found in FPC families without any occurrence of melanoma, and genetic testing for *CDKN2A* mutations is therefore recommended to these families as well.[51,52]

TABLE 1. Proposed referral criteria for germline *CDKN2A* diagnostics

Familial melanoma (diagnostic criteria)	▪ family with three relatives with melanoma, of which two are first-degree relatives (all first- and second-degree relatives)
	▪ family with two first-degree relatives with melanoma, of which one has multiple primary melanomas
Other families	▪ family with two first-degree relatives with melanoma *with a mean age at diagnosis <50 years*
	▪ family with two first- or second-degree relatives with melanoma and one first- or second-degree relative with pancreatic cancer *or head and neck cancer (larynx, pharynx, oral region)*
	▪ person with three or more primary melanomas
	▪ person with a juvenile melanoma (<18 years)
	▪ person with both melanoma and pancreatic cancer *or head and neck cancer (larynx, pharynx, oral region)*

The parts in *italic* are the proposed additions to the current referral criteria.

Although the *CDKN2A* gene is the most important melanoma predisposition gene that should be part of any genetic test for hereditary melanoma, several other high- and medium-penetrance melanoma predisposition genes are currently known and could potentially be tested in addition to *CDKN2A* (see *table 2* in chapter 1).[1,2] Pathogenic germline mutations in these genes are however much more rare and the yield when tested separately is very low (approximately 0-1%; *MITF* 0-3%).[53-57] But when these genes are incorporated in

† In chapter 7, the term upper airway cancer (UAC) is used as a synonym for cancers of the head and neck region (larynx, pharynx, oral region).

a multi-gene panel test for hereditary melanoma, the diagnostic yield of genetic testing can increase significantly. We performed such a multi-gene panel test in **chapter 8**, and report an additional 4% diagnostic yield in established melanoma predisposition genes in a cohort of 451 non-*CDKN2A/CDK4* melanoma families. The most important genes in our panel were *MITF* (p.E318K variant) and *BAP1*, in which we found pathogenic mutations in 15 (3.4%) and 3 (0.7%) families, respectively. Conversely, in the genes involved in telomere integrity, we only detected two variants of uncertain significance (VUS) in this large cohort, suggesting a minor role for these genes in the Dutch population. The additional yield of 4%, a number comparable to that reported by similar studies in non-*BRCA1/2* breast cancer families,[58,59] sufficiently justifies multi-gene panel testing in familial melanoma, but the increased chance of finding a VUS in one of the genes should be carefully discussed with the patient before such a test is requested.[60] When a patient is hesitant about multi-gene panel testing, or when there is a particularly high probability for a germline *CDKN2A* mutation based on the presence of PCs and/or head and neck cancers in a family (and consequently a high *CM-Score*, as discussed in chapter 7), then targeted *CDKN2A* diagnostics might be more appropriate as an initial test. The referral criteria for *CDKN2A* diagnostics proposed in *table 1* can also be applicable when a multi-gene panel test is being considered in a melanoma patient or family, because most patients with a germline mutation in one of these genes are expected to have a (familial) melanoma phenotype. One clear exception is *BAP1*, which we also recommend to test if there are occurrences of uveal melanoma, malignant mesothelioma, renal cell carcinoma or multiple basal cell carcinomas in a melanoma patient or his/her family members.[61,62] In our multi-gene panel test, we additionally included several candidate melanoma predisposition genes, of which the most interesting variants were found in the genes *OCA2*, *BRIP1* and *POLE*. Because of the exponential increase of WES/WGS technologies and possibilities in recent years, new candidate genes are continually being discovered by research groups worldwide.[63-66] These candidate genes have the potential to be added to a diagnostic gene panel test for hereditary melanoma in the future, which will likely result in a further increase in diagnostic yield of the panel. Moreover, adding a melanoma-specific polygenic risk score (PRS) to the panel will enable us to even better differentiate between patients with a higher and lower genetic risk for melanoma. This individual risk stratification is an integral part of personalized medicine and will further enhance the selection of patients that should or should not be offered dermatologic surveillance. The development of a PRS model based on common susceptibility SNPs derived from large Genome Wide Association Studies (GWAS) is part of a current research project of our group and recently, promising results of melanoma PRS models have been published by others.[67-69] Altogether, there is great potential for the individual patient to undergo multi-gene panel testing and future research will undoubtedly give more insight in the genetic background of melanoma families with a yet unknown hereditary predisposition.

