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Environmental and genetic factors in the pathogenesis of melanoma and melanoma associated other primary malignancies

Ph.D. Thesis

Zsófia Borbála Hatvani M.D.

Semmelweis University

Doctoral School of Clinical Medicine

Supervisor: Sarolta Kárpáti M.D., D.Sc.

Official reviewers: Judit Oláh M.D., Ph.D.

Tibor Krenács M.D., Ph.D.

Head of the comprehensive exam committee:

Ilona Kovalszky M.D., D.Sc.

Members of the comprehensive exam committee:

Bálint Nagy Ph.D.

Zsuzsanna Szalai Med.habil.

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ABBREVIATIONS

6,4 PP: ……..…….……6-, 4 pyrimidin-pyrimidon
AC: …….……..…..…….Adenylate cyclase
ACTH: ……..……..……..……..….Adrenocorticotropic hormone
AD: ……..……..……..…….Autosomal dominant
ALM: ……..……..……..…..……Acral-lentiginous melanoma
α-MSH: ……..……..……..……...Alfa-melanocyte stimulating hormone
BCC: ……..…..……..……Basal cell cancer
Bp:……..…..……..……Base pair
BRRS: ……..……..……..…….. Bannayan-Riley-Ruvalcaba syndrome
CCUO: ……...…...Cancer of unknown origin
CDK4: ……..……Cyclin-dependent kinase 4
CDKN2A: ……..……..……Cyclin-dependent kinase 2A
CLL: ……...……..……..…Chronic lymphocytic leukemia
CPD: ……...…..……..…….Cyclobutane pyrimidin dimer
CREB: ……...….cAMP-response element binding protein
CS: ……..……..……Cowden syndrome
CLS: ……..……Cowden-like syndrome
CSD: ……..……..……...……Chronic sun damage
DDD: ……...…….Dowling Degos Disease
DS: ……..…..……...……Dermoscopy
DSB: ……...……Double strand break
EB:..……...……..……..Epidermolysis bullosa
HDM2: ……..……..Human homolog of murine Mdm2
HGVS: ……..……..……Human genome variation society
HHD: ……..……Hailey-Hailey Disease
HR: ……..…...…….Homolog recombination
IVS: ……..…….... Intervening sequence
LC: ……..……Lung cancer
LFS: ……..…….Li-Fraumeni syndrome
LI: ……...…….Lamellar ichthyosis
LMM: ……...…….……..Lentigo maligna melanoma

MC1R: ……..……..…….Melanocortin 1 receptor
MEN-1: ……...…….……Multiple endocrine neoplasia-1
miRNA: ……..…….….microRNA
Mis: ……....Melanoma in situ
MITF: …….……Microphtalmia associated transcription factor
MM: ……...……...……Malignant melanoma
MMF: ……...…….Mycophenolate mofetil
MP: ……..……...Methylprednisolone
MPM: …….……Multiple primary melanoma
mTOR: ……...……..mammalian target of rapamycin
mTORi: ……...…….…….mammalian target of rapamycin inhibitor
NER: ……..……..Nucleotide excision repair
NHL: ……..…….……..Non-Hodgkin lymphoma
NLS: ……..……..Nuclear localization signal
NM: ……..…….……Nodular melanoma
NMSC: ……..…….……Non-melanoma skin cancer
NRHC: ……..……Non-red hair color
OMIM: ……..……...……On-line Mendelian Inheritance in Man
OR: ……..……..Odds ratio
OTR: ……..…….…….Organ transplant recipient
PaC: ……..……..Pancreatic cancer
PHTS: ……...…….….PTEN hamartoma tumor syndrome
PKA: ……..……cAMP dependent protein kinase A
PLS: ……..…….……Proteus-like syndrome
PrC: ……..……..Prostate cancer
PS: ……..……..…..……Proteus syndrome
PTEN: ……..……Phosphatase and ptensin-homolog
Rb: ……..……...…Retinoblastoma protein
RCC: ……..…….Renal cell cancer
RHC: ……..…….Red hair color
ROS: ……..……...……..Reactive oxygen species
RR: ……..……..……Relative risk

SIR: ……..……Standardized incidence ratio
SPM: ……..……..Single primary melanoma
SRL: ……..…..……Sirolimus
SSM: ……..…….Superficial spreading melanoma
SU: ……..…….Semmelweis University
TAC: ……..……..……Tacrolimus
TILs: ……..……..…Tumor infiltrating lymphocytes
UTR: ……..…….Untranslated region
UVA: ..……..……Ultraviolet-A
UVB: ……..……..…….Ultraviolet-B
UVR: ……..……..…….……Ultraviolet radiation
WHO: ……..……..…….World Health Organization

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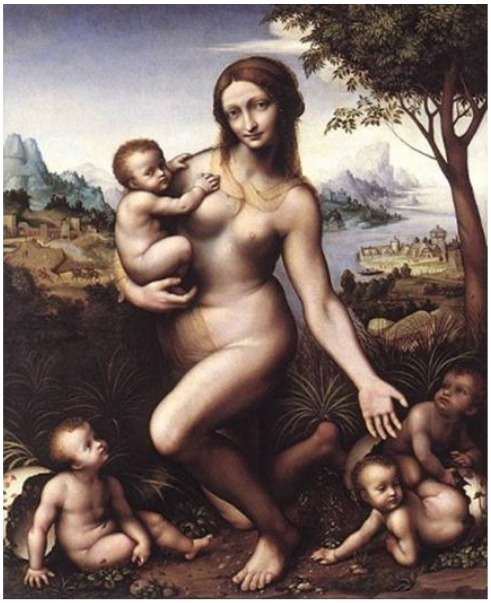
I. INTRODUCTION

I.1. Historical/cultural aspects of pigmented lesions

Before the 18th century, in most cultures immaculate skin with the aesthetics of smooth, white complexions of its ladies represented high value, health, beauty, innocence and perfection. Also in the art, skin marks (moles, birthmarks) or any disfiguring skin changes hadn't been portrayed on paintings (**Figure 1**).

Tiziano: Flora (1515)



Leonardo da Vinci: Leda (1530)

Velazquez: Venus at her mirror (1651)

François Boucher: Portrait of Madame de Pompadour (1759)

Figure 1. Representations of the skin on paintings between the 16th-18th century

There had been many beliefs concerning moles and birthmarks. Also in the first English book published on skin diseases (Turner 1714), an individual chapter analyses the

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concepts of birthmarks. That time, fetal skin development was considered to be directly associated with the mother's mental condition, and birthmarks as a consequence of maternal harmful impressions (desire, fright, accidents). Already from the sixteenth century localization of moles served as a basis of divination. As a few example, a mole on the belly denoted whoredome, luxury and gluttony; while a mole on the throat threatened the person with diseases such as asphyxia, or violent death. Top-to-bottom, left-right orientation and gender differences were also considered in mole reading. For instance a man with a mole on the upper lip was believed to have good fortune, while a woman to be debauched (summarized from Connor 2004).

Interestingly, on the basis of the favorable meanings of mole readings, in the eighteenth century, application of beauty spots from black silk to the face became a trend of fashion (**Figure 2**).

Daniel Dumoustier: Portrait of a Young Man with a Beauty Spot on his Cheek (1632)

Henry Morland: The Fair Nun Unmasked (about 1769)

Miguel Cabrera: Doña María de la Luz Padilla y (Gómez de) Cervantes (about 1760)

Schematic picture from the 18th century about the meanings of localizations of beauty spots

Figure 2. Artificial moles - application of beauty spots in the 18th century

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Later, from the late nineteenth century, as knowledge about dermatological conditions especially about moles and melanoma got augmented and accessible (first description of melanoma is from 1812 by Rene Laennac), visualization of pigmented lesions in art works became also more common. Unfortunately a parallel unfavorable trend of a new body culture evolved with a higher ultraviolet radiation (UVR) exposure of the skin. In the last decades, there is a significant awareness of pigmented lesions in the general public, which trend is also reflected in art works with a great importance regarding public health (**Figure 3**) (Sources of paintings are detailed in the references).

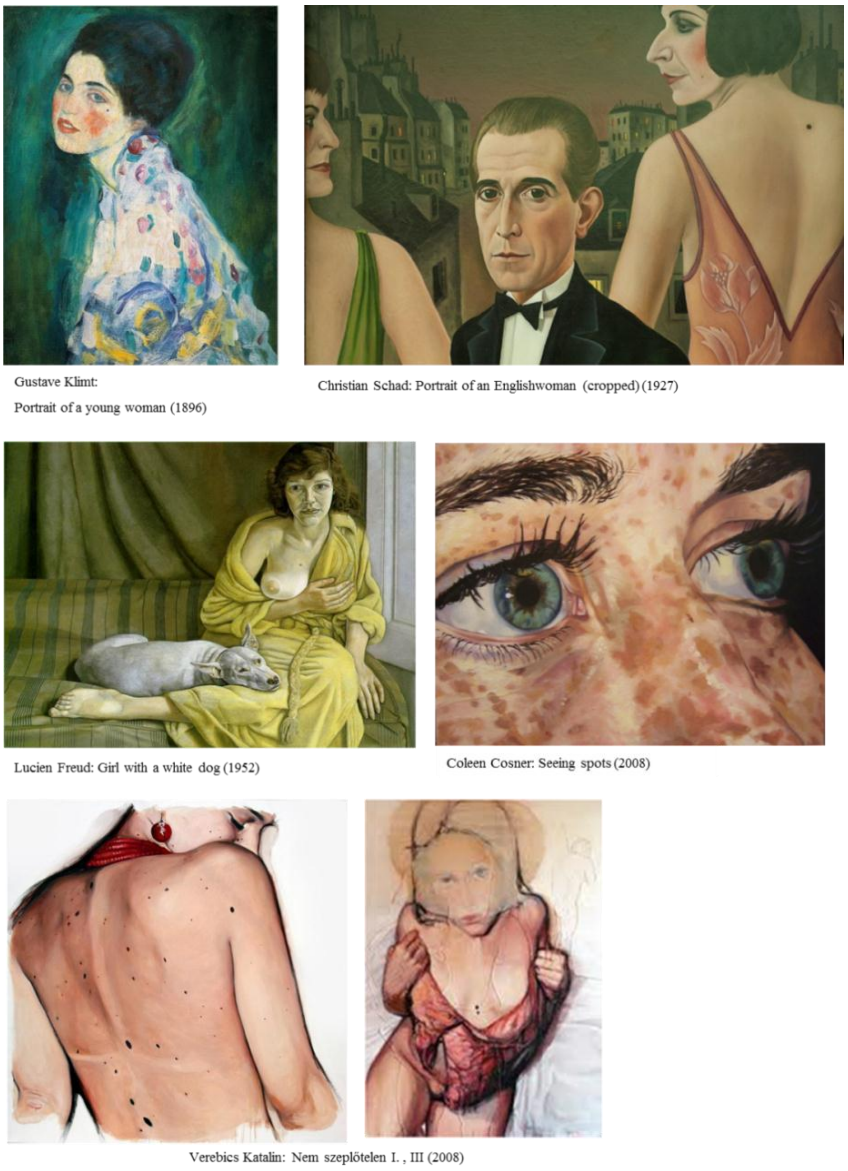

Figure 3. Moles as commonly visualized skin marks after the 19th century

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I.2. Malignant melanoma

Malignant melanoma (MM) is a tumor originating from melanocytes, the pigment cells with major function of synthesizing melanin pigment that determinates skin, eye and hair pigmentation. During embryogenesis melanocytes are originated from neural crest cells then they migrate to their targeted ectodermal locations. There are still debated results whether MM cells originate from the differentiated melanocytes, or from undifferentiated melanocyte-precursor stem cells. In any ways, sequential genetic events, inherited and acquired, are required for MM development (reviewed in Meyle and Guldberg 2009). MM occurs not only in the skin, but in mucosal membranes and in the uvea of the eye; however they are not elemental topics of this work.

MM incidence has dramatically risen in the last decades, affecting even younger generations (Purdue et al. 2008). There are strong efforts to lower this incidence rates due early recognition and patient education, as therapy of advanced stage MM is still not that promising. Over the last 10 years in Hungary (National Cancer Registry), and also at Semmelweis University (SU) Department of Dermatology, Venereology and Dermatooncology (own data), the number of identified new cases of MM and melanoma in situ (Mis) cases clearly demonstrates a rising trend (**Figure 4**).

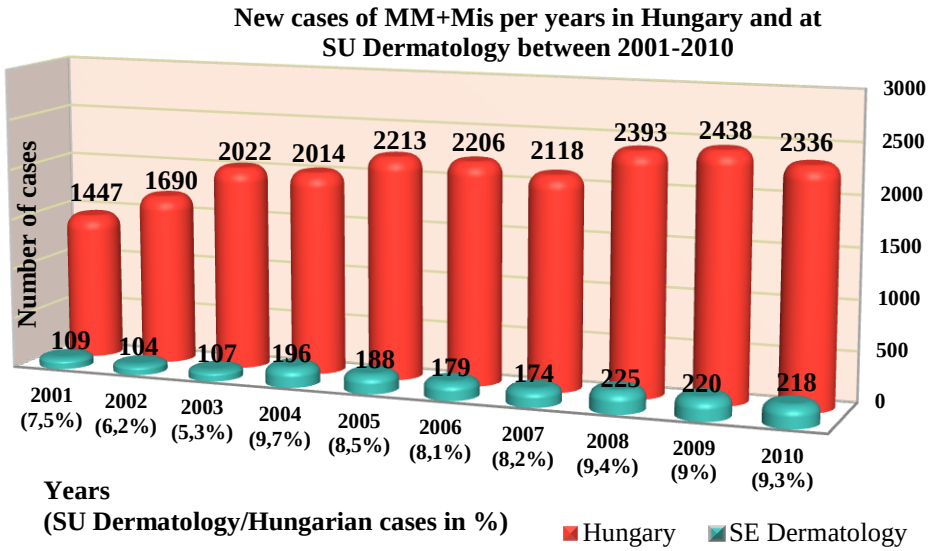

Figure 4. MM and Mis new cases in Hungary and at SU Dermatology between 2001 and 2010.

Using demographic data of Hungarian Central Statistical Office (KSH 2012), MM+Mis incidence rates within this time interval ranged between 12-20/100.000 with a rising trend, similarly to other Hungarian reports (Balatoni et al. 2011).

Traditional MM classification is based on tumor clinocopathology and includes major subtypes such as superficial spreading melanoma (SSM), nodular melanoma (NM), acrolentiginous melanoma (ALM) and lentigo maligna melanoma (LMM) (Clark 1967), supplemented with some less common subtypes (desmoplastic-, naevoid-, mucosal MM, MM arising from a blue naevus-, or from a congenital naevus, MM in childhood and persistent MM) (World Health Organization-WHO Classification 2006). However these subtypes own neither prognostic nor therapeutic importance. Recent molecular findings opened the opportunity of personalized targeted therapies with promising efficacy that points out the urgent need of a new up-to-date classification system (Scolyer et al. 2011). MM subgroups are also classified by body site and sun exposure (MM arising in chronically sun exposed, intermittently sun-exposed, or sun protected areas), moreover certain tumor locations show specific molecular characteristics regarding *BRAF, RAS, c-KIT* mutation status (see later in **Table 3** on page 26).

I.3. Non-genetic factors in the etiology of MM

MM is a multifactorial cancer with identified environmental and hereditary predisposing factors. Prevalence rates and gene-environment interactions vary along geographical locations upon latitude. While the highest prevalence is observed in Australia and New-Zeeland, followed by the United States and some European populations (Little and Eide 2012), mutation frequencies of predisposing genes are detected inversely with the incidence rates, suggesting that in regions with the higher incidences, sporadic cases make up the majority.

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I.3.1. Environmental predisposing factors

I.3.1.1. Ultraviolet light

Figure 5. Ferenc Gál: Beach

Fundamental role of UVR in MM genesis has long been hypothesized (summary in Hocker and Tsao 2007). Besides the original concept, that history of sunburns are the predominant UV related risk factors (Gilchrest et al. 1999, Noonan et al. 2001), recently more lines of evidence suggest that all types (intermittent, chronic, sunburns) of sun exposures together with the age at exposition may play role in MM genesis, especially those suffered during the most vulnerable early childhood.

Ninety-five percent of UVR reaching the ground is UVA (315-400nm) that is far less energetic than UVB (280-315nm). Previously, solar UVR induced DNA mutation formation and skin carcinogenesis was exclusively linked to UVB effects, however recently UVA has also been classified as class I carcinogen by the WHO (El Ghissassi et al. 2009).

I.3.1.1.1. UVB

Shortwave UV light generates DNA photoproducts by direct DNA absorption. These photoproducts are mainly *cis-syn* cyclobutane pyrimidine dimers (CPDs), pyrimidine 6-4 pyrimidone photoproducts (6-4 PPs) and Dewar valence isomers (DewPPs). CPDs, mostly formed as C to T transitions (also known as UV signature mutations), are the most common UVB lesions. If DNA damage response mechanisms are sufficient, these premutagenic lesions are repaired, and don't cause mutations and subsequent carcinogenesis (reviewed in Rünger and Kappes 2008). Upon different UVB exposures, activation of MAPK pathway, elevated tyrosinase expression in melanocytes, higher MM cell motility through interleukin-8 activation, and a number of clinical and experimental observations on animal models confirmed the pathogenic effects of UVB in MM genesis (reviewed by von Thaler et al. 2009).

I.3.1.1.2. UVA

UVA was originally hypothesized to create different DNA lesions than UVB due to reactive oxygen species (ROS) induction. Recent findings suggest that the majority of UVA mutations particularly of UVA2 (315-340nm) induce also C to T transitions and subsequent CPDs. UVA also generates oxidative DNA damage through singlet oxygen or other ROS, predominantly on guanine. The most mutagenic product is 7,8-dihydro-8-oxyguanin (8-oxoG). The carcinogenic effect of UVA is now mainly linked to CPDs and to the finding that UVA-generated CPDs are more mutagenic than UVB-induced ones (summarized in Rünger and Kappes 2008). This is also supported by our results, showing that antimutagenic cellular responses are much weaker upon UVA than UVB induction (Rünger et al. 2012), however on fibroblasts. When skin is exposed to the sun, the amount of UVB is sufficient to induce damage response mechanisms and also to minimize the effects of UVA induced damages. Pure UVA exposure (sunbed use, sun exposure through window glass, UVA phototherapy, use of non-broad spectrum sunscreens) is getting more frequent in the modern civilization, when the UVA induced DNA damage responses are usually not sufficient to prevent the mutation formation (reviewed in Rünger and Kappes 2008).

In primary MMs, besides oxidative DNA damages, predominantly UV signature mutations (C to T) are detected on tumor suppressor genes (*p53, p16/INK4a, PTEN*) at various percentages (52-68%). Whether these mutations are induced by UVA or UVB is unclear (reviewed in Rünger and Kappes 2008), however the role of UVA in MM genesis was repeatedly confirmed by clinico-epidemiological studies on sunscreen-, and sunbed use (Héry et al. 2010, Autier et al. 2011, Boniol et al. 2012).

I.3.1.2. Obesity

Obesity has been proven as a risk factor for several cancer types, although in terms of MM, debated results are available. A recent meta-analysis has proved the association with increased MM risk but only among men (Sergentanis et al. 2013).

I.3.1.3. Socioeconomic status, occupation

MM incidence is higher among people with larger income. The most likely explanation is the higher amount of recreational sun exposures during sunny holidays throughout a year (Kirkpatrick and White 1990, MacKie and Hole 1996). Moreover socioeconomic

status has been shown to have impact on MM survival as well due to better access to health services especially in countries where huge gap exist between the different socioeconomic layers (Quintella Mendes and Koifman 2013). As another factor, MM diagnosis in patients with lower socioeconomic level is more likely to be delayed with advanced Breslow thickness and disease stage (Pollitt et al. 2012).

Association between occupation and MM risk has been widely examined; early studies proved that indoor workers are at higher risk (Cooke et al. 1984), however if stratified those occupations by education level and training requirements, again higher socioeconomic status appeared to explain the differences. Indoor workers are more prone to get sunburn as they spend much less time in the sun than outdoor workers do.

A number of studies (Gundestrup and Storm 1999, Pukkala et al. 2012) reported that airline crew had a higher-than-expected rate of MM, and explained it by more recreational sun exposures between flights, but also by the regular exposure to cosmic radiation and magnetic field. Other studies did not confirm any difference between aircrew and random Icelandic population (Rafnsson et al. 2003).

Firefighters are reported to have an excess risk of certain cancers including MM. Mostly they are not related to carcinogenic inhalations (Ma et al. 1998, Milham 2009), but to exposures of electromagnetic field and radio-frequency radiation during their work, (Milham 2009).

Role of ionization radiation in skin cancer development is supported by the observed elevated incidence rates among radiologists (Wang et al. 1990). Studies on MM incidence among nuclear industry workers showed inconsistent results (Cardis et al. 2007, Gun et al. 2008). Our data from the Hungarian nuclear power plant did not support the occupational hazard to MM development (Tóth V. et al. 2013).

I.3.1.4. Pharmacological agents

I.3.1.4.1. Vitamin D

Serum 25-hydroxyvitamin D3 level and its role in cancer development and outcome is thoroughly investigated. Sun exposure is necessary to vitamin D synthesis, but also acts as a major risk factor for MM. Some studies indicate that increased level of vitamin D is associated with excess risk of MM (Afzal et al. 2013), while others fail to prove it (van der Pols et al. 2013).

I.3.1.4.2. Statins, non-steroidal anti-inflammatory agents, others

Statins show no association between drug use and MM development (Jagtap et al. 2012).

Long term use of *non-steroidal anti-inflammatory drugs (NSAIDs)* decrease the MM risk (Curiel-Lewandrowsky et al. 2011a).

Oral contraceptives and hormone replacement therapy have no impact on MM risk. As phototoxic and photoallergic drugs intensify the UVR effect on the skin their contribution to MM development has been suspected; even short term use of quinolone and propionic acid derivative NSAIDs may increase the risk of MM (Siiskonen et al. 2013).

I.3.1.5. Pesticide exposure

More lines of evidence suggest that pesticide exposure may be additional risk factor for MM development (Leslie et al. 2010, reviewed by Weichenthal et al. 2012).

I.3.2. Immunosuppression and MM development

Organ transplant recipients (OTR) undergoing combined, long-term immunosuppressive therapy are prone to develop cancers, especially non-melanoma skin cancers (NMSC) (Otley and Pittelkov 2000). Although it is clearly documented that squamous cell carcinoma (SCC) (65-fold) (Jensen et al. 1999) and basal cell cancer (BCC) (10-fold) (Hartevelt et al. 1990) incidences are highly elevated among these patients, data on MM incidence rate is contradictory. Based on large studies, as high as 8-times elevated risk for MM development among OTRs have been observed (Le Mire et al. 2006), while in other studies the risk was not elevated at all (Lindelöf et al. 2000). In general a three-, to five-fold elevated MM risk is concluded (Jensen et al. 1999, Hollenbeak et al 2005, Moloney et al. 2006, Zwald et al. 2010), and men are more frequently affected than women, reflecting the statistical fact, that most renal transplant recipients are men (Le Mire et al. 2006, Laing et al. 2006a). Reduction or cessation of immunosuppressive therapy results in a relative good outcome even in metastatic disease (Le Mire et al. 2006, Laing et al. 2006b). According to a Hungarian database, 3141 OTRs have been reported between 1973 and 2009, from who 7 developed MM during immunosuppressive therapy (Somlai et al. 2009).

The eruptive nevi phenomenon in immunosuppressed patients (López et al. 2010, de Boer and Kuyvenhoven 2011) further supports a significant association between melanocyte proliferation and immunosuppression. Chronic lymphocytic leukemia (CLL) or HIV positive patients are also more prone to MM development.

The suspected mechanisms behind the elevated cancer risks among OTRs are 1) a weaker immune surveillance against tumors and oncogenic viral agents, 2) direct oncogenic effects of certain immunosuppressive drugs, and 3) in rare cases transmission of primary MMs via transplanted organs.

I.4. Genetics of MM

The role of inherited and acquired genetic events in cancer development is well studied. Cancer arises from clones of cells due to germline and/or somatically acquired mutations, and mature cancer clone development is further influenced by environmental factors and genomic sequence variants (reviewed in Stratton 2011).

Germline mutations are already present in the fertilized egg, and therefore in all somatic cells. These mutations are able to influence cancer susceptibility in a number of ways, like cancer clone development, mutation rate in somatic cells, and carcinogen metabolism.

Germline genetic variations include single nucleotide polymorphisms (SNPs), small microdeletions and/or insertions (indels), polymorphisms, and mutations according to their frequency and strength with disease penetrance. Certain regions in the genome may have copy-number polymorphisms that result in chromosomes with more than one or no copies of a certain gene. Epigenetic changes (DNA methylation, histone modification) and regulatory effects of miRNAs play also role in cancer development.

Both oncogenes and tumor suppressor genes might be targeted by genetic events. Oncogenes are regulators of cell division; an activating mutation in only one allele is sufficient for initiating oncogenesis by bypassing the regulatory mechanisms and overactivation (dominant effect). In contrast, tumor suppressor genes inhibit cellular proliferation and initiate apoptosis if necessary. In general, loss-off function mutation only in one allele is insufficient to alter these functions, acting in a recessive manner, therefore mutations in both copies required to the oncogenic effect. More than 80% of the currently known cancer genes are dominantly acting. The proteins encoded by these

genes serve a wide variety of key functions in cell cycle regulation, apoptosis, chromatin modification and remodeling epigenetic processes (reviewed in Stratton 2011).

Before the era of next generation sequencing, the main purpose of cancer genetics studies was to identify rare, high penetrance gene mutations. With the new technical approaches, a huge number of high frequency, low penetrance, simultaneously inherited gene variants (not even mutations) are getting identified, and new model of cancer inheritance is emerging (‘common disease common variant’) (reviewed in Pfeifer and Hainaut 2011).

