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Melanoma Markers and New Treatment Perspectives

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

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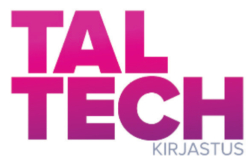

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List of Publications

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Author’s Contribution to the Publications

Contribution to the papers in this thesis are:

I Marina Teras collected the clinical data and was responsible for ethics approvals and consent of patients to participate in the study. She also read and has approved the final manuscript.

II Marina Teras conceived and designed this study, analysed the data, and supervised this study. She also contributed to interpretation the data, was one of the main contributors in writing the manuscript, drafting and revised it. She read and approved the final manuscript.

III Marina Teras designed the conseption of the project.

Introduction

Worldwide, the incidence rates of skin cancer, like other types of cancer, are increasing. People in Europe and the Nordic countries are at a higher risk of developing skin cancer due to their light and sun-sensitive skin type. The number of new cases of skin cancer in the Nordic countries including Estonia is rizing, but has not jet reached the same level as in several southern–countries including Australia, New Zealand, and Florida. Melanoma frequency cases in Northern Europe increased especially after the year 2000: people got more frequently exposed to strong ultraviolet radiation (UVR) doses over short periods such as sunbeds or holidays in tropical countries. The increasing trend among Estonians to travel to southern countries is also likely to play a role in this. However, in Estonia, the frequency of melanoma in comparison with the level in Nordic countries and the number of fatal cases is decreasing due to better early diagnostics.

Of all the types of skin cancer, melanoma has the worst prognosis and it is considered to have the highest metastatic potential.

Skin melanoma is one of the most spread type of all tumours and very aggressive. Since melanoma exhibits a high rate of mutations, it is always one step ahead of the immune system. An important risk factor for melanoma development is skin exposure to UVR in combination with other carcinogens. The usage of artificial sources of UVR, such as sunbeds should be avoided. Early diagnosis (thin melanoma, less than 1 mm) of melanoma and surgical treatment leads to almost 100% recovery and survival. It is very important to educate medical staff to recognise early melanoma, especially in elderly patients. Men usually have worse prognoses of melanoma compared to women, especially considering the thickness of primary melanoma lesions. When melanoma is thicker than 4mm, it is fatal in ~50% of cases.

What could help us to avoid melanoma?

1. Prevention of skin damage caused by UV radiation: people should know that using protective clothing offers better protection than solar creams.

2. Improved early diagnosis, for example by using dermatoscopy.

3. A better understanding of melanoma pathogenesis, mechanisms of spread and capacity of the immune system in fighting cancer in every individual case.

4. Better understanding of the role of the microbiome in melanoma development and during treatment.

5. Integrating epidemiological, pathological, clinical, and molecular features, to establish distinct subtypes of melanoma, which should lead to improved treatment accuracy and become a powerful tool in disease management.

The question of the thesis is: how the understanding of novel molecular markers and melanoma mechanisms, as well as the implementation of novel principles for treatment, can assist in better understanding of the disease and lead to enhanced patient survival and disease outcomes.

Abbreviations

ALM	Acral lentiginous melanoma
Anti-PD-1	Anti-Programmed cell death-1
BAP1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)
BRAFi	Serine/threonine-protein kinase B-Raf inhibitor
CDK	Cyclin-dependent protein kinases
CNS	Central nervous system
CTC	Circulating tumour cells
ctDNA	circulating tumour DNA
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
ELISA	Enzyme-linked immunosorbent assay
EGFR	Epidermal growth factor receptor
FAMMM	Familial atypical multiple mole melanoma
FDA	Food and Drug Administration
GALT	Gut-associated lymphoid tissue
GVHD	Graft versus host disease
IFN	Interferon
IL	Interleukin
LDH	Lactate dehydrogenase
LMM	Lentigo malignant melanoma
LN	Lymph node
mAb	Monoclonal Antibody
MAGE	Melanoma associated antigen
MAPK	Mitogen activated protein kinase
MART1	Melanoma antigen recognized by T-cells 1
MC1R	Melanocortin 1 receptor
MDSCs	Myeloid derived suppressor cells
MEKi	Mitogen-activated protein kinase kinase enzyme inhibitor
MM	Malignant melanoma
NM	Nodular melanoma
OS	Overall survival
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-ligand-1
PET	Positron emission tomography
PFS	Progression-free survival
POT1	Protection of telomeres protein 1
RAF	Proto-oncogene serine/threonine-protein kinase
RANK	Receptor activator of NF-kB
ROS	Reactive oxygen species
SCF	Stem cell factor
SLNB	Sentinel lymph node biopsy
SMG	Significantly mutated genes
SPF	Specific pathogen-free
SSM	Superficial spreading melanoma
TAM	Tumour-associated macrophages (TAM)
TME	Tumour microenvironment

Tregs	Regulatory T cells
UV	Ultraviolet
UVR	Ultraviolet radiation
WT	Wild type

1 REVIEW OF THE LITERATURE

1.1 What is melanoma?

1.1.1 What is cancer?

Cancer is one of the leading causes of death worldwide. The incidence of cancer is influenced by many factors such as genetics, age, gender, race, diet, and environmental factors. Cancerogenesis can be driven by ultraviolet (UV) light from sunlight or other carcinogenic agents such as X-rays, asbestos, tobacco, benzene, acrylamide, and some food additives. Several viruses can also trigger gene mutations, such as the human papillomavirus that can cause cervical cancer (Sadozai et al. 2017). It is estimated that 15–20% of cancer cases are driven by infectious agents, 20–30% of cancer cases are largely due to tobacco use, and 30–35% cases are associated with diet, physical activity, and/or energy balance (e.g., obesity) (Bhatt et al. 2017). UV radiation from sunlight in combination with many other carcinogenic substances: asbestos, benzene, radon (i.e., mixed exposures) may have a cumulative effect. The relative risk for cancer development is dependent on the combination, dose, and duration of each exposure and the genetic background of each individual.

Every individual is exposed to cancer-causing substances during the lifetime or can be the carrier of genes that are linked to genetic forms of cancer, but most people do not develop cancer due to lack of stimulus for activation of these mutations or genes. Most individuals also possess a normal immune system that controls and eliminates cells which could potentially become cancer cells. In addition, even certain dietary lifestyles play a significant role in conjunction with the immune system to allow or prevent cancer cell survival. That is why it is difficult to assign a specific cause of cancer to most individuals.

Cancer is the failure of normal cooperation between cells and tissue systems. Within multicellular tissues there exists cooperation among cells, which is essential for the development, reproduction, and maintenance. Cancer can be characterized by a breakdown of the central features of cooperation: disturbances in inhibition of proliferation, cell death, and maintenance of the extracellular environment, suggesting that deregulation of differentiation is a universal aspect of carcinogenesis (Aktipis et al. 2015). Although each cancer varies in tissue and cell type, also from patient to patient, the common features among all cancers at the systems level remain the same. Cancer is the transition of homeostasis to deregulated behaviour within a tissue, being induced by altered stromal signalling, failure of the immune response, ageing, and inflammatory events. If a cell has turned cancerous, it continues to divide until a mass of cells forms a tumour. As this tumour mass progresses, more cells break off and spread, or metastasise, around the body via the lymphatic system and blood vessels (Gandini et al. 2005, Kong et al. 2010, Paul 2019).

Cellular senescence is the state of permanent growth arrest, regulated by telomerase. Enzyme telomerase is elongating telomeres, which are series of repeating TTAGGG nucleotides of chromosomal ends that progressively shorten with each mitosis. When the critical length is reached it causes the cell to undergo apoptosis (Rachakonda et al. 2018). The role of telomeres in cancer development is unclear and may be cancer type-specific. When telomeres have sufficient length, they can prevent DNA modification and chromosomal instability through DNA repair mechanisms (Maciejowski et al. 2017). However, some cancer cell types with impaired DNA damage

responses (for example melanoma through the loss of functional p53) continue to divide in the presence of dysfunctional telomeres (Terzian et al. 2010). In general, telomere shortening is still typical in most human cancers, however, longer telomeres are associated with increased melanoma risk (Rachakonda et al. 2018). Telomere dysfunction can produce the opposing pathophysiological states of degenerative ageing. In most advanced cancers, telomerase is reactivated to maintain telomere length and regulate cancer-promoting pathways (Artandi et al. 2010). Short telomeres influence p53, which is a tumour suppressor and cellular stress sensor protein, able to enforce cell cycle arrest in response to DNA damage and hypoxia (Krizhanovsky et al. 2008). In humans, p53 has 15 isoforms, all encoded by the TP53 gene, which is the most frequently mutated gene (>50%) in human cancer. All p53 isoforms can bind to DNA and regulate gene expression to prevent mutations of the genome.

1.1.2 Melanogenesis – production of melanin

Melanoma is a malignant tumour of melanocytes, that produce a pigment called melanin. Melanocytes originate from neural crest cells (NCC). Human multipotent dermal stem cells (DSCs) express the neural crest stem cell markers NGFRp75 and nestin, can differentiate into several mesenchymal and neuronal lineages and melanocytes, indicative of their neural crest origin (Zabierowski et al. 2011). DSCs migrate to the basement membrane region of the skin and differentiate into melanocytes. After migration, proliferation, and differentiation they reside in the epidermis, hair follicles, the choroid of the eye, the iris, meninges, the inner ear, respiratory, gastrointestinal, genital-urinary tracts and oral mucosa (Hu et al. 2008). Normal human melanocyte that populates the epidermis and dermis are biologically different cell populations (Zabierowski et al. 2011). During normal homeostasis, epidermal melanocytes reside in the basal layer within epidermal melanin units where one melanocyte cooperates with 30–40 associated keratinocytes (Haass et al. 2005). The ratio of melanocytes to keratinocytes is 1: 10 in the epidermal basal layer (Haass et al. 2005). Mature melanocytes are dendritic-like cell, but darker and smaller than keratinocytes. In the cytoplasm, they exhibit membrane-bound melanin-producing organelles called melanosomes (Seiji et al. 1961, Whiteman et al. 1999).

The epidermal layer of skin is composed of keratinocytes, melanocytes, and immune cells. The proliferation and differentiation of melanocytes are influenced by keratinocytes and fibroblasts and being regulated by genetic and epigenetic factors, hormones, and several environmental factors (Cichorek et al. 2013). Normal melanocytes that populate the hair follicle are located in the proximal bulb, with a ratio of 1:5 melanocytes to keratinocytes and these melanocytes are larger and more dendritic than the epidermal ones (Fitzpatrick et al. 1963, Haass et al. 2005).

Melanocytes are long-living cells in the basal layer that proliferate extremely rarely under normal circumstances. Melanocyte cell division happens during the expansion of the skin surface in childhood or wound-healing and at a low rate upon stimulation by sunlight exposure (Bennett et al. 2002). To undergo mitosis, they detach from keratinocytes and the basement membrane. After division, they migrate along the basement membrane to form a new melanin unit with keratinocytes (Hirobe 2011). It is still not known whether cells from earlier developmental stages or mature melanocytes are more tumouricidal (Zabierowski et al. 2011).

Pigmentation determining hair colour is regulated by adrenocorticotropic hormone (ACTH), melanocortin receptor 1 (MCR1), melanocyte-stimulating hormone α-MSH,

receptor c-Kit and its ligand stem cell factor (SCF), cytokines, neurotransmitters, fibroblast growth factor (FGF) and several other regulators (Haass et al. 2005). In addition, melanocytes secrete a number of signalling molecules that target skin immune cells (Lu et al. 2002). The secretion of all the above-mentioned factors is increased under ultraviolet radiation (UVR) and leads to melanocyte proliferation, melanin production, and skin pigmentation (Larribere et al. 2019). FGF is the most mitogenic of the above-mentioned factors for melanocytes *in vitro* and can even cause melanoma-like lesions when combined with UVB, but not UVA (Czyz 2019). Higher levels of FGF2 in the microenvironment of dermal nevus-derived melanocytes allow *in vivo* melanocytes to adapt to growth in the dermis, which might also be an important factor for the development of melanoma (Paul 2019).

Melanogenesis takes place in melanocytes and melanin is present in the cytoplasm of melanocytes, but also the extracellular space, as free "naked melanin" called melanocore. Naked melanin is internalized by keratinocytes, where it acts as a natural sunscreen and protects the DNA by UVR damage (Boissy 2003, Dediol et al. 2011)

Melanocytes produce two types of melanin that differ in colour and the way of synthesis. Eumelanin is black-brown and pheomelanin (PM) is yellow-red. Human skin contains a mixture of both. The ratio of eumelanin to total melanin is responsible for skin colour and its photo protecting properties (Slominski et al. 2004, Yamaguchi et al. 2007). About 45% of melanoma metastases are pigmented. Eumelanin is predominantly expressed in primary lesions, whereas pheomelanin is associated with disease progression. Therefore, melanin could be used as a potential target for radionuclide therapy of metastatic melanoma (Dadachova et al. 2005).

The density of melanocytes in the skin depends on the environment and age. After 30 years of age, 10–20% of epidermal melanocytes are lost with every decade. In older people also melanocyte morphology is changed: they are larger, more dendritic and their tyrosinase activity is reduced (Tobin 2011, Cichorek et al. 2013). Senescent melanocytes are replaced by melanocyte stem cells that comprise a melanocyte reservoir for hair and skin pigmentation (Nishimura 2011).

1.1.3 Melanoma

Melanoma is the cause of 4% of all skin cancers and 80% of skin cancer-related deaths (Ferlay et al. 2019). It is a heterogeneous disease with specific genetic alterations within several functionally related molecular pathways. Melanoma is most common in white-skinned individuals, but may rarely develop with dark skin as well (<5%). The highest incidence rates overall are observed among white males and females over 65 years of age with the male to female ratio 3:2 (American Cancer Society, 2020).

DNA repair capacity, as well as environmental factors, mainly dose and timing of UV exposure during early periods of life, are risk factors for malignant transformation. Melanomas may develop in or near the previously existing precursor lesions or in healthy appearing skin (75%). Melanoma can develop on skin solar exposed or unexposed areas. Certain lesions are considered to be precursor lesions of melanoma: common acquired nevus, dysplastic nevus, congenital nevus, and cellular blue nevus (Cichorek et al. 2013).