SUMMARY AND CONCLUDING REMARKS

The studies in this thesis were aimed at I) better understanding the clinical phenotype of *CDKN2A*-p16-*Leiden* mutation carriers and improving the PC surveillance program for these carriers, and II) improving genetic testing for hereditary melanoma. In **part I**, we have shown that *CDKN2A*-p16-*Leiden* mutation carriers have an increased risk for several types of cancer including head and neck cancers, and therefore we have introduced the term '*CDKN2A*-associated cancer predisposition syndrome'. We have demonstrated that smoking is an important modifier of cancer risk in p16-*Leiden* mutation carriers and we therefore argued that smoking should be actively discouraged. We have also shown that, when compared to individuals from FPC families, p16-*Leiden* mutation carriers have a higher risk for (aggressively growing) PC and a lower frequency of precursor lesions of PC, but precursor lesions in these carriers might have a particular high malignant potential. Future studies should assess the feasibility of a more intensive PC surveillance program for this group. Since it is very likely that p16-*Leiden* mutation carriers that have been curatively treated for PC have a substantial risk for developing a second primary PC in the longer term, we have recommended to consider a total pancreatectomy when an early-stage and prognostically favourable PC is diagnosed in these carriers. Furthermore, we have demonstrated that a specific proteomic biomarker test for the early detection of PC is a very promising candidate for implementation in the PC surveillance program. In **part II**, we created a scoring system (*CM-Score*) to predict *CDKN2A* mutation probability and, based on several strong predictive features in this model, we have proposed to update the current Dutch referral criteria for *CDKN2A* diagnostics. Lastly, we have demonstrated that multi-gene panel testing in non-*CDKN2A* melanoma families results in an additional 4% diagnostic yield and that *MITF* and *BAP1* are important genes to include in such a panel. We have hypothesized that future incorporation of melanoma candidate genes in the panel, supplemented with an individual PRS calculation, will most likely increase the diagnostic yield of the panel (although more VUSs will be found as well) and will improve individual cancer risk assessment and individual recommendations for cancer surveillance. Together with further improvement of complex cancer surveillance programs such as that for PC, we are confident that these developments will eventually lead to better survival and quality of life for these individuals.

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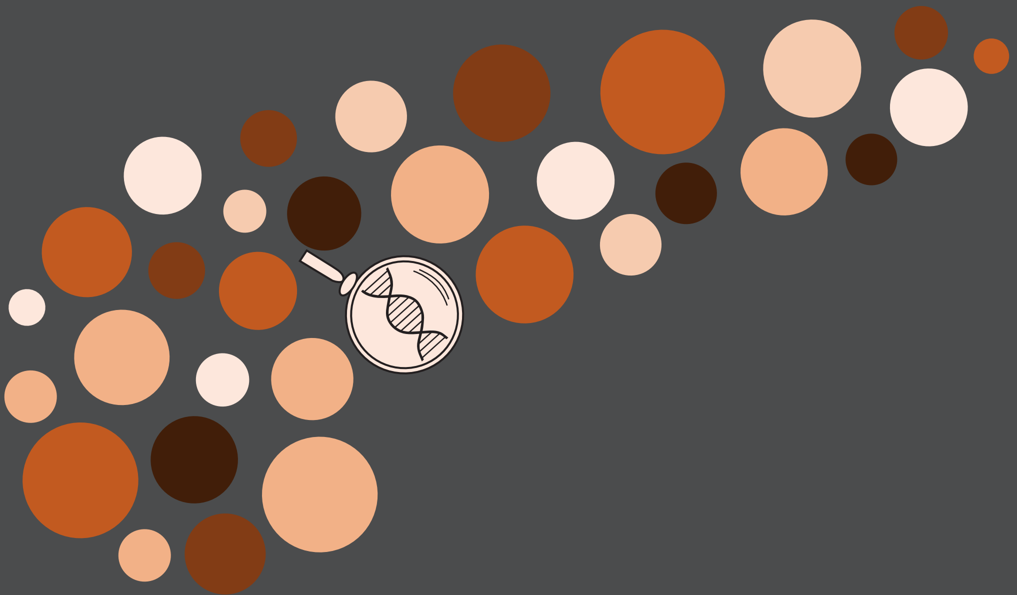

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SUMMARY

Approximately 5-10% of melanoma patients have a familial predisposition for melanoma, and up to 40% of familial patients harbor a germline mutation in the major high-risk melanoma susceptibility gene *CDKN2A*. Carriers of a germline *CDKN2A* mutation have a 70% lifetime risk for developing (multiple) melanoma, which generally occurs at a young age (<40 years). As *CDKN2A*-mutated melanoma families show pancreatic cancer (PC) as a second major type of cancer, the condition is sometimes referred to as familial melanoma-pancreatic cancer syndrome. In the Netherlands, most of these *CDKN2A* families have a specific Dutch founder mutation known as p16-*Leiden*. In the first part of this thesis, our studies focused on patients with this p16-*Leiden* founder mutation. Our first aim was to investigate the full cancer phenotype associated with the p16-*Leiden* mutation and to study potential genetic and non-genetic modifiers of cancer risk. In addition to regular dermatologic surveillance, p16-*Leiden* mutation carriers are offered yearly MRI-based PC surveillance at Leiden University Medical Center. The second aim of this thesis was to evaluate and improve the p16-*Leiden* PC surveillance program by focusing on a) the role of precursor lesions (PanIN, IPMN) in the development and early detection of PC, b) the surgical management of early-stage screen-detected PC, and c) potential biomarkers for possible implementation in the PC surveillance program. The third and final aim of this thesis was to to evaluate and improve genetic testing for hereditary melanoma in general, a subject that will be addressed in the second part of this thesis.