I.4.1. Germline alterations in MM genesis

I.4.1.1. High penetrance genes

A proportion of MM patients belong to MM prone families or have MPM; who are more prone to harbor rare, high-risk, high penetrance MM predisposing alleles. To date, two such genes are identified in MM genesis, cyclin-dependent kinase 2A (*CDKN2A*), and cyclin-dependent kinase 4 (*CDK4*).

I.4.1.1.1. *CDKN2A* gene

This locus (MIM#600160) is located on 9p21 and encodes two proteins through alternate reading frames, namely p16/INK4a and p14/ARF. The two tumor suppressor proteins act on different pathways, but both regulate cell cycle. As a result of two different promoters, p16 is formed from exon 1α exon 2 and 3, while p14/ARF is composed of a different exon 1 (1β) located 13 kb upstream of exon 1α and a shared exon 2 with an alternative reading frame.

The protein p16/INK4A consists of 156 amino acids and exhibits a structure with four ankyrin repeat motifs. As functionality, it inhibits the CDK4/6 mediated phosphorylation of retinoblastoma protein (Rb), thus resulting in a dephosphorylated, active Rb, that binds to E2F repressing its transcriptional function and arresting G1 checkpoint in cell cycle. When *CDKN2A* is mutated, phosphorylation of Rb is not inhibited, resulting in phosphorylated-inactive Rb state that cannot bind to E2F, so E2F is able to induce G1/S phase activating gene transcriptions and cells are undergoing uncontrolled cell divisions.

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In contrast, p14/ARF is regulating p53 mediated apoptosis pathway through binding to and inhibiting the human homolog of murine Mdm2 (HDM2) that is involved in ubiquitin mediated degradation of proteins. Mutation in p14/ARF activates HDM2 function, so marking of proteins that undergo ubiquitination and subsequent degradation in proteasome becomes uncontrolled, resulting in p53 degradation and subsequent loss of p53 mediated apoptosis (summarized in Kim and Sharpless 2006)(**Figure 6**).

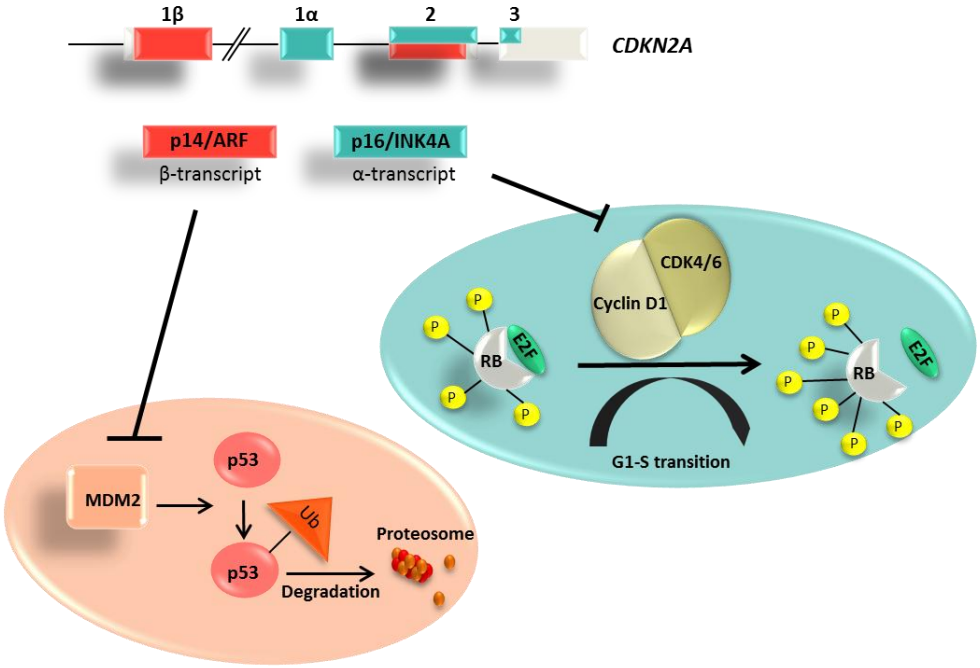

Figure 6. Structure and function of *CDKN2A* gene. Two distinct tumor suppressor proteins are encoded in the *CDKN2A* gene. P14/ARF (β-transcript-red) inhibits MDM2 mediated p53 ubiquitination, while p16/INK4A (α-transcript-blue) inhibits CDK4/6-mediated phosphorylation of Rb protein.

CDKN2A gene mutations may affect only p16/INK4A, only p14/ARF protein sequence or both; however those uniquely targeting p14/ARF (exon 1β) are relatively rare (Binni et al. 2010).

CDKN2A locus is frequently altered in many cancer types at somatic level, but the germline mutations are mostly associated with a phenotype of familiar MM or MPM and with an elevated risk of pancreatic cancer (PaC) (Lynch et al. 2002, 2008).

The mutation prevalence is about 0.2% among sporadic MM cases (Aitken et al. 1999); while approximately 10-40% of MM prone families are carriers, with geographical

differences (Berwick et al. 2006, Goldstein et al. 2006, Goldstein et al. 2007). Areas with the highest MM incidence (Australia) own the lowest mutation rates with high penetrance. Within Europe, in populations with low MM incidences (South-Europe, Mediterranean countries), *CDKN2A* mutations are far more frequent (Goldstein et al. 2007). Patients carrying *CDKN2A* mutation have a younger age at diagnosis of first MM (Goldstein et al. 2007, Pedace et al 2011), are more prone to develop subsequent MMs (Pedace et al. 2011) and are more likely to have history of MM within their family (Pedace et al. 2011, Maubec et al. 2012).

I.4.1.1.2. *CDK4* gene

CDK4 (MIM#123829) gene is located on 12q14 and contains 8 exons (**Figure 7**).

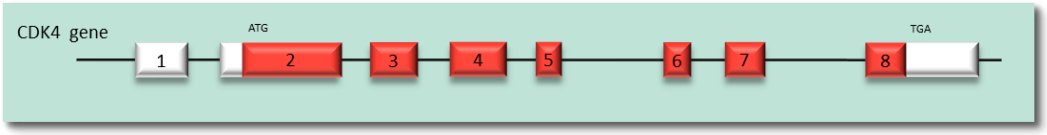

Figure 7. Structure of *CDK4* gene. Red color represents transcribed exons.

The encoded oncogene is a downstream target of p16/INK4, therefore changes in structure and function may induce similar phenotypes to those caused by *CDKN2A* mutations.

Identified mutations occur solely in exon 2 at codon 24 (Zuo et al. 1996, Soufir et al. 1998), resulting in a majority of cases in R24C (Zuo et al. 1996) amino acid change, however R24H has been also detected in some families (Soufir et al. 1998, Molven et al. 2005). To date only a small number (<15) of MM families have been identified with *CDK4* mutations (Zuo et al. 1996, Soufir et al. 1998, Helsing et al. 2008, Pjanova et al. 2007, Puntervoll et al. 2013). Phenotypes of these families do not differ from those of *CDKN2A* carriers' (propensity to MPM, younger age of onset, increased occurrence of atypical nevi) (Puntervoll et al. 2013).

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I.4.1.2. Intermediate penetrance genes

I.4.1.2.1. Melanocortin 1 receptor (*MC1R*) gene

MC1R (MIM#155555) gene is located on 16q24.3 and encodes for melanocortin 1 receptor, a 317 amino-acid G protein-coupled receptor with seven transmembrane domain that is expressed predominantly in epidermal and uveal melanocytes with a major role of regulating hair-, skin and eye pigmentation. Expression is also detectable in pituitary cells, leukocytes, mast cells and pro-monocytes. The receptor is a member of the melanocortin receptor family that contains five differentially expressed G-protein-coupled receptors (MC1R-MC5R). Under physiological conditions MC1R is activated by binding of its ligands alfa-melanocytes stimulating hormone (α-MSH) or adrenocorticotropic hormone (ACTH). The subsequent cAMP production via adenylate cyclase (AC) activates cAMP dependent protein kinase A (PKA) and a number of downstream targets like cAMP response element binding protein (CREB), microphtalmia-associated transcription factor (MITF). These events lead to proliferation and to a switch from red/yellow pheomelanin to the brown/black eumelanin production. Eumelanin has a major role to protect DNA from UVR as a shield, while skin pigmentation is subsequently augmented (**Figure 8**). In contrast pheomelanin has a weak shield effect to protect DNA of melanocytes against UVR, and has shown to amplify UVA-induced ROS production (Rouzaud et al. 2005, Hill HZ and Hill GJ 2000).

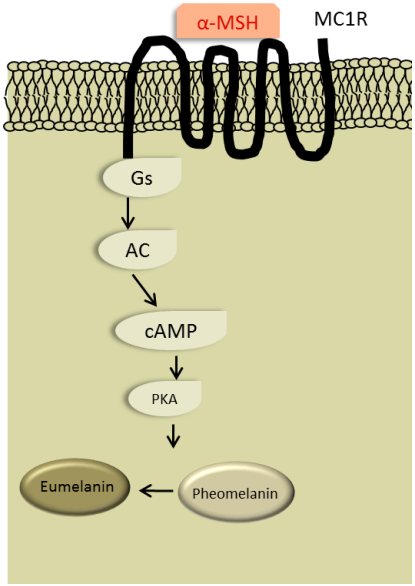

Figure 8. *MC1R* receptor function; See details in text. Abbreviations: Gs:G-proteins, AC: adenylate cyclase, PKA: cAMP dependent protein kinase A.

The *MC1R* is a highly polymorphic gene (Gerstenblith et al. 2007). The non-synonymous variants have different effect on the receptor function. While gain-of-function mutations are not reported in human to date (reviewed in Rees 2004), loss-of-

function alleles can either result in impaired ability to bind α-MSH (R163Q) or to impaired activation of the G protein-coupled pathway of cAMP dependent kinases (V60L, R142H, R151C, R160W) (Ringholm et al. 2004). In some variants both distresses are detectable (D84E, V92M, D294H) (Ringholm et al. 2004). As carrying more variants is also common in homozygous or even compound heterozygous fashion, effect of the different allele combinations may result in a wide range of functional failures and in an elevated pheomelanin/eumelanin ratio in melanocytes.

The constitutive pheomelanin production by the genetically and subsequently functionally impaired receptor in certain *MC1R* variant carriers may manifests in red hair color (RHC) phenotype with fair skin, freckles and poor tanning ability (Valverde et al. 1995, Smith et al. 1998). These variants are referred as 'R' variants. Recent findings suggest that complete loss-of-function effects are rare even among these 'R' allele carriers (Newton et al. 2005). Variants with less destructive effects on receptor function and phenotype called non-RHC (NRHC) alleles, and labeled as 'r' alleles. The most frequent *MC1R* variants and their effect on RHC phenotype and MM risk are summarized in **Table 1**.

Table 1. The most frequent *MC1R* variants and their individual associations (Odds ratios: OR) to RHC phenotype and MM risk.

***MC1R* variants**	**Nucleotide change at cDNA level**	**Amino acid change**	**Strength of association with RHC phenotype (OR)**	**Strength of association with MM risk (OR)**	**References**
'R'	252 C>A	D84E	2.99	2.4	Box et al. 1997
	425 G>A	R142H	4.96	1.66	Duffy et al. 2004
	451 C>T	R151C	8.1	1.78	**Raimondi et al. 2008**
	464 T>C	I155T	1.2	2.45	Beaumont et al. 2007
	478 C>T	R160W	5.00	1.43	
	880 G>C	D294H	5.92	1.77	
'r'	178 G>T	V60L	0.5	1.15	Cust et al. 2012
	274 G>A	V92M	0.42	1.22	Sturm et al. 2003
	488 G>A	R163Q	0.35	1.42	Ibarrola-Villava et al. 2012

Abbreviations: OR: odds ratio

It has long been suspected that MM risk among *MC1R* variant carriers isn't only via an UV dependent way, as MMs often develop on sun protected body sites, moreover UV signature mutations are uncommon driver mutations (Curtin et al. 2005). The altered pheomelanin synthesis (pheomelanin or its any intermediate-, or by-product) might result in a perturbed intrinsic susceptibility of certain carcinogenesis (Mitra et al. 2012). A number of meta-analyses specified the role of *MC1R* variants in MM development. Certain 'r' variants carry also increased MM risk independently of the phenotype effects (Raimondi et al. 2008, Fargnoli et al. 2010, Kanetsky et al. 2010, Williams et al. 2011). The number of 'R' or 'r' variant alleles may also enhance MM risk (Goldstein et al. 2005, Pastorino et al. 2008, de Torre et al. 2010). Most recent meta-analysis on *MC1R* variants and MM risk confirmed ORs for 'R' variants between 1.00 to 4.64, and for 'r' variants between 0.58-3.00 (Williams et al. 2011) (**Table 1**).

The frequency and distribution of variants vary among populations and along continents. Frequency in African populations is very low, therefore it is also suspected that eumelanin production represent an evolutionary benefit in those geographical regions (summarized in Makova and Norton 2005). In Asians the R163Q is an exceptionally common variant with a 70% average frequency in contrast with other locations where it is observed at a much lower incidence (almost absent in European populations, observed at a frequency of 7% in Indians) (Rana et al. 1999). In European populations the *MC1R* gene is especially polymorphic, with more than 70 variants identified to date (Gerstenblith et al. 2007). Among Caucasians, variant frequencies are higher among lightly pigmented populations than among darkly-pigmented ones (reviewed in Makova and Norton 2005), in accordance with findings suggesting a decreasing gradient of 'combined R variant' frequency from Northern to Southern Europe (Gerstenblith et al. 2007).

I.4.1.2.2. *MITF* gene

The gene (MIM#156845) is located on chromosome 3p14-p13, exhibits nine promoters with corresponding different MITF isoforms that own different first exons and shared exons 2-9. The encoded protein is a basic helix-loop-helix-leucine zipper protein. Only isoform M is melanocyte-specific and expressed exclusively in melanocytes and MM cells (reviewed by Levy et al. 2006).

MITF-M is key regulator of melanocyte development, survival and function by regulating a number of differentiation and cell-cycle progression genes. MITF directly binds to the promoter of p16/INK4A and regulates positively its transcription (Loercher et al. 2005). During activation of *MC1R* by α-MSH, phosphorylation of CREB occurs, that then binds directly to *MITF* promoter and stimulates it's transcription (Bertolotto et al. 1998).

Germline loss-of-function mutations in *MITF* gene results in Waardenburg syndrome IIA, an autosomal dominantly inherited disease that is characterized by melanocyte deficiencies of the eye, forelock and inner ear, the latest of which causing sensorineural hearing impairment.

The role of MITF as an oncogene in MM genesis was first described by identification of copy gains at *MITF* locus in MM cell lines using SNP arrays (Garraway et al. 2005). Moreover tissue microarrays proved that 10-20% of MMs exhibit amplification of *MITF* that is proven to be a late event in MM genesis, therefore is more common in metastatic cases, and represents a worse outcome (Garraway et al. 2005). Recently a germline missense mutation (c.G1075A; p.E318K) was identified to be associated with a 4-fold elevated MM risk and with MPM formation, while carriers exhibited a 14-fold risk to MM and renal cell cancer. Impairment of MITF SUMOylation with insufficient cellular stress management leads here to initiation of tumor formation (Bertolotto et al. 2011). Furthermore certain phenotypes such as increased nevi number and susceptibility to amelanotic melanoma (Sturm et al. 2014) are also observed among carriers of this mutation. Interestingly most patients harboring *MITF* E318K and amelanotic melanoma exhibited *MC1R* genotypes of homozygous 'R' variants, suggesting that an altered MC1R receptor together with the this *MITF* point mutation may result in this relatively rare MM subtype (Sturm et al. 2014).

I.4.1.3. Low penetrance genes

MM susceptible host factors such as nevi count, skin pigmentation, ability to tan and freckles are inherited with a polygenic trait. Some of these genes, together with other immune related-, DNA repair-, and metabolic genes together with vitamin D3 receptor polymorphisms are also linked to MM formation however by lesser strength (summarized in **Table 2**).

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Table 2. Low penetrance MM susceptibility and prognostic genes (According to reviews Wiesner et al. 2011, Ward et al. 2012).

Pigmentation/ naevus count genes	Immune genes	DNA-repair genes	Metabo-lism genes	*VDR* polymor-phisms	Others
ADTB3A *ASIP* *ATRN* *CHS1* *EDNRB* *HERC2* *HPS* *IRF* *KITLG* *MGRN1* *MLANA* *MYO5A* *MYO7A* *NID1* *OCA2* *PAX3* *PLA2G6* *SLC24A4* *SLC45A2 (MATP)* *SOX10* *TYR* *TYRP1*	*DQB1*03 01* *HLA class II allele* *ICAM-1* *IFN-γ* *IL-10* *IL-1β* *IL-6R* *LT- α* *TNF-α*	*APEX1* *BAP1* *ERCC1* *MDM2* *MGMT* *TERT1* *TERT-CLPTMIL* *TRF1* *XPD/ERCC2* *XPF* *XRCC1* *XRCC3*	*CYP2D6* *GSTM1* *GSTP1* *GSTT1*	A-1012G ApaI Bsml Fokl Taql	*EGF* *RB1*

I.4.2. Somatic mutations and polymorphisms in MM

Somatic mutations may occur in the genomes of normal dividing cells during fetal development or in postnatal life. The rate and type of these mutations are determined by exogenous and endogenous exposures inducing DNA damage or altered DNA repair processes. Somatic mutations are distributed throughout the genome more or less randomly. In the cells that undergo clonal expansion to become a cancer, the original so called driver mutations disturb the control of cell proliferation, differentiation, apoptosis or homeostatic interactions with the tissue microenvironment. Driver mutations confer growth advantage of neoplastic clones therefore they can expand more than the normal cells, invade the healthy tissues or metastasize. Most mutations are passenger mutations,

which usually do not confer growth advantage (reviewed in Hanahan and Weinberg 2000, and in Stratton et al. 2011).

Somatic events during MM formations may occur along multiple signal pathways simultaneously (**Figure 9**), namely:

1) Activation of the MAPK pathway,

2) Inactivation of the p16/INK4A-CDK4/6-Rb senescence barrier,

3) Inactivation of p53/ARF barrier or

4) Activation of the PTEN/PI3K/AKT pathway.

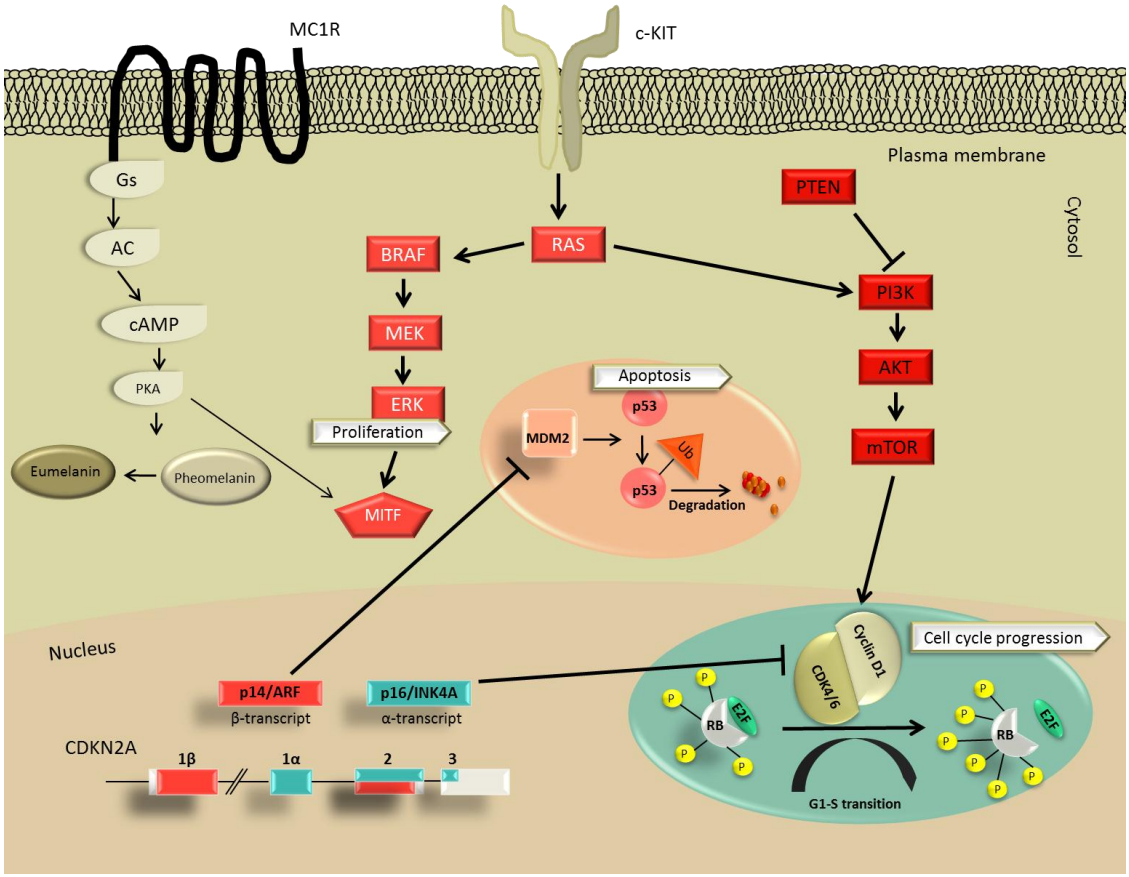

Figure 9. Summary of signal transduction pathways in MM development.

To date *BRAF*, *NRAS*, *c-KIT* and *MITF* somatic mutations are considered as driver mutations during MM development and progression (Curtin et al. 2005). The first therapeutic experiences with BRAF inhibitors in MM showed that MAPK and PI3K/AKT concomitant pathway activations induce acquired resistance during the course of therapy (reviewed in Hocker and Tsao 2008 and Lo 2013).

A number of additional genes might harbor somatic driver mutations in MM (*PPP6C, RAC1, SNX31, TACC1, STK19*) (Hodis et al. 2012).

The most common genes affected at somatic level in MM development and their association with other tumor features is summarized in **Table 3**.

Table 3. Most common genes with somatic alterations in MM genesis and their associated characteristic clinical features.

Genes	*BRAF*	*RAS (NRAS)*	*c-KIT*
Frequency	60-70%	15-33%	
Body site specificity	Intermittently sun exposed	Non-CSD Head and neck	Glabrous skin Nail/mucosal MM, CSD
Histopathologic specificity	SSM	NM /LM > SSM	Lentiginous growth pattern
Mutations in nevi	Occur	In congenital nevi	No
Other characteristics	Younger patients with high naevus count		Older patients, poorer survival
Germline effect	Cranio-facio-cutan syndrome (OMIM#115150)	RASopathies	Familial gastrointestinal stromal tumors, Piebaldism
References	Davies et al. 2002, Bauer et al. 2011	Chin et al. 1999, Platz et al. 2007	Jin et al. 2013, Giebel et al. 2004, Isozaki et al. 2000

Abbreviations: CSD: chronic sun damage, SSM: superficial spreading melanoma

I.4.2.1. MAPK pathway activation in MM

MAPK pathway is a key regulator of cell proliferation, differentiation, survival and death, and also has a significant role in MM development. The most frequently mutated member of this pathway is BRAF, but almost every MM harbors mutations in any of this signal pathway's proteins (Fecher et al. 2008). Type of mutations (*BRAF, NRAS*) varies upon body site and histology (**Table 3**).

I.4.2.1.1. *BRAF* in MM

BRAF is an oncogene, a protein kinase located in the cytosol. Somatic *BRAF* mutations are detected in 60-70% of MMs and the V600E substitution accounts for more than 90% of them (Davies et al. 2002). As many benign and dysplastic nevi (~80%) express also V600E (Pollock et al. 2003), it is questionable whether it is an early event in MM

genesis (reviewed in Palmieri et al. 2009), or might be a marker from ‘in situ’ to invasive progression state (Greene et al. 2009). It is however evident now that this *BRAF* mutation alone is not sufficient for MM formation.

I.4.2.1.2. *RAS* in MM

RAS is one of the most studied oncogene in human cancers; it is a 21kDa protein with a GTPase activity regulating receptor tyrosine kinase-induced MAPK activation. It binds to and activates PI3K, and its downstream targets (AKT), and interacts with p16/INK4A and p53 too (summarized in Chin et al. 1999).

Activating mutations in *NRAS* are found in approximately half of congenital nevi, and one third of MMs (Chin et al. 2006). Besides the most prominent *NRAS* mutations in MM, other RAS family members, such as *HRAS* (1%) and *KRAS* (2%) are also altered in MM (Forbes et al. 2008). *HRAS* mutations were detected to be at a higher frequency (7.7%) in NM (Jafari et al. 1995).

Germline mutations of RAS proteins cause RASopathies, and these patients are not reported to have elevated risk to MM development.

I.4.2.2. p16/INK4A-CDK4/6-RB inactivation

I.4.2.2.1. CDKN2A

Somatic loss of the 9p21 chromosome region encoding *CDKN2A* locus may manifests via deletion, point mutation or even by epigenetic changes and is a common event in many human cancer types such as MM, PaC, lung cancer (LC), high-grade glioma, colon adenocarcinoma, breast adenocarcinoma, biliary tract-, head and neck tumors and transitional cell carcinoma of the bladder (Dahl and Guldberg 2007, Bennett 2008). In MM, somatic *CDKN2A* alterations are detected in 30-70% of sporadic MMs (Bartkova et al. 1996, Walker et al. 1998).

