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

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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.

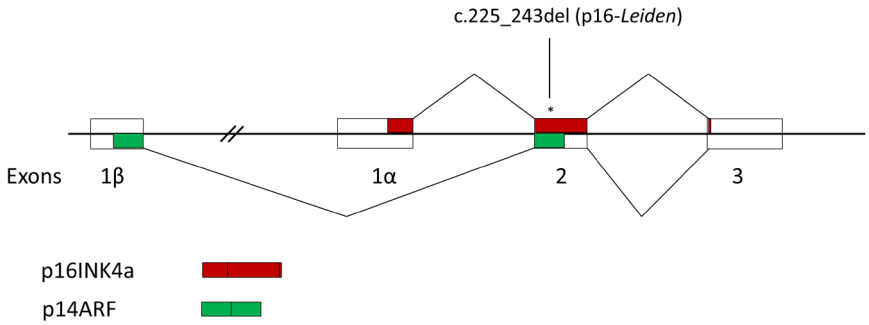

FIGURE 1. The *CDKN2A* gene and its two products, p16INK4a and p14ARF. The p16-*Leiden* mutation is located in exon 2 and affects both p16INK4a and p14ARF.
Adapted with permission from Pigment Cell Melanoma Research, 28, Aoude LG, Wadt KA, Pritchard AL, Hayward NK, Genetics of familial melanoma: 20 years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

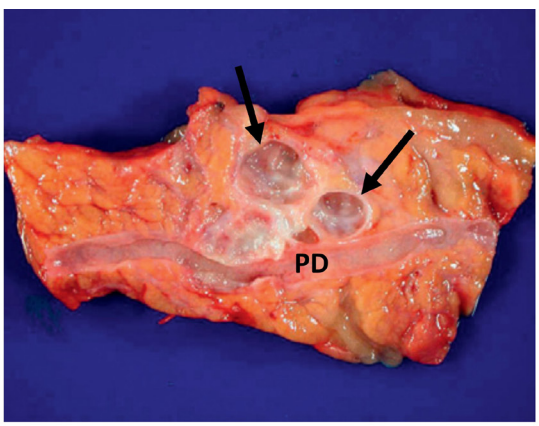

FIGURE 2. Surgical pathology specimen of resected pancreas that includes a branch-duct IPMN (arrows)
PD = main pancreatic duct
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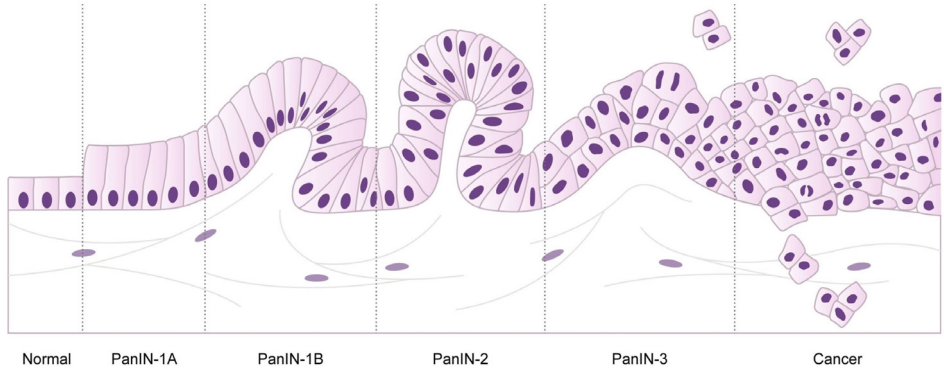

FIGURE 3. Progression model of pancreatic cancer from PanIN lesions. Normal ductal epithelial cells are short and cuboidal, while PanIN-1A lesions are flat and columnar. PanIN-1B lesions are identical to PanIN-1A, although papillary architecture can be observed in these lesions. PanIN-2 lesions can be 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.

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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.

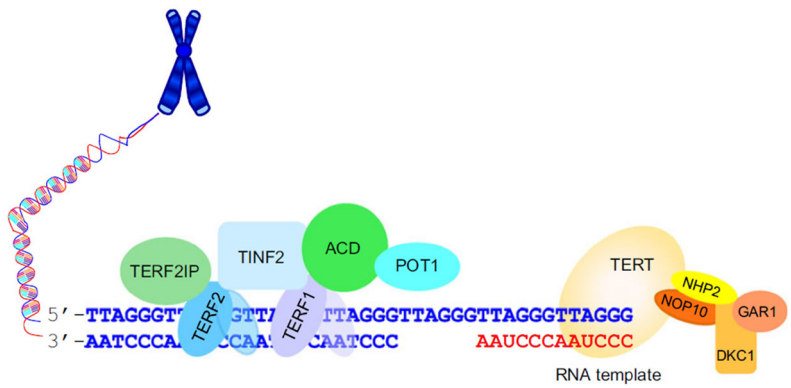

FIGURE 4 Schematic view of the telomere. The shelterin complex (TERF1, TERF2, TERF2IP, TINF2, ACD, POT1) is depicted on the left and the telomerase complex (TERT and other associated proteins) is depicted on the right. The telomerase complex adds telomere repeat sequences to the 3' end of the telomere. 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]

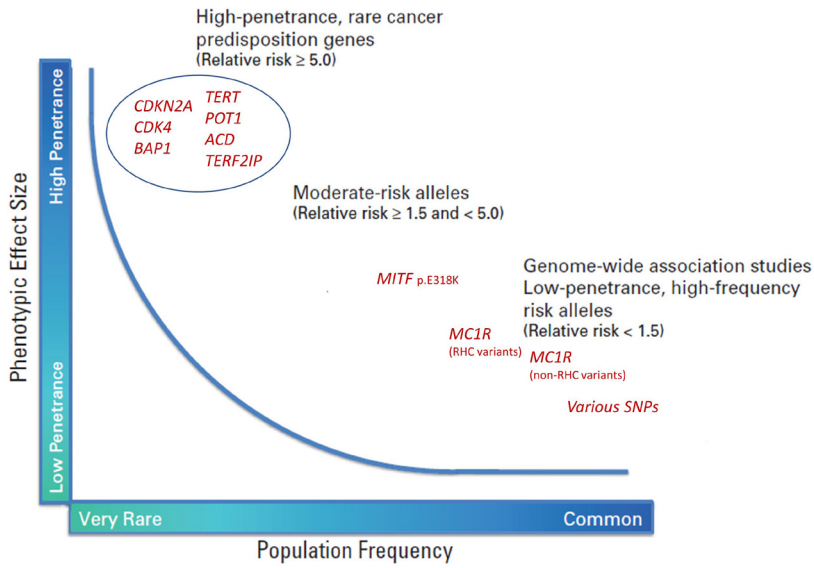

FIGURE 5. *[Legend on the next page]*

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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