In **chapter 1**, we provide an introduction to familial and hereditary melanoma, the p16-*Leiden CDKN2A* founder mutation, and the PC surveillance program in Leiden.

In **chapter 2**, we studied the prospective risk of cancer in a cohort of 150 p16-*Leiden* mutation carriers participating in the PC surveillance program. As expected, melanoma and PC were the most frequently observed cancers in these individuals, but we also found an overall increased risk for other cancers, most notably cancers in the head and neck region (lip, mouth, pharynx, and larynx). Moreover, a higher than expected number of (unrelated) individuals had a carcinoid tumor or a medical history of sarcoidosis. The risk for developing a tobacco-related cancer such as PC or head and neck cancer was increased fourfold for current smokers compared to former- and never-smokers. We concluded that active intervention to quit or refrain from smoking is very important to the prevention of these frequently occurring cancers, and that p16-*Leiden* mutation carriers should be advised to contact their doctor if they have complaints of hoarseness, dysphagia or ulcers in mouth or throat. An annual inspection of the mouth and throat might potentially contribute to the early detection of head and neck cancers in these individuals.

Among p16-*Leiden* families, there can be striking variability in the number of family members diagnosed with PC. Although tobacco use might explain some of this variability, it is also possible that there are additional genetic risk factors (*modifiers*) in these carriers that influence PC risk. In **chapter 3**, we performed a case-control study of 185 p16-*Leiden* mutation carriers, of whom 50 were diagnosed with PC, and investigated whether a diagnosis of PC was associated with any of seven selected Single Nucleotide Polymorphisms (SNPs) that were derived from large genome wide association studies (GWAS) of sporadic PC. In our analyses we found no significant associations with any of these SNPs. We then hypothesized that either the cohort was too small to detect an effect, that some of the controls might develop PC in the future and therefore could have biased the results, or that modification of PC risk in p16-*Leiden* mutation carriers might actually be due to other SNPs that were not selected in this study.

The studies presented in chapters 4 to 6 focused on the p16-*Leiden* PC surveillance program. In **chapter 4**, we compared the frequency and behavior of precursor lesions and PC in the p16-*Leiden* PC surveillance cohort (n=116) to a German Familial Pancreatic Cancer (FPC) surveillance cohort (n=125). We showed that the frequency of PC was ten times higher in the p16-*Leiden* cohort, but MRI-detected cystic lesions of the pancreatic ducts were much more frequent in the FPC cohort. Resected specimens in the FPC cohort also frequently revealed IPMN and PanIN2-3 precursor lesions, which were only rarely seen in the p16-*Leiden* cohort. However, as precursor lesions in p16-*Leiden* mutation carriers more often progressed to PC, these lesions appear to have a higher malignant potential in p16-*Leiden* mutation carriers. These findings suggest that a more intensive PC surveillance program might be considered for this high-risk group.

In **chapter 5**, we described two high-risk patients (one p16-*Leiden* mutation carrier and one *BRCA2* mutation carrier) who developed a second primary PC two to four years after a partial resection of an early-stage (T1-2N0M0) PC. These cases point to a probable increased risk for developing a second primary PC in high-risk patients who survive long enough after the initial diagnosis. We therefore discussed the advantages and disadvantages of total pancreatectomy (TP) and concluded that TP should be considered in high-risk patients (p16-*Leiden* mutation carriers in particular) diagnosed with an early-stage and prognostically favorable PC.

An important limitation of current PC surveillance programs is the suboptimal diagnostic performance of imaging modalities. Deciding whether a patient actually needs pancreatic surgery can therefore be challenging. In **chapter 6**, we evaluated a previously identified proteomic biomarker signature for PC for its potential inclusion in the Leiden PC surveillance

program as an additional, non-invasive, screening modality. Using this biomarker signature, we could accurately distinguish cases with PC (n=5) from controls without PC (n=61). We could also distinguish the only patient with histologically confirmed precursor lesions (multifocal PanIN1-2 and IPMN) from other controls. Importantly, the biomarker signature was not disturbed by a (recent or non-recent) medical history of melanoma. Since this was a preliminary study with limited sample size, additional studies will be needed before this biomarker test can be implemented in the current PC surveillance program.

Chapters 7 and 8 comprise the second part of this thesis, in which we focused on genetic testing for hereditary melanoma. In **chapter 7**, we studied the association between germline *CDKN2A* mutations and the presence of five clinical features in a melanoma family. One of these features, the presence of head and neck cancer(s) in a family, had not been previously studied in relation to the probability of a *CDKN2A* mutation. Using multivariate logistic regression analysis, significant associations were found for every feature in a large cohort of 1227 Dutch melanoma families (13.7% with a *CDKN2A* mutation). For practical purposes we further developed *CM-Score*, a non-computerized scoring system derived from the logistic regression model. In a predominantly Swedish familial melanoma validation cohort (n=421; 9.0% with a *CDKN2A* mutation), *CM-Score* showed a good ability to discriminate between families with and without a *CDKN2A* mutation (Area under the Curve 0.94). The commonly used threshold of 10% mutation probability was approximated to a *CM-Score* of 16 out of 49 points; the mutation probability below this score was very low (≤4%). We therefore concluded that *CDKN2A* diagnostics should at least be recommended to families with a *CM-Score* ≥16 points.