I.4.2.2.2. CDK4, Rb1

Somatic mutations in *CDK4* are common at the 24th (Wolfel et al. 1995), as well as at the 22nd amino acid position (Bennett 2008, Dahl and Guldberg 2007), while amplification of this oncogene is also observed in MMs (Muthusamy et al. 2006).

Rb1 protein is less commonly affected in MM and mainly by nonsense mutations at somatic level (Bartkova et al. 1996).

I.4.2.3. ARF/p53 inactivation in MM

ARF deletion occurs around 50% of MMs (Curtin et al. 2005), while protein p53, the common tumor suppressor gene of many cancer types, is altered only in 10-30% (Albino et al. 1994, Zerp et al. 1999).

I.4.2.4. PTEN /PI3K/AKT pathway activation in MM

PTEN is altered in 30-40 % of cultured MM cell lines and approximately 10% of primary MMs (Guldberg et al. 1997, Tsao et al. 1998) by mutation, allelic loss, epigenetic silencing by methylation (Mirmohammadsadegh et al. 2006) or impaired subcellular localization (Trotman et al. 2007). It is still controversial whether *PTEN* loss is an early or late event in MM genesis (summarized in Palmieri et al. 2009). As PTEN downregulates AKT and MAPK pathways, it is possible that RAS activation and PTEN loss confers a reciprocal mutation status (Nogueira et al. 2010).

I.4.2.5. MC1R in MM at somatic level

Besides the well-known effects of *MC1R* germline variants on MM predisposition, no somatic genetic or copy number alterations have been proved so far in any type of MM. (Valverde et al. 1996, Kim et al. 2008).

I.4.2.6. Novel signaling pathways in MM

Further genes with lesser significance might also undergo somatic mutations, deletions, amplifications, or epigenetic changes (promoter hypermethylation) (**Table 4**).

Table 4. List of further genes involved in MM genesis at somatic level
(On the basis of Dahl and Guldberg 2007, with personal changes).

Gene type	Gene	Altered signaling pathway	Most frequent genetic changes	Frequ-ency %	Ref.
Oncogenes					
	CTNNB1		Mutation	2-23	
	CCND1/ Cyclin D		Amplification	6-44	Sauter et al. 2002
	MITF		Amplification	10-16	
	AKT3	PI3K	Amplification	40-60	

Gene type	Gene	Altered signaling pathway	Most frequent genetic changes	Frequ-ency %	Ref.
	ERBB4	AKT	Mutation	19	Prickett et al. 2009
Tumor suppressor genes					
	MTAP		SNP		Bishop et al. 2009 Stevens et al. 2009
	STK11/ LKB1		Mutation	10	Forbes et al. 2011
Others					
Non-receptor tyrosine kinase (glutamate) receptors	*GRIN2A*		Mutation	25	Wei et al. 2011
	GRM3		Mutation	16.3	Prickett et al. 2011

Abbreviations: Ref: reference, SNP: single nucleotide polymorphism

I.4.2.7. MicroRNAs (miRNA)

miRNAs are small noncoding RNAs with posttranscriptional regulatory function. They modulate the gene expression mainly by translational repression or mRNA degradation and might also contribute to MM genesis and/or progression. Dysregulation of miRNAs by genetic or epigenetic alterations and aberrant miRNA biogenesis may lead to down-, or upregulated miRNAs. More miRNAs regulate *MITF* expression (miR-137, miR-148 and others) promoting development and invasiveness of MM (reviewed by Bell and Levy 2011). miRNA expression profiles of primary and metastatic MMs are associated with somatic *BRAF* or *NRAS* mutational status and predict survival and sites of distant metastasis (Caramuta et al. 2010).

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I.5. MM-associated other primary malignancies

MM survivors are at an increased risk of developing subsequent primary tumors with an incidence rate of 6.4-20% (Levi et al. 1997, Bhatia et al. 1999, Wolff et al. 2000, Schmid-Wendtner et al. 2001, Crocetti et al. 2008, Bradford et al. 2010, Spanogle et al. 2010). The most frequently observed high risk tumor is second and subsequent MM.

I.5.1. Multiple primary melanomas (MPM)

I.5.1.1. Incidence, etiology

Pack described firstly the phenomenon of MPM in 1952 (Pack et al. 1952). Among patients with a history of primary MM, second primary MM occurs in 3.4-8.3% (Slingluff et al. 1993, Giles et al. 1995, Blackwood et al. 2002, Ferrone et al, 2005, Helsing et al, 2008, Savoia et al, 2012).

It is important to ensure that subsequent MMs are primary tumors and not cutaneous epidermotropic metastasis of previous MM (Heenan et al. 1991, Bengoechea-Beeby et al. 1993). A recent report on somatic mutation patterns of first and subsequent MMs excluded any common clonal origin, and proved that subsequent MMs are indeed clinically and histologically independent primary tumors (Orlow et al. 2009). Estimating the real MPM incidence on large MM populations is limited by the difficulty of follow-up for a long period of time.

The majority (63-88%) of patients develop two primary MMs (Ferrone et al. 2005, Savoia et al. 2012), however higher number of primaries are also observed (Ferrone et al. 2005, Savoia et al. 2012, Hwa et al. 2012)

Most strongly associated risk factors include positive family history of MM (Giles et al. 1995, Blackwood et al. 2002) and personal history of dysplastic naevus (Blackwood et al. 2002, Titus-Ernstoff et al. 2006, de Giorgi et al. 2010) or a higher number of common nevi (de Giorgi et al. 2010), all suggesting an underlying genetic contribution factor. Additional risk factors are 1) high UV irradiance at birth and before 10 years of age, 2) lifetime recreational sun exposure, 3) beach and waterside activities and 4) vacations in sunnier climate (Kricker et al. 2007).

I.5.1.2. Clinicopathological characteristics

The mean age at diagnosis of first MM is lower if number of primary MMs rise (Helsing et al. 2008). Second MM develops often within the first 1-2 years from the initial MM diagnosis (Ferrone et al. 2005, Hwa et al. 2012, Savoia et al. 2012), and there is also a strong site concordance (42-56%) (Ferrone et al. 2005, Savoia et al. 2012, Slingluff et al. 1993, Giles et al. 1995, Bower et al. 2010, Manganoni et al. 2012, Hwa et al. 2012). The subsequent MMs are showing a trend of being thinner, the proportion of Mis rises and they exhibit histological features with more favorable prognostic value (less ulceration, more regression) (Brobeil et al. 1997, Ferrone et al. 2005, Murali et al. 2012a, Hwa et al. 2012). SSM is the most frequently presented histological subtype among MPM, while NM is far less frequent (Vecchiato et al. 2013).

MPM patients have a better survival than single primary melanoma (SPM) patients (Doubrowsky et al. 2003, Bower et al. 2010, Murali 2012b, Kricker et al. 2013). Two possible explanations are 1) the careful follow-ups of these patients, that have been proven to be a reason why the subsequent MMs are thinner; and 2) a distinct biological behavior of these tumors (summarized in Hwa et al. 2012). Comparing prognostic features such as mitotic rate, ulceration and pathologic stage of the primary MMs in SPM and MPM patients, no significant difference have been detected (Hwa et al. 2012).

I.5.1.3. Genetics of MPM

I.5.1.3.1. CDKN2A

Familial MM and MPM patients own a higher genetic predisposition, therefore are particularly good candidates exploring germline genetic background of MM susceptibility. Many studies have been conducted in order to determine predisposing gene mutation frequency among MPM patients. Mutation rate of *CDKN2A* among MPM patients is higher than among SPM patients, varying between 2.9-32.6%, from which sporadic MPMs exhibit 8-15% of mutation rate, while it can be much higher (even 63%) if family history is positive for MM (summarized in **Table 5**). Factors such as selection bias, geographical differences of MM incidences, overrepresentation of familial MM cases, founder effects and sensitivity differences in terms of mutation detection may explain this wide range of prevalence (Helsing et al. 2008). Risk of

germline *CDKN2A* mutation raises with the number of MMs (Puig et al. 2005, Helsing et al. 2008, Pastorino et al. 2008) irrespectively of positive family history. Type of *CDKN2A* mutations doesn't differ between MPM, SPM or familial MM cases. *CDKN2A* mutation carrier MPM patients develop their first MM at an earlier age than mutation negative patients (Helsing et al. 2008, Pastorino et al. 2008).

Table 5. Published frequencies of *CDKN2A* mutations among MPM patients.

Reference	Year	Origin	Type of sample	Number of studied MPM patients	*CDKN2A* mutation frequency among MPM patients (%)		
					Total	Sp	Fam
Monzon et al.	1998	USA		33 sp	-	15	-
Hashemi et al.	2000	Sweden	P	80 total: 65 sp 15 fam	11.25	3	46.6
Auroy et al.	2001	France		100 sp	-	9	-
Mantelli et al.	2002	Italy, Liguria		23 fam	-	-	47.8
Blackwood et al.	2002	USA		96 total	-	8.3	-
Peris et al.	2004	Italy	H	14 sp	-	21.4	-
Puig et al.	2005	Spain, Catalonia	H	104 total: 73 sp 31 fam	16.3	8.2	35.5
Berwick et al.	2006	GEM study*	P	1189 total: 929 sp 260 fam	2.9	1.8	6.9
Pastorino et al.	2008	Italy		95 total	32.6	18.5	63.3
Helsing et al.	2008	Norway	P	390 total: 309 sp 81 fam	6.9	3.2	21
Majore et al.	2008	Italy	H	155 total: 61 sp 18 fam	-	4.9	44.4
Lukowsky et al.	2008	Germany		40 sp	-	2.5	-
Grazziotin et al.	2012	Brazil		5 sp	-	0	-

*GEM (Genes, Environment and Melanoma) study population consists of 8 population-based cancer registries from nine geographic regions: Australia (New South Wales and

Tasmania), Canada (British Columbia and Ontario), Italy (Piemonte), and United States (Orange County and San Diego County from California, Michigan, New Jersey, North Carolina).

Abbreviations: P: population based; H: hospital or clinic-based; sp: sporadic, fam: familial

Besides *CDKN2A* gene mutations, the c.442 G>A resulting p.A148T amino acid change located in exon 2, as the third most common polymorphism in the gene, seems to occur also statistically more frequently in MPM patients than in healthy controls: 13.5% versus 5.45%; p=0.05 (Puig et al. 2005), and 15.7% versus 6.6 %; p=0.011 (Pastorino et al. 2008). Frequency differences between healthy compared to SPM patients are debated; Pastorino et al found no difference (Pastorino et al. 2008), while in Brazil this variant was more frequent in MM patients than in controls (12.6% versus 3.9%; p=0.009), and was also more frequent if family history of cancers other than MM was positive (Bakos et al. 2011).

I.5.1.3.2. MC1R

Carrying multiple variant alleles of *MC1R* gene is in association with the development of subsequent primary MMs both in *CDKN2A* mutation positive (Goldstein et al. 2005, Fargnoli et al. 2010) and negative MM patients (Pastorino et al. 2008, de Torre et al. 2010). Kanetsky and co-workers found another, more qualitative link between MPM formation and carrier status of 1 or 2 'R' alleles, or 'R'/'r' alleles, while carrier status of 2 'r' alleles did not follow this trend (Kanetsky et al. 2010). In *CDKN2A* mutation positive MPM patients, as number and type of variants increase, the median age of onset decrease significantly (Goldstein et al. 2007).

There are only a few studies on *MC1R* variants among pure MPM patients (Kanetsky et al. 2006, Helsing et al. 2008), therefore so far no pattern or variant specificity among this patient population have been observed.

Role of *CDK4* and *MITF* E318K mutation in MPM are discussed above (I.4.1).

I.5.1.4. Other non-melanoma primary malignancies among MPM patients

Second primary cancer among MM survivors are commonly explored, however risk of developing subsequent non-melanoma primaries among MPM patients is rarely

discussed. Manganoni and co-workers reported recently, that non-melanoma primaries are more common among MPM than SPM patients (OR: 2.1; 95%CI 1.11-3.97) (Manganoni et al. 2012). Majority of these tumors were NMSC, while breast cancer and prostate cancer (PrC) were also observed among others. When comparing SPM and MPM patients regarding MM associated malignancies MPM patients have significantly more co-occurred malignant tumors (Pollio et al. 2013).

I.5.2. MM and co-aggregation of other cancers

Further tumors that are most consequently established with increased risk among MM survivors are NMSC, pancreas, non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL), female breast-, kidney-, bladder-, corpus uteri-, small intestine-, brain-, oropharynx-, salivary gland-, soft tissue-, and thyroid-, prostate-, and bone cancers (**Table 6**). Interestingly some malignant tumors are less frequently observed among MM survivors, namely LC (Crocetti et al. 2008, Balamurugan et al. 2011), and liver cancer (Crocetti et al. 2008).

Table 6. Summary of studies on MM associated subsequent primary malignancies.

Reference	Type of sample	Patient number	SIR	Incidence rate %	Overall risk %	Cancers with significantly increased risk
Wassberg et al. 1996	P	20,354	1.45	7.9		Nervous system tumors, leukemia Colon cancer in men
Wolff et al. 2000	H	554	-	11		MM, BCC
Schmid-Wendtner et al. 2001	H	4597		6.4		Kidney cancer in men
Wu et al. 2006	H	955		6.2		NHL and renal cell cancer in men
Crocetti et al. 2008	P	14,560	1.27	-	27	NMSC, bone, kidney
Bradford et al. 2010	P	89,515		12.1	28	Breast, PrC, NHL
Spanogle et al. 2010	P	16,591		11	32	Soft tissue, MM of the eye and orbital, non-epithelial skin, salivary gland, bone and joint, thyroid, kidney, CLL, brain

Reference	Type of sample	Patient number	SIR	Incidence rate %	Overall risk %	Cancers with significantly increased risk
						and nervous system, NHL, PrC, and female breast
Balamu-rugan et al. 2011	P	40,881 Mis 6041 MM			32% men, 35% women 57% men 64% women	CLL CLL CLL, NHL thyroid CLL, NHL, thyroid

Abbreviations: SIR: standardized incidence ratio, NHL: non-Hodgkin lymphoma, CLL: chronic lymphocytic leukemia, NMSC: non-melanoma skin cancer, Mis: melanoma in situ, P: population-based study, H: hospital-based study

In our previous study analyzing subsequent primary tumor development in 740 MM survivors showed an extremely elevated risk of subsequent MM (SIR men:160; women: 93), Mis (SIR men: 342, women: 77) and NMSC (SIR men: 17, women: 18), while regarding non-skin malignancies, significantly elevated risk was observed for CLL, colon-, and kidney cancers in both genders, NHL and cervical cancer in women only, and bladder cancer in men (Tóth V, Hatvani Z. et al. 2013).

Co-occurring tumor types may indicate common environmental and/or genetic background, while coincidental risk factor exposures must be also taken into account. Early MM diagnosis is in most cases followed by only surgical therapy, therefore irradiation and systemic chemotherapy in the genesis of subsequent primaries may not play significant role. Socioeconomic status may also have an impact as it represents a pattern of environmental risk factors; and certain risk factors may coincidentally affect individuals with higher socioeconomic status (e.g.: late pregnancies and holiday/recreational sun exposure habits to elevated risk of breast cancer and MM) (summarized in Spanogle et al. 2010).

Common genetic and environmental factors of the most frequent tumors associated with MM are summarized in **Table 7**.

Table 7. Shared genetic and environmental factors contributing to co-occurrence of MM and other primary malignancies.

	Shared risk factors with MM			**Reference**
Type of malignancy	Genetic	Environmental	Other	
NMSC	*MC1R* variant number and type	UV	Skin screening effect	Crocetti et al. 2008, Bastiaens et al. 2001, Ferrucci et al. 2012
PaC	*CDKN2A* *MTAP* *RAS* *STK11* *BRCA2*	Obesity (?)	-	Soufir et al. 1998, Goldstein et al. 2004, Borg et al. 2000, Almoguera et al. 1988, Guldberg et al. 1999, Su et al. 1999, Hustinx et al. 2005
NHL	*CDKN2A* (9p21)	1.UVR 2.Tumor related immunodeficiency 3.UVR-related immunodeficiency 4.Pesticides	Surveillance bias	Pollack and Hagemeijer 1987 Spanogle et al. 2010 Adami et al. 1995 Jadayel et al. 1997 Kato et al. 2004
Breast cancer	*BRCA2* *CDKN2A* *BAP1*	Socioeconomical status		Goggins et al. 2004 Goggins et al. 2006, Wiesner et al. 2011
PrC	*CDKN2A* *TSPY* *PTEN* somatic *BRCA1* *BRCA2*	Pesticides	Age	Van Maele-Fabry and Willems 2004, Monahan et al. 2010, Belinsky et al. 1998, Komiya et al. 1995, Lee et al. 1999, Oram et al. 2006, Birck et al. 2000, Li et al. 1997, Ford et al. 1994
Kidney cancer	*MITF* E318K (RCC)	-	Common immunologic background	Bertolotto et al. 2011, Rosenberg 2000
Thyroid cancer	*BRAF*	-	-	Goggins et al. 2006 Davies et al. 2002
CLL **Hairy cell leukemia**	*BRAF*	Pesticides	-	Davies et al. 2002 Alavanja and Bonner 2012

Abbreviations: NMSC: non-melanoma skin cancer, PaC: pancreatic cancer, NHL: non-Hodgkin lymphoma, PrC: prostate cancer, RCC: renal cell cancer, CLL: chronic lymphoid leukemia, UVR: ultraviolet radiation

There are some limitations concerning data on subsequent cancer types among MM survivors. Patients with MM are under a strict follow up focusing on skin-, and regional lymph node status together with distant metastasis imaging and obtained laboratory tests. This enhanced surveillance assures early cancer detection.

I.5.3. Hereditary cancer predisposing syndromes and MM

Approximately 5-10% of all cancers develop in individuals with higher susceptibility to certain cancers due to an inherited genetic background. Tumor clustering is more common than expected in these kindred. Tumor predisposing syndromes with any link to MM are summarized in **Table 8**, and those with significant relevance to this work are detailed below.

Table 8 on the next page. List of tumor predisposing syndromes that are reported with any associations with MM (based on Tsao 2000, Garber and Offit 2005 with personal completions).

Abbreviations: MM: malignant melanoma, chr: chromosome, NMSC: non-melanoma skin cancer, BCC: basal cell cancer, SCC: squamous cell cancer, PaC: pancreatic cancer, PrC: prostate cancer, CS: Cowden syndrome, RR: relative risk, ALL: acut lymphoid leukemia, AML: acut myeloid leukemia.

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Syndrome	OMIM entry	Mode of inheritance	Underlying genes (chr. locations)	Protein function	Tumor spectrum	Observed MM risk	References
Familial MM	606719	Dominant	*CDKN2A* *CDK4*	Tumor suppressor	**MM**, PaC	-	
Xeroderma pigmentosum (XP)	194400 133510 278700-278800	Recessive	*XPA, B,C,D,E,F,G, POLH*	Nucleotid excision repair (NER), global genome repair	**NMSC** (SCC, BCC), MM Oral cancer	600-8000x	Kraemer et al.1994
Hereditary retinoblastoma	180200	Dominant	*RB1* (13q14.2)	Tumor suppressor	**Retinoblastoma** Osteosarcoma	4-80x	reviewed in Nelson and Tsao 2009
BRCA-associated breast and ovarian cancer syndromes	113705, 600185	Dominant	*BRCA1* (17q21.31) ***BRCA2*** (13q13.1)	Tumor suppressor Tumor suppressor	**Breast** **Ovarian** PrC PaC BCC	*BRCA2*: RR: 2.58 MM	Easton et al. 1997, Iscovitch et al. 2002, Ford et al. 1994, Liede et al. 2004, The Breast Cancer Linkage Consortium 1999, Ginsburg et al. 2010
Li-Fraumeni Syndrome	151623	Dominant	*p53* (17p13.1) *CHEK2* (22q12.1)	Tumor suppressor Tumor suppressor	Soft tissue sarcoma Breast cancer Osteosarcoma Leukemia Brain tumors	Rare, contro-versial	Curiel-Lewandrowski et al. 2011b
Lynch syndrome (Hereditary non-polyposis colorectal cancer)	120435	Dominant	mismatch repair (MMR) genes: *MLH1,* *MSH2,* *MSH6,* *PMS2*	DNA repair at replication	Colon cancer Endometrial cancer Ovarian cancer Renal pelvis cancer Ureteral cancers PaC Stomach, small bowel cancer Hepatobiliary cancer	Incidental	Alvino et al. 2002, Hussein et al. 2001 Ponti et al. 2008
MEN-1 syndrome	131100	Dominant	*MEN-1* (11q13)	Tumor suppressor	Pancreatic islet cell tumor Pituitary adenoma Parathyroid adenoma	Incidental	Baldauf et al. 2009, Nord et al. 2000
Peutz-Jeghers syndrome	175200	Dominant	*STK11/LKB1* (19p13.3)	Tumor suppressor	Colon cancer Small bowel cancer Breast cancer	Incidental	Braitman 1979, Wong and Rajakulendran 1996 Guldberg et al. 1999, Rowan et al. 1999, Liu et al. 2012
PTEN hamartoma tumor syndrome (PHTS)	153480 158350 158350	Dominant	*PTEN*	Tumor suppressor	Hamartomas	CS: 5% lifetime risk	Greene et al. 1984 Eng 2012
LEOPARD syndrome (RASopathy)	151100	Dominant	*PTPN11,* *RAF1,* *BRAF*	Oncogene Oncogene Oncogene	Juvenile myelomonocytic leukemia like disease, Neuroblastoma, ALL and AML	Incidental	reviewed in Hernández-Martín and Torrelo 2011, Martínez-Quintana and Rodríguez-González 2012, Seishima et al. 2007
Type-1 Neurofibromatosis	162200	Dominant	*NF1*	Tumor suppressor	Neurofibrosarcoma Pheochromocytoma Optic glioma Meningeoma	Incidental	reviewed in Hernández-Martín and Torrelo 2011,

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I.5.3.1. Breast-ovarian cancer syndrome

BRCA1 and *BRCA2* are tumor suppressor genes, whose protein products play essential role in different cellular pathways including cell cycle checkpoint activations and DNA damage repair processes (summarized in Venkitaraman 2002, O'Donovan and Livingston 2010) (**Figure 10/b**).

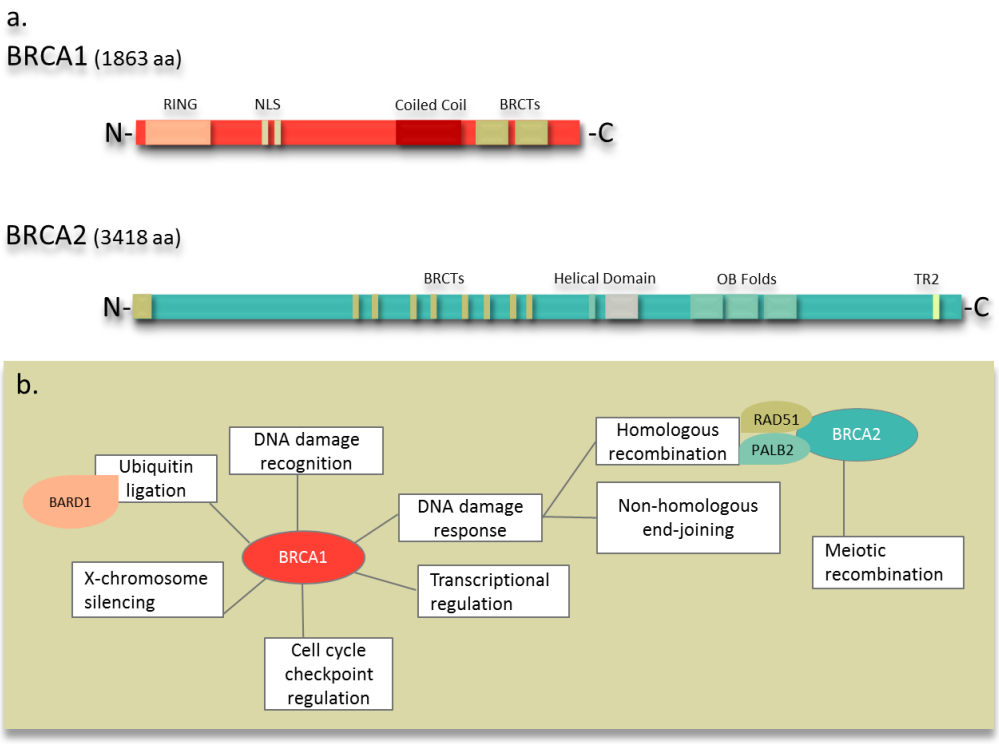

Figure 10 a.) Structure of BRCA 1 and 2 proteins with most important binding sites regarding functionality. **b.**) Major functions of BRCA1 and BRCA2 tumor suppressor proteins. Color codes are representing binding sites of interacting proteins.

Homolog recombination (HR) is one of the processes (next to non-homolog end joining) that is activated upon DNA double strand break (DSB). Both proteins have a significant role in HR therefore also in maintaining genome integrity, thus loss of function mutations of either of them may lead to genomic instability and subsequent tumor geneses.

BRCA1 gene is located at 17q21.31 chromosome, contains 24 exons, and encodes for the 220 kD BRCA1 protein (1863 amino acid) product. The protein owns a RING finger domain in the N-terminal end, a nuclear localization signal (NLS), a coiled-coil domain interacting with PALB2 and BRCT domains in the C-terminal end.

The protein is only stable in a heterodimer state with BARD1 binding to its RING domain (reviewed in O'Donovan and Livingston 2010), while interaction with PALB2 protein is essential to bind BRCA2 (**Figure 10/a**). BRAC1 is a member of a number of essential supercomplexes promoting genome integrity and cell survival, most importantly of DSB recognition and HR. Other functions and structure are visualized in **Figure 10/b**. Besides its significant linkage to breast and ovarian cancer syndrome, elevated PaC (al-Sukhni et al. 2008) and PrC (Ford et al. 1994) susceptibility are also documented.