Melanoma arises from all kinds of melanocytes wherever in the body through progressive accumulation of genetic and epigenetic alterations that disrupt homeostatic pathways. That is followed by uncontrolled tumour cell proliferation resulting in invasion and lymphatic or haematogenous dissemination of the tumour

cells to distant sites (Monteagudo et al. 2019). Melanoma cells can have different morphology and in different body regions, they react to different growth factors. For this reason, they differ by their tumourigenicity: influenced by location, environment, or gene expression in this location (Bastian 2014). **There is an interesting observation that sometimes melanoma appears later in the location of previous trauma or chronic pain (Marina Teras unpublished data).**

Melanocytic cell mutations can be either genetic: cyclin-dependent kinase inhibitor 2A (CDKN2A) etc. (Chandler et al. 2013) or oncogene-induced senescence following oncogene activation in normal cells (Monteagudo et al. 2019). Two major pathways are well established to be responsible for signalling arrest in human cell senescence: the p16 pathway and the p53 pathway, that arise from different causes (Salama et al. 2014).

Melanomas can be divided into several subtypes and multiple distinct categories to precursor lesions, locations, histologic presentation, typical mutations and pathogenitic role of UV radiation, for exaple chronic sun damage (CSD), non-CSD melanoma (Curtin et al, 2005) and all of them will be discussed later in this thesis.

1.1.4 Main risk factors of melanoma

The etiology of melanoma is multi-factorial, resulting from gene-environment interactions (exposure to sunlight and UVR). The mechanism of mutations in cutaneous melanoma is based on the modification of DNA by UVR exposure (Coricovac et al. 2018).

Ultraviolet B (UVB, wavelengths 290 to 320 nm) radiation appears to be a more important risk factor for the development of melanoma than ultraviolet A (UVA, wavelengths 320 to 400 nm), and a causal link to UVA exposure is also supported by data from patients using tanning beds or treated uncontrolled with psoralen plus UVA (PUVA) for psoriasis (Noonan et al. 2012).

Both UVA and UVB radiation have been shown to accelerate serine/threonine-protein kinase B-Raf (BRAF)-mediated melanoma genesis through gene TP53 mutation, but the molecular mechanisms by which UVR drives melanoma genesis remain unclear. The most common somatic mutation in melanoma is V600E substitution in BRAF, which is an early event (Viros et al. 2014, Eckhart et al. 2019).

The pattern and timing of sun exposure appear to be important for skin cancer: melanomas are associated with intense, intermittent sun exposure and sunburns and they frequently occur in areas exposed to the sun only sporadically (e.g., the back in men, the legs in women) (Zanetti et al. 2006). Red hair, freckling tendency, and poor tanning ability are associated with UV-independent mutagenesis in melanoma. They cover the occurrence of melanomas at cutaneous locations that are not sun-exposed, as well as in the eye and mucous membranes. Pheomelanin can have carcinogenic activity, indicating that both the relative degree and type of pigmentation are key factors in melanoma susceptibility. Pheomelanin produces reactive oxygen species (ROS) that cause DNA damage and eventually lead to melanomagenesis (Tanaka, Yamashita, et al. 2018). Light skin pigmentation, red or blond hair, blue or green eyes, freckling tendency, and poor tanning ability are associated with a two- to fourfold increase in melanoma risk (Bishop et al. 2009).

Chronic sun exposure may contribute to the development of melanoma in other sites; such as the head and neck, and those melanomas are often on the surface and with very slow growth. A history of multiple severe sunburns in childhood is also

associated with increased melanoma risk (Dinnes et al. 2018, Ferrante di Ruffano et al. 2018). After severe UVR-induced DNA damage, keratinocytes undergo apoptosis. In contrast, melanocytes are resistant to this level of radiation, and their survival results in the propagation of mutated genes, especially if damaged DNA is not fully repaired (Gilchrest et al. 1999).

UVR can also alter populations of adaptive immune cells in the skin. This may be of particular relevance to novel immunotherapies that focus on potentiating the antitumour effect of the adaptive immune system through T cell checkpoint inhibition (Wong et al. 2013). Phototherapy (UVR) is used as therapy for a number of inflammatory dermatoses and is typically given as either UVA or broadband or narrow-band UVB therapy. Phototherapy can reduce autoinflammation by altering the number of B and T cells present inside the skin (Ceovic et al. 2007). UVB can also drive intraepidermal T cell apoptosis and reduce the number of Langerhans cells, potentially by driving them into the systemic circulation (Seite et al. 2003). Langerhans cells express high levels of Receptor activator of NF-kB (RANK) and induce regulatory T cell (Treg) formation. Besides, UVR induces keratinocyte apoptosis through p53, forming 'sunburn cells' that can be seen to peak 48 h post exposure (Lee et al. 2002).

Oral supplementation of L-glutathione prevents UVB induced melanogenesis and oxidative stress in mice (Balic et al. 2019, Davis et al. 2019, Nagapan et al. 2019). This observation indicates that dietary antioxidant supplements like L-glutathione may possess antiaging, antimelanogenic, and anticancer properties and may be used as a photoprotection agent against UVB-induced oxidative stress and melanogenesis.

1.1.5 Nevi

Nevus (**nevi** if multiple) is a morphological variant of melanocytes that has more epithelioid and less dendritic phenotype with a variety of histologic, anatomic, architectural, and cellular patterns. The term originates from nævus ("birthmark"– *in Latin*) and can be either congenital (present at birth) or acquired. **Common acquired nevi** are usually less than 5 mm in diameter and can be flat or slightly raised and typically with uniform colour and round shape (Ackerman et al. 1992). Only approximately one-third of melanomas arise from preexisting nevi. However, there is a strong association of high total body nevus counts with melanoma (Borg et al. 2000). Individuals with the propensity to develop fewer melanocytic nevi require greater sun exposure to promote the development of melanoma and tend to develop melanoma on chronically sun-exposed sites (e.g., head or neck) (Czajkowski et al. 2004). Conversely, individuals with large numbers of nevi may require less solar stimulation for the development of melanoma and often in sites where large numbers of nevi are found, such as the back (Lynch et al. 2002). This "divergent pathway" model suggests that melanomas on different sites of the body may occur via different mechanisms (Vourc'h-Jourdain et al. 2013) including inheritance and gene mutations.

Congenital melanocytic nevi (CMN) are defined as melanocytic nevi present at birth or within the first few months of life. For patients with large CMN, the risk of developing melanoma (cutaneous or extracutaneous) is approximately 2 to 5 percent over a lifetime (Ross et al. 2011, Vourc'h-Jourdain et al. 2013).

However, **atypical nevi s**hare some features of melanoma, such as mixed colour, asymmetry, irregular borders, and they are usually larger than 5 mm in diameter. These nevi are strong phenotypic markers of an increased risk of melanoma, especially in individuals with numerous nevi and a family history of melanoma (Cust et al. 2019).

According to some meta-analysis the relative risk of melanoma correlated with the number of nevi, being 1.5 for the presence of a single atypical nevus and 6.36 (95% CI 3.80-10.33) for five atypical nevi, versus none for typical nevi (Gandini et al. 2005, Cust et al. 2019). The familial atypical multiple mole melanoma (FAMMM) syndrome is showing concordance for malignant melanoma and a cutaneous phenotype characterized by multiple large moles of variable size and colour (reddish-brown to bright red) with pigmentary leakage. Their personal history of melanoma is associated with a two- to threefold increased risk of developing a second primary cutaneous melanoma (Pomerantz et al. 2015).

1.2 Melanoma-initiating process

1.2.1 Cancer Stem Cell

Melanoma, as an aggressive cancer, may contain a higher percentage of cancer stem cells (CSCs) which are characterized by unlimited self-renewal potential (Kumar et al. 2017). Tumour progression and metastasis can be initiated by genetic, epigenetic, and environmental factors or their combinations (Bruttel et al. 2014). Progression and metastasis of solid tumours are driven by CSCs that migrate from the primary tumour into surrounding tissues, blood, and lymph nodes (Gray et al. 2015). CSCs are multipotent and can differentiate into adipocytes, chondrocytes, osteocytes, and also into several melanoma subtypes. This genotypic and phenotypic variability helps melanoma to escape from the immune system attack (Bruttel et al. 2014).

Skin Adult Stem Cells and melanomagenesis

Adult stem cells exist in essentially all tissues, they are multipotent and capable of self-renewal. Since they are long-lived, they can accumulate all genetic transformations. A major reservoir for melanocytic cell precursors is the hair follicle. Melanocytes isolated from the hair follicle are pigmented mature and amelanotic immature cells (Tobin 2011). The amelanotic population has a slower growth rate, but higher long-term proliferation ability than the pigmented mature cell population. Melanocyte stem cells are located in the hair follicle bulge region and may migrate from hair follicles to the epidermis.

Stem-like melanoma cells can grow as spheres in embryonic stem cell media. They can differentiate into multiple lineages: adipocytes, chondrocytes, and osteocytes. Stem-like melanomas are more resistant to chemotherapeutic reagents (Kumar et al. 2017). A small population of them is positive for CD20, a marker that is usually expressed by B lymphocytes (Fang et al. 2005). Another putative population of melanoma-initiating cells is characterized by the expression of the ABC (ATP-binding cassette) drug transporter family (ABCB5). ABCB5+ cell population has an enhanced tumour-forming ability *in vivo* and they can survive when transplanted into animals (Abel et al. 2011). A monoclonal antibody against ABCB5 reduces melanoma growth (Schatton et al. 2008). Another minor population of melanoma stem cells is positive for CD133 (that is the ABC transporter protein ABCG2) (Kumar et al. 2016).

1.2.2 Melanomagenesis. Genetic models for melanoma progression

The following four steps can be characterized in the generation of metastatic melanoma. Each step adds a further genetic or epigenetic component, but not all melanomas develop in this order.

1. A mitogenic driver mutation is usually the first step in any cancer, and it will stimulate a normal cell to proliferate. Drivers mutations are most common in the following genes: BRAF; MYC; NRAS; ERBB4; PTPs, NF1, KIT, etc /benign nevus/
2. These changes suppress the primary senescence and this step is mediated by CDKN2A, CDK4, CCND1, APC, etc genes
3. Mutations in the following genes suppress cellular apoptosis: APAF1, PTEN, PTPs, PREX2, PTKs, AKT, TP53, etc /
4. Maintenance of telomere length is mediated by TERT (or ALT)

In principle, a melanoma could metastasize as soon as it generates cells that are able to survive outside the epidermis. Melanoma cells in the epidermis are sessile and they do not migrate (Richetta et al. 2018).

Growth factors

Primary melanomas are characterized either by a phase of radial growth without competence for metastasis or by the vertical growth phase with metastatic potential. Six growth factors show biological significance in the melanocyte system and they are EGF, NGF, FGF, PDGF, insulin, and TGF-beta (Herlyn et al. 1987, Mancianti et al. 1988).

Since tumour cells are constitutively producing multiple growth factors, they do exhibit growth autonomy. Basic fibroblast growth factor (bFGF) is consistently expressed in cultured melanoma cells, but not in normal melanocytes and is the main factor produced for autocrine stimulation. Other growth factors are not involved in autocrine loops, but are important for paracrine roles: initiating angiogenesis, for triggering of an inflammatory reaction and for activating proteolytic enzymes produced by normal cells (Bernardini et al. 2019).

Inhibition of epidermal growth factor receptor (EGFR) improves the antitumour efficacy of vemurafenib in BRAF-mutant human melanoma in preclinical studies (Kenessey et al. 2018). Efficacies of EGFR- tyrosine kinase inhibitors (TKIs) showed significant differences, and irreversible inhibition had the strongest antitumour potential. Interestingly, BRAF-mutant cells are sensitive against EGFR inhibitor gefitinib, while *wild type (WT)* BRAF cells are not (Kenessey et al. 2018, Spirina et al. 2019). This data suggest that EGFR is a potential target in the therapy of BRAF-mutant malignant melanoma.

The fibroblast growth factor receptor (FGFR) family consists of four conserved transmembrane receptors (FGFR1–4) mainly localized at the cell surface. In the skin, fibroblasts and keratinocytes control the proliferation of melanocytes in a paracrine manner via FGFs. Melanoma progression is linked to increased FGF2 expression (Czyz 2019). FGF/FGFR signalling is higher during wound healing, and skin protection from UV light damage. FGFR1 is expressed in the majority primary of melanomas at a high level (Giehl et al. 2007). Melanoma that synthesizes FGFs can influence the neighbouring cells and contribute to intratumoural angiogenesis. Therefore, inhibitors of aberrant FGF/FGFR signalling can be potentially considered as drugs for melanoma (Czyz 2019).

1.2.3 Cell senescence and genetics of melanoma progression

Gene **CDK4** and **CDKN2A** products control cell division. Mutations in CDKN2A comprise a high risk of melanoma and the prevalence of this mutation varies by geographic area. The reasons for these differences are not completely understood. There may be differences in the amount of UV the individuals receive or other individuals' genetic differences can be involved. CDKN2A, encodes two effectors of cell senescence, while other familial melanoma genes are mostly related to telomeres (Horn et al. 2013).

The melanocortin-1 receptor (MC1R) gene is a regulator of skin pigmentation. Some products of this gene carry an increased risk of melanoma independently from sun exposure. Carriers of two or more MC1R variants are linked to a twofold increased risk of melanoma, compared with *WT* carriers (Wendt et al. 2016).

Xeroderma pigmentosum (XP)- disorder patients have a mutation in a gene needed for repair of UV induced damage. These patients have an extremely high rate of skin cancers, including melanoma (Daya-Grosjean 2008).

BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase) **(BAP1)** mutations have been described in an autosomal dominant tumour predisposition syndrome. Histologic assessment of cutaneous melanomas in this group has demonstrated spitzoid and nevoid features (Wendt et al. 2016).

The Phosphatase and TENsin (**PTEN)** gene is one of the frequently inactivated tumour suppressor genes in sporadic cancers. Inactivating mutations of the PTEN gene are found in many types of cancers, including melanoma (Aguissa-Toure et al. 2012, Trujillo et al. 2019).

A large cohort sequencing study has also demonstrated key drivers and significantly mutated genes (**SMGs)** in melanoma, including both known (BRAF, NRAS, TP53, CDKN2A, and PTEN) and new genes (NF1, RAC1, PPP6C, IDH1, and ARID2) (Hodis et al. 2012, Krauthammer et al. 2015, Watson et al. 2015).

Melanoma samples also exhibit high numbers of passenger mutations caused by UV exposure (Hodis et al. 2012). To overcome this, Hodis et al. controlled for UV-induced mutational load and identified six novel genes: PPP6C, RAC1, SNX31, TACC1, STK19, and ARID2. Some of them, PPP6C, RAC1, and STK19 are thought to be potentially targetable (Hodis et al. 2012).