Although the *CDKN2A* gene is the most important melanoma predisposition gene that should be part of any genetic test for hereditary melanoma, several other high- and medium-penetrance melanoma predisposition genes are currently known and could potentially be tested in addition to *CDKN2A*. In **chapter 8**, we performed multigene panel testing of 30 established and candidate melanoma predisposition genes in a cohort of 451 Dutch melanoma-prone families without a known *CDKN2A* (or *CDK4)* mutation. We found pathogenic mutations in *BAP1* (n=3 families) and *MITF* (p.E318K variant) (n=15 families), which together resulted in a diagnostic yield of 4.0%. In the *BAP1* families, there were no reported diagnoses of *uveal* melanoma or malignant mesothelioma, both of which are major *BAP1*-associated cancers. Based on these findings, we concluded that both *BAP1* and *MITF* genes should always be included in a multigene panel test for *cutaneous* melanoma. In the known melanoma predisposition genes involved in telomere integrity (*POT1*, *ACD*, *TERF2IP*, *TERT*), we only identified two variants of uncertain significance, in *ACD* and *TERF2IP*, suggesting only a minor role for these genes in the Dutch melanoma population.

Additionally, we found several variants of interest in candidate melanoma predisposition genes, in particular in *OCA2*, *BRIP1* and *POLE*, but more research is warranted before these or other candidate genes can be included in regular diagnostic testing for hereditary melanoma.

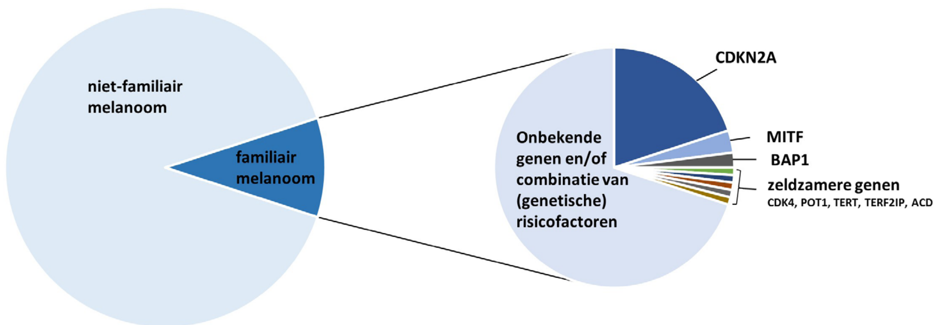
In the final chapter, **chapter 9**, we discuss the main findings of the studies presented in this thesis in the context of the most recent literature. In this chapter, we introduce the term "*CDKN2A*-associated cancer predisposition syndrome" and we propose a revision of the current referral criteria for *CDKN2A* diagnostics in familial melanoma. We emphasize that *CDKN2A* mutation carriers are a distinctive group within PC surveillance programs and we discuss the possible implications that this may have for clinical practice. In the future, improved referral criteria combined with increasing possibilities for elaborate multigene panel testing will enable us to better identify individuals with a high genetic risk for melanoma and other cancers. Together with further improvement of complex cancer surveillance programs such as that for PC, we are confident that these developments will eventually lead to better survival and quality of life for these individuals.

NEDERLANDSE SAMENVATTING

Melanoom is de meest agressieve vorm van de frequent voorkomende typen huidkanker. Als melanoom niet vroegtijdig ontdekt en behandeld wordt, kan het in potentie uitzaaien naar andere organen zoals hersenen, longen, lever en botten. In Nederland worden er jaarlijks ongeveer 6000 patiënten met melanoom gediagnosticeerd. Van alle patiënten met melanoom heeft ongeveer 5-10% een naast familielid die ook melanoom heeft gehad. Bij maximaal 30-40% van deze patiënten met een familiaire belasting kan een erfelijke aanleg voor het ontwikkelen van melanoom worden vastgesteld (zie *figuur)*. De belangrijkste erfelijke eigenschap (gen) waarin afwijkingen (mutaties) worden gevonden in deze families is het *CDKN2A*-gen. *CDKN2A*-mutatiedragers hebben een sterk verhoogd risico (tot 70%) op het ontwikkelen van melanoom en krijgen vaak meerdere melanomen. De gemiddelde leeftijd waarop het eerste melanoom wordt vastgesteld is 39 jaar; ruim 15 jaar jonger dan de gemiddelde leeftijd van diagnose in de algemene bevolking. Naast een verhoogd risico op melanoom hebben *CDKN2A*-mutatiedragers ook een verhoogd risico op andere vormen van kanker, met name alvleesklierkanker *(pancreatic cancer*). Daarom spreekt men in dit kader ook wel van het "*familial melanoma-pancreatic cancer syndrome*". In Nederland komt een hele specifieke mutatie in het *CDKN2A*-gen veel voor, namelijk een 19 baseparen deletie in exon 2 die p16-*Leiden* wordt genoemd. Van alle families met een *CDKN2A*-mutatie gaat het in 80% om de p16-*Leiden* mutatie. Deze specifieke mutatie is terug te voeren tot een gemeenschappelijk verre voorouder (anno 1707) woonachtig in de omgeving van Leiden en heeft zich verspreid onder vele nakomelingen waarvan het merendeel in de regio is blijven wonen. De p16-*Leiden* mutatie wordt daarom een *founder mutatie* genoemd, en de regio waar de meeste p16-*Leiden* mutatiedragers wonen een *genetisch isolaat*. Omdat het merendeel van de p16-*Leiden* mutatiedragers in de regio Leiden woont, is een groot deel van deze personen onder periodieke dermatologische controle in het Leids Universitair Medisch Centrum (LUMC).