BRCA2 gene is located on 13q13.1 chromosome location, contains 27 exons that encode the 3418 amino acid protein. Its major function is also DNA damage repair, specifically HR, in which it binds to RAD51 through the eight BRCT repeat domains and TR2 domain. PALB2 and subsequent BRCA1 interaction occurs via the N-terminal of the protein, the helical domain is essential in meiosis, while the OB folds are also important to bind single stranded DNA during HR process (**Figure 10/a**) (reviewed in O'Donovan and Livingston 2010). In *BRCA2* carrier families, beyond the elevated risk of breast and ovarian cancers, male breast, PaC and PrC develop also at an elevated risk (Moran et al. 2012). Major functional and structural features of both proteins are visualized in **Figure 10**. In terms of MM, *BRCA2* carriers show some associations with elevated uveal MM likelihood (Easton et al. 1997, Iscovitch et al. 2002). However epidemiological studies suggest that MM and breast cancer co-occur at a higher frequency, only *BRCA2* mutation harboring families exhibited a relative risk (RR) of 2.58 to MM according to the Breast Cancer Linkage Consortium (The Breast Cancer Linkage Consortium 1999).

I.5.3.2. PTEN hamartoma tumor syndrome (PHTS)

The PTEN hamartoma tumor syndrome includes different phenotypes with common genetic background (germline *PTEN* mutations), including Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS) and adult Lhermitte Duclos disease. All syndromes share the phenotypic characteristics of hypertrophic hamartomatous and neoplastic lesions of the skin, breast, thyroid, gastrointestinal, genitourinary system and brain, while some other characteristics are syndrome-specific (see in **Table 9** on page 52).

The *PTEN* gene is located in 10q23.3, encodes a dual-specificity tumor suppressor protein. Exons 1-6 in the N-terminal, especially exon 5 encodes for the phosphatase domain that has fundamental significance in the mechanism of the disease that is also proven by the clustered germline mutations within this region. The C-terminus contains the lipid-binding C2 domain among other important structural elements (**Figure 11**) (reviewed in Orloff and Eng 2008). PTEN protein function in cell cycle and in subsequent carcinogenesis within the PTEN/PI3K/AKT signal pathway is visualized on **Figure 9** (on page 25).

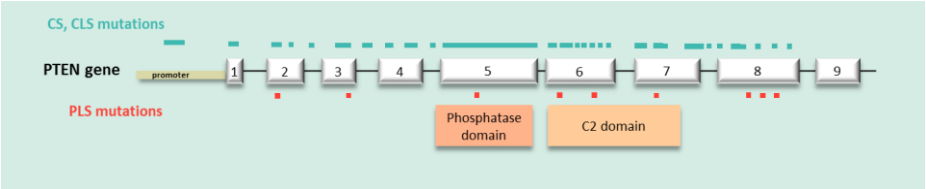

Figure 11. Schematic presentation of reported *PTEN* mutations in phenotypes CS, Cowden-like syndrome (CLS) and Proteus-like syndrome (PLS) (based on Orloff and Eng 2008).

Germline *PTEN* alterations include large deletions, point mutations together with promoter mutations and methylations and show genotype-phenotype associations (**Figure 11**), however significant proportion of patients (15% of CS), who meet the criteria of any of the phenotypes, are without identified germline *PTEN* mutation (reviewed in Orloff and Eng 2008). In CS phenotype *PTEN* promoter mutations between c.-1344 to c.-745 have also been identified and linked to an abnormal protein translation (Teresi et al. 2007). On the other hand, genes coding subunits (B,D) of succinate dehydrogenase (SDH) enzyme, member of the mitochondrial complex II that participate in electron transfer chain and Krebs's cycle, revealed to be linked with *PTEN* negative CS (Ni et al. 2008). *SDHB* gene is located on 1p36.1-p35, contains 8 exons, that codes for a 280 amino acid protein, while *SDHD* gene is on the 11q23 in 4 exons, and its' protein product consists of 159 amino acids. Heterozygous mutations in both genes were originally linked to phaeochromocytoma-paraganglioma syndrome (Baysal et al. 2000, Neumann et al. 2004), while homozygous or compound heterozygous mutation are linked to Leigh syndrome, a fatal neurodegenerative disease. Interestingly *SDHD* mutation positive *PTEN* negative CS exhibit a higher frequency of renal cell -, thyroid -, or breast cancer compared to *PTEN* positive CS (Ni et al. 2012). Germline

PIK3CA and *AKT1* mutations are also reported in CS patients as susceptibility factors (Orloff et al. 2013), while recently germline epigenetic mutations in Killin (*KLLN*) gene with a subsequent down-regulation of the encoded protein expression was also reported (Bennett et al. 2010).

Lifetime risk of MM in CS patients is around 5-6% (reviewed in Eng 2012 and Pilarski et al. 2013), and increasing evidence suggest the association between MM and PHTS (reviewed in Pilarski et al. 2013).

I.6. Classic genodermatoses and MM

I.6.1. Epidermolysis bullosa (EB)

Epidermolysis bullosa is a heterogeneous group of bullous genodermatoses. Different genes encoding cutaneous structural proteins are known to be affected causing blister formation between the subsequent epidermal layers and the underlying dermo-epidermal junction. In the mildest types (EB Simplex), structural proteins above the basal membrane in the epidermis (Keratin 5, 14) are affected. If the mutated proteins are member of the dermo-epidermal junction (collagen XVII, laminin 332), or the hemidesmosoms (plectins, integrins) (Junctional EB), the clinical picture is getting more severe (lethal form: laminin 332). In the sublamina densa region (collagen VII-Dystrophic EB), blisters heel with significant scarring and milia formation. As the whole disease spectrum is characterized by blister formation and the recurrent dermo-epidermal tissue renewal, cancer formation occur with a higher incidence, especially among patients with recessively inherited EB forms. SCC even with metastatic outcome influences life expectancies in patients (reviewed in Intong and Murrell 2012). Large, acquired melanocytic nevi occur relatively common in all types of EB; in younger age with atypical, sometimes MM mimicking clinical and dermoscopic features, described as epidermolysis bullosa naevus. Their incidence is around 14% of EB patients based on an Austrian EB registry data (Bauer et al. 2001). The large size and atypical, irregular pattern are alarming and a careful follow up is necessary. The alarming dermoscopic findings are explained by the pathogenesis of EB: the repeated disruption of dermo-epidermal junction and the subsequent fibrosing inflammation, scar formation, and neovascularization. The damage of melanocytes at the site of blister formation is followed by re-epithelization and repeatedly induced melanocyte proliferation

(summarized in Bauer et al. 2001, Cash et al. 2007). MM development on a basis of EB naevus is rare, although incidence rates are somewhat higher in comparison to the general population. In a retrospective study conducted from a United States based EB Registry among dystrophic type Hallopou-Siemens (collagen VII) patients, they showed a cumulative MM risk of 2.5% by age 12 (Fine et al. 2009).

I.6.2. Ichthyoses

Inherited ichthyoses, as a heterogeneous group of cornification disorders do not confer to any elevated occurrence of melanocytic naevus or MM. There are a few lamellar ichthyosis (LI) cases with *ABCA12* mutations and early onset MM formation reported (Natsuga et al. 2007). Among LI and non-bullous ichthyosiform congenital erythroderma patients, an elevated number of melanocytic lesions were found, rarely with features of dysplasia, mimicking MM (Fernandes et al. 2010).

I.6.3. Dowling-Degos disease (DDD)

Dowling-Degos disease is a rare autosomal dominant genodermatosis, characterized by adult onset reticulate hyperpigmentation with keratotic papules on the flexures and great skin folds. The underlying genetic defect is in keratin 5, a keratin type expressed in the basal layer of the epidermis that have significant role in melanosome transport between melanocytes and keratinocytes. Subsequently, melanosome distribution is disturbed within the keratinocytes, while keratin cytoskeleton is remained intact (Betz et al. 2006). Besides the characteristic pathomechanism of the disease, no reports have been published about coexistence of MM and DDD.

I.6.4. Hailey-Hailey disease (HHD)

Hailey-Hailey disease (HHD) is a rare autosomal dominant skin disease caused by mutation in the ATP2C1 gene, encoding a human secretory pathway Ca/Mn ATPase protein that is located in Golgi apparatus and expressed abundantly in keratinocytes (Sudbrak et al. 2000). While SCC and BCC development in lesions of HHD have been described in the literature (summarized in Mohr et al. 2011), MM formation was detected only in 2 patients so far. Both patients exhibited MPM, and had a history of previous cancer. Thus according to the authors chemotherapy and /or immunosuppression could play significant role in MM etiology. (Mohr et al. 2011).

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II. AIMS

1. Genetic background of MPM patients significantly contributes to disease development and shows interesting geographical differences. As data on mutation frequency or variant distribution of MM predisposing genes was not yet available from Hungary, our purpose was to analyse MM (MPM) predisposing genetics, and discuss a comparison to other countries' results focused on *MC1R* variants. The analysis of MPM patients (see as ***MPM study***) was obtained from an institution based (SU Department of Dermatology, Venereology and Dermatooncology) registry of an 11-year period (2001-2011) using

a. clinicopathological features including patients' phenotypes, medical history, family history of malignancies, MPMs' characteristics with detailed histologies, disease course and outcome;

b. Genetic results of sequence analysis of the major MM predisposing genes: *CDKN2A*, *CDK4*, *MC1R*, and *MITF* E318K; further analysis of the results was obtained to find associations between available clinical, pathological and genetic data using statistical methods focused on *MC1R* variants.

c. Analysis of *MC1R* variant status by creating patient groups in terms of *MC1R* germline status as 'r' carriers (00, r0, rr) and 'R' carriers (R0, rR, RR), and to compare these two groups of genotype carriers regarding MM-related clinical and/or pathological characteristics by statistical methods.

2. During patient care and data analyses, some patients presented with unique clinical course and highly positive medical -, and family history of benign and malignant tumors. Therefore second part of the study focused on three unique MM-associated cases (see as ***Unique MM-associated cases***), in which we separately obtained clinical exploration of the detected tumor constellations and pedigrees together with

a. sequence analysis of the most suspicious genes,

b. discussion of the relevant etiological factors including the identified genetic results, documented environmental factors, and further suspicious, herein not examined genetic events.

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III. MATERIALS AND METHODS

III.1. Clinical data and patient characteristics of MPM study

The study was approved by the Ethical Committee of SU (SE TUKEB 32/2007) and was conducted according to the Declaration of Helsinki Principals.

Subjects were recruited from the SU Department of Dermatology Venereology and Dermatooncology, among those had been diagnosed or treated with more than one primary MM between January 2001 and December 2011. While 1855 patients were diagnosed with primary MM in this period, 108 developed MPMs (5.8%). Out of the 108 MPM patients, 43 agreed to participate in the genetic studies: 16 women (40%) and 27 men (60%). They developed altogether 106 MMs (with an average of 2.47): 33 patients with 2-, 6 patients with 3-, 2 patients with 5-, and 2 patients with 6 primaries were registered. Mean follow-up time was: 6.7 (0-34.2) years. All participating subjects who provided biological sample (peripheral blood) for DNA extraction signed informed consent and completed an interview about general medical history and MM specific familial and individual factors. Those who had only Mis without invasive MM were excluded. Synchronous MMs were diagnosed within 30 days of each other. Tissue sections have been revised by the same histopathologist (J.H). All MPMs were carefully checked again to exclude the presence of intraepidermal epidermotropic metastasis. Anatomical site, MM subtype, Breslow thickness, Clark level, mitotic rate (number of mitotic cells per mm^2), tumor ulceration or erosion, tumor infiltrating lymphocytes (TILs) (non-brisk or brisk), presence of satellite tumors as well as vascular/neural/lymphatic invasion were documented. Less common MM subtypes, namely SSM with nodular component, desmoplastic MM and unclassified MM were categorized statistically as ‘others’. MM or other cancers of family members were self-reported, relying on patient’s knowledge about first and second degree relatives. Familial MM was defined, if one first-degree or two second-degree relatives were diagnosed with MM. Non-melanoma malignancies before and after the first MM were also documented. Patients’ pigmentation phenotype (hair-, eye color and skin phototype according to Fitzpatrick I-IV) and an approximate number of common and dysplastic nevi were recorded. Hair color was categorized as dark (black or brown), blond or red. Localization of first and subsequent MMs was estimated as i) chronically sun exposed (head and neck, lower arm), ii) intermittently sun exposed (trunk, legs, upper arm) and

iii) rarely sun-exposed (mucosal, toe, subungual) body sites. Smoking habit and occupation (indoor, outdoor or mixed) were also recorded.

Patients were followed-up according to the always current guidelines. Nodal propagation was recorded if histology verified the metastatic MM cells within the suspicious lymph node, while distant metastases were considered if detection by ultrasound was further confirmed by computer tomography (CT) scan or magnetic resonance imaging (MRI). During detection of distant metastases, if the possibility raised that subsequent primary tumor development is more potential than distant MM metastasis, histological verification was attempted. MM-related deaths were also recorded.

III.2. Clinical data of Unique MM-associated cases

III.2.1. Two cancer prone families (Figure 12)

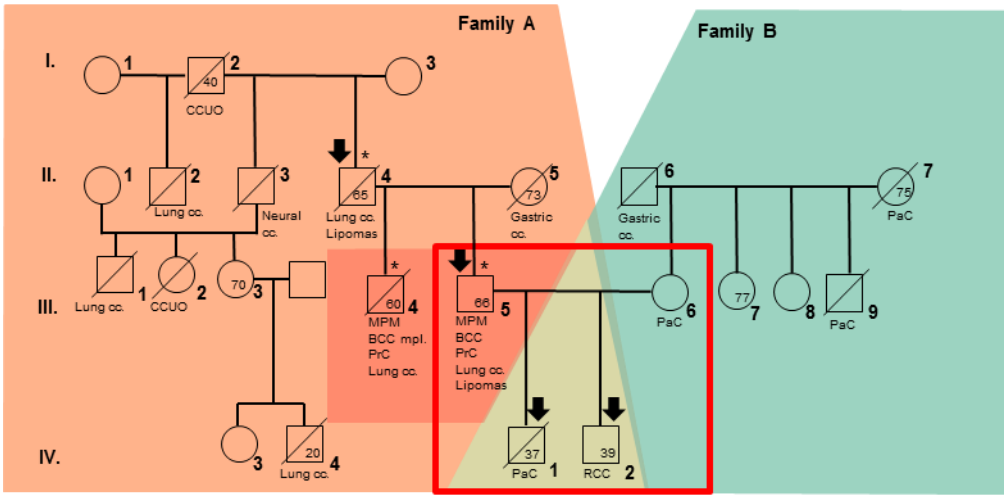

Figure 12. Four-generation pedigree of the cancer prone family A and B with the documented tumors. Family members within the red rectangular were subjected to genetic analysis.

Arrows indicate firefighters, stars indicate heavy smokers.

Abbreviations: CCUO: cancer of unknown origin, cc: cancer, MPM: multiple primary melanoma, BCC: basal cell cancer, PrC: prostate cancer, PaC: pancreatic cancer, mpl: multiple, RCC: renal cell cancer.

Family A

Sibling 1 (III/4), a pensioner office worker was diagnosed with a primary MM on the back at the age of 44 (SSM, Clark II, Breslow thickness 0.3 mm, mitotic rate:0, inflammation: mild lymphocytic) and with a second MM 9 years later on the lumbar region (ulcerated SSM, Clark IV, Breslow 2.4 mm, mitotic rate:7, no inflammation) without clinical signs of propagation. That time low-dose interferon therapy was introduced. In the same year, PrC was also diagnosed, and the therapy was completed by goserelin and flutamide. Shortly thereafter interferon was discontinued due to fatigue and muscle pain. He was 56 when a BCC from the back was excised and 57 when primary LC was diagnosed in the background of recurrent pneumonias. The patient died at age of 60 due to the LC propagation. He had been smoking for 15 years earlier.

Sibling 2 (III/5, the index person) presented with two synchronous MMs at age of 49 on the right upper arm (I) and the left side of trunk (II). Histology showed (I): SSM, Clark III, Breslow thickness 0.64 mm, mitotic rate:2, strong lymphocytic inflammation and a satellite tumor; (II): SSM upon a dysplastic naevus, Clark III, Breslow thickness 0.6 mm, mitotic rate: 2. Block dissection proved the right axilla tumor free. He presented a BCC on the face when he was 56, a PrC when he was 61 and underwent a radical prostatectomy. 3 years later he was treated for LC by lobectomy and chemotherapy. No evidence of any malignant propagation at the age of 66. Additionally he has multiple subcutaneous lipomas as well as cysts in both kidneys. He has a Fitzpatrick skin type II, blue eyes and blond hair and had been smoking for 15 years. Pedigree with the documented malignancies is visualized on **Figure 12**.

Regarding parents of these brothers (of index patient: III/6 and III/5), their mother (II/5) developed gastric adenocarcinoma at age of 73; father (II/4) died at age of 65 in LC.

The index patient had two sons: IV/1 died at age of 37 in PaC, while IV/2 developed renal cell cancer (RCC) at the age of 35 (grade II, stage: T1NxMx) and is propagation free 5 years after disease identification. He has Fitzpatrick skin type I, green eyes and blond hair.

A common environmental factor in family A, that II/4, III/5, IV/1 and IV/2 were firefighters; II/4, III/4 and III/5 had been smoking for years.

Family B

The wife of index patient (III/6), reported 3 first-degree relatives with PaC. At the time of consultation she was 65 years old in great general condition with excellent laboratory values and denied any symptoms of malignancy. An abdominal ultrasonography and MRI verified a 2.3x2.4 cm large pancreatic tumor at the border of head and body without signs of local or distant propagation. The immediately performed surgical attempt identified an inoperable PaC with mesenterial propagation. She has skin type II-III, less than 20 nevi, no dysplastic naevus, brown eyes and blond hair.

Sibling 2 (=index person, III/5) as MPM patient is also participant of the MPM study.

III.2.2. Six primary MMs

The female patient had renal transplantation at age of 59 after two years of hemodialysis because of chronic renal failure. Her family history was negative for any malignancies. She has been working decades in a public canteen mainly indoor. She had Fitzpatrick skin type I, more than 20 nevi, red hair and blue eyes. She reported an excessive sunbath habit before the first MM appeared, that was also reflected by the degree of photodamage along her skin. After renal transplantation, she got immunosuppressive therapy according to protocols with tacrolimus (TAC), methylprednisolone (MP), and mycophenolate mofetil (MMF). However OTR patients require regular dermatological follow-up, patient's compliance was not fully adequate; her first dermatological visit was three years after transplantation that might partly explain the simultaneously detected first three MMs that time. Two years later a fourth MM was detected, when the immunosuppressive therapy has been revised and TAC was exchanged to sirolimus (SRL), an agent with proven antitumor effect. She denied dermatological visits again for two years, when two simultaneous MMs were detected, with an already visible and palpable regional lymph node conglomerate in the loco-regional submandibular triangle. The nodal progression was verified by histology, but patient denied the surgical removal therapy because of the possible subsequent cosmetic destruction. Further propagation at this time was not identified; therefore palliative loco-regional radiotherapy was applied in a total dose of 50 Grays. As radiotherapy did not result in any regression, dacarbazine monotherapy was introduced, but had to be discontinued after 3 months because of hematological side effects. At this point, even though patient agreed to the surgical intervention and parotidectomy with 2nd level blockdissectomy was performed, at the 6

months check-up, regional cutaneous metastases together with distant propagation in lungs, spleen and liver were detected. Three month later the patient deceased due to the multiple propagations. Postmortem analysis verified all secondary tumors as MM metastases. During the immunosuppression, two BCCs were also detected and treated. The clinical course of patient is summarized in **Figure 13**.

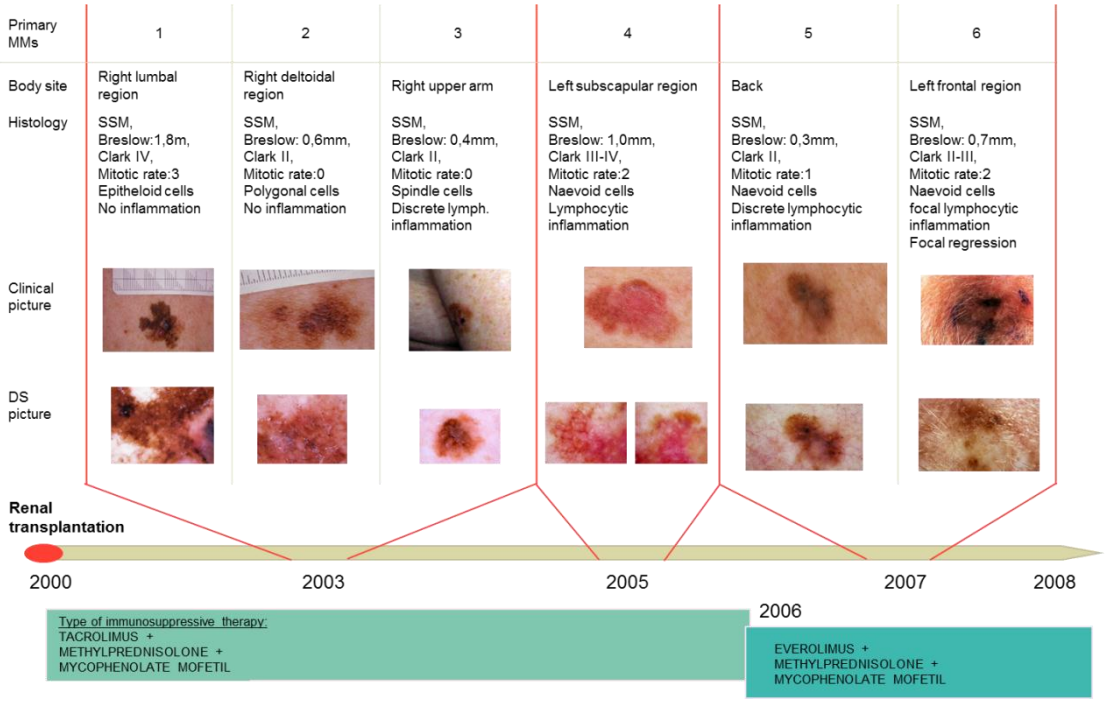

Figure 13. Clinical course of this case. Localization, histology, clinical and dermoscopy appearance of the six primary MMs with timeline and therapies are presented.
Abbreviations: DS: dermoscopy, SSM: superficial spreading melanoma, MM: malignant melanoma

Patient, as MPM patient is also participant of the MPM study.

III.2.3. MM and phenotype suggesting PHTS/CS

The female patient had no remarkable diseases before the age of 17 (she even danced ballet). In her twenties, she had uterus removal because of myoma uteri. In her middle ages a right mastectomy and irradiation was conducted to treat a breast cancer. Besides the progressive skeletal deformities she reported a number of interventions in her medical history: total colectomy because of acquired megacolon, exstirpation of a

rectovaginal fistulae, left nephrectomy due to renal stone development, inguinal hernia reconstruction and removal of parathyroid adenoma. At age of 61 years, she was admitted to our hospital with a pigmented lesion on the right side of the forehead that was a 1x0.8 cm sized asymmetric, almost evenly pigmented black thin plaque (**Figure 14**), confirmed as Mis by histology.

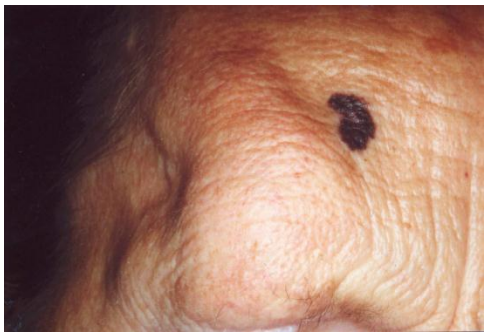

Figure 14. Pigmented lesion on the right forehead

As for her detailed phenotype, on the forehead the next to the pigmented lesion a huge bony bulge (**Figure 14**) were observed and similar deformities at the parietal and occipital regions. The patient had dolichocephaly and long face, and on the abdomen, right side of the chest, right thigh (**Figure 15/a**) and above the 7th collar vertebra (**Figure 15/b**) multiple, soft, subcutaneous masses sizing 10-15 cm.

Besides the pigmented lesion, other skin findings were also detected; 1) around the right nostril and medial canthus some sharply bordered, infiltrated, skin-colored flat plaques with 3-6 mm sized nodules on their surface; 2) on the right arm, abdomen and on the sacral region extending to the right thigh flat, slightly pigmented 3-5 mm sized confluent papules (**Figure 15/c**). Both these symptoms (1, 2) proved to be tissue nevi with lipomatous and collagenous parts by histology. These and the severe scoliosis significantly deformed her body contour (**Figure 15/a**). Her intellectual functions were normal and she had been working with a university degree at a responsible position.