Additional factors involved in melanoma progression

The role of dietary factors (antioxidants, vitamins C, E, and retinoid) have not shown a consistent impact on the incidence of melanoma. A case-control study has suggested that diets rich in vitamin D and carotenoids and low in alcohol may be associated with a reduced risk for melanoma (Millen et al. 2004).

An epidemiologic study has shown an increase in melanoma in women with **endometriosis.** Endometriosis is a clinical condition when a tissue similar to the tissue that normally lines the inside of the uterus – the endometrium – grows outside of the uterus. This extra tissue has no way to exit the women's body during menstruation and it becomes trapped, irritated and eventually is developing scar tissue or occasionally also melanomas (Kvaskoff et al. 2007, Farland et al. 2017).

Parkinson disease (PD) and levodopa therapy for PD is a potential factor for an increased risk of melanoma (Inzelberg et al. 2009). PD patients have a 60% higher risk of melanoma than healthy individuals (Wirdefeldt et al. 2014).

A personal history of prostate cancer is also linked to an increased risk of melanoma. There is a hypothesis of a potential role for androgens in the aetiology of melanoma (Li et al. 2013).

There is a central role of the immune system and immunosuppression in melanoma pathogenesis. The immune system has long been recognized as the main force in cancer control. Defects in immunity contribute to poor responses in cancer treatment. The tumour microenvironment includes infiltrating immune cells that either stimulate or inhibit an immune response (such as myeloid-derived suppressor cells) (Sharma et al. 2017, Ostrand-Rosenberg et al. 2018). Melanomas occur with increased frequency in immunosuppressed patients, including organ transplant recipients solid organ transplant recipient (OTR), lymphoproliferative disease patients including lymphoma, for example, chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma (NHL), radiation and Human immunodeficiency virus (HIV) infection (Hoffmann et al. 2005, Kubica et al. 2012). In all those cases the acquired melanoma is associated with a poorer prognosis (Brewer et al. 2011, Brewer et al. 2012).

1.2.4 Immunosenescence

The name 'immunosenescence' has been used to describe the loss of immune functions in elderly individuals (>65 years old). Immunosenescence is associated with increased susceptibility to infections, poor response to treatments and vaccination. With progressive age, the immune system will change fundamentally. Ageing declines both adaptive and innate immunity. Cancer, infections and autoimmune diseases occur more frequently in the elderly. Older individuals commonly present chronic low-level inflammation and increased pro-inflammatory cytokine production by memory and senescent T cells (Ferrucci et al. 2018). Ageing is associated with a state of increased autoimmunity and inflammation that is surprisingly coexistent with a state of immunodeficiency.

There are three theories of immunosenescence:

1. The autoimmunity theory: the immune system tends to lose efficiency and experiences widespread dysfunction, (immune reactions against single proteins). Increased CD5+ B lymphocytes in the elderly population play a role as producers of autoantibodies that lead to an imbalance tolerance (Bulati et al. 2011).
2. The immunodeficiency theory: In elderly people, the body is unable to defend itself from pathogens and this results in detrimental harm. There is an impaired ability to mount immune responses to new antigens that cause a high risk of infectious diseases (Aw et al. 2007, Weiskopf et al. 2009).
3. The deregulation theory: Downregulation of toll-like receptors (TLRs) and nod-like receptors (NLRs) during ageing contribute to the lack of effective recognition of pathogens and commensal flora. This leads to aberrant secondary immune cell activation and contributes to morbidity and mortality at an advanced age (Aiello et al. 2019).

Effects of ageing on the immune system

During ageing, immune senescence is a process that affects the entire immune system and results in a higher rate of infectious diseases and cancer. Skin and mucous are the first line of defence of our body and constitute the main part of the **innate immune**

system. With age, the replacement of skin cells decreases, sweat production is reduced, and the number of Langerhans cells and melanocytes are decreased (Campisi et al. 2007, Chilosi et al. 2014). In the skin, ageing is associated with epidermal thinning, the pro-inflammatory state and gradual deterioration of the epidermal immune response (Kinn et al. 2015). The mucus membranes of hair cells, which play an important role in removing pathogens, are reduced in quantity and movement (Kim et al. 2019, Kim et al. 2019. Dendritic cells- (DC) in ageing persons are functionally impaired concerning the uptake of antigens, and migration into lymph nodes (Gupta 2014). Also, a decrease in interleukin (IL)-15 interferon-alpha (INF-α) levels and tumour necrosis factor-alpha (TNF-α) has been seen in DC of elderly people (Tucci et al. 2019a, Tucci et al. 2019b). Age-related alteration of the adaptive immune system is the decrease of de novo generation of T and B cells (Stervbo et al. 2015).

Microglial cells (MG) in the central nervous system (CNS) comprise 12% of the brain cells and serve as the main brain's immune defence (Dheen et al. 2007). They also play an important role in CNS homeostasis during development, adulthood, and ageing. Ageing specific production of cytokines (TNF-α, NO, IL1-β), by MG cells and ROS, is damaging to neurons, especially in the case of Parkinson's disease, a condition where microglia multiply and adopt an activated – also priming state. This makes the microglia susceptible to a secondary inflammatory stimulus (Wang et al. 2015).

Gut-associated lymphoid tissue (GALT) is the largest component of the immune system and influences local and systemic immune responses. Gut mucosa has a single epithelial cell layer made up of intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IEL) that facilitate interaction with the immune system. Gut epithelia contain Paneth cells that secrete antimicrobial peptides and goblet cells that secrete mucus (Vaishnava et al. 2011, Pelaseyed et al. 2014).

During ageing, mucus production is diminished, and it makes gut susceptible to a secondary inflammatory stimulus. In the elderly, the microbiota composition changes gradually but can maintain similar physiological functions (O'Toole et al. 2015).

The **adaptive immune system** is based on the generation of a diverse repertoire of B and T cell receptors and subsequent activation of lymphocytes and their clonal expansion. The induction of adaptive immunity depends on the recognition of the particular antigen receptor and is in most cases activated by the innate immune system (Schenten et al. 2011).

The primary function of B lymphocytes is the production of antibodies in response to infection from a pathogen (Tedder et al. 1997, Gitlin and Nussenzweig 2015). This B cell function undergoes significant modifications with age. Elderly people are characterized by impaired B cell responses and defective antibody production and a reduced ability to respond to microbes. In addition, the total plasma cell number decreases in the bone marrow (mostly the memory B cells) of old people (Pritz et al. 2015).

Both CD4+ and CD8+ T lymphocytes are educated in the thymus to recognize antigens in the context of major histocompatibility complex (MHC). Ageing is also associated with reduced functionality of T cells. Age-related regression of the thymus is associated with a decline in naive T cell output (Moro-Garcia et al. 2012, Chou et al. 2013). This contributes to a reduction in T cell diversity seen in older individuals and is linked with increased susceptibility to infection, autoimmune diseases, and cancer (Palmer 2013). CD4+ T cell lymphopenia is a well-recognized T cell defect associated with ageing (Martinet et al. 2014).

Biomarkers of senescence

The CD8+CD28−CD27+ cell and anti-thymocyte globulin (ATG)-induced T helper cell lymphopenia is used as a biological marker for immunosenescence (Crepin et al. 2015).

Another marker of ageing is the telomere length. Telomere shortening is common in highly differentiated effector memory CD8+ T cells, that are CD28− T cells. Intensive replication in an ontogenic period of the cell will thus induce a shortening of telomeres, which put these cells at risk of DNA mutations (Laberthonniere et al. 2019). Telomere dysfunction is a risk of developing melanoma. To date, several mutations have been identified: to the TERT promoter (Harland et al., 2016; Horn et al., 2013), as well as to the protein-coding sequence of the sheltering complex members POT1 (Pandiani et al. 2017).

Cancer patients also display the expansion of inactive effector memory type CD8+CD28− T-cells. This expansion indicates that chronic antigenic stimulation is driving CD8+ T cells towards end-stage differentiation and functional disability. CD8+CD28− T cells also exhibit suppressor activities (Reiser et al. 2016).

The incidence of cancer increases with advancing age due to a cumulative number of accumulation of mutations and exposure to carcinogens. One of the most important cell types for cancer-killing is the NK cells, which functionality is reduced with ageing. During ageing, there is a significant decrease in naive T-cell population and an increase in memory T-cells, especially CD8+ cells. The immunological space, called a niche, is filled by virus-specific memory T cells, thus a reduced immune repertoire enables the identification and fights against new antigens, such as cancer cells (Cambier 2005, Nikolich-Zugich 2008).

1.3 Melanomas and microbes

Genetic mutations are required for the proliferation of cancer cells and their fitness within the tissue in which they originate (Hausser et al. 2019). Progression and invasion of other tissues are also dependent on the host response in terms of antitumour immunity. This host response provides both a tumour-promoting environment and an immune barrier to tumour progression, which the tumour needs to neutralize or overcome in order to progress (Gonzalez et al. 2018). In addition, clear experimental evidence indicates that inflammation provides a tumour-promoting environment in which stromal cells and infiltrating inflammatory cells, such as macrophages and other myeloid cells, produce growth and angiogenic factors, as well as tissue remodelling enzymes. However, no clear role of pathogen microbes in the induction of melanoma has been shown so far.

1.3.1 Possible impact of the microbiome on the immune system and melanoma development

The individual microbiome is composed of a collection of microbes within a host organism. During their evolution, bacteria acquire virulence factors that confer pathogenicity. Most of the disease-promoting and pro-carcinogenic effects of pathogens depend on these virulence factors (Gagniere et al. 2016). The symbiotic interactions between resident microbes and the body, contribute to maintaining gut and skin homeostasis. However, all alterations to the microbiome caused by environmental changes, for example, diet, infections or lifestyle, can disturb the

balance between the host and its microbiome, thereby promoting disease, including cancer.

The first eukaryotes evolved surrounded by microorganisms, such as viruses, bacteria, archaea, and fungi. Symbiotic cross-signalling between eukaryotic cells and commensal microbes regulates metabolism, nutrition, and morphogenesis. In our bodies, commensal microorganisms inhabit all the barrier surfaces, with the largest number present in gut and colon (Dzutsev et al. 2015). The microbiota which colonizes the skin and the mucosal surfaces interacts with the host directly or through the release of products, for example, carbohydrates, lipids, proteins, and nucleic acids, all of which exhibit innate conserved pattern recognizing receptors (PRR) and cytoplasmic sensors in hematopoietic, epithelial and stromal cells, regulating local immunity and inflammation (Chen et al. 2018).

The role of the microbiome in response to cancer and its therapeutic implementations are becoming increasingly apparent. Modulating the gut microbiome may have a positive impact on cancer therapy. It is very important to understand factors influencing the gut microbiome and strategies to manipulate the microbiome to lead to therapeutic responses (Gopalakrishnan et al. 2018). Recent studies highlight also the impact of microbiota on responses across several cancer therapies. Patients who previously failed standard therapies were responding well after microbiota transplantation from patient donors who had responded to immunotherapy (Baruch et al. 2019).

Disruption of the balance of commensal microbiota leads to dysbiosis, which is characterized by the instability of the microflora, with potential enrichment of opportunistic pathogenic bacteria (Frosali et al. 2015). Dysbiosis can lead to impaired local or systemic immune responses. Dietary intake can also promote the differential composition of the microbiome, and changes in dietary regimens and components can alter the gut microbiota in a relatively short amount of time (Picardo et al. 2019).

The gut Microbiome and Immunity

Bacterial metabolites from the gut can activate local DCs that migrate to the draining lymph nodes, to activate naïve T cells which can differentiate into T17 effector T cells or T-regulatory cells and migrate back to the gut mucosa. Depending on the type of the microbe, the DCs will direct the T cells towards a T-regulatory versus Th17 phenotype. T-regulatory cells function in secreting IL10 and TGF-b, creating a local anti-inflammatory cytokine milieu. Th17 cells, produce IL17 which can increase Paneth cell production of antimicrobial peptides and activate the pro-inflammatory milieu (Kamada et al. 2013).

Investigation of microbial communities

DNA sequencing in combination with proteomics or metabolomics helps investigate the microbial communities. A microbial community consists fundamentally of a collection of individual cells, each carrying a distinct complement of genomic DNA, but these communities differ from person to person (Goodman et al. 2018, Picardo et al. 2019).

The microbiome clearly represents a key component of future personalized medicine. The number and diversity of phenotypes linked to the composition of the microbiota are immense: obesity, inflammatory bowel disease, diabetes, allergies, autism, cardiac function, fibromyalgia, various cancers, and depression have all been reported to correlate to the composition of the microbiome. The microbiome is plastic;

it changes metagenomically within hours and metatranscriptomically within minutes, in response to antibiotics or certain food intake. For any phenotype to which the microbiome is causally linked, opens the possibility of pharmaceutical, prebiotic, or probiotic treatments (Goodman et al. 2018).

The skin of humans hosts relatively few microbes (e.g. *Propionibacterium),* the nasal cavity somewhat more (e.g. *Corynebacterium*), the oral cavity (dominated by *Streptococcu*s) several hundred taxa with remarkable diversity even among saliva, tongue, teeth, and other substrates, and the gut over 500 taxa with densities over 10^{11} cells/g. Unlike in the gut, skin microbes are not required for the development of the associated lymphoid tissue, but they are required in order to maintain, through the production of IL1α, sustained activation of Th17 cells and Th1 cells in the derma, and allow a protective immune response to skin pathogens.

The gut microbiota has an important role in immune health

The onset of acute- graft versus host disease (GVHD) after transplantation is associated with significant shifts in the composition of the microbiota with a loss of overall diversity and reduction of health-promoting obligate anaerobes, such as *Faecalibacterium, Ruminococcus, Lactobacillus,* and *Blautia*, and enrichment of *Enterococcus* and *Clostridiales* (Biagi et al. 2015). The administration of the probiotic *Lactobacillus rhamnosus* during transplantation in mice was associated with reduced rates of GVHD and improved overall survival (OS) of transplanted animals (Gerbitz et al. 2004). The responsiveness of 15 specific bacterial groups and their downstream metabolites to a variety of nutrients and immune parameters is known, and it provides preliminary insight into how dietary modulation could be used as a strategy to enrich the gut microbiome and immune health (Ma et al. 2018, Ma et al. 2019), that definitely has a positive impact also in cancer treatment.

Dietary fibres are one component that has a profound influence on the composition of the gut microbiome (Ma et al. 2018). For example, supplementation of the diet with prebiotic inulin (plant polysaccharide) results in a significant increase of good bacteria: *Faecalibacterium and Bifidobacterium* species (Ma et al. 2019). Conversely, the elimination of animal fats in the human diet was associated with a decrease in unfavourable *Bacteroidales* bacteria. This indicates that careful administration of certain probiotics may provide a more feasible method of microbial manipulation in the clinical setting.