Het is gebleken dat het risico op alvleesklierkanker bijzonder hoog is voor p16-*Leiden* mutatiedragers, ongeveer 15-20% met een gemiddelde leeftijd van diagnose op 58 jaar. Alvleesklierkanker is een van de meest dodelijke vormen van kanker. Het wordt meestal pas in een gevorderd stadium ontdekt en heeft dan een zeer slechte prognose (5-jaars overleving <5%). Vanwege dit sterk verhoogde risico en de sombere prognose komen alle personen met een aangetoonde p16-*Leiden* mutatie in aanmerking voor periodiek onderzoek (surveillance) naar alvleesklierkanker vanaf de leeftijd van 45 jaar. In het LUMC bestaat deze surveillance uit een jaarlijks MRI-onderzoek gecombineerd met *magnetic resonance cholangiopancreatography* (MRCP) en optioneel een endo-echografie (EUS). Het doel van dit onderzoek is om alvleesklierkanker of voorlopers daarvan (precursors) in

een zo vroeg mogelijk stadium te ontdekken en te behandelen. De belangrijkste precursors van alvleesklierkanker zijn *pancreatic intraepithelial neoplasms* (PanIN, graad 1 tot 3) en *intraductal papillary mucinous neoplasms* (IPMN). Deze precursors, in het bijzonder IPMN, kunnen worden gedetecteerd met beeldvorming van de alvleesklier omdat ze zich manifesteren als kleine cysteuze afwijkingen van de afvoerkanalen van de alvleesklier. Een eerste evaluatie van het Leidse surveillanceprogramma voor alvleesklierkanker in 2011 toonde aan dat kleine tumoren en mogelijke precursors ontdekt en geopereerd kunnen worden, maar dat p16-*Leiden* mutatiedragers ook vaak agressieve (snelgroeiende) tumoren hebben.



FIGUUR Bij ongeveer 5-10% van alle mensen met melanoom komt melanoom ook in de familie voor. Bij slechts een deel hiervan kan een erfelijke oorzaak worden vastgesteld, meestal een verandering (mutatie) in het *CDKN2A*-gen.

Hoewel het *CDKN2A*-gen het belangrijkste hoog-risico gen voor melanoom is, en als zodanig ook altijd getest zou moeten worden in families met een reële verdenking op een erfelijke aanleg voor melanoom, zijn er een aantal andere, veelal nieuw ontdekte genen die eveneens een sterke of matig-sterke associatie hebben met familiair melanoom. Dit zijn: *CDK4*, *BAP1*, *MITF*, *TERT*, *POT1*, *ACD* en *TERF2IP* (zie *figuur)*. Mutaties in deze genen zijn echter veel zeldzamer dan mutaties in het *CDKN2A*-gen en voor de meeste van deze genen is er nog veel onduidelijk over de precieze risico's op melanoom en eventuele andere vormen van kanker. Bovendien is het nog niet bekend wat de frequentie is van mutaties in deze genen in de Nederlandse populatie. Ook zijn er meerdere kandidaat melanoomgenen beschreven die vaak gevonden zijn met genoombrede sequencing (*whole exome sequencing; WES*) van grote melanoomfamilies, maar waarvan nog niet duidelijk is wat hun precieze rol is in de erfelijkheid van melanoom. Samen met het *CDKN2A*-gen kunnen deze nieuwe (kandidaat)melanoomgenen worden opgenomen in

een genenpanel voor erfelijk melanoom en worden getest in melanoomfamilies. Hierdoor kan de opbrengst van genetisch onderzoek naar erfelijk melanoom verbeteren.