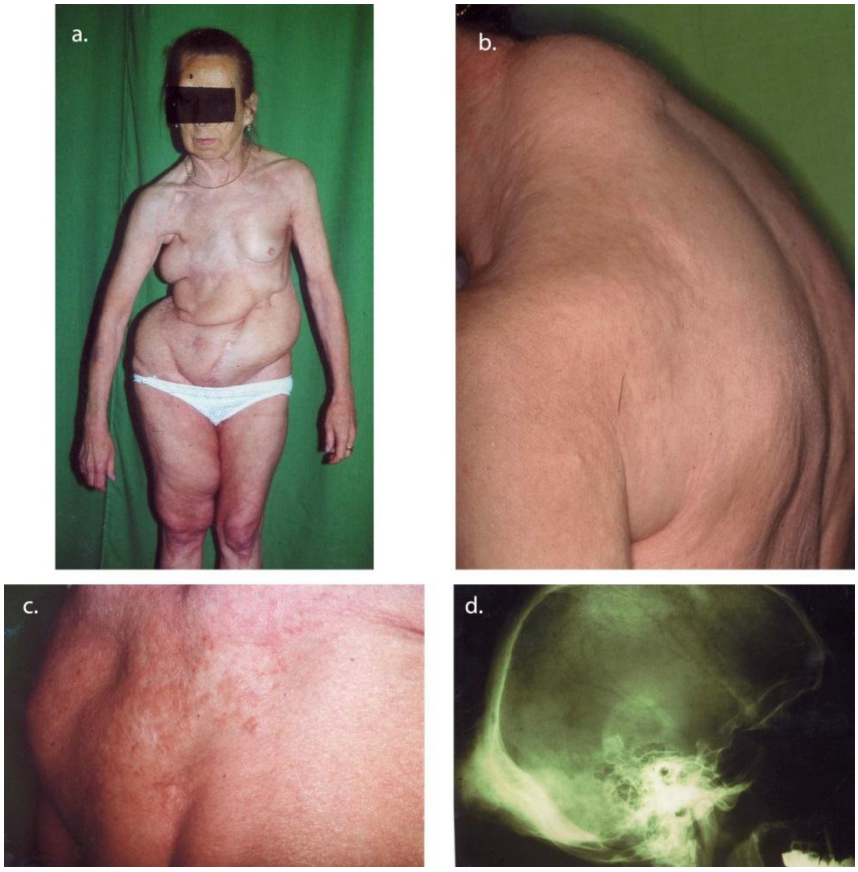

Figure 15. Clinical findings of this case.

a) Appearance of the female patient with the significant deformities, and the pigmented lesion on the forehead.

b) Soft subcutaneous mass above the vertebra

c) Tissue nevi on the abdominal skin

d) X-ray of the skull with hyperostosis in the occipital region

In her family no one have any similar phenotype; however multiple malignancies occurred (LC, uterine cancer, and BCCs).

Along her general check-up, severe scoliosis, osteoporosis, spondylosis with megaspondylodysplasia (chest X-ray), and external hyperostoses in the occipital (**Figure 15/d**) and parietal region (skull X-Ray) have been detected. Abdominal, pelvic and thyroid ultrasound was also performed with findings of: 1) a solid tumor in the central part of the remaining kidney; 2) two solid tumors in the urinary bladder wall; 3) a large inhomogeneous mass behind and under the bladder (10×8 cm in size); 4) two, adenoma suspicious partly calcified regions in the thyroid gland; and 5) a superficial,

mobile, solid tumor at the right mandibular angle. Any further examinations to clarify all the ultrasonography findings and to exclude Lhermitte-Duclos disease were refused by the patient.

Clinical diagnosis of CS is difficult, that is also supported by the convergent statistical results on the rate of identified *PTEN* germline mutations. Since consensus criteria have been developed in 2000, there have been many updates and revisions to rationalize the wide range of symptoms and specify the diseases by evidence-based experiences (Pilarski et al. 2013). Based on the 2006 revision, one of the following criteria is sufficient for an operational CS diagnosis: certain combination of mucocutan lesions; 2≤ major criteria; one major and 3≤ minor criteria; or 4≤ minor criteria. Although in this case some findings could not have been clarified thoroughly, patient indeed met the diagnostic criteria of CS (**Table 9**).

Table 9. Diagnostic criteria of CS. Color-coded symptoms represent the fulfilled criteria.

Syndrome	Diagnostic criteria (based on Eng 2012)	Germline *PTEN* mutation rate	Other germline genes
CS	Pathognomonic criteria • Adult Lhermitte - Duclos disease • Mucocutan lesions ▪ Trichilemmomas ▪ Acral keratoses ▪ **Papillomatosus lesions** ▪ Mucosal lesions Major criteria • **Breast cancer** • Epithelial (non-medullary) thyroid cancer • **Macrocephaly** • Endometrial carcinoma Minor criteria • **Other thyroid lesions** • Intellectual disability • Hamartomatous intestinal polyposis • Fibrocystic breast disease • **Lipomas** • Fibromas • **Genitourinary tumors (RCC)**	25-85%	*SDHB* *SDHD* *KLLN* *PIK3CA* *AKT1*

Syndrome	Diagnostic criteria (based on Eng 2012)	Germline *PTEN* mutation rate	Other germline genes
CS	• **Genitourinary malformation** • Uterine fibroids		

Abbreviations: CS: Cowden syndrome, RCC: renal cell cancer

III.3. Mutation analysis

DNA isolation

DNA was isolated from peripheral lymphocytes with Roche Magna Pure Compact (Roche Magna Pure Compact Nucleic Acid Isolation Kit Large Volume, Indianapolis, USA). With this method, 1000 ml peripheral blood is used to isolate a final volume of 200 µl DNA with a concentration ranging between 25-150 µg/ml. Isolated DNAs were stored at -20°C.

In cases, where DNAs were isolated from tissue sections, formalin fixed paraffin embedded (FFPE) sections were used as source of genetic material and was obtained with QIAamp DNA FFPE Tissue Kit (Hilden, Germany) according to manufacturer's manual.

Polymerase chain reaction (PCR)

The examined genes regarding studies are summarized in **Table 10**.

Table 10. List of genes examined within this work. In "Case 1", family members are represented according to their position on the pedigree (see on **Figure 12** on page 46).

Genes	Exon(s)	MPM Study	Unique MM-associated cases						
			Case 1					Case 2	Case 3
			III/5	III/6	III/6 t	IV/1 t	IV/2		
CDKN2A	1-4	x	x	x	exon 2	exon 2	x	x	x
CDK4	2	x	x	x	-	-	x	x	x
MC1R	1	x	x	x	-	-	x	x	x
MITF E318K	10	x	x	x	-	-	x	x	x
PTEN	1-9	-	x	x	-	-	exons 2, 8	x	x
SDHB	1-8	-	-	-	-	-	-	-	x
SDHD	1-4	-	-	-	-	-	-	-	x

Genes	Exon(s)	MPM Study	Unique MM-associated cases						
			Case 1					Case 2	Case 3
			III/5	III/6	III/6 t	IV/1 t	IV/2		
BRCA1	1-24	-	exons 2,11*, 20*	x	exons 2,11*, 20*	exons 2,11*, 20*	x	-	-
BRCA2	1-27	-	11*	x	11*	11*	x	-	-

Abbreviations: t: tissue level. * Exons were sequenced only partially focusing hot-spot regions. Case 1: Two tumor prone families; Case 2: Six primary MMs, Case 3: MM and phenotype suggesting PHTS/CS.

Primers were either obtained from previous publications, or designed using Primer3 open online program (http://frodo.wi.mit.edu/). While designing primer sets, it was of high priority to take care of some important details, such as 1) primer lengths are between 19-24 base pairs; 2) melting temperature of the forward and reverse primers do not differ significantly; 3) GC:AT ratio is around 53:63%. Functionality of primers was checked with an in silico online program (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/).

Invitrogen™ synthetized our designed primer sets. The lyophilized primer products were then attenuated to 10 μM concentration with distilled water and stored at -20°C until use.

For the genes that have been analyzed in their entire coding region (*CDKN2A*, *MC1R*, *PTEN*, *SDHB*, *SDHD*), primers were designed or selected to cover not only the entire coding region, but also the exon-intron boundaries and to extending to the 5' and 3' untranslated regions (UTRs).

The 5'-end M13-tailed universal primers of the 4 exons of *CDKN2A* gene (not of 2* that were self-designed) and *MC1R* primer set number 3 were obtained from http://www.ncbi.nlm.nih.gov/probe.

Primer sequences for *CDK4* exon 2 (Soufir et al. 2004) and segment of *MITF* exon 10 (containing amino acid position 318) were used as described (Yokoyama et al. 2011). All primer sequences and annealing temperatures with references (if appropriate) are summarized in **Table 11**.

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Table 11. Primer sequences (5'-3'), annealing temperatures, PCR product sizes and references.

Genes/exons/primers	Annealing temperature (°C)	PCR product size (bp)
CDKN2A (RSA M13 tails are marked in Italic)		
1β-forward: TGTAAAACGACGGCCAGTTTCGCCTCAGTTTCCCACGA 1β-reverse: CAGGAAACAGCTATGACCAGGAGTGGCGCTGCTCACC	62 62	498
1α-forward: TGTAAAACGACGGCCAGTCGCCAGGAGGAGGTCTGTGA 1α-reverse: CAGGAAACAGCTATGACCCCACATTCGCTAAGTGCTCGGA	66 66	558
2-forward : TGTAAAACGACGGCCAGTGAGACTCAGGCCGTCCCACC 2-reverse: CAGGAAACAGCTATGACCTGGAAACTGGAAGCAAATGTAGGG	62 62	343
2*- forward: GGAGGGCTTCCTGGACAC 2*- reverse: GCAGGTACCGTGCGACAT	60 60	131
3-forward: TGTAAAACGACGGCCAGTCGATGCTGTCTTCCATGCGAT 3-reverse: CAGGAAACAGCTATGACCTGACCTCAGGTTTCTAACGCCTGT	62 62	580
CDK4 (Soufir et al. 2004)		
2-forward: GGATGGGATGCTGGTGGTGTT 2-reverse: TTCCCTTTACTCCCCACGCCC	62 62	392
MC1R		
1-forward: CAGCACCATGAACTAAGCAGGACACCTG 1-reverse: CCAGCATAGCCAGGAAGAAGACCACGAG	58 58	693
2-forward: TGGGTGGCCAGTGTCGTCTTCAGCA 2-reverse: AAGGGTCCGCGCTTCAACACTTTCAGA	58 58	610
3-forward: *TGTAAAACGACGGCCAGT*CCCATGTACTGCTTCATCTGCTGC 3-reverse: *CAGGAAACAGCTATGACC*GGGCAGAGGACGATGAGTGTG	62 62	590
MITF (Yokoyama et al. 2011)		
10-forward: CAGGCTCGAGCTCATGGA 10-reverse: TGGGGACACTATAGGCTTGG	62 62	232
PTEN		
1-forward:CTTCAGCCACAGGCTCCCAGAC 1-reverse:GGAGCAGCCGCAGAAATGG	62 62	124
2-forward:CCATGTGGAAGTTACCTTTTA 2-reverse:TACGGTAAGCCAAAAAATGA	62 62	431
3-forward:AATGACATGATTACTACTCTA 3-reverse:TTAATCGGTTTAGGAATACAA	58 58	366
4-forward:CATTATAAAGATTCAGGCAATG 4-reverse:GACAGTAAGATACAGTCTATC	58 58	205
5-forward:AGTTTGTATGCAACATTTCTAA 5-reverse:TCCAGGAAGAGGAAAGGAAA	58 58	371
6-forward:ATATGTTCTTAAATGGCTACGA 6-reverse:CTTTAGCCCAATGAGTTGA	62 62	426
7-forward:ACAGAATCCATATTTCGTGTA 7-reverse:TAATGTCTCACCAATGCCA	62 62	428
8-forward:TGCAAATGTTTAACATAGGTGA 8-reverse: CGTAAACACTGCTTCGAAATA	62 62	468
9-forward: CCTCTTAAAGATCATGTTTG 9-reverse: AGATTGGTCAGGAAAAGAGAA	58 58	474

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Genes/exons/primers	Annealing temperature (℃)	PCR product size (bp)
BRCA1		
2-forward: CGTTGAAGAAGTACAAAATGTCA 2-reverse: TCAATTCTGTTCATTTGCATAGG 11/3-forward: CCTGTGAATTTTCTGAGACGG 11/3-reverse: GCCTCATGAGGATCACTGG 11-forward: AGGGGCCAAGAAATTAGAGTC 11-reverse: AAGTTTGAATCCATGCTTTGCTCT 20-forward: ATATGACGTGTCTGCTCCAC 20-reverse: TGCAAAGGGGAGTGGAATAC		
BRCA2		
11-forward: GTCTGGATTGGAGAAAGTTT 11-reverse: CTCTTGTGAGCTGGTCTGAA		
SDHB (McWhinney et al. 2004)		
1-forward: GCGGCTACTGCGCTATTG 1-reverse: GCTTTCCTGACTTTTCCC	56 56	285
2-forward: TCTGTTGTGCCAGCAAAATG 2-reverse: GCCTTCCAAGGATGTGAAAA	55 55	287
3-forward: ACATCCAGGTGTCTCCGATT 3-reverse: CTATCAGCTTTGGCCAGC	55 55	247
4-forward: GTCAGTGCTGCCCCTGAT 4-reverse: TGCAAATAAAAACAAAACCA	55 55	284
5-forward: GCTGAGGTGATGATGGAATCT 5-reverse: CCACACTCCTGGCAATCATC	55 55	249
6-forward: ATGCACTGACCCCAAAGGTA 6-reverse: CAGCAATCTATTGTCCTCTTG	55 55	271
7-forward: CTTTCCTCTGCACTCCCAGA 7-reversed: TTGTGAGCACATGCTACTTC	55 55	283
8-forward: GGAAGGAGTTTCACCCAAGA 8-reversed: 777TGCTGTATTCATGGAAAACCAA	55 55	263
SDHD (McWhinney et al. 2004)		
1-forward: ACCTTCCGACAGCTGTGTTT 1-reverse: GAGTGGGGAGCATTCTAGGG	55 55	267
2-forward: AAGTAGCTTACCTATGGTCATTTAGAA 2-reverse: AAGATGGCTAGAGCCCAGAA	55 55	249
3-forward: CTTTTATGAATCTGGTCCTTTTTG 3-reverse: CAACTATATTTGGAATTGCTATAC	55 55	200
4-forward: TGATGTTATGATTTTTTCTTTTTCT 4-reverse: CAATTCTTCAAAGTATGAAGTCA	55 55	224

Abbreviations: bp: base pair.

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PCR amplifications were performed with the above detailed primer sets in a total volume of 30 μl using 15 μl Immomix Red (Bioline, London, UK), 1 μl of the primers in both directions, 1 μl template and 12 μl distilled water and the following conditions using Techne TouchGene Biometra T3 Thermocycler (Biometra GmbH, Göttingen, Germany):

- Initialization at 95^{0}C for 5 minutes,
- Denaturation at 90^{0}C for 45 seconds
- Annealing at 57-66^{0}C for 45 seconds } for 40 cycles.
- Extension at 72^{0}C for 45 seconds
- Final elongation at 72^{0}C for 10 minutes.

Analyzing quality and quantity of PCR reaction, agarose gel electrophoresis of the PCR products were performed on 2% agarose gel that contains 100 ml 1x TAE- buffer, 2 grams of agarose (NA Agarose, Amersham Biosciences) and 7 μl GelGreen™ (Biotium, Hayward, USA) for staining. The electrophoresis is obtained in 1x TAE-buffer on 120V for 15-20 minutes. Results were analyzed with gel documentation imaging system.

Purification of PCR products

PCR mixture directly after the amplification is not pure enough for high quality sequencing results. The remaining dNTPs and primer remnants must be removed, for what ExoSAP-IT (USB-Affymetrix, Cleveland, Ohio) enzymatic system was used according to the protocols.

Sequencing reaction

Using the purified PCR products sequencing reaction were carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, Texas) followed by purification of the products from dye-terminators with NucleoSEQ (Macherey-Nagel GmbH, Düren, Germany) gel-filtration technology platform as suggested.

As a final step, samples were mixed with 20 μl of Hi-Di™-formamide (Applied Biosystems, Austin, Texas) and have been denaturized on 95^{0}C for 1 minute, then were placed into ice to avoid DNA strand renaturations. For automated sequence analysis, ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) was used.

Sequence analysis

Sequence results were visualized in FinchTV Softver (www.geospiza.com), than were compared to the following NCBI reference sequences (**Table 12**) using Vector NTi Software (Life Technologies).

Table 12. NCBI reference sequences.

Genes	NCBI reference sequences
CDKN2A	NM_000077.3
***CDK4* (exon 2)**	NM_000075.3
MC1R	NM_002386.3
***MITF* (exon 10)**	NM_000248.3
PTEN	NM_000314
BRCA1	NM_007294.3
BRCA2	NM_000059.3
SDHB	NM_003000
SDHD	NM_003002.2

Mutation nomenclature was considered using nucleotide numbering starting from the first ATG (Start codon) within the coding region. The *BRCA1* and *BRCA2* nomenclature follows the nomenclature of Human Genome Variation Society (HGVS).

Mutation verification, control analysis

As all sequence reactions were carried out in both directions, possible failure of polymerase enzymes were excluded, therefore if our findings (mutation, SNP or variants) have been already described elsewhere, further verification was not performed. In case of new observations, locus specific restriction endonuclease enzymes were matched and digestions were performed:

1. In terms of D117G variant of *MC1R*, PCR products were digested with PsyI (Tth111I) restriction endonuclease (Fermentas Life Science, EU), that cleaves the wild type alleles into 2 detectable products (approx. 440bp+80 bp), but leaves the mutant alleles unaffected (520 bp). Two hundred healthy alleles from randomly selected 100 controls were also examined with the method.

2. In case of *MITF* V320A SNP, PCR products were digested with BsrBI (MbiI) restriction endonuclease (Fermentas Life Science, EU). The enzyme cleaves the mutant alleles into two detectable products (144+20 bp), while leaves the wild type alleles intact (164 bp). Here 100 healthy alleles from randomly selected 50 controls were digested.

III.4. Statistical analysis

In the MPM study, to the comparison of continuous variables between groups we used the independent Student's t test. Categorical variables were compared by the Chi-square test or the Fisher-exact test whichever appropriate. The levels of significance were set to $p<0.05$. Data were analyzed using the IBM SPSS Statistics version 20.0.

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IV. RESULTS

IV.1. MPM study

IV.1.1. Clinicopathological attributes

IV.1.1.1. Patient characteristics

The mean age at initial diagnosis was 61 years (36-86), that was significantly lower in women than in men (55.3 versus 65.4 years; p=0.016). First MM developed earlier in those with history of other malignancy (56.8 versus 64.4 years; p=0.054) and with family history of MM (55.4 versus 62.0 years; p=0.333). The average age at MM onset in patients with 2 MMs was 62 years (36-86), and 60 years (37-74) in those with more than 2 MMs. Family history of MM was positive in 4 patients (9.3%).

IV.1.1.2. Clinicopathology of first and subsequent MMs (Table 13)

Mean time interval between first and second MM was 26.3 months; 39.3 in men, 13.2 months in women (p=0.118). This period was shorter in patients with second Mis compared to second invasive MMs (7.7 versus 41.6 months; p=0.06) and with additional NMSC in history (14.6 versus 55.9 months; p=0.06) (non-significant differences). Second MM was observed synchronously in 48.8% (21/43) of patients, and within the first year of initial diagnosis in 16% (7/43).

Phenotype with higher number of common nevi (>20) or any dysplastic naevus was presented in 51.1% of patients; they had significantly more MMs (2.8 MMs versus 2.4 MMs; p=0.026), moreover their first MM developed earlier (59.1 versus 65.2 years; p=0.121).

The first MMs localized mostly on the trunk (60.5%). Twenty patients (47%) had the first and second MMs on the same body site. Vast majority of all MMs (84%) developed on intermittently sun exposed body areas.

More Mis were observed among the second (37%)-, or subsequent (25%) than among first tumors (9%). SSM was the most common subtype (60.4%), while NM was observed only in four cases (3.8% of all MMs). First MMs exhibited significantly thicker Breslow thickness (2.16 versus 1.16mm; p=0.013), more ulceration (15% versus 9.5%; p=0.372) and regression (26% versus 19%; p=0.423) than subsequent ones. TILs presented in the same proportion of first and second MMs (48%) (**Table 13**).

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Table 13. Clinical and histological characteristics of first, second and subsequent MMs.

All MMs (Mis and invasive MM) n=106	First MMs n=43	Second MMs n=43	Sub-sequent MMs n=20	Difference between first and second MM
	n (%)	n (%)	n (%)	
Anatomical site of MM				
-Head and neck	3 (7)	5 (12)	3 (15)	
-Trunk	26 (60)	25 (58)	10 (50)	p=0.684
-Extremities: -Arms	11 (26)	8 (19)	4 (20)	
-Legs	3 (7)	5 (12)	3 (15)	
Site of MM by sun exposure				
-Chronically	7 (16)	7 (16)	3 (15)	
-Intermittently	36 (84)	36 (84)	17 (85)	p=0.896
-Rarely exposed	0	0	0	
Histology subtype				
-Mis	4 (9)	16 (37)	5 (25)	
-SSM	29 (67)	25 (58)	10 (50)	
-NM	3 (7)	0	1 (5)	
-LMM	0	2 (5)	1 (5)	p=0.662
-ALM	0	0	0	
-Others	5 (12)	0	3 (15)	
-No data	2 (5)	0	0	
Presence of regression				
-Absent	28 (65)	25 (58)	13 (65)	
-Present	11 (26)	9 (21)	3 (15)	p=0.705
-No data	4 (9)	9 (21)	4 (20)	
MM coexisting naevus				
-Absent	34 (79)	35 (81)	17 (85)	
-Present	3 (7)	8 (19)	3 (15)	p=0.174
-No data	6 (14)	0	0	
Only invasive MMs n=82	**n=40**	**n=27**	**n=15**	
Breslow thickness (mm)				
-0,01-1,00	19 (48)	20 (74)	11 (73)	
-1,01-2,00	9 (23)	4 (15)	2 (13)	
-2,01-4,00	4 (10)	1 (4)	0	p=0.246
->4,00	6 (15)	2 (7)	1 (7)	
-No data	2 (5)	0 (0)	1 (7)	
Mean Breslow thickness (mm)*	2.16	1.16		**p=0.013**
Ulceration				
-Absent	31 (78)	25 (93)	13 (87)	
-Present	6 (15)	2 (7)	2 (13)	p=0.293
-No data	3 (8)	0	0	

Only invasive MMs n=82	First MMs n=40	Second MMs n=27	Sub-sequent MMs n=15	Difference between first and second MM
Presence of mitoses **-Absent** **-Present** **-No data**	 4 (10) 27 (68) 9 (23)	 4 (15) 22 (81) 1 (4)	 3 (20) 12 (80) 0	p=0.788
Mitotic rate (/mm2) **-0** **-1-2** **-3-10** **-10+** **-No data**	 4 (10) 10 (25) 12 (30) 5 (13) 9 (23)	 4 (15) 10 (37) 9 (33) 3 (11) 1 (4)	 3 (20) 8 (53) 3 (20) 1 (7) 0	p=0.920
TILs **-Absent** **-Present** **-No data**	 13 (33) 19 (48) 8 (20)	 13 (48) 13 (48) 1 (4)	 3 (20) 11 (73) 1 (7)	p=0.475

*Mean Breslow thickness was calculated of first and subsequent MMs.

Abbreviations: ALM: acral-lentiginous melanoma, LMM: lentigo maligna melanoma, Mis: Melanoma in situ, MM: malignant melanoma, NM: nodular melanoma SSM: superficial spreading melanoma, TIL: tumor infiltrating lymphocytes.

IV.1.1.3. Second non-MM primary malignancies among MPM patients

Of the 43 MPM patients, 18 (41.9%) had non-melanoma tumors from which 83.3% had cutaneous, 16.7% extracutaneous, while 27.8% had both. Men were more prone to NMSC than women (42% versus 24%; p=0.18), while more women had extracutaneous malignancies (12% versus 4%; p=0.34); although these results are non-significant because of the small sample sizes. Eight patients developed altogether 11 extracutaneous malignancies: PrC in three patients; LC, cervix and colon cancers each in two patients, while thyroid cancer and RCC occurred once among the MPM patients.

IV.1.2. Genetic results

***CDKN2A*:** Two out of 43 MPM patients (4.7%) had *CDKN2A* mutations. One carried the c.296 G>C - p.R99P, the other the c.206 A>G-p.E69G mutation, both located in exon 2. These mutation positive patients were under 50 years at initial diagnosis. The well-known c.-191 G>A SNP was present in 88.3% of MPM patients with a 62.8% allele frequency. The p.A148T variant was carried only by 2 patients (4.6%). In the 3'UTR, SNPs c.500 C>G was observed in eleven patients (25.6%) with a 12.8% allele frequency, while c.540 C>T in 7 (16.3%) with 8.1% allele frequency.

***CDK4*:** None of the patients carried any of the previously described amino acid changes at position 24 (R24H, R24C).

***MITF*:** The c.1075 G>A;p.E318K mutation was not detected in our patients. We identified the SNP c.1082 T>C;p.V320A (rs2055006) in the same exon in one patient. None of the 50 healthy controls (100 alleles) carried this SNP.

***MC1R*:** Nine different variants (8 non-synonymous and 1 synonymous) were detected in 37/43 (86%) patients. See variant frequencies in **Table 14**.

Table 14. *MC1R* variant carrier and allele frequencies in the MPM study.

Nucleotide change	Amino acid change	*MC1R* variant type ('R' or 'r')	Carrier frequency (%) (n=43)	Allele frequency (%) (n=86)	Overall non-synonymous allele frequency (%)
c.178G>T	V60L	'r'	20.9	11.6	64.0
c.274G>A	V92M	'r'	20.9	10.5	
c.350A>G	D117G	unkonwn	2.3	1.2	
c.451C>T	R151C	'R'	30.2	16.3	
c.464T>C	I155T	'R'	4.6	2.3	
c.478C>T	R160W	'R'	20.9	12.8	
c.488G>A	R163Q	'r'	16.2	8.1	
c.880G>C	D294H	'R'	2.3	1.2	
c.942A>G	T314T	-	25.6	12.8	-

MPM patients in this study had 0 to 2 *MC1R* variants. The number of MPMs rose with the number of carried variants: 2.2 MMs in carriers of 0 variant (6/43), 2.5 MMs in carriers of 1 variant (24/43), and 2.6 MMs in of 2 variants (13/43).