The gut microbiota and immunotherapy of cancer

Immune checkpoint inhibitor, CTLA-4 blockade, induces large changes in gut microbiota in mice (Vetizou et al. 2015) with a relative increase in *Bacteroidales* and *Burkholderiales*, and a decrease in *Clostridiales*. The efficacy of anti-CTLA-4 therapy was markedly reduced in germ-free (GF) mice and specific pathogen-free (SPF) mice treated with antibiotics. Supplementation with an oral probiotic containing *Bifidobacterium* restored anti-tumour efficacy in mice, resulting in enhanced tumour-specific $CD8^+$ T-cell activity (Routy et al. 2018; Sivan et al. 2015).

Another study revealed that patients treated with antibiotics before or during anti-programmed death-1/programmed death-ligand-1 (PD-1/PD-L1) treatment had significantly lower progression-free survival (PFS) compared to patients who had not received antibiotics. This shows that disrupting the gut microbiota could potentially impair anti-tumour immune responses or the response induced by immune checkpoint

blockade (Gopalakrishnan et al. 2018, Routy et al. 2018, Routy et al. 2018). Moreover, recently it was shown that patients who responded to anti-PD-1 therapy had a significantly higher diversity of bacteria in their gut microbiota, as well as a higher relative abundance of *Clostridiales, Ruminococcaceae,* and *Faecalibacterium* (Routy et al. 2018).

Melanoma-related changes in skin microbiome are controversial:

1. Mrázek et al (2019) used a minipig model to demonstrate a strong relationship between melanoma development and skin microbiome changes. Moreover, the abundances of *Fusobacterium* and *Trueperella genera* were significantly increased in melanoma samples (Mrazek et al. 2019).
2. However, Salava et al. used cutaneous microbiome high-throughput DNA sequencing to showed that there is no correlation between melanoma and the skin microbiome. This result indicates that the skin microbiome may not be a useful diagnostic tool for melanoma and melanocytic nevi (Salava et al. 2016)**.**

Immune response to melanoma may be dependent on the gut microbiome

Iida and colleagues showed that commensal bacteria control cancer response to therapy by modulating the tumour microenvironment (TME). Using mice with transplanted tumours that were treated either with chemo- or immunotherapy, they demonstrated that commensal flora is a requisite to obtain the oxidative burst or the inflammatory necrosis induced by oxaliplatin or immune therapy, respectively. **Commensal bacteria that augmented immunotherapy included *Bifidobacterium spp* (*breve, longum,* and *adolescentis Ruminococcus* and *Alistipes shahii*, while *Bacteroidetes* and *Lactobacillus fermentum* dampened the immunotherapy response (Iida et al. 2013).**

How does the gut microflora manage to affect distant tumours? Tumour-associated macrophages (TAM) (Chanmee et al. 2014) cells communicate closely by T cells, via Toll-like receptors (Kawasaki et al. 2014). These TAMs are highly plastic cells that can differentiate into various phenotypes and are involved in tumour progression or regression, depending on the molecular signals received. Many of the signals that TAM cell use to elicit the immune response of the tumour, require the presence of intact gut microbiota. In the experiments already mentioned, Iida and colleagues showed that when the gut was sterilized, TAMs were not capable of producing the requisite oxidation for oxaliplatin cytotoxicity (Iida et al. 2013).

Checkpoint blockade agents and microbiota

PD-1, PD-L1, and cytotoxic T lymphocyte-associated protein-4 (CTLA-4) are checkpoint blockade proteins expressed by cancer cells, by immune and other cells in the TME.

Normally, once cross-linked, PD-1, PDL-1, or CTLA-4 deliver an inhibiting signal to the cell. When one of the monoclonal antibodies binds to the checkpoint protein, it allows the immune response to proceed with the activation state (Frankel et al. 2017).

In a small study of metastatic melanoma patients (n=26), faecal microbiota composition was analysed at steady-state and before each ipilimumab treatment. Patients whose baseline microbiota was driven with *Faecalibacterium genus* and other *Firmicutes* (cluster A; n=12) had longer PFS (P=0.0039) and OS (P=0.051) than patients whose baseline microbiota was enriched by *Bacteroides* (cluster B; n=10). Those with

Firmicutes-dominant flora also had fewer T-regs, less colitis, and overall better benefit of treatment (Chaput et al. 2017).

In another study of tumours in mice, *Bifidobacterium breve, B. longum,* and *B. adolescentis* enhanced dendritic cell activation and improved response to PD-1 inhibitor treatment (Sivan et al. 2015). In Iida's experiment mentioned above, commensals that augmented immunotherapy where *B. breve, B. longum,* and *B. adolescentis, Ruminococcus* and *Alistipes shahii.* While *Lactobacillus fermentum* surprisingly dampened the response to immunotherapy (Iida et al. 2013).

Clinical Considerations and Conclusions

1. *Bacteroidetes* are often used for reducing colitis. Current data suggests this could undermine the cancer treatment response.
2. Antibiotics should be reserved as a last resort in those undergoing cancer treatment.
3. It is too early to recommend specific probiotics or prebiotics; scientists are in a position to optimize microbiota diversity in an effort to create the healthiest micro-ecosystem possible.
4. Evidence implies that diversity may be one of the keys to immune recognition of cancer cells and the efficacy of anticancer treatments

By using probiotics as adjuvants for checkpoint immunotherapy (or by targeting microbial enzymes) microbiota can be harnessed to improve cancer care.

Faecal Microbiota Transplant (FMT)

FMT represents the most direct means to manipulate the microbiota, and FMT preparations can be administered to patients via oral administration of lyophilized or frozen pills or via direct delivery by colonoscopy or gastroscopy (Gopalakrishnan et al. 2018). Based on the available literature, there are clearly bacterial taxa that are associated with response and also toxicity – with some overlap in the bacterial signatures across the studies. However, this data is indicating that the gut microbiota could be targeted to improve therapy while attenuating adverse reactions (Dzutsev et al. 2017).

Conclusion: Microbiota has a possible immune and treatment response role in melanoma, but the exact impact and mechanisms are not clear yet.

1.4 Molecular markers of melanoma

1.4.1 Definition and classification of melanoma markers

The most relevant molecular markers expressed by melanoma cells include:

1. Differentiation antigens or tumour-associated antigens (**TAAs) expressed** by both normal and malignant melanocytes, but at different levels. Tumour differentiation antigens are expressed by normal melanocytes in diverse periods of cell differentiation and melanin synthesis. **These proteins are unmutated but generally overexpressed on malignant melanoma** cells (Renkvist et al. 2001). Melanoma antigens such as **gp100, MART-1, TRP-1, TRP-2, and tyrosinase** belong to this group. Unfortunately, these markers are poorly immunogenic and they often result in T-cell tolerance.

2. Cancer/testis antigens that are typical of several tumour types and germline cells derived from normal adult testis include **melanoma-associated antigen A (MAGE-A)**. MAGE-A represents a class of tumour antigens that are expressed in a variety of malignant tumours (bladder, lung, skin and breast malignancies), however, their expression in healthy normal tissues is restricted to the fetal ovary, placenta and germ cells of the testis. The immunogenicity and restricted expression of these antigens make them ideal targets for immunotherapy in human cancer. The MAGE-A antigens were initially identified as tumour antigens that can be recognized by cytotoxic T-lymphocytes in melanoma patients (Rother et al. 2009).
3. Tumour-derived **neo-antigens** result from somatic mutations in the genome of tumour cells during tumourigenesis. These molecular markers of tumour cells are determined by modifications of gene expression, due to loss of function mutations of the following genes: CDKN2A, PTEN, MC1R gene, BAP1, TYR, TYRP1, MDm2, CDK4, and ASIP genes.

Gene mutations differ between melanoma subtypes as demonstrated by recent whole-genome sequencing studies from melanoma samples (Hartman et al. 2019, Laikova et al. 2019, Newell et al. 2019).

The mechanism of **mutations in cutaneous melanoma is linked to UVR** exposure, while acral and mucosal melanomas tend to have lower mutation burdens, with different genes implicated in mutagenesis. For example, CDKN2A, BRAF, RRAS and TP53 are mutations common in cutaneous melanoma, while NF1 mutations are more consistently found in acral melanoma. Additionally, KIT mutations are identified more often in mucosal and acral melanomas as compared to cutaneous melanomas.

1.4.2 Genetic predisposition markers for melanoma

Genetically inherited predisposition to melanoma is generally based on only one mutation per patient, typically in BRAF (50%), NRAS (13.25%), MEK1 (6%), KIT (2.6%), EGF, CTNNB1 (2%–3%), GNA11 (2%) or GNAQ (1%) in eyes (Davis et al. 2019). The first two genes BRAF and NRAS belong to the mitogen-activated protein kinase (MAPK) signal transduction pathway, which plays a central role in regulating cell growth, survival, and cell proliferation. The BRAFV600E mutation is in the BRAF kinase domain that induces continuous stimulation of cell proliferation and growth by activating phosphorylation of the extracellular signal-regulated kinase (ERK). ERK cascade regulates cell proliferation, differentiation, apoptosis, and stress response as well. However, oncogenic activation via BRAFV600E is necessary, but not sufficient since this BRAF mutation can be found also in common nevi (Davies 2002, Davies et al. 2002), indicating that BRAF cooperates with members of other activation pathways, which for example are controlled by CDKN2A.

The microphthalmia-associated transcription factor (MITF) seems to play also a relevant role in melanoma because MITF participates in controlling proliferation and differentiation of melanocytes (Giehl 2005).

cKIT mutations are not common and mainly have been reported in acral (10% of cases), mucosal (15–20% of cases), and chronically sun-exposed (5% of cases) melanomas (Smalley et al. 2009).

Interestingly, the genetic contribution to melanoma, differs between populations, indicating that it may be linked to the major histocompatibility complex (MHC).

1.4.3 Markers of melanoma progression identified by gene expression studies

Gene expression changes during tumour progression can be due to activating mutations in pathways that modulate transcription or by epigenetic regulation. Melanoma at sun-exposed body locations may more frequently show UV-induced mutation, whereas melanomas arising at non-sun-exposed sites may more frequently use epigenetic regulation. During melanoma progression, multiple different tumour suppressor genes are silenced. Genetic and epigenetic alterations in the proteins regulating the G1 to S transition in the cell cycle and loss of p16 appear to be common to most melanoma subtypes, including superficial spreading, mucosal and nodular melanoma (NM) cases. However, multiple abnormalities in the G1-S proteins can occur simultaneously in the same melanoma (Suzuki et al. 2007). Another major cellular pathway that becomes dysregulated in melanoma progression is the G2-M mitotic transition.

1.4.4 Genes mutated during non-sporadic melanomagenesis

High-throughput sequencing has now revealed more than 515 genes that were mutated in at least 10% of melanomas. However, not all of these mutations are linked to malignancy. Melanomas have a median of 13–17 mutations per Mbp of DNA, which is the highest frequency among 21 cancer types analyzed (Hodis et al. 2012, Lawrence et al. 2014), even without considering rearrangements, copy number alterations, and epigenetic changes. These mutations predominantly carry the signature of ultraviolet light mutagenesis (Khan et al. 2018). Every melanoma specimen has a different set of mutations, many of these are random events, while fewer comprise the key drivers' (Pandiani et al. 2017, Helgadottir et al. 2018, Newell et al. 2019).

Cyclin-dependent kinase 4 (CDKN2A) is the major high-penetrance susceptibility gene, with germline mutations identified in 20%–40% of melanoma families. A positive CDKN2A mutation status has been associated with, multiple primary melanomas, pancreatic, breast or lung cancer, and early age at melanoma onset. The CDKN2A tumour suppressor gene encodes 2 different proteins, p16INK4A (p16) and p14ARF (p14), both regulating cell cycle. When CDKN2A is mutated it produces 2 dysfunctional proteins whish are inducing cell cycle progression and avoiding p53 degradation. CDKN2A mutation penetrance varies between geographical areas, according to the population incidence rate of melanoma, ranging from 58% in Europe to 76% in the United States and 91% in Australia, by age of 80 years (Bishop et al. 2009). **The recommendation to avoid smoking** has been recently suggested for CDKN2A mutation carriers, following the description of an increased prevalence of tobacco-associated cancers in CDKN2A-mutated families.

CDKN2A mutation carriers should be included in skin examination programs including scalp, oral and genital mucosa, and be performed every ~ 6 months. They are also candidates for annual pancreatic cancer screening via endoscopic ultrasonography or magnetic resonance cholangiopancreatography (Rossi et al. 2019).

Mutations in the other melanoma predisposition genes: CDK4, BAP1, TERT, Protection of telomeres protein 1 (POT1), ACD, TERF2IP, and MITF are much rarer, contributing to about 10% of melanomas. Laboratory genetic testing for melanoma is currently recommended only for CDKN2A and CDK4 mutations. Individuals, who have this mutation (and are from melanoma families) should regularly perform skin self-examination and dermatological screening. The CDK4 oncogene plays an important role in the G1/S phase cell cycle checkpoint. When CDK4 is mutated, p16 cannot inhibit

the CDK4 kinase activity resulting in a higher E2F release. E2F activates the transcription of pro-S phase cell cycle genes, promoting G1/S phase transition (Bishop et al. 2009, Papakostas et al. 2015).

The **BAP1** gene regulates the differentiation of melanocytes. Cutaneous melanoma is associated with the BAP1 cancer susceptibility syndrome, characterized by skin-coloured spitzoid melanocytic tumours, uveal melanoma, and cutaneous melanoma

The **TERT** gene encodes the catalytic subunit of telomerase maintains telomere length. TERT mutations induce increased expression of telomerase.

Protection of telomeres 1 (POT1) is a crucial member of the sheltering complex proteins, important for telomere maintenance. Mutations in the POT1 gene have been recently identified in a total of 12 CDKN2A-negative melanoma families.

Overall, germline mutations in TERF2IP, POT1, and ACD are detected in approximately 9% of families which often present with MPM and early-onset melanoma.

Unlike other cancer predisposition syndromes, melanoma is not linked to a single gene, but several high- and intermediate-penetrance melanoma susceptibility genes have been identified to date (Table 1). Penetrance relates to the lifetime risk for a mutation carrier for developing melanoma.

The MITF gene is a master regulator of melanocyte homeostasis, encoding a lineage-specific transcription factor, involved in cell survival, differentiation, and proliferation. Mutation of MITF leads to the development of variant IIa Waardenburg syndrome that is characterized pigmentation abnormalities of the eyes, hair and/or skin and varying degrees of deafness (Koludrovic et al. 2013).