Dit proefschrift heeft drie doelstellingen:

1) Het bestuderen van het gehele spectrum aan kankers bij p16-*Leiden* mutatiedragers, en onderzoeken of er andere genetische en niet-genetische factoren van invloed zijn op de hoogte van het risico op alvleesklierkanker
2) Het evalueren en verbeteren van het surveillance programma voor alvleesklierkanker bij p16-*Leiden* mutatiedragers
3) Het evalueren en verbeteren van genetische tests voor erfelijk melanoom

De studies in het eerste deel van dit proefschrift (hoofdstukken 2 tot en met 6) zijn gericht op personen met de p16-*Leiden* mutatie en hebben betrekking op de eerste twee doelstellingen. De studies in het tweede deel (hoofstukken 7 en 8) zijn gericht op personen met familiair melanoom in het algemeen, het merendeel zonder *CDKN2A*/p16-*Leiden* mutatie, en hebben betrekking op de derde doelstelling.

Hoofdstuk 1 is een inleiding waarin de huidige kennis over familiair en erfelijk melanoom, de p16-*Leiden* founder mutatie in het *CDKN2A*-gen en het surveillance programma voor alvleesklierkanker in Leiden wordt besproken.

In **hoofdstuk 2** bestuderen we het risico op kanker in een cohort van 150 p16-*Leiden* mutatiedragers die in de tijd werden gevolgd (prospectief). Zoals verwacht kwamen melanoom en alvleesklierkanker het meeste voor, maar er werd ook een globaal verhoogd risico op andere vormen van kanker gezien, met name kankers in het hoofd-hals gebied (lip, mond- en keelholte). Daarnaast had een hoger dan verwacht aantal (niet-verwante) personen een carcinoïde tumor of een medische voorgeschiedenis van sarcoïdose. Het is bekend dat zowel alvleesklierkanker als kanker in het hoofd-hals gebied sterk geassocieerd is met roken in de algemene bevolking. In deze studie hadden p16-*Leiden* mutatiedragers die rookten een viermaal verhoogd risico op deze vormen van kanker vergeleken met niet-rokers. We concluderen daarom dat het belangrijk is om roken actief te ontmoedigen om zo deze frequent voorkomende kankers te kunnen voorkomen. Bovendien moet aan p16-*Leiden* mutatiedragers geadviseerd worden om laagdrempelig contact op te nemen met hun (huis)arts als er aanhoudende klachten zijn van heesheid, slikklachten of zweren in de mond- en keelholte. Jaarlijkse inspectie van de mond- en keelholte zou kunnen helpen om kanker in dit gebied in een vroeg stadium te ontdekken.

Het aantal personen met alvleesklierkanker in een p16-*Leiden* familie kan sterk variëren. Roken zou een deel van deze variabiliteit kunnen verklaren, maar het zou ook kunnen dat

er bij sommige p16-*Leiden* mutatiedragers additionele genetische risicofactoren aanwezig zijn die het risico op alvleesklierkanker beïnvloeden. In **hoofdstuk 3** onderzoeken we in een cohort van 185 p16-*Leiden* mutatiedragers, waarvan 50 met alvleesklierkanker, of het hebben van alvleesklierkanker geassocieerd is met zeven verschillende genetische risicovarianten (Single Nucleotide Polymorphisms; SNPs) die we selecteerden op basis van grote genoomwijde associatiestudies van niet-erfelijk alvleesklierkanker. We vonden daarbij geen associatie met deze SNPs. We veronderstellen dat het cohort wellicht te klein was om een associatie te vinden, dat sommige personen ten onrechte in de controlegroep zaten omdat ze misschien op latere leeftijd alvleesklierkanker krijgen en daardoor mogelijk een vertekenend beeld hebben kunnen geven, of dat er geheel andere SNPs zijn die het risico op alvleesklierkanker bij p16-*Leiden* mutatiedragers daadwerkelijk beïnvloeden.

De studies in hoofdstukken 4 tot en met 6 zijn gericht op het surveillance programma voor alvleesklierkanker bij p16-*Leiden* mutatiedragers. In **hoofdstuk 4** vergelijken we de frequentie en het beloop van precursors van alvleesklierkanker en alvleesklierkanker zelf tussen het Leidse p16-*Leiden* surveillance cohort (n=116) en een Duits surveillance cohort van personen uit families met familiair alvleesklierkanker (*familial pancreatic cancer;* FPC) (n=125). We toonden aan dat de frequentie van alvleesklierkanker in het p16-*Leiden* cohort tien keer hoger was, maar dat op de MRI zichtbare cysteuze afwijkingen van de afvoerkanalen van de alvleesklier (oftewel precursors) veel vaker voorkwamen in het FPC-cohort. Resectiepreparaten in het FPC-cohort toonden vaak IPMN en PanIN2-3 precursors, die slechts zelden werden gezien in het p16-*Leiden* cohort. P16-*Leiden* mutatiedragers ontwikkelden echter vaker alvleesklierkanker uit een precursor. Precursors bij p16-*Leiden* mutatiedragers hebben daarom mogelijk een hogere kwaadaardigheid in vergelijking met precursors bij personen uit FPC-families. We concluderen daarom dat een intensiever surveillance programma kan worden overwogen voor p16-*Leiden* mutatiedragers.