The overall allele frequency was 30% for 'r' variants and 33% for 'R' variants (see later in **Table 23** on page 79). The most common variant was R151C (30%). One novel non-synonymous variant (c.350 A>G; p.D117G) was identified in one MPM patient. The R163Q was observed in 16.2% of patients with an allele frequency of 8.1%. The synonymous T314T was present in 11/43 (25.6%) patients.

The association of RHC phenotype (4 patients) with *MC1R* 'R' genotype (23 patients) was not informative, although 3 out of 4 red head carried one or two 'R' variants.

IV.1.3. Analysis of *MC1R* variant status

To further investigate the association between *MC1R* 'R' or 'r' status and the clinicopathological features of the patients, two patient groups were formed: one with wild type or only 'r' variants (V60L, V92M, R163Q) ('r' carriers) and another with any 'R' variants (R151C, I155T, R160W, D294H) ('R' carriers) (Beaumont et al. 2007, Cust et al. 2012). Results of the compared variables are listed in **Table 15**.

Table 15. Comparison of clinical and histological data of *MC1R* 'r'-, and 'R' carrier patients.

Patient groups by *MC1R* carrier status*	***MC1R* genotype**			
	00+r0+rr 'r' carriers n=20	rR+R0+RR 'R' carriers n=23	OR / differ-rence	p**
Medical history positive for:				
Non-melanoma malignancy	10%. (2/20)	26% (6/23)	3.18	0.169
BCC	35% (7/20)	35% (8/23)	0.99	0.619
More than 1 BCC	15% (3/20)	26% (6/23)	2.0	0.31
MM-specific clinical data:				
Age at first MM	63.0 years	60.1 years	2.90	0.463
Time period between 1st and 2nd MMs	17.7 months	38.9 months	21.20	0.322
Average MM number	2.3	2.6	0.3	0.344
More than 2 MMs	15% (3/20)	30% (7/23)	2.48	0.203
1st and 2nd MM localization on the same body site	30% (6/20)	48% (11/23)	2.14	0.19

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Patient groups by *MC1R* carrier status*	*MC1R* genotype			
	00+r0+rr 'r' carriers n=20	rR+R0+RR 'R' carriers n=23	OR / differ-rence	p**
1st and 2nd MM localization on the same body site by sun exposure	35%(7/20)	57% (13/23)	2.41	0.135
1st MM localization on the back	55% (11/20)	35% (8/23)	2.29	0.153
Histologic features of 1st and 2nd MMs:				
2nd MM invasive	55% (11/20)	70% (16/23)	1.87	0.252
1st MM with mitotic rate >0	88% (15/17)	71% (12/17)	0.32	0.199
2nd MM with mitotic rate >0	55% (11/20)	50% (11/22)	0.82	0.494
1st MM with ulceration	11% (2/19)	21% (4/19)	2.27	0.33
2nd MM with ulceration	10% (2/20)	0% (0/22)	4.89	0.221
1st MM with regression	32% (6/19)	25% (5/20)	0.72	0.46
2nd MM with regression	20% (4/20)	22% (5/23)	1.11	0.595
1st MM with TILs	59% (10/17)	50% (9/18)	0.70	0.427
2nd MM with TILs	27% (3/11)	67% (10/15)	4.72	**0.035**
1st MM with local invasion	6% (1/18)	5% (1/19)	0.94	0.743
2nd MM with local invasion	5% (1/20)	4% (1/23)	0.86	0.72
Disease course:				
Mean follow-up time	5.9 years	7.5 years	1.6	
Death	12% (2/17)	30% (6/20)	3.21	0.174
Propagation	26% (5/19)	38% (8/21)	1.72	0.325
5-year overall survival	1/20	3/23	73.9	0.611

*Cases with missing data are excluded. **Student t-test or Fisher's exact test

Abbreviations: MM: malignant melanoma, OR: odds ratio, TIL: tumor infiltrating lymphocytes

Younger age of MM onset was recorded in 'R' carriers (60.1 versus 63.0 years; p=0.463); moreover they had more non-melanoma malignancies in their medical history (26% versus 10%; p=0.169). BCC occurrence was the same in both groups (35%), however multiple BCCs were more prevalent among 'R' carriers (26% versus 15%; p=0.31). Time period between first and second MM was longer in 'R' carriers (38.9 versus 17.7 months; p=0.322), although non-significantly. Also 'R' carriers had their first and second MM more frequently on the same body site (48% versus 30%; p=0.19) and their first MM less frequently on the back (35% versus 55%; p=0.153).

MPM histologies in 'R' carriers was non-significantly associated with more i) invasive second MMs (70% versus 55%; p=0.252), ii) ulcerated first MMs (21% versus

11%; p=0.33), and iii) non-ulcerated second MMs (10% versus 0%; p=0.221), but correlated significantly with TILs in second MMs (67% versus 27%; p=0.035). Regression or mitotic rate showed no considerable differences between 'R' and 'r' carrier patients. Although propagation and death were more frequent among 'R' carriers, 5-year overall survival was not significantly worse in 'R' carriers (87% versus 95%; p=0.611).

IV.2. Genetic results of the Unique MM-associated cases

IV.2.1. Two cancer prone families

Genetic results of family members are summarized in **Table 16**, for pedigree see **Figure 12** on page 46.

Table 16. Results of germline/somatic genetic analyses of family members.

Level of genetic analysis				Germline			Somatic from PaC	
Gene	**Loca-tion**	**cDNA; amino acid position**	**Variant type**	**Index person (III/5)**	**Offspring 2 (IV/2)**	**Wife (III/6)**	**Wife (III/6)**	**Offspring 1 (IV/1)**
CDKN2A	1α	c.-191 G>A	SNP (rs3814960)	het	het	het	NO	NO
	1β	c.193+174 A>G	SNP (rs2811711)	wt	wt	het	NO	NO
	2	c.296 G>C; p. R99P	Mutation	het	wt	wt	wt	het
	3'UTR	c.*69 C>T	SNP (rs3088440)	het	het	wt	NO	NO
CDK4	2			wt	wt	wt	NO	NO
MC1R		c.178 G>T; p.V60L	'r' variant	het	wt	wt	NO	NO
		c.274 G>A; p.V92M	'r' variant	het	het	wt	NO	NO
		c.942 A>G; p.T314T	Synonymous variant	het	het	wt	NO	NO
MITF	10	c.1075G>A; p.E318K	mutation	wt	wt	wt	NO	NO
PTEN	IVS1	c.80- 96 A>G	SNP (rs1903858)	het	het	wt	NO	NO
	IVS8	c.1026+32 T>G	SNP (rs555895)	het	het	wt	NO	NO
BRCA1	11	c.1067 A>G; p.Q356R	SNP (rs1799950)	het	wt	wt	wt	wt
	11	c.2311 T>C; p.L771L	SNP (rs16940)	NO	wt	het	NO	NO
BRCA2	5'UTR	c.-26 G>A	SNP (rs1799943)	NO	het	het	NO	NO
	IVS7	c.631+183 T>A	SNP (rs3752451)	NO	het	het		
	11	c.3396 A>G; p.K1132K	SNP (rs1801406)	NO	wt	het		
		c.3807 T>C; p.V1269V	SNP (rs543304)	NO	het	het		
		c.5744 C>T; p.T1915M	SNP (rs4987117)	NO	het	het		

Level of genetic analysis				Germline			Somatic from PaC	
Gene	Loca-tion	cDNA; amino acid position	Variant type	Index person (III/5)	Offspring 2 (IV/2)	Wife (III/6)	Wife (III/6)	Offspring 1 (IV/1)
	11	c.7242 A>G; p.S2414S	SNP (rs1799955)	NO	het	het		
	IVS16	c.7806-14 T>C	SNP (rs9534262)	NO	het	het		
	IVS21	c.8755-66 T>C	SNP (rs4942486)	NO	het	het		
	27	c.*105 A>C	SNP (rs15869)	NO	het	het		

Abbreviations: wt: wild type, NO: analysis not obtained, het: heterozygous, mut: mutation, SNP: single nucleotide polymorphism, var: variant, IVS: intervening sequence

***CDKN2A*:** The index person (III/5) carried the c.296 G>C missense mutation resulting in amino acid change p.R99P. For this mutation, offspring IV/2 appeared to be a non-carrier, while IV/1 carried it at somatic level in FFPE PaC sample (DNA for germline analysis was not any more available). III/6 did not carry any mutations along the gene. The identified genetic alterations are summarized in **Table 16**.

***CDK4, MITF*:** None of the three examined patients (III/5, IV/2, III/6) exhibited any mutations in the *CDK4* exon 2; neither carried the *MITF* E318K point mutation in exon 10.

***MC1R*:** The index person (III/5) carried the c.178 G>T; p.V60L and the c.274 G>A; p.V92M non-synonymous together with the c.942 A>G; p.T314T synonymous variants; IV/2 had only the p.V92M and the p.T314T variants; while III/6 didn't carry any variants.

***PTEN*:** Index patient (III/5) had only two germline intronic SNPs (c.80-96 A>G, c.1026+32 T>G), that were also detected in his offspring IV/2, while III/6 exhibited whole wild type sequence.

***BRCA1*:** Index person (III/5) carried a heterozygous base substitution at position c.1067 A>G resulting in amino acid change p.Q356R, an SNP not found in any of his offspring (IV/1, IV/2). He did not carry any other alterations in the examined hot spot regions of *BRCA1* (exon 2, and segments of exon 11 and 20). III/6 and IV/2 have been genotyped regarding the whole coding region of the gene. III/6 carried the c.2311 T>C ;p.L771L SNP, that did not penetrate to IV/2. IV/2 exhibited a wild type gene.

***BRCA2*:** Index person (III/5) had wild type sequence in the hot-spot prone segmentes of exon 11. III/6 and IV/2 have been sequenced along the whole coding regions of the gene. Both of them had a number of SNPs (c.-26 G>A, c.631+183 T>A, c.3807 T>C; p.V1269V, c.5744 C>T; p.T1915M, c.7242 A>G; p.S2414S, c.7806-14 T>C, c.8755-66 T>C, c.*105 A>C), but no mutations. The c.3396 A>G; p.K1132K was found only in III/6 but not in IV/2. Sequence results of III/5 are visualized on **Figure 16**.

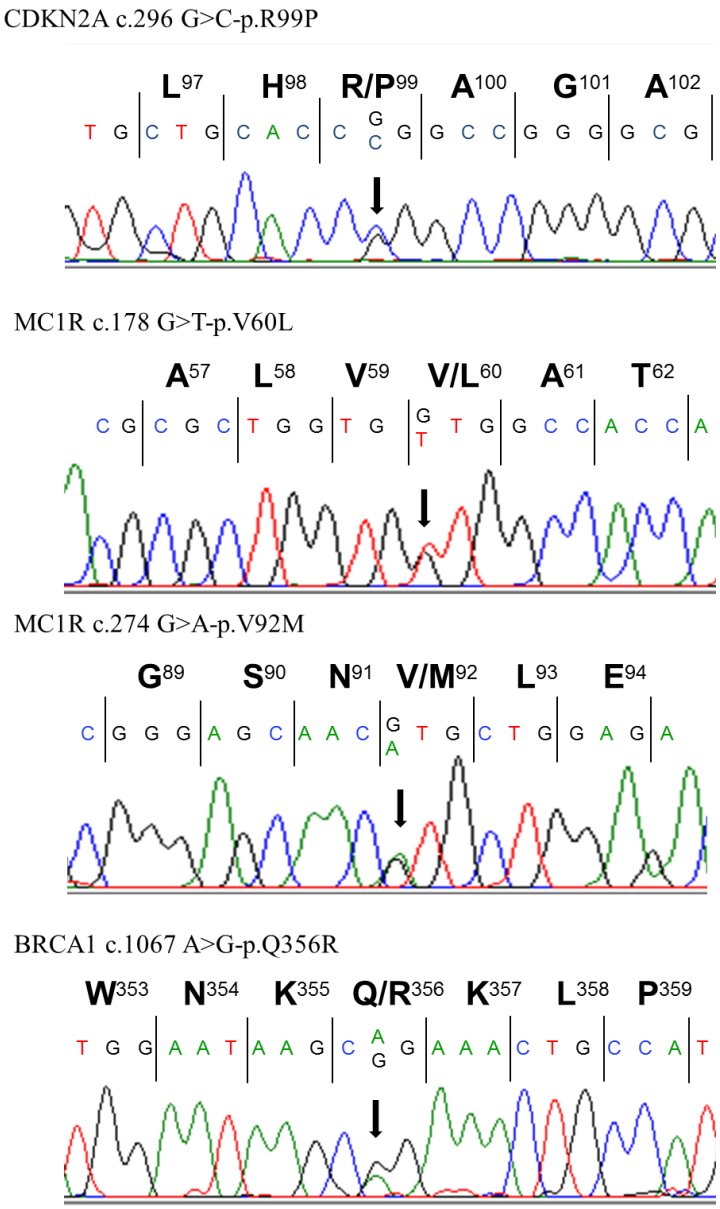

Figure 16. Identified mutations and variants in index person (III/5) from family A. Localization of mutations and variants are marked with arrows. Amino acid sequences with positions are illustrated above the nucleotides.

IV.2.2. Six primary MMs

This patient's data are also included in the MPM study except the gene analysis of *PTEN*. Genetic results are summarized in **Table 17**.

Table 17. Results of germline genetic analysis.

Gene	Exon	Position	Allele genotype
CDKN2A	5'UTR	c.-191 G>A	heterozygous
	3'UTR	c.*29 C>G	heterozygous
MC1R		p.R151C	homozygous

No *CDKN2A* mutation, only two SNPs was detected: in the 5'UTR region (c.-191 G>A) and in the 3'UTR (c.*29 C>G).

In *MC1R* gene, the R151C variant has been detected in homozygous state. This variant is considered as an 'R' variant. No mutations in exon 2 of *CDK4* gene was observed, and she did not carry the *MITF* E318K mutation. The whole coding region of *PTEN* was wild type, without any sequence variants neither.

The patient's clinical course suggested that the secondary propagation originated from the latest, sixth MM. Somatic mutation profiling from that MM was attempted, but quality of DNA obtained from FFPE MM tissues were not sufficient to complete the analysis.

IV.2.3. MM and phenotype suggesting PHTS/CS

Based on the unique phenotype characteristics, at first, genotyping of *PTEN* coding regions with exon intron boundaries was obtained. As no mutations, only two SNPs were found along the sequenced regions, further analysis in terms of *SDHB* and *SDHD* genes were completed, moreover MM predisposing *CDKN2A* and *MC1R* were also examined. Results are summarized in **Table 18.**

Table 18. Results of germline genetic analysis. Not listed exons are considered as wild type.

Genes	Exon	Position	Allele genotype	Categorization
PTEN	IVS1	c.80-96 A>G	heterozygous	SNP (rs1903858)
	IVS8	c.1026+32 T>G	heterozygous	SNP (rs555895)
SDHB	1	c.18 C>A; p.A6A	homozygous	SNP (rs2746462)
SDHD	IVS1	c.52+136 G>T	heterozygous	SNP (rs7121782)
	IVS3	c.314+15 T>A	heterozygous	first report
CDKN2A	IVS1β	IVS1β +174 A>G (c.-19138 A>G)	heterozygous	SNP (rs2811711) uncertain significance
	3' UTR	c.*69 C>T	heterozygous	SNP (rs3088440)
MC1R		R160W	heterozygous	'R' variant

Abbreviations: IVS: intervening sequence, SNP: single nucleotide polymorphism

In the *SDHD* gene, the identified intronic base substitution (IVS3: c.314+15 T>A) is a newly observed alteration within *SDHD* gene, therefore its effect on protein translation is questionable and needs further studies.

As no pathogenic mutations have been detected along the selected genes, the role of somatic mosaicism was also hypothesized. Unfortunately the patient was not any more available for additional tissue sampling. The FFPE archived tissue samples weren't sufficient neither in quality nor in quantity to obtain genetic analysis. FFPE archived sample of a previously removed lipoma was used for DNA isolation, however amplification of only some exons of *PTEN* gene (No. 1,3,8) was successful, without any detected mutations. We only found the *PTEN* c.1026+32 T>G SNP in heterozygous state.

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V. DISCUSSIONS

V.1. MPM study

The aim of this work was to statistically analyze prognostic clinical and histological factors together with genetic data of 43 MPM patients diagnosed and followed by the same team during an 11-year period.

V.1.1. Clinicopathological attributes

MPM was observed in 5.8% (108/1855) of MM patients, that is in accordance with previous findings (5-9.5%) (Hashemi et al. 2000, Ferrone et al. 2005, Helsing et al. 2008, Savoia et al. 2012). The two major clinical factors of MPM development were also evaluated:

1) Family history for MM presented in 9.3% of MPM patients, which data is far below the published values (20-31.6%) (Ferrone et al. 2005, Berwick et al. 2006, Helsing et al. 2008, Pastorino et al. 2008). We hypothesize that this is due to the relatively low patient number or to the type of study population (hospital-based).

2) High naevus count (>20) or presence of dysplastic nevi phenotype (de Giorgi et al. 2010) was presented in 51.1% of patients, and showed significant association with higher MM number (2.8 versus 2.4 MMs; p=0.026) and non-significant correlation with younger age of onset (59.1 versus 65.2 years; p=0.121).

In accordance with previous findings, in our study women were significantly younger than men at the time of first MM (55.3 versus 65.4 years; p=0.016) (Ferrone et al. 2005, Savoia et al. 2012), and higher order MMs were more commonly i) Mis (Ferrone et al. 2005, Savoia et al. 2012), ii) thinner (Ferrone et al. 2005, Savoia et al. 2012) and iii) localized on the same body site as the first MM (Ferrone et al. 2005, Savoia et al. 2012). As indicated in the literature (Ferrone et al. 2005, Puig et al. 2005, Hwa et al. 2012, Savoia et al. 2012), most MPM patients developed two MMs (76.7%) in our study as well; moreover in 47% of the cases the first two MMs developed on the same body site (Ferrone et al. 2005, Savoia et al. 2012, Hwa et al. 2012).

In our sample synchronously diagnosed first and second MMs were more frequent (48.8%) than in other studies (25.9-31.4%) (Ferrone et al. 2005, Murali et al. 2012a, Savoia et al. 2012). Non-melanoma malignancies among MPM patients occurred also at

a higher frequency (41.9%) than in previous reports (16-23%) (Slingluff et al. 1993, Manganoni et al. 2012).

V.1.2. Genetic results and their clinicopathological relevance in MPM patients

V.1.2.1. *CDKN2A* in MPM patients

Identified mutations and SNPs of *CDKN2A* are visualized in **Figure 17.**

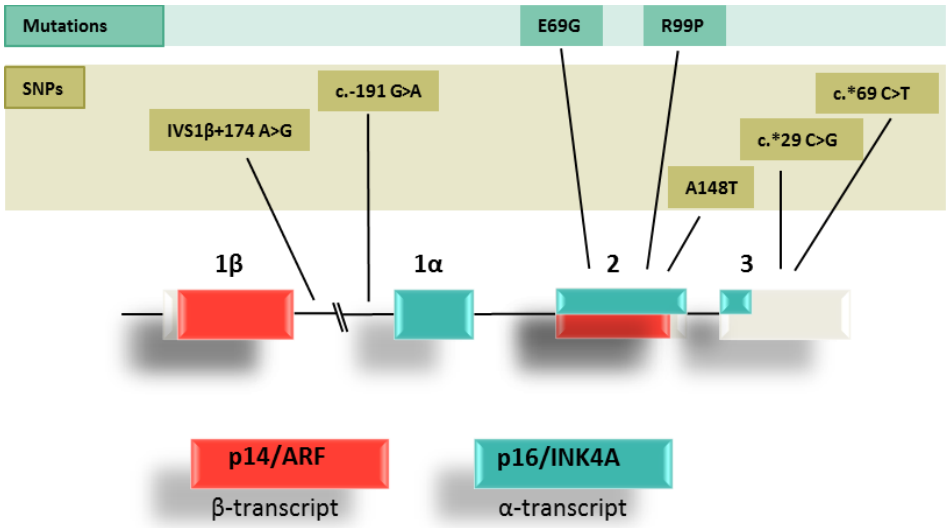

Figure 17. Identified mutations and SNPs along the *CDKN2A* gene in the MPM study.

V.1.2.1.1. *CDKN2A* mutations

Two of the 43 MPM patients (4.7%) carried mutations in the major high-risk MM predisposing *CDKN2A* gene. The published mutation frequencies in MPM patients are 2.9-32.6% (Hashemi et al. 2000, Blackwood et al. 2002, Puig et al. 2005, Berwick et al. 2006, Helsing et al. 2008, Pastorino et al. 2008), depending highly on the type of sample (population-, versus hospital-based) and elevating significantly with the presence of family history of MM.

So far only two *CDKN2A* mutations from carrier families have been identified from Hungary (P48T, IVS1+37 G>C) (Széll et al. 2007, Balogh et al. 2012), but none of these were detected among our patients.

Relevance of R99P mutation is discussed later (V.2.1). The other identified mutation, E69G is also reported in a few MM cases (Goldstein et al. 2006, Kannengiesser et al. 2009, Cust et al. 2011). ARF protein structure or function is not altered by this base substitution (G83G). Concerning the effect of mutation on protein function, there are computational algorithms (Grantham score, BLOSUM62, POLYPHEN, SIFT, Panther, SNPs3D, Pmut, GV GD programs, UMD-Predictor) and *in vitro* functional studies available. In silico predictions are also useful for determine pathogenicity; however there are still doubts about the correct integration and clinical translation of all these data. In terms of *CDKN2A*, most relevant functional studies include binding affinity to CDK4 and to CDK6, cell cycle arrest capacity or effect on proliferation by measuring Ki67 expression in transfected cells. Additionally epidemiological studies using data of previous mutation reports from disease affected and healthy controls together with segregation analysis are also useful methods. A new algorithm integrating a number of genetic evidences has recently been developed to define pathogenicity of *BRCA1*/2 variants (Bayesian analysis), that has recently been tested in terms of *CDKN2A* variants too (summarized in Miller et al. 2012). The most recent in silico and functional results regarding E69G mutation are summarized in **Table 19**.

Table 19. Functional significance of E69G mutation by different methods.

Methods		Significance	References
In silico	Sumarized prediction score[a]	4/7	Kannengiesser et al. 2009
	Grantham score prediction	Deleterious	McKenzie et al. 2010
	BLOSUM62 prediction	Deleterious	McKenzie et al. 2010
Functional	*CDK4* binding	69% (partial)	Kannengiesser et al. 2009
	Functional conclusion	Deleterious	Kannengiesser et al. 2009
	Cell cycle arrest experiments	No data	Miller et al. 2011

Methods		Significance	References
Classification by clinical and epidemiological criteria		Uncertain	Miller et al. 2011
Bayesian analysis		No data	Miller et al. 2011

[a] Predictions of deleterious effects based on seven prediction tools (Grantham score, POLYPHEN, SIFT, Panther, SNPs3D, Pmut, GV GD programs, UMD-Predictor)

Considering genotype-phenotype associations of the E69G carrier patients', the well reported characteristic features of *CDKN2A* mutation carriers have been evaluated. In our carrier patient, besides the MPM development, only the early age of onset was remarkable clinical feature (summary in **Table 20**).

Table 20. Clinical characteristics associated with E69G mutation carrier status

Clinical characteristics of the *CDKN2A* E69G carrier patient	
Number of MMs	2 (simultaneous)
Age of onset at first MM	36 years
Family history for MM	Negative
PaC in family	No
History of non-melanoma malignancy	No
Additionally carried *MC1R* variants	1 'R' variant (R151C)
Other genetic findings	None

Abbreviations: MM: malignant melanoma, PaC: Pancreatic cancer

V.1.2.1.2. *CDKN2A* SNPs and sequence variants

In the 5'UTR, the association of the c.-191 G>A SNP with MM is debated. Some authors reported its prevalence similar to that in the general population (Harland et al. 2000, Bisio et al 2010), while others found MM patients more prone to carry it (Mantelli et al. 2002), albeit without significant disease phenotype segregation (Harland et al. 2000). The allele and carrier frequencies observed in our series are far above the reported incidences on controls, sporadic-, and familial MM cases. To assess the relevance of this observation, further studies should be obtained. It would be also interesting to determine incidence rates in sporadic MM and normal control cases from Hungary. Allele and carrier frequencies from the literature are summarized in **Table 21.**

Table 21. Allele and carrier frequencies of *CDKN2A* c.-191 G>A SNP in previous reports

Allele/ Carrier frequency (%)	Size of population (per individual)	Normal controls (nc)		MM		Familial MM		MPM		diff.
		AF	CF	AF	CF	AF	CF	AF	CF	
Our data	MPM: 43	-	-	-	-	-	-	63	88	-
Bisio et al. 2010	FAMM:125 Nc:75	59				53				ns
Goldstein et al. 2008	MM:704 Nc: 691	38		41						ns
Mantelli et al. 2002	FAMM:41 Nc: 65		26				41			0.0182
Harland et al. 2000	FAMM:135 Nc: 120	38	62	39	61					ns

Abbreviations: AF: allele frequency, CF: carrier frequency; nc: normal control; ns: not significant, diff.: difference between control-, and MM populations

In the ARF transcriptome in IVS1β, the c.193+174 A>G substitution (rs2811711) was detected in 8 patients (18.6%). The relevance of this alteration is not found in the literature; therefore analysis of further studies on bigger and other sample sizes would be interesting.