Table 1. Most common mutations in sporadic cutaneous melanoma

Mutated gene	Mutation type	Tumour type and mutation frequency %	Reference
NRAS	Activating mutation; Q61R most common mutation	Congenital nevi 81%	(Bauer et al. 2007)
NRAS	Activating mutation; Q61R most common mutation	Primary tumours 25%	(Houben et al. 2004)
NRAS		Metastases 33%	(Houben et al. 2004)
BRAF	Constitutive activation; V600E most common mutation	Melanomas 66%	(Davies, Bignell, et al. 2002)
BRAF	Constitutive activation; V600E most common mutation	Benign nevi 82%	*(Pollock et al. 2003)*
PTEN	Inactivation	Melanomas 10%	*(Stahl et al. 2003)*

AKT3	Activation	Sporadic melanomas 43–60%	*(Stahl et al. 2004)*
AKT3	Activation	Benign nevi 4%	*(Stahl et al. 2004)*
mTOR	Activation	Melanomas 73%	*(Karbowniczek et al. 2008)*
mTOR	Activation	*In situ* melanomas 78%	*(Karbowniczek et al. 2008)*
mTOR	Activation	Invasive melanomas 67%	*(Karbowniczek et al. 2008)*
mTOR	Activation	Metastases 76%	*(Karbowniczek et al. 2008)*
cKIT	Activating mutation and amplification	Benign nevi 0–18%	*(Curtin et al. 2006)*
cKIT	Activating mutation and amplification	Melanomas on sun-damaged skin 28%	*(Curtin et al. 2006)*
cKIT	Activating mutation and amplification	Acral melanoma 36%	*(Curtin et al. 2006)*
cKIT	Activating mutation and amplification	Mucosal melanoma 39%	*(Curtin et al. 2006)*
MITF	Amplification	Primary tumours 10%	*(Garraway et al. 2005)*
MITF	Amplification	Metastases 21%	
TP53	Inactivation	Primary tumours 1–5%	*(Akslen et al. 1998)*
TP53	Inactivation	Metastases 11–25%	*(Papp et al. 1996)*

1.4.5 Genes mutated during sporadic melanomagenesis (RAS-RAF-ERK pathway)

NRAS and BRAF mutations are mutually exclusive events in melanoma development, indicating that mutant BRAF or NRAS alone is able to activate the MAPK pathway. Abnormal activation of the MAPK cascade plays a pivotal role in melanoma cell proliferation and evasion of apoptosis (Edlundh-Rose et al. 2006). Constitutive activation of this pathway is seen in up to 90% of melanomas, which makes it an ideal pathway for the development of small-molecule inhibitors (Cohen et al. 2002).

RAF – a downstream effector of RAS

There are 3 isoforms of RAF in human cells (A, B and C): ARAF, BRAF, and CRAF; however, mutations in BRAF are the most frequent and occur in 50% to 70% of melanomas. Substitution of valine by glutamic acid at codon 600 in exon 15 (**V600E)** takes place in more than 90% of all BRAF mutations of melanomas. This introduces a conformational change in the kinase domain, which leads to a 10- to 480-fold increase in the kinase activity compared with that of *WT* BRAF. BRAF mutations are also common in papillary thyroid cancer (44.2%), ovarian serous carcinomas (30%), and colorectal carcinomas (30%).

Mutant BRAF transmits survival signals and initiates nuclear transcriptions, resulting in expression of cancer-associated genes, including those for cyclin D (cell cycle genes associated with growth promotion), hypoxia-inducible factor–1α (HIF-1α), vascular endothelial growth factor (angiogenesis), matrix metalloproteinase (MMPs), urokinase and integrins (tissue invasion and metastasis), and mouse double minute 2 (apoptosis evasion and angiogenesis) (DeLuca et al. 2008).

Although BRAF mutations are a critical step in the initiation of melanocytic neoplasia, the exact role of mutant BRAF in human melanocytic tumour initiation is unclear. About 80% of benign nevi displace the V600E mutation, suggesting that it is an early mutational event, that by itself is not sufficient for malignant transformation: expression of BRAFV600E, combined with p53 knockdown or PTEN tumour suppressor gene silencing, elicits development of melanoma in the model systems, with metastases to lymph nodes and lungs (Dankort et al. 2009).

NRAS is the most commonly mutated gene in congenital nevus typically with Q61R mutation, leading to the substitution of glutamine for arginine. 30% of primary and metastatic melanomas harbour NRAS mutations, and they are more common in NM. Mutations in NRAS correlate with poor outcome. NRAS mutations have been documented in most congenital nevi but they are rarely seen in dysplastic nevi, suggesting that congenital nevi and dysplastic nevi may arise through activation of different pathways in melanocytes (Kong et al. 2010).

The PI3K/AKT and PTEN Pathway

The PI3K (phosphatidylinositol 3-kinase)/Akt pathway is often activated in melanoma because of mutations in the tumour suppressor gene PTEN or activation of AKT (Guldberg et al. 1997).

AKT/protein kinase B, a serine/threonine kinase, is a core component of the PI3K signalling pathway activated through phosphorylation of Ser473/474 and Thr308/309. Activation of AKT has been found in 60% of melanomas (Cully et al. 2006). Phosphorylated AKT (pAKT) immunoreactivity is increased in severely dysplastic nevi and melanomas (66.3% positivity) compared to dysplastic nevi.

PTEN encodes a phosphatase that regulates intracellular levels of the lipid phosphatidylinositol-3,4,5-trisphosphate (PIP3). Loss of functional PTEN in tumour cells causes increased AKT activity, leading to decreased apoptosis. Mutations or homozygous deletions in PTEN are found up to 40% of melanoma cell lines, but only in 10% of uncultured melanoma specimens (Wu et al. 2003).

KIT (CD117) encodes a tyrosine kinase receptor for the SCF and plays a key role in melanocyte development. KIT is a member of the platelet-derived growth factor family of receptor tyrosine kinases. Its activating mutations are found in mast cell and

gastrointestinal stromal cell tumours. KIT mutations are typical acral or mucous area melanomas with little UV exposure. The KIT mutation is in 88% of oral mucosal melanomas and 15% of anal melanomas. Studies have shown that KIT reconstitution promotes melanocyte migration but it is not associated with tumourigenic transformation.

Patients with high KIT expression respond well to imatinib that is receptor tyrosine kinase inhibitor (Curtin et al. 2006, Cheung et al. 2019).

MITF, a basic helix-loop-helix leucine zipper transcription factor acts as a master regulator for differentiation, morphology, proliferation, and survival of melanoblasts, melanocytes, and melanoma. MITF is also a melanoma oncogene (Garraway et al. 2005, Levy et al. 2006).

In humans, mutation of MITF causes Waardenburg syndrome characterized by a deficiency of melanocytes in the eye, inner ear, and forelock, with varying degrees of hearing loss. MITF regulates cellular proliferation, and both too high or too low levels of MITF induce cell cycle arrest. MITF is amplified in 10% of primary and 21% of metastatic melanomas, but not in nevi. Its amplification is correlated with decreased overall patient survival and resistance to chemotherapy (Levy et al. 2006).

The p53 protein is a tumour suppressor gene and cell cycle control transcription factor that responds to a variety of stresses, including DNA damage, genomic stability, and hypoxia. During the transformation mutation of the gene of p53 gene are common in 50% of cancers but surprisingly rare in melanomas (1-5% in primary and up to 25% in metastatic melanomas) (Akslen et al. 1998, Ticha et al. 2019).

Knowing that melanoma cells are highly resistant to apoptosis the expression of normal or high levels of p53 protein in melanoma cells surprisingly and indicate that the p53 pathway is dysfunctional in melanoma. Recently Thiem et al showed that IFNγ induced PDL1 depends on p53, making this cancer sensitive to checkpoint inhibitor drugs (Thiem et al. 2019).

The additional factor for sporadic melanomagenesis: Hypoxia-Inducible Factors (HIF) is an important player in the development of tumour microenvironment. Skin is physiologically hypoxic that is one of the -promoting factor in melanomagenesis. The hypoxic response is stabilizing the hypoxia-inducible factors (HIFs), which is heterodimeric transcription factor consist of an oxygen-sensitive α subunit, which forms an active complex with HIF-1β/ARNT. During normal oxygen levels, the HIF-α subunits are degraded but under hypoxic conditions, it becomes stable (Keith et al. 2007).

HIF-1α and HIF-2α activate transcription of target genes involved in angiogenesis, invasion, and metastasis (Wiesener et al. 2003). Both of them are overexpressed in melanomas. HIF-1α activates genes involved in glycolysis and apoptosis while HIF-2α activates stem cell factor gene Oct494 and ABCG2. Tumour hypoxia occurs early during tumour development, about a diameter of 1 millimeter, because of poor vascularization of the tumour tissue (Keith et al. 2007).

Hypoxia is critical for the formation of the premetastatic niche, tumours with hypoxic regions have a poorer prognosis therefore, HIFs play an important role during melanoma progression (Zbytek et al. 2013).

1.4.6 Molecular classification of melanoma and conclusion of 1.4 sections

These genomic subtypes describe how melanoma subtypes differ in their characteristics and clinical presentation and focusing on the prognostic and predictive significance (Cancer Genome Atlas, 2015).

Melanoma can be broadly divided into four heterogeneous molecular subtypes based on recurrent mutations in the MAPK pathway and the pattern of the most frequently mutated genes: *BRAF*, *NRAS*, *NF1*, and a triple *WT* (non-*BRAF*, non-*NRAS*, non-*NF1*) subtype. However, in primary melanomas, BRAF and NRAS mutations are mutually exclusive.

Several specific treatments are created for BRAF mutation of melanoma: mutations in the V600 codon of BRAF (35%–50% of melanomas) and Q61 codons (less frequently, the G12 or G13 codon) of NRAS (10%–25%) lead to the development of highly selective kinase inhibitors that target the MAPK pathway (Tsao et al. 2012).

1. BRAF subtypes BRAF, as a serine/threonine kinase, is involved downstream of RTKs and RAS proteins signalling. 30–70% of melanomas show BRAF point mutations (V600E mostly) (Tsao et al. 2012). **V600 and K601** subtype are hot-spot mutations: (52%) of studied mutations (Pollock et al. 2003). *BRAF* subtype carrying patients are usually younger than patients in the other subtypes.

BRAF mutations are most common in the nodular and SS types and rare in AL (5–10% of cases) and non-cutaneous melanomas (Long et al. 2011).

BRAF mutations also arise more commonly in patients with younger age at presentation and lymph node metastasis (rather than satellite tumours or visceral metastasis). Furthermore, BRAF-mutant tumours metastasize much more to the brain than BRAF *wild type* (*WT)* tumours. BRAF-mutated melanoma is linked to shorter overall survival in patients with stage IV cancer compared to those with BRAF *WT* disease (Ribas et al. 2011).

2. NRAS somatic mutation was found in 28% of studied melanomas. There are less-frequent mutations in other RAS family members, including HRAS and KRAS. Patients who have NRAS-mutated melanomas are usually males and older (>55 years of age) with a chronic pattern of ultraviolet exposure compared to patients with BRAF-mutated melanoma. These patients with NRAS-mutated melanomas have to have thicker tumours that are typically located at the extremities and have greater rates of mitosis (more aggressive).

3. NF1 is a tumour suppressor gene that is mutated in 14% of melanoma patients (usually in spradic melanomas). *NF1* subtype has the highest mutation prevalence (39 mutations/Mb, more than double that of the other three subtypes) and strongest UV mutation signature. It is a GTPase-activating protein that can downregulate RAS activity through its intrinsic GTPase activity (Ratner et al. 2015). *NF1* mutations are common in 50% of desmoplastic melanomas, which have a very strong association with UVR damage, but there are multiple secondary driver genes too. NF1 subtype carrying melanoma patients are usually older and their risk to acquire malignant and benign tumours and pigmentary disorders is higher (Kiuru et al. 2017, Zoller et al. 1997, Seminog et al. 2013).

4. Triple *WT* subtype, comprising about 5–15% melanoma cases, is a heterogeneous subgroup of melanomas characterized by a lack of hot-spot *BRAF, N/H/K-RAS*, or *NF1* mutations. Triple *WT* are quite common melanomas in mycus surfaces (6%) and they have lower mutation rates (6,5mut/Mb versus 14,8/Mb otherwise) and lower PD-L1 expression. These melanomas may have IGFaR and KIT mutations and in generally Triple-WT is with bad prognosis (Hayward et al. 2017).

The different mutational pattern between metastatic and not-metastatic melanoma cases covering 15 most frequently mutated genes in solid tumours (by NGS technology) shows that the most frequent mutations identified were BRAF mutations (52.4%), followed by mutations in NRAS (19.0%) and TP53 (14.3%) (Richetta et al. 2018).

Conclusion: BRAF mutations were mainly detected in metastatic melanoma, confirming a more aggressive behaviour for melanomas associated with BRAF mutations, including thin lesions. Chronic exposure to UVR is highly correlated with mutation rate in the epidermis, patients with melanomas with a higher mutation burden are more likely to respond to novel immunotherapies.

1.5 Diagnosis of melanoma

1.5.1 Diagnosing of different lesions, stages and clinical considerations

The routine physical examination of the skin provides a good opportunity for the identification of early malignant melanomas. In most cases, the diagnosis of melanoma starts with a visual examination of the patient. **The visual examination involves a** physical examination of the skin, as well as the personal and medical history of the patient. Comparative analysis of nevus patterns in an individual patient, visual analysis, pattern recognition, and dynamic analysis may identify suspicious lesions.

Identification of suspicious lesions

The following primary clinical features and parameters are examined clinically for suspicious lesions:

Asymmetry,
Border irregularity,
Colour variation,
Diameter >6mm,
Evolving

This clinical prediction rule is also called “the ABCDE rule of melanoma“. It was first described in 1985 at NY University by the Friedman group. ABCDE rule (asymmetry, border irregularity, colour variegation, diameter >6mm, evolving) characterizes well surface spreading melanomas (Figure 1) adequately described by Robinson et al (Robinson et al. 2009).