In **hoofdstuk 5** beschrijven we twee hoog-risico patiënten (één p16-*Leiden* mutatiedrager en één *BRCA2* mutatiedrager) die een tweede primaire alvleesklierkanker ontwikkelden na een gedeeltelijke alvleesklierverwijdering vanwege een vroeg-stadium (T1-2N0M0) kanker twee tot vier jaar eerder. Deze gevallen illustreren dat er zeer waarschijnlijk een verhoogd risico is voor het ontwikkelen van een tweede primaire alvleesklierkanker bij hoog-risico patiënten die lang genoeg overleven na de eerste diagnose. We hebben daarom de voor- en nadelen van een totale alvleesklierverwijdering (totale pancreatectomie; TP) besproken en geconcludeerd dat een TP moet worden overwogen bij hoog-risico patiënten (p16-*Leiden* mutatiedragers in het bijzonder) die gediagnosticeerd worden met een vroeg-stadium en prognostisch gunstige vorm van alvleesklierkanker.

Een belangrijke beperking van huidige surveillance programma's voor alvleesklierkanker is het feit dat de gebruikte beeldvormende technieken (MRI/MRCP en EUS) een suboptimaal vermogen hebben om relevante afwijkingen in de alvleesklier betrouwbaar te detecteren of te onderscheiden van minder relevante afwijkingen. De beslissing of een patiënt wel of geen alvleesklieroperatie moet ondergaan kan daarom soms erg moeilijk zijn. In **hoofdstuk 6** bestuderen we of een eerder geïdentificeerde, op eiwitten gebaseerde (*proteomics*) biomarker signatuur voor alvleesklierkanker in bloed toegevoegd zou kunnen worden aan het Leidse surveillance programma, als een additionele niet-invasieve test bovenop de gebruikte beeldvormende technieken. We konden met deze biomarker signatuur een nauwkeurig onderscheid maken tussen p16-*Leiden* mutatiedragers met alvleesklierkanker (*cases*; n=5) en p16-*Leiden* mutatiedragers zonder alvleesklierkanker (*controles*; n=61). De enige patiënt met histologisch (door de patholoog) bevestigde precursors (meerdere PanIN1-2 en IPMN) kon ook onderscheiden worden van andere controles. Bovendien werden de biomarker profielen niet verstoord door een (recente of niet-recente) diagnose van melanoom. Hoewel de resultaten veelbelovend zijn, is dit is een kleine voorstudie met een beperkt aantal patiënten. Er is daarom meer onderzoek met een groter aantal patiënten nodig voordat deze biomarker test ingevoerd kan worden in het huidige surveillance programma voor alvleesklierkanker.

De hoofdstukken 7 en 8 vormen het tweede deel van dit proefschrift, waarin we ons richten op personen met familiair melanoom in het algemeen met als doel het verbeteren van genetische tests voor erfelijk melanoom. In **hoofdstuk 7** bestuderen we de associatie tussen *CDKN2A*-mutaties en de aanwezigheid van vijf klinische kenmerken in een melanoomfamilie, namelijk 1) het aantal familieleden met melanoom, 2) het aantal familieleden met multipele melanomen, 3) gemiddelde leeftijd van diagnose van melanoom in een familie, 4) aanwezigheid van alvleesklierkanker in een familie en 5) aanwezigheid van kanker in het hoofd-hals gebied in een familie. Het laatstgenoemde kenmerk was nog niet eerder in dit kader onderzocht. Met een specifieke statistische analyse (multivariate regressieanalyse) vonden we significante associaties voor elk klinisch kenmerk in een groot cohort van 1227 Nederlandse melanoomfamilies (13.7% met een *CDKN2A*-mutatie). Op basis van dit regressiemodel ontwikkelden we een praktisch en niet-computergestuurd scoresysteem genaamd *CM-Score*. Met behulp van een grotendeels Zweeds validatiecohort (n=421 melanoomfamilies; 9.0% met een *CDKN2A-mutatie*) toonden we aan dat *CM-Score* goed onderscheid kan maken tussen families met en zonder een *CDKN2A*-mutatie (*Area under the Curve* 0.94). In de praktijk wordt, voor het verrichten van genetisch onderzoek in een familie met een mogelijke erfelijke aanleg voor kanker, vaak een drempelwaarde van 10% kans op het vinden van een mutatie aangehouden. Deze drempelwaarde werd bereikt bij een *CM-Score* van 16

uit 49 punten. De kans op een *CDKN2A*-mutatie is erg laag bij families met een lagere score (<4%). We concluderen daarom dat analyse van het *CDKN2A*-gen in ieder geval aangeboden moet worden aan families met een *CM-Score* van 16 punten of meer.