In the 5'UTR, the c.-34 G>T substitution is a reported alteration in a subset of *CDKN2A* mutation-negative MM families. As it creates a novel AUG translation initiation codon, a decreased translation occurs from the original AUG codon (Liu et al. 1999). Although our primers were designed to cover this point, none of the patient carried this alteration.

The most frequently observed SNPs in *CDKN2A* gene are located in the 3'UTR: c.*29 C>G and c.*69 C>T. The prevalence of c.*29 C>G is correlated with both familial (Aitken et al. 1999) and individual (Kumar et al. 2001) risk-, while the c.*69 C>T carries only an elevated individual risk for MM (Kumar et al. 2001) and may be associated with a better survival (Straume et al. 2002). The frequency we observed regarding these SNPs are quite similar compared to previous reports (**Table 22**).

Table 22. Carrier frequencies of the SNPs located in the 3'UTR of *CDKN2A* gene in our study and in previous reports

Reference	Population	Carrier frequency (%)	
		c.*29 C>G	c.*69 C>T
Our results	MPM	25.6	16.3
Kumar et al. 2001	MM	28.3	26.6
Puig et al. 2005	MPM	25.2	15.4
Pjanova et al. 2007 et al.	MM	18	20
Orlow et al. 2007	MPM	26.4	19.7

Abbreviations: MM: malignant melanoma, MPM: multiple primary melanoma

The third most common SNP of *CDKN2A*, the c.442 G>A resulting A148T amino acid change is located in exon 2. In previous studies, this SNP was statistically more frequent in MPM patients than in healthy controls: 13.5% versus 5.45%; p=0.05 (Puig et al. 2005), and 15.7% versus 6.6%; p=0.011 (Pastorino et al. 2008), although no differences between healthy compared to SPM patients (Pastorino et al. 2008) or healthy compared to individuals with atypical naevus phenotype or family history of MM (Bertram et al. 2002) have been observed. In a Brazilian study, this variant was more frequent in MM patients than in controls (12.6% versus 3.9%; p=0.009), moreover patients with positive family history of cancer were more prone to carry this SNP (Bakos et al. 2011). In our MPM series, two patients (5%) carried the A148T variant allele that represents a very low rate compared to any of the previous findings. None of the carriers reported any history of cancer in their own, nor in their families. Interpretation of this result would be rational, if allele frequencies of healthy individuals and/or SPM patients from our population were available.

V.1.2.2. CDK4

Lack of any patients harboring *CDK4* mutation is in concordance with previous findings, that *CDK4* germline mutations are very rare even in familial MM kindreds.

V.1.2.3. MITF

Although *MITF* E318K mutation is associated with MM and MPM development (Yokoyama et al. 2011) we did not detect it in our series. Within the same exon (exon

10) we found the V320A SNP in one patient, who had two MMs on the same body site at age of 64 and 67 years. She had no family history of MM or non-melanoma malignancy, but had multiple BCCs in her medical history. She had brown hair and skin type II; and didn't carry any alterations in *MC1R* gene, only the c.-191 G>A SNP in *CDKN2A* gene. There is no data about the frequency of V320A in any Hungarian case series, but we did not find this SNP in 50 healthy individuals. The role of this variant concerning MM is not known.

V.1.2.4. *MC1R* variants

V.1.2.4.1. Discussion of indentified *MC1R* variants

There is a documented pattern diversity of *MC1R* variants depending on geography (Harding et al. 2000, Gerstenblith et al. 2007). By analysis of the whole coding region of *MC1R*, we identified nine different variants (8 non-synonymous and 1 synonymous), while we could not identify the 'R' variants D84E and R142H. The most frequent variant was R151C that was recently proved to be significantly more frequent among MPM patients than among controls (Helsing et al. 2012).

Localization of the identified variants within the protein structure is presented in **Figure 18.**

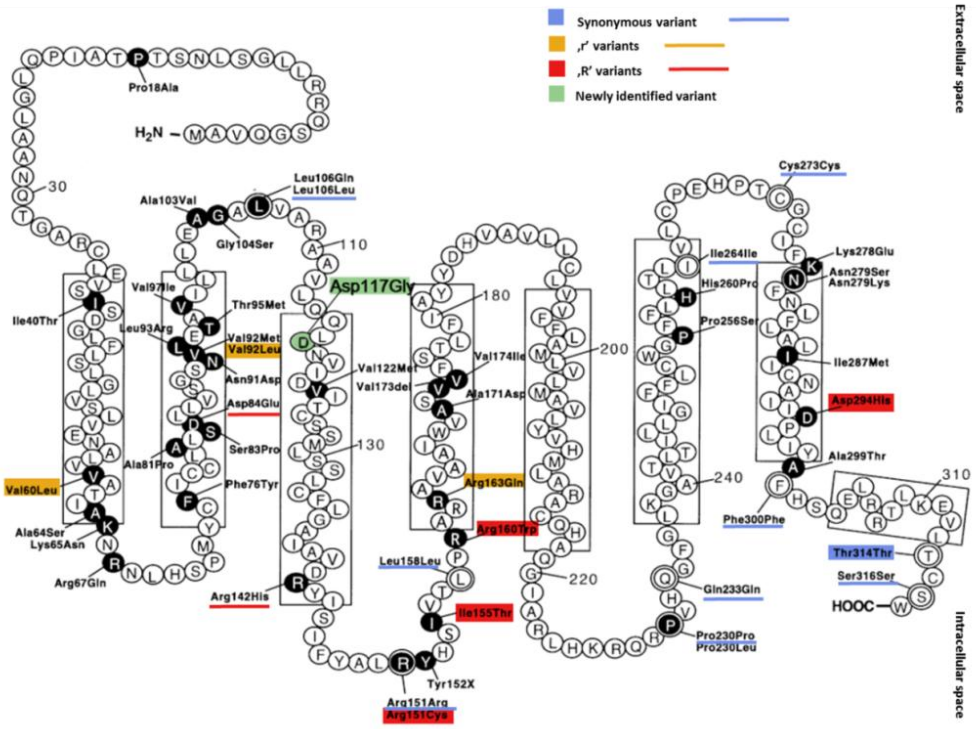

Figure 18. The *MC1R* protein with seven transmembrane domains. Amino acids are represented within the circles with markings of variant locations (not all identified variants are marked). Colored rectangular reflect detected variants within this study -, while lines indicate variants not detected in this study. Variants without color marks represent rare variants without linkage either to red hair, fair skin or to MM risk (original source is Rouzaud et al. 2005, here with personal changes).

Within the *MC1R* coding region we identified a new variant c.350 **A>G**;p.D117G in one MPM patient with brown hair color, blue eyes and skin type III; who developed two synchronous primary MMs at age of 65, without history of NMSC. The variant was not detected in 100 healthy randomly enrolled controls. The amino acid position 117 is an important ligand binding site as it is the first amino acid of the third transmembrane region that owns key position in rotation during the signal transduction process (Lu et al. 1998). At the same, 350th nucleotide position, two other substitutions **A>T**-p.D117V (Kanetsky et al. 2006) and **A>C**-p.D117A (Frändberg et al. 1994) have already been reported also among MM patients but with unknown significance. The D117A variant has an altered binding affinity to the L-isomer forms of melanocortin peptides (eg.:α-MSH) (Frändberg et al. 1994). The position is conserved among the

MCR families, but non-conservative among species. One can speculate why the 117G is reference sequence for the Bamboo and Ruffed lemur Genuses, while within the superfamily Lemuroidea the only published MM case occurred on a Grey mouse lemur (Remick et al. 2009) that belongs to different taxonomic family and genus than Bamboo or Ruffed lemurs (to our knowledge no data is available about its' *MC1R* reference sequence).

V.1.2.4.2. *MC1R* variant frequency

The overall allele frequency of non-synonymous *MC1R* variants in our study (64.0%) was around as expected (56-65%)(Kanetsky et al. 2006, Pastorino et al. 2008). The allele frequencies of 'R' variants (R151C, R160W, D294H) (30.2%) were higher than in other healthy populations (15.9-21.5%)(Gerstenblith et al. 2007), but was close or similar to that reported in MPM populations (25.5%) (Helsing et al. 2012) and (30.2%) (Kanetsky et al. 2006) (**Table 23**).

Table 23. *MC1R* variant allele frequencies in this MPM study and in another multinational MPM sample.

***MC1R* variants**	***MC1R* variant type**	**This study (Hungary)**		**Kanetsky et al. 2006 (USA, Canada, Italy, Australia)**		**OR**	**p**
n (alleles)		**86**		**2198**			
		n	**%**	**n**	**%**		
V60L	'r'	10	11.6	288	13.1	0.873	0.870
V92M	'r'	9	10.5	222	10.1	1.04	0.856
R151C	'R'	14	16.3	349	15.9	1.03	0.881
I155T	'R'	2	2.3	30	1.4	1.721	0.341
R160W	'R'	11	12.8	237	10.8	1.214	0.595
R163Q	'r'	7	8.1	109	5.0	1.698	0.204
D294H	'R'	1	1.2	78	3.5	0.32	0.366
T314T	synonymous	11	12.8	270	12.3	1.047	0.867
'R' variants		28	32.6	694	31.6	1.046	0.906
'r' variants		26	30.2	619	28.2	1.106	0.714

Abbreviations: OR: odds ratio

As no other Hungarian data was available regarding *MC1R* variant frequencies, we compared our data to published results from different countries, to assess, whether our

MC1R allele frequencies show any pattern similarity with other MM populations (**Table 24**).

Table 24. Odd ratios (OR) gained by comparison of our *MC1R* allele frequencies to other populations' allele frequency results.

Population (Ref)	Origin	Popu-lation type	Our results (Hungary)							
			V60L	V92M	R151C	I155T	R160W	R163Q	D294H	T314T
German (Scherer et al. 2009)	WE	MM	1.061	1.057	1.758 *	2.414	1.326	1.69	0.591	1.486
Dutch (Kennedy et al. 2001)	WE	MM	1.163	1.033	2.197 **	nd	0.884	1.365	1.435	nd
French (Matichard et al. 2004)	WE	FAMM, MPM	0.658	1.987	2.139 *	2.548	1.717	1.825	0.306	nd
Spanish (de Torre et al. 2010)	SE	FAMM	0.774	1.558	2.378 *	2.024	3.007 **	2.451	0.277	1.43
Spanish (Scherer et al. 2009)	SE	MM	0.6	1.825	3.712	1.163	4.789	4.329 **	0.225	1.072
Italian (Fargnoli et al. 2006)	SE	spMM	0.666	1.442	2.583 **	9.5 *	3.113 **	4.342 **	0.38	nd
Slovenien (Peric et al. 2008)	SE	FAMM, MPM	1.4	1.034	1.88	3.024	1.56	1.802	1.494	1.194
Polish (Brudnik et al. 2009)	CEE	MM	1.222	1.031	2.276 **	2.548	0.875	2.646 *	1.259	nd
Australian (Cust et al. 2012)	O	MM	0.938	1.106	0.956	1.011	1.258	1.837	0.305	nd
American (Kanetsky et al. 2010)	O	MM	0.776	1.148	1.748 *	2.295	1.367	2.212 *	0.304	1.159
Izraeli (Galore-Haskel et al. 2009)	O	FAMM, MPM, spMM	0.318 **	1.643	1.72	0.738	1.939	2.18	3	1.297

OR>1 means that Hungarian allele frequency is higher than in the compared population, OR <1 means that Hungarian allele frequency is lower than in the compared population. * indicates p<0.1, ** indicates p<0.05. Darker colored results are the most similar findings to our results. R163Q is clearly more common in our series than in any other studies.

Abbreviations: WE: Western Europe, SE: Southern Europe, CEE: Central and Eastern Europe, O: others, non-Europe; nd: no data, ref: reference, spMM: sporadic MM.

Interestingly the pattern among our MPM patients was most similar to the Australian, a little bit to Polish, German, Dutch results, but not to other European countries.
Additionally R163Q variant, rare in Europeans (0.6-4.9%)(Gerstenblith et al. 2007) ,but frequent (70%) in Asians (Rana et al. 1999), was observed at a much higher allele frequency (8.1%) in our MPM series than in any other previous MM or MPM studies (**Table 23 and 24**).

V.1.3. Analysis of *MC1R* variants status

Further analysis was carried out by comparing MPM patients by their *MC1R* carrier status; therefore two groups have been established:

1. 'r' carriers consisted of patients with genotypes 00, r0 or rr (n=20)
2. 'R' carriers consisted of patients with genotypes R0, rR or RR (n=23).

While characterization of certain *MC1R* variants regarding their 'r' or 'R' significance are obvious (V60L, V92M, R163Q as 'r' and I155T, R160W, D294H as 'R' variants), the definition of some variants are still under debate (R151C). In our study, grouping of the variants followed methods of the latest significant papers', considering R151C as 'R' variant (Beaumont et al. 2007, Cust et al. 2012).

We confirmed the previous findings that both 'R' variants (Kanetsky et al. 2006) and multiple variants regardless of their type (Goldstein et al. 2005, Pastorino et al. 2008, Fargnoli et al. 2010) are associated with MPM development.
Among our MPM patients the presence of any 'R' variants suggested similar trends as seen in *CDKN2A* mutation carriers: first MM at a younger age and propensity to develop more than 2 MMs. Additionally 'R' variant carriers were more prone to develop extracutaneous tumors and multiple BCCs (non-significant correlation).

From histopathological point of view, 'R' carriers exhibited a non-significant trend to own invasive first-, ulcerated first-, and not ulcerated second MMs. The only significant association was that second MMs of 'R' carriers exhibited more likely signs of TILs. Presence of TILs in MM is independent predictor of survival among MM patients (Azimi et al. 2012, Thomas et al. 2013). As in MPM patients mostly the first MM is the thickest one (Ferrone et al. 2005, Savoia et al. 2012), the prognosis is mainly determined by the first tumors' characteristics (Savoia et al. 2012). Evaluating 5-years overall survival there was no significant difference between the 'r' and 'R' carriers,

supporting the phenomenon, that however pathology of second MMs shows histology signs reflecting better prognosis, survival may not be influenced consequently.

As for limitations of this work, the studied population was a single clinic-based sample, so the number of genotyped patients was relatively small, therefore many aspects of the analysis and comparisons were either not applicable or didn't reach significance. Due to missing population-based data from Hungary, we could only compare our results with previous publications from other countries.

V.2. Unique MM-associated cases

V.2.1. Two cancer prone families

Two cancer prone families (Family A and B) have been identified and analyzed in this part of the work (for pedigree see **Figure 12** on page 46). Sequence analysis of the most relevant genes has been obtained, and these results were taken into account with the additionally suspicious environmental factors that could contribute to the unique tumor constellations.

V.2.1.1. Discussion of genetic results

Family A

Our first aim was to clarify the genetic background of the unique set of tumors detected in the two non-twin brothers (including index patient) in family A. They both developed the same four primary malignancies (MPM, BCC, PrC, LC), a combination not fitting into any known cancer predisposition syndromes.

Given the early onset MPM (<50 years) in both brothers, *CDKN2A*, *CDK4*, *MC1R* gene analyses were performed in one of them (index patient-III/5), as III/4 deceased earlier. *MITF* E318K is a newly identified germline point mutation that seems to be more common among patients with MPM and renal cell cancer. Moreover carriers with personal and/or family history of pancreatic cancer and kidney cancer have an elevated risk of MM development (Ghiorzo et al. 2013), therefore this point mutation was also screened in the family members.

Family A: *CDKN2A*

Index patient harbored the *CDKN2A* R99P mutation, reported so far only in a few MM families/cases (Soufir et al. 1998, Kannengiesser et al. 2009). This mutation is located

in exon 2, within the third ankyrin repeat, but does not alter the ARF/p14 amino acid translation (P113P). Regarding consequences of R99P mutation in p16/INK4A protein function, all in silico, functional and epidemiological methods provided evidence of a significant pathogenic effect (**Table 25**).

Table 25. Functional significance of R99P mutation examined by different methods.

Methods		Significance	References
In silico	Sumarized prediction score[a]	3/7	Kannengiesser et al. 2009
	Grantham score prediction	Deleterious	McKenzie et al. 2010
	BLOSUM62 prediction	Deleterious	McKenzie et al. 2010
Functional	*CDK4* binding	6% (loss)	Kannengiesser et al. 2009
	Functional conclusion	Deleterious	Kannengiesser et al. 2009
	Cell cycle arrest experiments	Loss of function	Miller et al. 2011
Classification by clinical and epidemiological criteria		Pathogenic	Miller et al. 2011
Bayesian analysis		Pathogenic	Miller et al. 2011

[a] Predictions of deleterious effects based on seven prediction tools (Grantham score, POLYPHEN, SIFT, Panther, SNPs3D, Pmut, GV GD programs, UMD-Predictor)

The same mutation was also detected in his son (IV/1) (at somatic level in PaC tissue), who died of PaC at the age of 37; but unfortunately no germline analysis could be implemented. Several studies have shown an increased risk of PaC in *CDKN2A* mutation positive MM-prone families (Goldstein et al. 2004, 2006, 2007). Our finding further support the previous observation that PaC development is very probable if the *CDKN2A* mutation affects the p16/INK4A, without effect on p14/ARF protein structure (Goldstein et al. 2007). In previously reported MM families with R99P mutation-segregation, PaC occurred in MM-affected (Soufir et al. 1998) and non-affected family members (Kannengiesser et al. 2009). Therefore the identified *CDKN2A* R99P mutation seems to be a genetic risk factor in family A for MM (III/5) and PaC (IV/1) development.

Family A: *MC1R*

MC1R ‘R’variants are known to contribute to MM genesis (**Table 1** on page 21). Recent studies showed that also ‘r’ variants (Kanetsky et al. 2010), and variants independently of their ‘R’ or ‘r’ type confer an elevated MM risk (Goldstein et al. 2005). Our index person carried two non-synonymous ‘r’ variants (V60L, V92M) and a frequent synonymous variant (T314T). The V60L has a reduced ability to stimulate cAMP, unlike V92M (Beaumont et al. 2007) that shows decreased affinity to α-MSH (Ringholm et al. 2004). The role of these two variants in MM risk is contradictory (Palmer et al. 2000, Matichard et al. 2004). A recent meta-analysis on *CDKN2A* carriers showed that both were significantly associated to MM: V92M in North America, whereas V60L in Australia (Demenais et al. 2010). In another study increased risk of MM was associated with V60L in a Spanish population (OR: 1.47), while V92M in both German (OR: 1.37) and Spanish (OR: 1.97) samples (Scherer et al. 2009). In a Greek study, both V60L (OR: 2.76) and V92M (OR: 1.58) were associated with elevated MM risk (Stratigos et al. 2006). Both variants are strongly associated with BCC risk too (Liboutet et al. 2006), therefore their significance in all the observed skin malignancies (MPM and BCCs) is relevant.

Family A: *PTEN*

Germline mutations of *PTEN*, the tumor suppressor gene of the MAPK pathway, are linked to PHTS and carry an elevated risk of non-hamartomatous malignancies and lipomas too. In our index person no germline mutation was detected, and the presence of heterozygous SNPs (c.80-96 A>G, c.1026+32 T>G) excluded gross deletions or total allele losses. The link between these germline SNPs and the herein associated malignancies is not known, albeit somatic *PTEN* mutations with protein function loss have been described in MM, PrC and gastric cancer among others (Li et al. 1997, Birck et al. 2000, Holbrook et al. 2011).

Family A: *BRCA1/2*

BRCA1/2 are tumor-suppressor genes with basic cellular functions mostly activated upon DNA damage response. Germline *BRCA1/2* mutations confer a substantial lifetime risk of breast (in male too) and ovarian cancer and may lead to genome instability with further malignant tumor formations including PrC and PaC, while only *BRCA2* mutation harboring families exhibited higher risk (2.58-fold) of MM (The Breast

Cancer Linkage Consortium 1999). Hot-spot prone areas in *BRCA1* (exon 2, 20 and segments of exon 11) and in *BRCA2* (segment of exon 11) were also analyzed in our index person III/5 and in IV/1. Only III/5 carried the Q356R, a reported SNP (rs1799950) with controversial associations of familial breast and ovarian cancer. In-silico studies confirmed this position within the ring-finger domain as 1) critical due to interaction with DNA, RAD51 and p53; 2) having a regulatory effect to alternative splicing; 3) affecting DNA repair functions, moreover 4) bioinformatics methods suggested it's deleterious effect (summarized in Ricks-Santi et al. 2013).

Family A: *MITF*

RCC developed relatively early in family member IV/2. An elevated risk for RCC among MM survivors (Bradford et al. 2010), and also for MM in RCC survivors (Beisland et al. 2006) are reported. No correlation regarding *CDKN2A* status has been identified so far in patients carrying both of these tumors (Maubec et al. 2012). In 16 patients with MM and RCC (1 developed also PrC), a significant familial MM predisposition was detected (Maubec et al. 2012). Based on a recent finding that a *MITF* missense substitution (E318K) is a predisposing mutation for MM and RCC co-occurrence and for familial MM and MPM (Bertolotto et al. 2011), we screened our patients for *MITF* E318K, but none of the examined family members carried the mutation.

Family B

Family B was strongly positive for PaC with a pattern of dominant inheritance, and the involved mother (III/6) didn't carry any *CDKN2A* mutation. While the early onset PaC formation in the son (IV/1) could be explained by the paternal R99P penetration alone, it does not explain the overrepresentation of PaCs in family B. Given that an elevated PaC risk is reported both in *BRCA1* and *BRCA2* mutation carriers, we analyzed both genes at germline level in the PaC positive mother (III/6) and her living son (IV/2). We could not identify any *BRCA1/2* mutations in family B, but we found ten SNPs (**Table 16** on page 66), two of which, *BRCA1* L771L and *BRCA2* K1132K, were present only in the PaC positive mother (III/6). Although the significance of these two SNPs in familial PaC is unknown, some other *BRCA1* SNPs are considered as significant risk factors in breast cancer development when interacting with environmental factors

(Ricks-Santi et al. 2013). The tumor spectrum of family B does not fit to any known familial cancer syndrome with elevated PaC risk (**Table 26**).

Table 26. Familial cancer predisposing syndromes with PaC as part of the tumor spectrum. (Based on Lynch et al. 2004 with completions)

Familial cancer syndrome	Underlying genes	Mode of inheri-tance	Estimated PaC risk (RR)	Proportion of PaC families	Reference
Breast and ovarian cancer syndrome	*BRCA1* *BRCA2*	AD	1.5-3.1 3.5-10	6-12%	Brentnall 2000, Brose et al. 2002 (b)
Peutz-Jeghers syndrome	*STK11*	AD	132	<1%	Giardiello et al. 2000
Familial pancreatitis	*PRSS1*, *SPINK1*, *CFTR*, *CTRC*	AD	87	<0.5%	Rebours et al. 2008
Familial MM	*CDKN2A*	AD	13-22	<1%	Lynch et al. 2008
Lynch syndrome	MMR genes: *MLH1*, *MSH2*, *MSH6*, *PMS2*	AD	4.5	-	Aarnio et al. 1999
Li-Fraumeni syndrome	*TP53*	AD	7.3	-	Ruijs et al. 2010
Familiar adenomatosus polyposis	*APC*	AD	4.5	-	Giardiello et al. 1993

Abbreviations: MM: malignant melanoma, RR: relative risk, AD: autosomal dominant

Recently further high risk genes (*PALB2*, *ATM*) have also been identified in PaC prone families (Jones et al. 2009, Roberts et al. 2012) but they were not studied in this family. The genetic basis of familiar PaCs remains unknown in about 80% of families.

Taking all these results together, in the non-twin siblings in family A with the unusual combination of four primary tumors (MPM, BCC PrC, LC), the R99P *CDKN2A* germline mutation could have contributed to the familial MPM and also to the early onset PaC. The co-occurrence of BCC and MM could be explained by their shared genetic (*MC1R* V60L, V92M) and environmental risk factors in family A, while in terms of the other two malignancies (PrC, LC), the presence of any common genetic background is questionable. The role of *BRCA1* Q356R SNP in any of the four malignancies is unclear, although functional studies termed the locus crucial for protein function.

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V.2.1.2. Further suspicious genes and environmental factors regarding detected tumor types

PrC is a common cancer type with Hungarian incidence rates similarly 79/100.000 among men inhabitants in the years of diagnoses of our patients (2003, 2006 according to National Cancer Registry). Familial aggregations are rare, in which cases certain germline gene mutations (*BRCA1/2*, *HPC1*, *HOXB13* etc.) or SNPs (reviewed in Dean and Lou 2013) have been suspected. *BRCA1* mutations are the most common (2-5% of familial cases) among them, while germline *PTEN* mutations brought inconsistent results (Cooney et al. 1999). PrC is more frequent among MM survivors than in the general population, while there are also PrC families with reported coexisting MMs (Hemminki and Chen 2005). Still the suspicious relevance of *CDKN2A* mutations or SNPs in PrC development is doubtful. Additional extrinsic etiological factors, such as age, pesticides, dietary and hormonal changes have also been characterized.

In Hungary **LC** is the most prevalent cancer among men, with incidence rates 148-151/100.000 among men in years 2007 and 2009 (National Cancer Registry) respectively. This is a cancer type where environmental factors play a major role in the pathogenesis, while genetic factors are not as well defined. It has recently been postulated, that genetic susceptibility factors differ in smokers and nonsmokers (Subramanian and Govindan 2008). A number of low penetrance genes have been characterized in pathways of smoke related carcinogen metabolism and DNA repair (Schwartz et al. 2007). In sporadic and familial LC chronic tobacco smoke and second hand smoke, among other carcinogenic exposures (ionizing radiation, cadmium, arsenic, radon exc) are significant environmental risk factors. According to reports on familial LC aggregations, a 1.5- to 2-fold elevated risk is associated if having a first-degree relative with LC (Matakidou A. et al. 2005). In terms of MM and LC, some studies proved a significant lower than expected incidence rates of lung or bronchus cancer among MM survivors compared to the general population (Wolff et al. 2000, Wu et al. 2006, Crocetti et al. 2008, Spanogle et al. 2010). Matthews and co-workers analyzed 18,000 families from a multi-institutional Cancer Genetics Network in terms of cancer

co-aggregations and found significant co-aggregations of LC with PrC and LC with PC (Matthews et al. 2008).