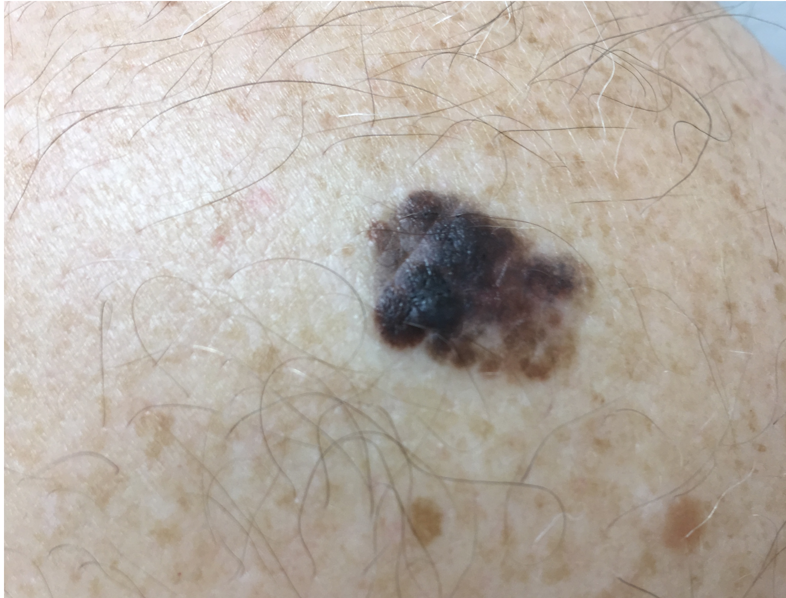


Figure 1. A typical melanoma with ABCDE rules: The melanoma is asymmetrical 'A', has irregular borders 'B', displays colour variation, ranging from brown to black, with a hint of blue 'C', has a diameter >6mm'D', and has a history of evolution 'E'. Photo: Dr. Marina Teras personal collection.

In parallel with the ABCDE rule, a sign called the "ugly duckling" is used to detect possible melanomas. A pigmented lesion that is obviously different from the others in a given individual is always suspicious, even if it does not fulfil the ABCDE criteria (Friedman et al. 1985, Robinson et al. 2009).

It is important to know, that all melanomas have at least one microscopic origin of neoplastic melanocytes, despite that one or more of the ABCDE criteria may be lacking. It is important to remember, that malignant neoplasms change over time by size, shape (elevated/nodular) and ulceration. The sensitivity and specificity of ABCDE criteria vary when used alone or in combination: sensitivity 57%–90% and specificity from 59%–90% (Robinson et al. 2009).

The mainstay of diagnosis is established by biopsy and pathohistologic assessment using the classical method (examination of tissue by a pathologist using lesion sectioning and the microscope) (Swetter et al. 2019).

The importance of detecting and diagnosing melanoma as early as possible.

Since the prognosis of melanoma is directly proportional to the depth of the neoplasm in the skin, the detection of melanoma is of critical importance in saving lives and minimising healthcare costs of treatment. Melanoma initially grows horizontally within the epidermis and in time even after years, it can penetrate the dermis, thereby starting the vertical growth phase. Micro invasive melanomas are characterized by microscopic papillary dermal extension. Vertical growing lesions are associated with an expansible nodule, filling the papillary dermis and invading the reticular dermis or fat tissue.

The vertical depth of the melanoma – the so-called "Breslow depth", is most dangerous and is a predictive factor for prognosis and survival. This thickness is shown in millimeters, from the granular cell layer of the epidermis (or overlying area of

ulceration) to the deepest malignant cell in the dermis or subcutaneous fat (Breslow 1970). Alexander Breslow was the first to show that metastases generally did not occur in melanoma lesions less than <0,76mm in thickness (Breslow 1970). Later the statistical data showed, that melanomas below this value have very little metastatic potential.

For NM, the Breslow thickness rule is not applicable. Mostly NM have no identifiable radial growth period or *in situ* phases, and appear to enter the vertical growth phase from their inception even stating from the dermal region of skin, resulting in thicker tumours at diagnosis. A vertical growth phase melanoma has at least one dermal mitosis or one dermal nest larger than the largest epidermal nest. This feature is the earliest histologic evidence of progression from a nonmetastatic to a potentially metastatic lesion. It has been shown that vertical growth phase melanomas have different cell surface antigen expression, cytogenetic profiles, and growth characteristics, as compared to horizontal ones (Balch 1981, Salhi et al. 2015).

1.5.2 Methods and diagnostic approaches

Dermoscopy:

Dermoscopy is a non-invasive method that allows the evaluation of colour and microstructure of the epidermis and the papillary dermis, which is not visible to the naked eye. The primary purpose of dermoscopy is the evaluation of pigmented and nonpigmented skin lesions, in order to decide whether or not a lesion should be biopsied or referred. The specialized oncodermatological settings then differentiate early melanoma from benign skin lesions, thus minimizing the unnecessary excision of benign nevi. Dermoscopy is performed with a handheld instrument called a dermatoscope, which consists of a transilluminating light source and magnifying optics.

Ambient light is reflected, scattered, or absorbed by objects. Under normal conditions, most of the light is reflected by the skin surface, but the reduction of skin surface reflection allows for visualization of deeper epidermal and dermal structures up to the stratum corneum. These structures are usually correlated to histologic features. The identification of specific diagnostic patterns related to the distribution of colours and dermoscopic structures can better suggest if a pigmented skin lesion is malignant or benign (Dinnes et al. 2018). Digital dermoscopy may also be useful for the follow-up of patients with multiple common and atypical nevi.

The most important epiluminescence microscopy (ELM) feature of melanocytic and nonmelanocytic lesions is the pigment network (PN) and vascular pattern changes over time. There are different algorithms and methods intended to differentiate nevi from suspicious lesions or melanoma. Vascular pattern analysis has superior specificity compared with other features, such as rising of the tumour and is preferred by most experienced clinicians.

In experienced practitioners, dermoscopy improves both the sensitivity and specificity of the clinical diagnosis of melanoma. The sensitivity of the ABCD rule of dermoscopy ranges from 78–90% and specificity from 45–90% among experts and non-experts (Argenziano et al. 1998, Blum et al. 2006). At a fixed specificity of 80%, sensitivity for dermoscopy plus visual inspection was 92% versus 76% for visual inspection alone (Blum et al. 2006, Dinnes et al. 2018). If the lesion is considered to be suspicious for melanoma, it should undergo excisional biopsy.

Other non-invasive techniques are reflectance confocal microscopy, electrical impedance spectroscopy, gene expression analysis, optical coherence tomography, and others. These methods can also be considered as an adjunct if they are possible and available (Dinnes et al. 2018).

1.5.3 Pathohistology of melanoma

The standard and confirmed method to rule out melanoma is biopsy followed by histopathological examination (Clark et al. 1969, Breslow 1970, Balch 1981). The histogenetic classification of cutaneous melanoma dates back to the 1969's when Wallace Clark and colleagues portrayed melanoma tumours with distinct macro-and microscopic features, as well as different biologic behaviour, by its level of invasion into the dermis or subcutaneous tissue in five categories, where tumour thickness was shown to be a powerful prognostic factor (Clark et al. 1969).

Diagnostic criteria

Components of the pathology report include the subtype and a description of cytomorphology and architecture of melanoma, including greatest thickness, anatomic or Clark's level, presence of ulceration, margin status, and for vertical growth phase lesions, the presence of neural or vascular invasion, lymphocytic infiltrates, mitoses and microsatellites. Melanoma cells phenotypically are divided into epithelioid and spindle cells like types (Lilyquist et al. 2017).

Immunohistochemistry – Immunohistochemistry is very helpful in difficult cases that lack compelling morphologic indicators, or in the case of nodal metastases. The most widely used markers are S-100 and Sox10, which are sensitive, but less specific. Melan-A/ MART-1, HMB-45 and tyrosinase are more specific for melanocytic differentiation. Other parallel tests that can be of value in selected cases include expression of proliferation marker Ki-67 and staining for p16, but they are less used.

Testing of primary cutaneous melanoma for oncogenic mutations, such as the BRAF proto-oncogene, is generally not recommended in the absence of metastatic disease and/or clinical trial consideration. TERT promoter mutations and protein expression are under investigation for diagnostic and prognostic value.

Histopathological types and subtypes of melanoma

There are many histological and clinical major subtypes of melanoma however, most common subtypes of melanoma are the following four:

- Superficial spreading melanoma (SSM)
- Acral lentiginous melanoma (ALM)
- Lentigo malignant melanoma (LMM)
- Nodular melanoma (NM)

In addition, less common variants of melanoma are desmoplastic melanoma, amelanotic melanoma, nevoid melanoma, melanoma arising in a giant congenital nevus, melanoma of childhood and melanoma not otherwise classified. Those melanoma types and subtypes have been well characterized a long time ago (McGovern et al. 1973, Balch 1981).

Five anatomical levels – the **so-called Clark's levels,** are recognized. Higher levels have a worse prognostic implication for patients (Figure 2).

- Level 1: Melanoma confined to the epidermis (melanoma *in situ*)
- Level 2: Invasion into the papillary dermis
- Level 3: Invasion to the junction of the papillary and reticular dermis
- Level 4: Invasion into the reticular dermis
- Level 5: Invasion into the subcutaneous fat

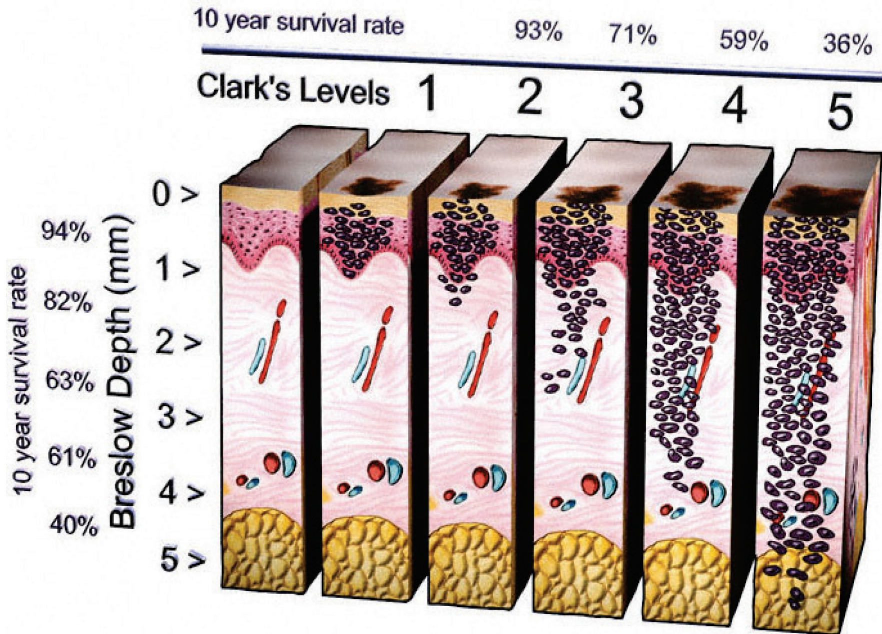


Figure 2. Differences and overlap between Clark levels and Breslow depth. Visualization of melanocytes throughout the various layers of the skin, depending on the stage of progression and Breslow depth. (Image by Med-Art).

1.5.4 Typical clinical subtypes and rare patterns. Evaluation of the tumour growth pattern.

Most melanomas arise as superficial tumours that are confined to the epidermis, where they may remain for several years. During this stage, the horizontal or "radial" growth phase (RGP) occurs and the melanoma is almost always curable by surgical excision alone. Melanomas that infiltrate into the dermis are in a "vertical" growth phase (VGP) and these have a higher metastatic potential.

SSM is the most common subtype: comprising 75% of all malignant melanomas. About 30% of SSM are found in association with a pre-existing nevus, while the majority appear to arise de novo. It can occur in any anatomic location, and also at any age (Pampena et al. 2017). Melanomas which arise as superficial tumours in the epidermis may remain silent for several years. SSM may be composed of the radial growth phase (RGP) only or RGP in combination with VGP melanoma (Figure 3), or combined with satellite metastasis.

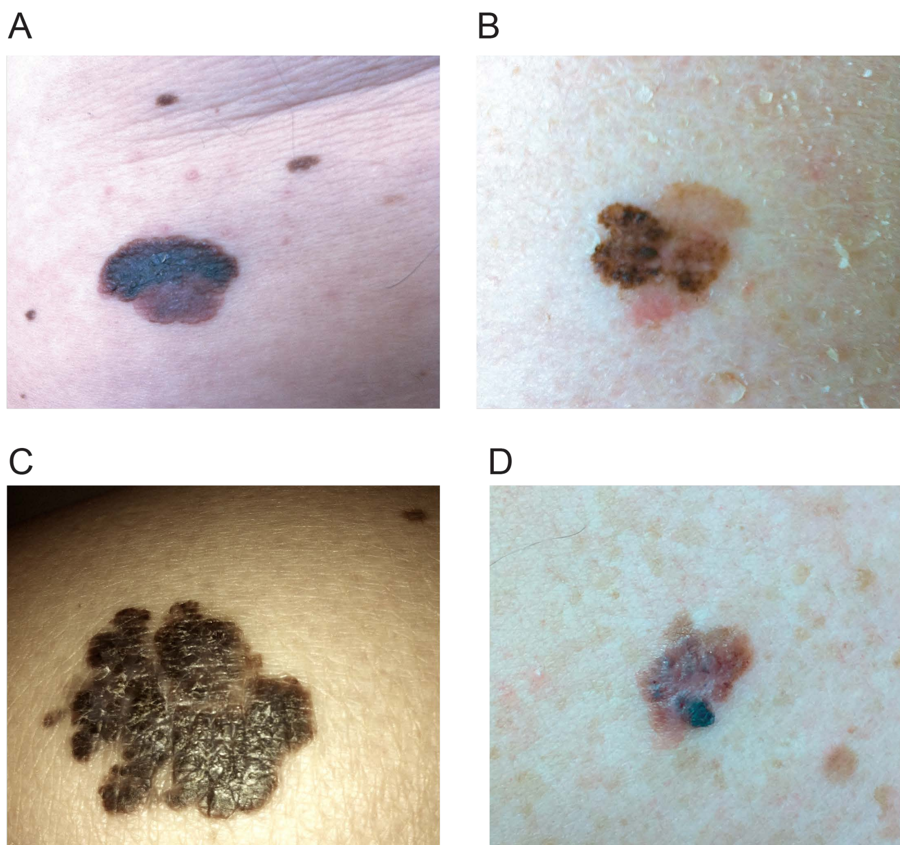


Figure 3. Typical image of a superficial spreading malignant melanoma. A-D: SSM appears as a flat or barely raised lesion, often with irregular borders and variations in colour. Photo: Dr. Marina Teras personal collection.

LMM often arises in sun-damaged areas of the skin in older individuals (Figure 4), and begins as a freckle-like macule, growing gradually **during 10 and even 50 years**, (very slow) before a vertical growth phase becomes apparent (Clark et al. 1969).