In **hoofdstuk 8** onderzoeken we wat de opbrengst in van een multi genen paneltest bij 451 Nederlandse melanoomfamilies zonder mutatie in het *CDKN2A*(of *CDK4*)-gen. In totaal includeerden we 30 genen in de paneltest, bestaande uit 9 bekende hoog-risico en matig-hoog risico melanoomgenen en 21 kandidaat melanoomgenen. In 18 families vonden we een pathogene (ziekte veroorzakende) mutatie in een van de 9 bekende melanoomgenen, wat resulteerde in een diagnostische opbrengst van 4.0%. Drie van deze families hadden een pathogene mutatie in het *BAP1*-gen en 15 families hadden een pathogene mutatie in het *MITF*-gen (p.E318K variant). Het *BAP1*-gen is geassocieerd met verschillende vormen van kanker, waaronder huidmelanoom. De twee belangrijkste en meest frequent voorkomende vormen van kanker bij *BAP1*-mutatiedragers, namelijk *oog*melanoom en maligne mesothelioom (borstvlieskanker), kwamen opmerkelijk genoeg niet voor in onze drie *BAP1*-families. Op basis van deze resultaten concluderen we dat *BAP1* en *MITF* altijd moeten worden opgenomen in een multi genen paneltest voor erfelijk *huid*melanoom. In de vier bekende melanoomgenen die een functie hebben in de integriteit van telomeren (*POT1*, *ACD*, *TERF2IP*, *TERT*) vonden we slechts twee varianten van onduidelijke betekenis (in *ACD* en *TERF2IP*). Waarschijnlijk spelen deze telomeergenen dus maar een kleine rol in de Nederlandse melanoompopulatie. Daarnaast vonden we diverse varianten in de 21 kandidaat melanoomgenen, in het bijzonder in *OCA2*, *BRIP1* en *POLE*. Er is echter meer onderzoek nodig voordat deze genen of andere kandidaatgenen in de reguliere genetische diagnostiek naar erfelijk melanoom opgenomen kunnen worden.

Tot slot bespreken we in **hoofdstuk 9** de belangrijkste bevindingen van de studies in dit proefschrift in de context van recente literatuur. We introduceren in dit hoofdstuk de term "*CDKN2A*-geassocieerd tumorpredispositie syndroom" en doen een voorstel voor aanpassing van de huidige verwijscriteria voor *CDKN2A* analyse bij familiair melanoom. Ook benadrukken we de bijzondere positie die *CDKN2A* mutatiedragers hebben binnen surveillanceprogramma's voor alvleesklierkanker en we bespreken wat dat zou kunnen betekenen voor de klinische praktijk. In de toekomst zal een verbetering van de verwijscriteria in combinatie met de toenemende mogelijkheden van multi genen paneltests ons in staat stellen om personen met een genetisch hoog risico op melanoom en andere vormen van kanker beter te kunnen identificeren. Samen met een verdere verbetering van complexe surveillanceprogramma's zoals dat voor alvleesklierkanker zal dat uiteindelijk leiden tot een betere overleving én een betere kwaliteit van leven van deze personen.

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ABOUT THE AUTHOR

Thomas Potjer was born on January 27th 1986 in Assen, the Netherlands. In 2004 he completed athenaeum secondary education at Dr. Nassau College in Assen. He then moved to Leiden and began his medical education at Leiden University Medical Center (LUMC). During a short intermezzo in 2008-2009, he successfully completed a propaedeutic year of Art History at Leiden University, after which he continued with the senior years of his medical education. In 2011-2012 he did his scientific internship with Prof. Hans Vasen and Drs. Ingrid Schot at the department of gastroenterology and hepatology of the LUMC, which led to his first scientific publication (*Clin Cancer Res*, 2013) and marked the start of his PhD research. He completed his medical education in 2012 after a senior clinical internship at the department of clinical genetics of the LUMC, where he subsequently started his postgraduate training as a clinical geneticist (residency) under supervision of Dr. Sarina Kant and Dr. Emilia Bijlsma. Meanwhile, he continued his PhD research, first at the Stichting Opsporing Erfelijke Tumoren (StOET) in Leiden under supervision of Prof. Hans Vasen and later at the department of clinical genetics of the LUMC under supervision of Prof. Christi van Asperen and Dr. Frederik Hes. In 2015, he received a personal research grant from the Dutch Cancer Society (KWF) for a project titled "Development of a gene panel test to improve tumor risk assessment in melanoma families" (project supervisor Dr. Nienke van der Stoep), with which he could further continue his PhD research aside his residency. In his PhD period, he orally presented his research at several national and international scientific conferences and he supervised two medical students during their scientific internships, both of which resulted in publications that are part of this thesis (*Eur J Hum Genet*, 2015 and *J Med Genet*, 2018). As part of his residency, he undertook an internship "Dysmorphology" in Paramaribo, Suriname and an internship "Hereditary Cancer in Children" at the Princess Máxima Center in Utrecht. He completed his residency in January 2019 and continues his career as a clinical geneticist at the LUMC with a special interest in oncogenetics and an ongoing involvement in familial melanoma research. He currently lives in Den Haag together with his partner Natalja.

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