The LC aggregation in 4 members of family A could have been the common result of smoke related genetic and environmental predisposition factors considering the number of firefighters and heavy smokers among them. Interestingly MM is also reported as a frequently seen cancer among firefighters, as a consequence of the excessive use of magnetic communication devices (Milham 2009). Cadmium could also be a suspicious environmental risk factor both from cigarette smoke and from firefighters' environment contributing to LC, PrC and kidney cancers (Hartwig 2013).

All suspicious common genetic and environmental factors are summarized in **Table 27**.

Table 27. Review of the published common environmental and genetic risk factors contributing to malignancies in family A.

Predisposing factors			**Cancers in**						**References**
			Medical history				**Family history**		
			MM	BCC	LC	PrC	PaC	RCC	
Environ-mental		Cadmium			+	+		+	Hartwig 2013
		Smoking			+	+	+	+	Zu and Giovannucci 2009 Kuper et al. 2002
		Pesticide	+		+	+	+		Alavanja and Bonner 2012
		UVR	+	+					Greinert 2009
Genetic	Germline	*CDKN2A*	+				+		Lynch et al. 2002, 2008
		MC1R	+	+					Liboutet et al. 2006
		MITF E318K	+					+	Bertolotto et al. 2011
		PTEN	+					+	Eng 2012
		BRCA 2	+			+	+		Moran et al. 2012
		CYP1B1			+	+			Xu et al. 2012, Gajjar et al. 2012
		BAP1	+		+			+	Njauw et al. 2013
	Somatic	*CDKN2A*	+		+	+	+	+	Monahan et al. 2010, Belinsky et al. 1998, Komiya et al. 1995, Bartsch et al. 1995, Kinoshita et al. 1995
		BRAF	+		+				Brose et al. 2002(a)
		RAS	(N)+		(N)+		(K)+	(H)+	Brose et al. 2002(a), Almoguera et al. 1988, Fujita et al. 1988, Waldman and Rabes 1996

Predisposing factors	Cancers in						References
	Medical history				Family history		
	MM	BCC	LC	PrC	PaC	RCC	
PTEN	+			+			Birck et al. 2000 , Li et al. 1997
MITF	+					+	Garraway et al. 2005, Camparo et al. 2008
p53	+		+		+	+	Chiba et al. 1990, Casey et al. 1993, Uchida et al. 1994
STK11	+		+		+		Guldberg et al. 1999, Ji et al. 2007, Su et al. 1999
TSPY	+			+			Oram et al. 2006
ARP/ ARMET			+	+	+	+	Shridhar et al. 1996(a), 1996(b), 1997

It is a limitation of this analysis, that only one of the index siblings (III/5) was available for genetic testing, therefore his germline data can only be hypothetically extrapolated to his brother (III/4) with the same phenotype. Genetic background of the familial PaC on the maternal side was not exhaustively investigated.

Next generation sequencing techniques would be interesting future options for these families to find more genetic link underlying the unique phenotypes.

V.2.2. Six primary MMs

V.2.2.1. Discussion of genetic results

Organ transplantation is now widely available, and while immunosuppressive therapy is required to avoid graft rejection, new generation pharmaceutical agents make it possible to this population to live longer even with the unfavorable consequences of the long-term immunosuppression. There is a clear association between immunosuppressed state and cancer development; MM risk is about 3-times elevated among OTRs.

Given the six primary MMs that developed in this patient, the role of additional genetic susceptibility has also emerged. We didn't find any mutations in the major MM predisposing genes (*CDKN2A*, *CDK4*), only two *CDKN2A* SNPs (c.-191 G>A, c.*29 C>G).

In *MC1R* gene, she carried homozygous 'R' variant alleles (R151C). This variant is reportedly associated with RHC phenotype (reviewed in Sturm 2002), BCC development (Box et al. 2001, Liboutet et al. 2006), and elevated MM risk (Raimondi et al. 2008, Williams et al. 2011). She indeed was a red haired individual, with vulnerable fair skin type and history of multiple BCCs, with an additional unfavorable sun tanning habit. In none of the further examined genes (*PTEN*) or gene segments (*CDK4*, *MITF*) did she harbor any germline alteration.

V.2.2.2. Discussion of environmental factors

Regarding immunosuppressive therapy, the initial combination that was introduced to the patient (MMF, MP, TAC) showed the lowest cancer incidence rates in a study that compared the different combinations in terms of cancer development during combined immunosuppressive therapy (Watorek et al. 2011). Analyzing effects of immunosuppressive agents on tumor formation is complicated, as most OTRs are exposed to combinations of drugs from different pharmacological groups.

MMF, with a major effect on inhibiting lymphocyte proliferation by blocking de novo purine synthesis pathway, is thought to have an anti-proliferative effect. This effect is partly explained by the purine synthesis blocking mechanism, and also by altering expression of certain integrins that play role in tumor cell invasion towards the vascular endothelia. Recent study confirmed that MMF has a significant inhibitor effect on tumor cell growth and angiogenesis in vitro, however showed undetectable effects against MM tumors in vivo (Koehl et al. 2007). In a clinical study, tumor development increased if Cyclosporine or TAC treatment was combined with MMF compared to the single treatments (Wimmer et al. 2007).

MP as a synthetic glucocorticoid, considered in general as anti-proliferative agent mainly due to a cell cycle arrest effect in G1 phase, however they also inhibit interleukin-1 and 6 (with subsequent suppression of both cellular and humoral immune responses), TNF-α, interleukin-2, and interferon-γ. There is no direct data whether MP has any effect on melanocyte proliferation or MM development (summary in Zattra et al. 2009).

TAC is a calcineurin inhibitor agent with a highly effective potential to prevent graft rejection, however without significant antitumor effects. Topical use of TAC is reported to result in enlargement of primary MM in a case (Mikhail et al. 2008). Human

cultured melanocytes showed activated cell migration and tyrosinase activation upon TAC treatment, moreover in another *in vitro* study obtained using keratinocytes, TAC created favorable conditions to melanocyte growth and migration. Therefore TAC might be considered to promote melanocyte proliferation both directly and by its immunosuppressive effect (summary in Zattra et al. 2009).

SRL that was her new agent after TAC, is an inhibitor of mammalian target of Rapamycin (mTOR), a protein kinase controlling cell growth via cell cycle arrest in G1 phase mainly in T-lymphocytes and also on vascular smooth muscle cells resulting in an immunosuppressive and anti-proliferative action (**Figure 9** on page 25). A number of mTOR inhibitor (mTORi) agents are already used in therapy of different primary tumors (pancreatic neuroendocrine tumor, RCC, mantle cell lymphoma), moreover studies on OTRs showed also lower incidence of malignancies under mTORi treatment (Tedesco Silva et al. 2010, Wimmer et al. 2007). Besides the clear anti-proliferative effects of mTORi in several tumor types, MM seems not to be a major candidate. However *PTEN* somatic mutations resulting in an overactivation of mTOR pathway, are commonly seen in primary MMs, early studies did not support their relevance as single therapeutic agent, while in combination with BRAF V600E inhibitors (as *BRAF* and *PTEN* mutations are commonly concurrent events during MM genesis) studies are already ongoing (sorafenib-BRAF inhibitor with temsirolimus-mTORi) (Jang and Atkins 2013).

In general, in OTRs switching from a calcineurin inhibitor agent to mTORi is highly recommended upon malignant tumor development (especially in cases of NMSC development) that was followed in this case by the transplantation team.

Reports about outcome of MM in immunocompromised patients are lacking consequent results. MM in other subset of immunosuppressed populations such as in HIV positive patients (Rodrigues et al. 2002) or in CLL (McKenna et al. 2003) noted a potentially more aggressive behavior, however were obtained on small groups and the type of immunosuppression differed from that of OTRs. Dapprich and coworkers reported a small retrospective case series but did not find differences between OTRs and prognostically matched not immunosuppressed MM patients (Dapprich et al. 2008). Another study also proved the lack of difference in MM outcome among these populations (Matin et al. 2008).

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In our patient, pathological characteristics of the latest MM would normally not suggest such a fast and aggressive outcome; however the presence of regression raises the possibility of a more advanced state at detection due devolution in Breslow depth. Interestingly regression should reflect the proper function of immune system (immunization effect) as a response to tumor, this phenomenon is also suspected to play role in MPM patients, where the subsequent MMs are reportedly thinner and shows favorable biological behavior (Doubrowsky et al. 2003, Ferrone et al. 2005).

In summary, considering the etiologies for both the MPMs and BCCs, we hypothesized that the susceptibility factors consisted of:

1. an intermediate risk genetic predisposition by carrying a homozygous 'R' variant of *MC1R* with a subsequent vulnerable phenotype (red hair, fair skin, light eye color),
2. a positive history of sun exposure and recreational suntan habit with resulting significant photodamage of the skin
3. the initial immunosuppressive therapy could have been not the most favorable in terms of MM development, and however revision and switching (TAC→SRL) was obtained after four primary MMs to an mTORi that is reportedly more convenient in terms of malignant tumor development, further MMs developed.

It must be however mentioned, as a limitation of this analysis that somatic mutation profiling of the tumors could serve us with interesting information about clonality and origin of the fatal propagation. Unfortunately, even though attempts were made to this direction, we could not fulfill these aims.

V.2.3. MM and phenotype suggesting PHTS/CS

V.2.3.1. Discussion of genetic results

Quantification and early detection of subsequent primary malignancies during patient management after diagnosis of MM is of a great importance, and also raises new opportunities to identify shared environmental and genetic backgrounds, or in rare cases certain cancer predisposing syndromes.

In this patient, analysis of MM predisposing genes revealed no pathogenic, high penetrance gene mutations (*CDKN2A*, *CDK4*). We found one *MC1R* 'R' variant (R160W), that owns some elevated risk for MM development.

Based on the patient's complex phenotype and medical history, principally CS from PHTS was suspected. Therefore germline *PTEN* mutation analysis was performed, in which we found no pathogenic mutations, only two SNPs (c.80-96 A>G-rs1903858 and c.1026+32 T>G-rs555895) along the full coding sequence. *PTEN* promoter analysis was not obtained in our case, albeit they are linked to CS phenotype. Also big deletion or duplication might have been missed by the test method we used (Sanger sequence analysis). However the two heterozygous SNPs identified at the beginning and the end of the *PTEN* gene could serve us as a proof against full gene deletion.

In *PTEN* negative CS and CLS patients, recently *SDHB* and *SDHD* germline mutations were identified and linked to the disease phenotype. By sequencing these two genes, in the *SDHB* gene we identified the c.18 C>A substitution resulting in p.A6A (rs2746462) that has a 2-4% prevalence in general populations (Cascón et al. 2002, Castellano et al. 2006, Korpershoek et al. 2006). In *SDHD* gene, two intronic sequence variants were identified, one of which was already interpreted as an SNP (c.52+136 G>T-rs7121782). The *SDHD* c.314+15 T>A has not been reported before, therefore is of unknown significance, however given the proximity to the exon boundary, it's effect on splicing or disease phenotype need to be further studied. Interestingly an overrepresentation of certain tumor types (female breast-, thyroid-, and kidney cancer) had been published among *SDHD* carrier CS patients compared to *PTEN* positive ones (Ni et al. 2012), all of which cancers were observed in our patient. Large germline deletions in *SDHB* or *SDHD* are usually reported in hereditary paraganglioma patients (McWhinney et al. 2004). Large deletion in *SDHB* gene cannot be ruled out in this case; however the two identified heterozygous SNPs in *SDHD* gene may again exclude the possibility of whole-, or large deletions of the gene.

V.2.3.2. Discussion of further suspicious genetic events

Germline analyses of this case resulted with no proof of CS, except the possible role of the newly identified *SDHD* intronic substitution. Analysis of further genes, that were identified as pathogenic in the disease development (*AKT1*, *PIK3CA*, *KLLN*) could verify the clinical diagnosis of CS. Based on the clinical findings, Proteus syndrome

(PS) would be another suspicious diagnosis regarding this case; diagnostic criteria are listed in **Table 28**.

Table 28. Diagnostic criteria for PS. Color-codes indicate the fulfilled criteria.

PS	Diagnostic criteria (based on Eng 2012)
	Mosaic distribution, progressive course and sporadic occurrence: • **Connective tissue nevi** Or two of the followings: • Epidermal nevus • **Disproportionate overgrowth of one or more:** ▪ **Limbs** ▪ **Skull-hyperostosis** ▪ External auditory meatus-hyperostosis ▪ Vertebrae ▪ Viscera: spleen/thymus • **Specific tumors before 2nd decade** • Bilateral ovarian cystadenomas • **Parathyroid adenoma** Or three of the followings: • **Dysregulated adipose tissue** (Lipomas, regional absence of fat) • Vascular malformations • **Facial phenotype** (dolichocephaly, long face, minor downslanting of palpebral fissures/minor ptosis, low nasal bridge, wide-anteverted nares, open mouth at rest)

Abbreviations: PS: Proteus syndrome

PS, however once considered as a PHTS with low incidence of germline *PTEN* mutations, recently have been proved to be a consequence of mosaic somatic activating mutations in *AKT1*, a downstream member of the same, PTEN/PI3K/AKT signal pathway (Lindhurst et al. 2011, Marsh et al. 2011). A recent study suggested that the level of *AKT1* somatic mutations confer association with the type of skin lesions in PS patients (Lindhurst et al. 2014). There are also reports of PS phenotypes with somatic *PTEN* mutations (Loffeld et al. 2006). The hypothesis that certain sporadic diseases with lethal outcome in autosomal dominant manner can survive in a unique distribution along the skin and body, reflecting somatically mutated mosaic state, was initially suggested by Happle (Happle 1987, 1999). As previously in some PS cases germline *PTEN* mutations have been detected (Smith et al. 2002), now they are referred as Proteus-like syndrome (PLS).

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VI. CONCLUSIONS

VI.1. MPM study

With this work (Hatvani et al. 2014), our major aim was to analyze Hungarian MPM patients for the first time from clinical and genetic point of view and to find associations between these results focusing on *MC1R* carrier status.

Based on our findings in this MPM study we can conclude, that

1. The following results were in accordance with previous data from the literature:

- Rate of MPM occurrence among MM patients
- Higher number of common nevi or presence of dysplastic naevus as susceptibility factors for MPM development
- Younger age of onset in women MPM patients than in men
- Subsequent MMs in MPM patients are thinner, more likely Mis than invasive MM, and develop commonly on the same body site with the first MM
- Rate of *CDKN2A* mutations among MPM patients
- Frequency of certain *CDKN2A* SNPs (c.*29 C>G, c.*69 C>T)
- Frequency of *MC1R* non-synonymous variants
- Frequency of 'R' variants (R151C, R160W, D294H) among MPM patients

2. The following of our observations differed from those reported in the literature, or brought novel results:

- Less frequent positive family history of MM among the MPM patients
- A higher rate of synchronous first and second MMs
- More frequent non-melanoma malignancy occurrence among MPM patients
- Two Hungarian MPM patients with *CDKN2A* mutations (p.E69G, p.R99P)
- Lower frequency of *CDKN2A* SNP A148T
- Higher allele and carrier frequency of *CDKN2A* SNP c.-191 G>A
- The lack of presence of two otherwise common *MC1R* variants (D84E, R142H) among the Hungarian MPM patients
- The *MC1R* R163Q variant to be exceptionally common among Hungarian MPM patients, a variant otherwise frequent in Asia, but not in Europe, supporting the previous findings on geographical differences regarding *MC1R* variant occurrence.

- Identification of a new *MC1R* variant firstly in humans (c.350 A>G;p.D117G)
- some new potentially unfavorable predictive observations among *MC1R* 'R' carriers compared to *MC1R* 'r' carriers
  - younger age of onset
  - MPM co-occurrence with more non-melanoma primary tumors, or with multiple BCCs
  - more ulcerated first MMs, less ulcerated second MMs
  - TILs in second MMs (significantly).

Therefore we hypothesize that as already suspected in terms of other MM predisposing genes (*CDKN2A*), *MC1R* genotype details may also carry additional useful information concerning patient survival and prognosis if confirmed on bigger sample sizes.

This work is the first Hungarian description of MPM populations regarding cliniopathological and genetic characteristics.

VI.2. Unique MM-associated cases

VI.2.1. Two cancer prone families

We examined two unique cancer prone families and their two common offspring. In family A, an unfavorable coincidence of inherited and environmental risk factors induced the reported new constellation of 4 primary malignancies (MPM, BCC, PrC, LC) in two non-twin siblings. In family B with a dominantly inherited PaC aggregation we could identify only a number of SNPs with unknown significance regarding PaC development. The inheritance of predisposing genetic events from the two families resulted in early onset malignant tumor formation in both of the offspring, in one of them even with fatal outcome. These data also indicate the necessity of a very close follow up of the uninvolved family members too in such cancer prone families.

VI.2.2. Six primary MMs

Immunosuppressed state (also due to immunosuppressive therapy in OTRs) makes one vulnerable to cancer and also to MM development by many mechanisms. Development of six primary MMs in an OTR patient however raises the possibility of an additional genetic susceptibility to MM too. There was a complex interplay between the diverse

environmental (UV, sun tan habit, combined long term use of harmful pharmacological agents, bad compliance) and genetic (*MC1R* 'R' variant) predisposing factors in the skin tumor formations. This case points out the importance of the careful dermatological follow-up of OTRs under immunosuppressive therapy, and also of their education about the subsequent harmful environmental predisposing factors.

VI.2.3. MM and phenotype suggesting PHTS/CS

As a rare co-aggregation, MM(Mis) was described in a patient with phenotype highly suspicious for PHTS/CS.

Identification of pathogenic genetic loci in rare, unique phenotypes with standard methods (Sanger sequencing) is time, and cost demanding, with many limitations (large deletion or insertions). Therefore such patients may remain without identified genetic background however with clear phenotype.

In this case, although the involvement of a particular signal transduction pathway (*PTEN*) and other genetic loci (*SDHB*, *SDHD*) were highly suspicious, we could not identify the exact underlying genetic disturbance. Germline involvement of further downstream genes in the PTEN/PI3K/AKT pathway (*PI3K*, *AKT1*) is possible, that would be of a great interest to study. Next-generation, high throughput techniques (exome-, genome sequencing) would serve to identify rare disease causing mutations in new genes in such cases. To prove PS as other suspicious diagnosis, somatic *AKT1* mutation analysis would be diagnostic.

VI.2.4. Conclusions of Unique MM-associated cases:

- In unique cancer cases with suspicious genetic predisposition, traditional techniques are helpful for identifying known, rare, highly penetrant disease causing gene mutations. With next-generation sequencing methods, many new data are already available about common, low penetrance alterations in a huge number of genes, and some of them are already linked to certain cancers as additional significant predisposing factors.

  In some of our analyzed cases we could not identify mutations in high penetrance genes, but detected a number of more common SNPs, however their

individual or aggravated significance is not determined. These findings also support the emerging need of further high throughput studies in cases like these, to find associations between common germline or somatic genetic factors and the development of particular tumors or tumor-constellations.

- In cancer development, gene-environment interactions are highly diverse with wide variety of causal burden. In our highly civilized lifestyle, many of the additional extrinsic factors and their potential harmful effect on malignant transformation might not have been identified. Even if an environmental factor is proved to be linked to a specific cancer development in general, the complexity of all extrinsic factors and their interplay with the harbored genetic events are hard to define.

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VII. SUMMARY

Malignant melanoma (MM) patients are more prone to develop subsequent malignancies; especially MMs (multiple primary melanoma: MPM). MM predisposing gene mutations (*CDKN2A*, *CDK4*, *MC1R*, *MITF* E318K) are more frequent among MPM patients than single MM patients, and exhibit geographical differences. Environmental factors, especially UV play also significant role in MM/MPM development. Next to UVB, the significance of UVA is emerging and is also supported by findings of weaker DNA damage response mechanisms compared to UVB.

The major aim of this work was to analyze a Hungarian MPM patient group regarding clinical, histologic and genetic aspects. In our institution in an 11-year long period, 108/1855 MM patients developed MPMs (5.8%); 43 participated in this study. Furthermore three unique cancer prone MM cases have been also analyzed in detail.

As a first analysis of Hungarian MPM patients, some of our results were consistent with the literature, while we got a number of new observations: family history of MM was less frequent (9%), synchronously diagnosed first and second MMs (49%) and occurrence of non-MM malignancies (42%) was found at a much higher rate than in other studies. We identified two MPM patients (4.7%) with *CDKN2A* mutations (E69G, R99P), while none of the patients carried *CDK4* hot spot mutations, neither the *MITF* E318K. We determined *MC1R* variant frequencies, and found a new variant (D117G) for the first time in humans. As a suspicious specific *MC1R* variant distribution pattern of Hungary, the frequent Asian variant R163Q was quite overrepresented in our series compared to other European reports. Comparing patients regarding their ‘r’ (n=20) or ‘R’ (n=23) *MC1R* variant status, ‘R’ carriers had younger age of onset; more non-MM tumors - especially multiple BCCs; showed a non-significant trend of a more progressive disease and had significantly more tumor infiltrating lymphocytes in their second MMs, suggesting that certain germline *MC1R* variants (‘R’alleles) might reflect prognostic relevance in MPM patients. By analyzing the three unique cancer prone MM cases, besides the identification of a pathogenic mutation (*CDKN2A* R99P) and some suspicious environmental factors, a number of other common SNPs have been identified with unknown significance regarding the specific tumor constellations, but supporting the relevance of parallel inherited common gene alterations and gene-environment interactions in malignant tumor development and aggregations.

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VIII. ÖSSZEFOGLALÁS

A melanomás (MM) betegek fokozott hajlamot mutatnak egyéb malignitások kialakulására, melyek leggyakrabban további MM(-ák)(multiplex primer MM: MPM). A MM-ra hajlamosító gének mutációi (*CDKN2A, CDK4, MC1R, MITF* E318K) jellegzetes földrajzi megoszlást mutatnak, és gyakoribbak a MPM-, mint az egyszeres primer MM-s betegek körében. Számos környezeti faktor -legjelentősebbként az UV sugárzás-, szintén szerepet játszik a MM/MPM kialakulásában. Az UVB mellett az UVA szerepe is felvetődik azon új eredmények tükrében, miszerint az UVA hatás sokkal gyengébb celluláris DNS javító mechanizmusokat indukál, mint az UVB.

Munkánk elsődleges céljaként egy magyar MPM populációt vizsgáltunk klinikai, hisztológiai és genetikai szempontból. A részletes vizsgálat részét képezte három unikális MM-s eset is. A klinika 11 éves MM-s beteganyagán 108/1855 MM betegnél alakult ki MPM (5,8%), akik közül 43-an vettek részt a genetikai analízisben.

Ezen első magyar MPM beteganyagról készített klinikai, hisztológiai és genetikai vizsgálat során megfigyeléseink egy része egybevág korábbi külföldi publikációkéval, azonban számos új konklúzió is született: MPM betegeink körében a famíliáris MM ritkábban (9%), míg az egyidejűleg diagnosztizált első két MM (49%)-, valamint az asszociált malignus tumorok (42%) gyakrabban fordultak elő. Két MPM betegnél (4,7%) azonosítottunk *CDKN2A* mutációt (E69G, R99P). Beteganyagunkon nem identifikáltunk *CDK4* hot-spot-, sem az *MITF* E318K mutációt. Meghatároztuk a *MC1R* variánsok hazai megoszlását, és egy, emberben teljesen új variánst (D117G) azonosítottunk egy betegnél. Az Ázsiára jellemző R163Q-t gyakoribbnak találtuk a publikált európiai eredményekhez képest. A MPM-s betegeket *MC1R* ‘r’ (n=20) vagy ‘R’ (n=23) státuszuk alapján összehasonlítva az ‘R’ hordozóknál fiatalabb átlegéletkort, több nem-MM tumort és többszörös basaliomát észleltük. Emellett nem szignifikánsan, de trend jelleggel klinikopathológiai adataik rosszabb prognózist sugalltak; míg a második MM-aikban gyakoribb volt a gyulladás jelenléte. Mindezek felvetik a *MC1R* genotípus (‘R’ allél) esetleges prognosztikai jelentőségét MPM-ban. A három egyedi eset vizsgálata során egy kóroki mutáció (*CDKN2A* R99P) mellett számos egyéb kérdéses jelentőségű SNP-t találtunk. Mindezek, a felderített környezeti hajlamosító faktorokkal együtt rávilágítanak az SNP-k és a gén-környezet finom interakcióinak szerepére a rosszindulatú daganatok és daganat-konstellációk kialakulásában.

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X. OWN PUBLICATIONS

Publications related to the thesis:

Hatvani Z, Brodszky V, Mazan M, Pinter D, Harsing J, Toth V, Somlai B, Karpati S. (2014) Genotype analysis in Hungarian patients with multiple primary melanoma. Exp Dermatol, 23: 361-364. **IF: 3.578**

Toth V, **Hatvani Z**, Somlai B, Harsing J, Laszlo JF, Karpati S. (2013) Risk of Subsequent Primary Tumor Development in Melanoma Patients. Pathol Oncol Res, 19: 805-810. **IF: 1.555**

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Tóth V, Somlai B, **Hatvani Z**, Szakonyi J, Gaudi I, Kárpáti S. (2013) Melanoma Screening in a Hungarian Nuclear Power Plant. Pathol Oncol Res, 19: 323-328. **IF: 1.555**

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