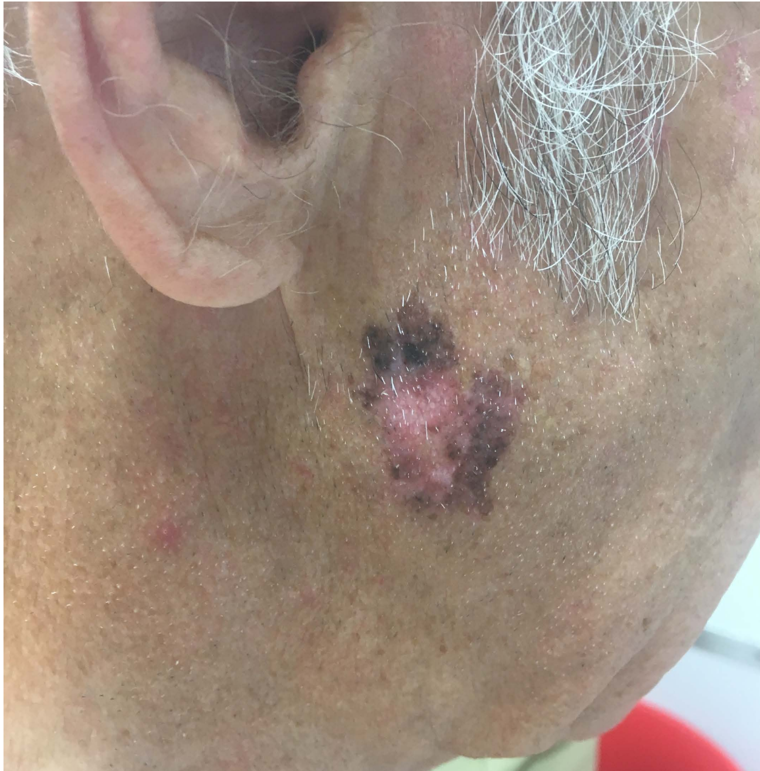
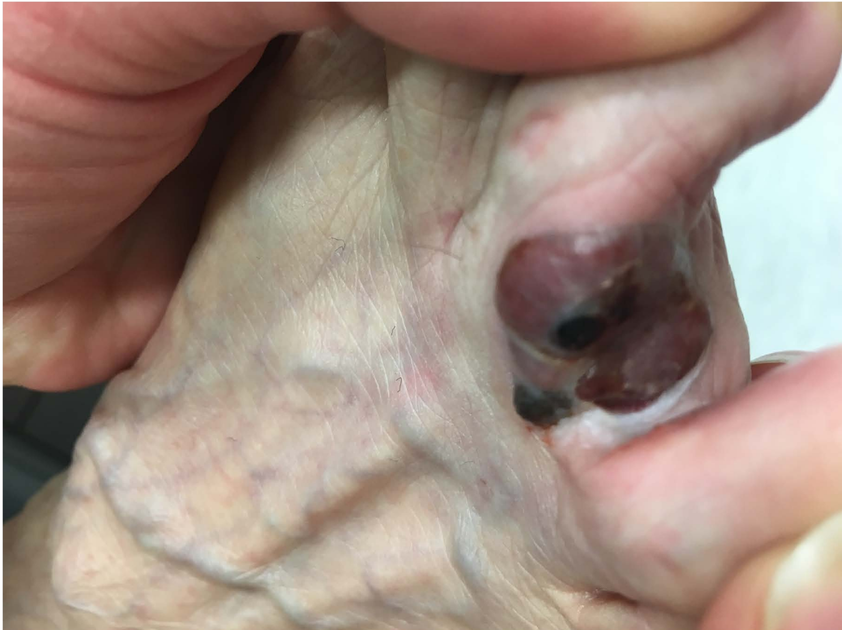


Figure 4. Lentigo malignant melanoma in regression phase. This melanoma begins as a spreading, flat, patch with irregular borders and variable colours of brown. As the lesion grows deeper into the skin it may become various shades of black and brown and begin to form nodules. Photo: Dr. Marina Teras personal collection.

ALM is a quite rare variant of radial growth phase melanomas, composing about 5% of all melanomas (Figure 5). They arise often on palmar, subungual, plantar, and seldom on mucosal surfaces. This subtype of melanoma is the most common among Asians and dark-skinned individuals.

A



B

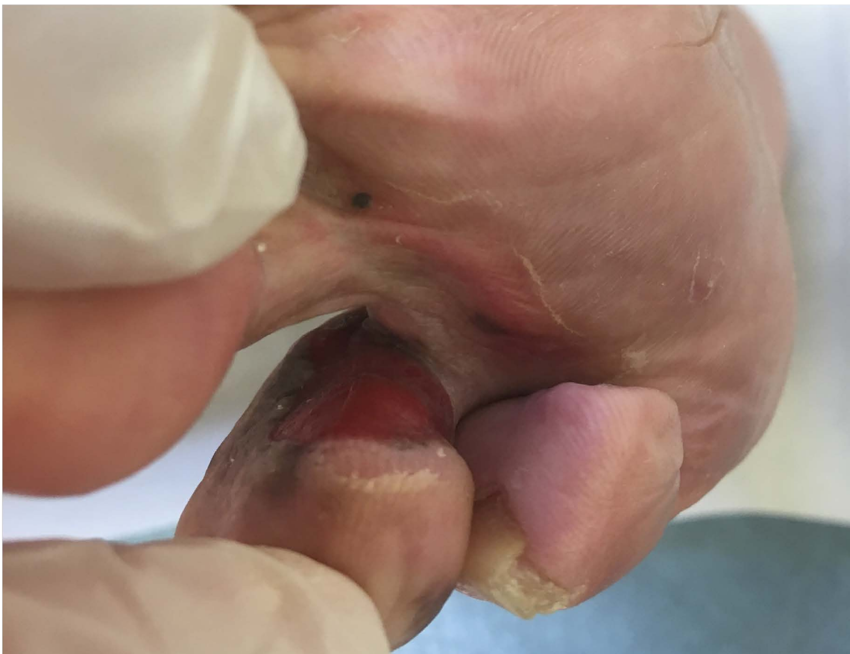


Figure 5. Acral malignant melanoma A: between toes; B: under foot between toes on previously inflammated place. The acral malignant melanoma often appears on the palms of the hand, soles of the feet or under the nails. Lesions are usually tan, brown, or black, with irregular borders and variations in colour. Photos: Dr. Marina Teras personal collection.

NM are vertical growth phase melanomas, that compose 15 to 30% of all melanomas. Clinically they are most commonly dark pigmented nodules and seldom the amelanotic variant, sometimes pedunculated or polypoid nodules (Figure 6). NM do not have the radial growth or *in situ* phases and they enter the vertical growth phase from their inception, resulting in thicker tumours at diagnosis (Rother et al. 2009).

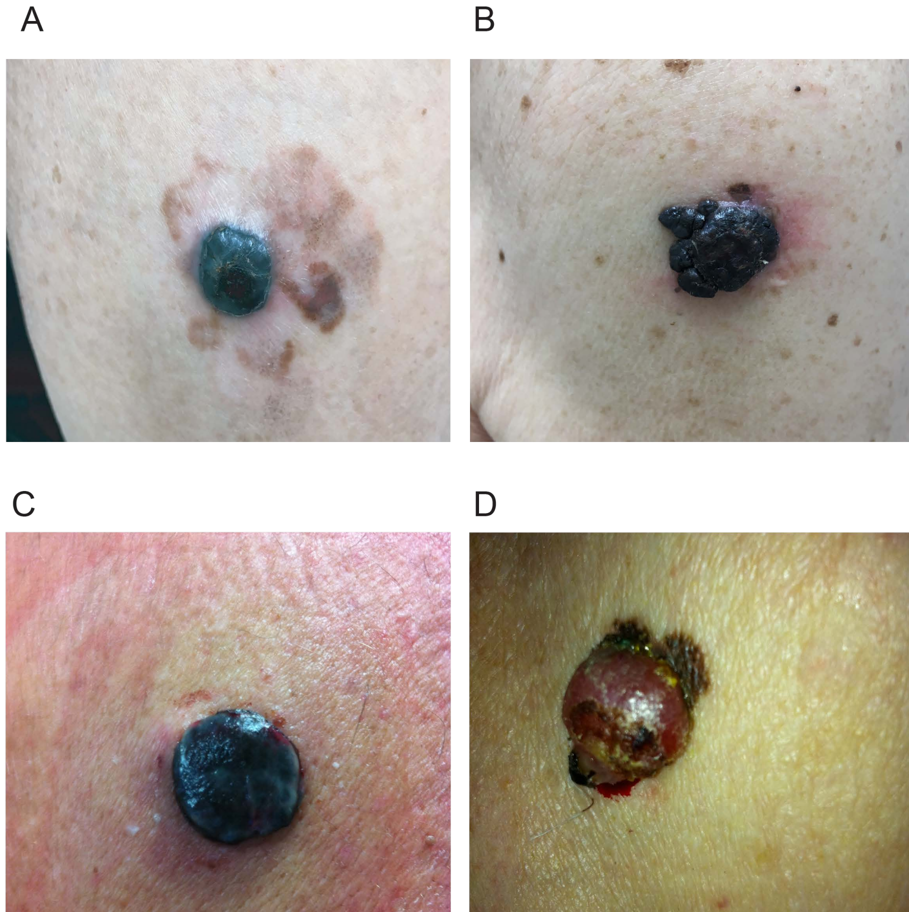
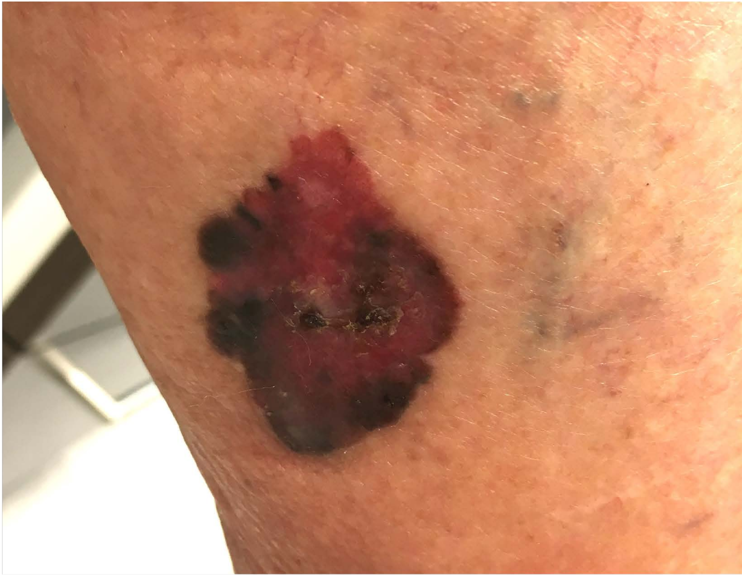


Figure 6. Typical images of nodular melanomas (NM). NM forms 15% of the cases and is typically very dark brownish black or black in colour but can be pink or red and forms a lump on the skin surface; also it invades deeper into the skin as displayed in this figure A-D. A: NM starting from surface spreading melanoma. B: NM with irregular inflammated border. C: Round NM with regular inflammated boarder. D: Round NM with regular inflammated border and with surface spreading phenotype. Photos: Dr. Marina Teras personal collection.

All melanoma subtypes may present as amelanotic or hypomelanotic variants of lesions clinically, and this is most commonly observed with nodular and desmoplastic subtypes which represent 5% of cases. Amelanotic melanomas are clinically pink or red macules, plaques, or nodules (Figure 7). They are often confused with benign lesions and this may lead to considerable delay in the diagnosis and potentially worse prognosis.

A



B

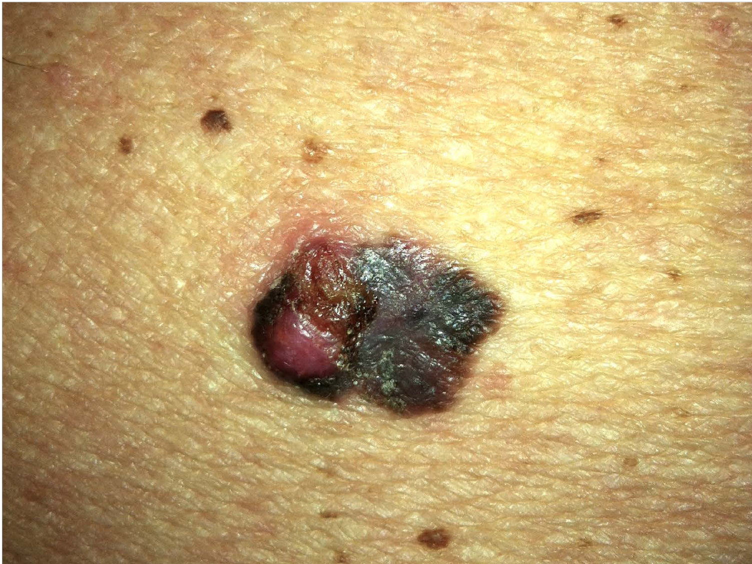


Figure 7. Amelanotic lesion of malignant melanoma. A: partly apigmented melanoma; B: partly apigmented nodular, combined with surface spreading melanoma. Photos: Dr. Marina Teras personal collection.

1.5.5 Melanoma clinical classifications and stages: the concept of stage/grade

Knowledge of staging is very important for melanoma diagnosis, for prognostic assessment, and the planning of surgical, adjuvant, and systemic treatment. For this reason, a staging system has been developed by the American Joint Committee on Cancer (AJCC) in collaboration with the Union for International Cancer Control (UICC), which is based upon the assessment of the primary tumour (T), the presence or absence of regional lymph nodes (N) and distant metastatic sites (M). AJCC melanoma staging system groups patients **into four prognostic stage groups** based upon the primary tumour, lymph node involvement, rate of metastases, and it distinguishes between clinical and pathologic staging:

Pathological Stage 0 (melanoma *in situ*) and T1a do not require pathological evaluation of lymph nodes to complete pathological staging; use cN information to assign their pathological stage

Stage I – Stage I melanoma is low-risk primary melanoma (T1a, T1b, and T2a) without evidence of distant or regional metastases. Stage I is divided into stages IA and IB based on the thickness of the primary tumour and the presence or absence of ulceration.

Stage II – Stage II disease includes primary tumours that are at higher risk of recurrence (T2b, T3a, T3b, T4a, and T4b) but do not have any evidence of lymphatic disease or distant metastases. Stage II is divided into stage IIA, IIB, and IIC depending upon tumour thickness and the presence or absence of ulceration.

Stage III – Stage III disease includes pathologically documented involvement of regional lymph nodes or the presence of in-transit or satellite metastases (N1, N2, and N3). Stage III disease is further subclassified according to the involvement of the lymphatic system: stage IIIA, IIIB, IIIC, or IIID.

Unknown primary tumour– Patients with isolated metastases identified in the lymph nodes, skin, or subcutaneous tissue who do not have an identifiable primary cutaneous melanoma (T0) are classified as pathologic stage III.

Stage IV – The presence of distant metastases defines stage IV disease (M1a to M1d). Central nervous system metastases (M1d) are associated with a very poor prognosis and tare no subgroups.

From the indicated parameters (T, N, and M) the Breslow thickness (T) is the most powerful predictor for developing metastatic disease. Moreover, none of TM with lymph-node involvement have Breslow thickness <0.6 mm. Most studies have shown that the presence of lymph-node metastases is very rare (<5%) in melanomas with Breslow thickness <0.8 mm and occurs in approximately 5% to 12% of patients with primary melanomas with Breslow thickness from 0.8 to 1.0 mm (Richetta et al. 2018). Breslow depth or **thickness** requires evaluation of the entire tumour from an excisional biopsy, rather than a wedge or punch biopsy. It is recommended to ensure the measurement of the thickest part of the lesion; a shave biopsy can compromise this evaluation and is therefore discouraged. The maximal tumour thickness is measured at a right angle to the surface of the skin over the tumour mass.

In conclusion, Breslow thickness as a valid prognostic factor is able to distinguish a high- or low-risk of developing metastases. Breslow thickness ≥0.8 mm is optimal as a threshold value where draining lymph node biopsy should be considered. Future studies

using omics approaches are needed to provide a larger genetic profile that could be useful as an even better prognostic and predictive factor.

1.5.6 Metastasing characteristics of malignant melanoma

Melanoma is a unique tumour, due to its capacity to metastasise to all possible tissues and body regions. Although regional lymph nodes are the first place where melanoma metastases appear – the secondary sites can be in rather diverging places such as skin (Figure 8), subcutaneous soft tissue, lung, brain, bones, heart, spleen, gall bladder, etc. This feature of melanoma makes it difficult to diagnose metastasis, involving clinically and radiologically expensive methods like all body positron emission tomography (PET) scan or similar.

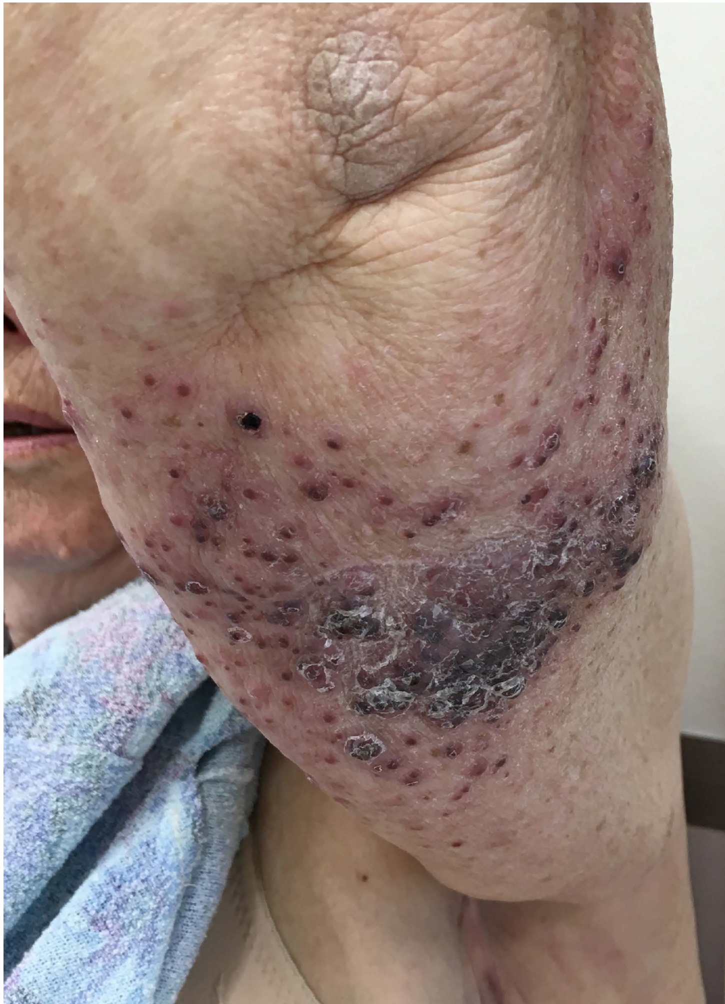


Figure 8. Typical in-transit metastases (ITMs). Multiple ITM are visible on the left arm before treatment. Photo: Dr. Marina Teras personal collection.

Micrometastasis

Circulating tumour cells (CTC) are cells of solid tumour origin, which have migrated (or passively released) to the circulation. Their appearance in the peripheral blood is a prerequisite step for establishing distant metastases. CTC number usually correlates with the clinical outcome (Khoja et al. 2015). The immune system constantly controls for CTC presence. Numerous studies have been published on CTC detection in melanoma at various stages of the disease, but this approach is not yet used clinically. Immunomagnetic-isolation techniques and qRT-PCR detection of melanocytic markers are limited due to possible loss of specific melanocytic antigens during tumour progression. Size-based cell separation is more promising since tumour cells are larger as compared to blood leukocytes. CTC count in blood (Khoja et al. 2015) or circulating tumour DNA (ctDNA) in the blood (Tan et al. 2019) is in correlation with early recurrence, shorter disease-free survival (DFS) or shorter OS. Circulating tumour DNA predicts relapse and correlates with survival in high-risk resected melanoma and could aid in the selection of patients for adjuvant therapy**.** Patients with detectable ctDNA had decreased disease- and metastasis-free interval versus patients with undetectable ctDNA (Lee et al. 2018). The detection of CTCs in circulation is an important prognostic marker, unfortunately, their number is very small, thus these cells are not easily detected. It is estimated that among the cells that have detached from the primary tumour, only 0.01% can form metastases.

Dormancy and early metastasis

Clinically, dormancy describes the status of microscopic metastases before cancer progression. Tumour dormancy depends on three elements: (1) cellular dormancy, during which tumour cells survive in a quiescent, slowly dividing state; (2) angiogenic dormancy, during which lack of vascularization holds the growth of micrometastases under control and promotes programmed cell death, known as apoptosis; and/or (3) immune-mediated dormancy, where the immune system continues to limit the tumour population. Melanoma cells can be shed early from a primary tumour and remain silent or “dormant” as micrometastatic foci for periods ranging from under a year to decades. Disruption of these processes can awaken dormant micrometastases and stimulate their expansion into overt metastases, leading to patient disease progression and eventually to mortality. Maintenance of a dormant state requires intrinsic factors and extrinsic microenvironmental cues. The latter can be derived from the vascular and lymphatic systems, as well as the presence and activity of inflammatory or stromal cells. During the dormant state, **immunoediting** may occur, which is a process in which most immunogenic tumour cells are eliminated, leaving a poorly immunogenic, dormant population (Recasens et al. 2019).

The relationship between melanoma dormancy and physiological stem cell dormancy requires further study. In tumour cells, a state of dormancy means the ability to maintain viability over a longer time without proliferation. This state is controlled by both cellular and environmental factors, such as intracellular signalling, immune surveillance, and tumour-stroma interaction. The quiescent or inactive period is important for cancer cells to acquire additional mutations, to survive in a new environment, to evade immune destruction, generate metastasis, and to become resistant to cancer therapy. Interference of dormancy to an active state of melanoma results in a relapse and the metastatic burst (Kleffel et al. 2013). Many of the biological mechanisms involved in controlling the dormant state can govern CSC behaviour,

like alteration of angiogenic processes, cell cycle modifications and modulation of antitumour immune responses (Senft et al. 2016). Consequently, dormant cancer cells are considered to be responsible for cancer progression.

Macro metastases

Histologically, macro metastasis (Macro-MTS) can appear in any tissue while cutaneous metastases appear as growing nodules in the dermis or subcutaneous fat without an epidermal connection (Figure 9). It has been shown, that the nodular growth pattern is the highest risk factor for disease progression. Sometimes growing nodules appear in the skin without any primary tumour, indicating that it may be already a metastasis (Lattanzi et al. 2019).

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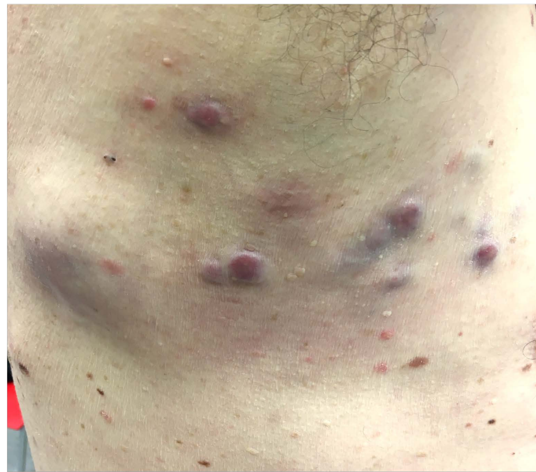

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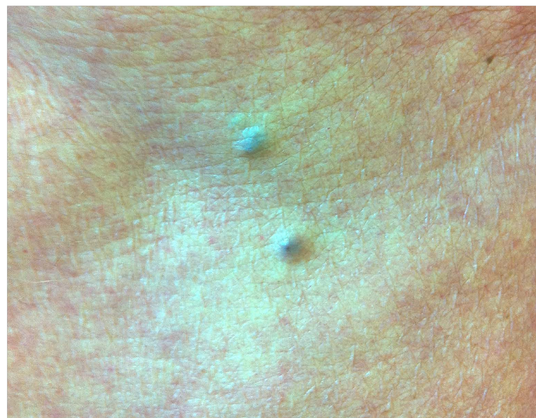

Figure 9. In transit metastases (ITMs) that differ in size. A: Macro metastases under arm. B: Tiny in ITMs with live size about 1-2 mm. Photos: Dr. Marina Teras personal collection.

Diagnosis of distant metastatic disease

In patients with newly diagnosed melanoma and with evidence of high risk for spreading, several different imaging studies have to be utilized, in order to rule out clinically distant metastasis, specifically if the Breslow depth is more than 4mm. Before imaging studies, some known melanoma markers like S100 may be assessed, and if the value is high, there is a clear indication for radiological assessment. Imaging possibilities for the surveillance of melanoma patients are variable and include ultrasonography, magnet resonance tomography (MRT), computed tomography (CT), PET and a combination of both (PET-CT). From those, the ultrasonography is the best imaging study to diagnose lymph node metastasis involvement. For staging of distant metastases, the PET-CT scanning has the highest sensitivity, specificity, and diagnostic odds ratio, as has been concluded by a meta-analysis (Xing et al. 2011).

1.6 The interest of omics (transcriptomics, proteomics, single-cell analysis) for understanding melanoma and treatment planning

Melanomas often have a high capacity to mutate and evolve quickly, losing the combinations of markers that describe their phenotype and define their classification. Transcriptomics (as well as proteomics) is a very useful tool to get a global and quantitative description of gene expression and to follow the evolution of this profile during genomic modification of the tumour. This comprehensive description can help define evolution patterns for melanomas, and large annotated databases of melanoma transcriptomes should lead to a dynamic classification of these tumours based on multiple parameters, in the future. Proteomics, microRNA and exosome studies also have provided new knowledge to complement the information generated by genomic studies. The application of such approaches is developing fast and will be the basis of personalized medicine in the near future (Hogan et al. 2018).

Biomarkers from next-generation sequencing, both from whole exome (WEseq) and RNA sequencing (RNAseq), are powerful methods for evaluating the genome of a tumour and its expression profile. Weseq captures all exonic gene regions, showing expressed mutations in coding regions, while RNAseq is useful for establishing gene signatures for specific cohorts within a patient group. Melanoma has one of the highest mutation rates of all cancers (Hayward et al. 2017). Most of the current cancer therapies, such as immune checkpoint blockers, act on molecules responsible for cellular communication that are dysregulated in cancer, trying to restore the normal behaviour of the cells (especially immune cells). In 2009, lactate dehydrogenase (LDH) was shown to be an independent predictor of survival in melanoma and was therefore added to the AJCC guidelines (Balch et al. 2009). Elevated LDH is a negative prognostic marker for patients treated with monoclonal antibodies targeting CTLA-4 (ipilimumab) (Kelderman et al. 2014) or programmed cell death protein 1 (PD-1) (pembrolizumab) (Jessurun et al. 2017). Thus, LDH has been validated as a useful marker to monitor disease progression and to help treatment decisions.

Another old but good marker to monitor melanoma is S100, which indicates a well clinical disease stage (Kaskel et al. 1999). C-reactive protein (CRP) is a negative prognostic factor for anti-CTLA-4 treatment, which is directly related to the immune response (Simeone et al. 2014).

The immunohistochemical evaluation of programmed cell death protein 1 ligand (PD-L1) expression in tumour cells is a predictive biomarker for a choice of treatment (Kythreotou et al. 2018). High intratumoural PD-1 expression indicates generally a positive effect for anti-PD-1 therapy, although there is no clear correlation with it because the PD-L1 expression in tumour cells varies al lot (Munari et al. 2018). For anti-PD-1-treated patients, the relative lymphocyte count and relative eosinophil count (REC), as well as the myeloid-derived suppressor cells (MDSC) count, seem to hold some predictive potential prior to treatment initiation (Weide et al. 2016).

For anti-CTLA-4 treatment, the absolute neutrophil, lymphocyte, monocyte, T-reg, MDSC and eosinophil count, have been described as predictive biomarkers (de Coana et al. 2017).

Immune checkpoints inhibitors that boost antitumour immunity, have already demonstrated a huge potential as anticancer treatments that target cells in the TME. However, as checkpoint blockade is currently beneficial only in a limited fraction of patients, there is an urgent need to understand why not all patients respond.

Overall, the mechanistic involvement of these markers in the biology of the tumour and anti-tumoural response remains poorly understood. Because these markers provide a comprehensive description of the tumour and its environment, one should hope that they will provide new insights into the mechanisms that explain the correlation between melanoma features and biomarkers. Of course, new biomarkers and combinations of markers are also under development.

Hence, these technologies and approaches aim at the development of novel multiparametric classifications of melanomas, based on combinations of markers of which biological functions are elucidated.

For example, in order to elucidate biomarkers of response to treatment, Hugo et al. used WES from 38 patients and RNAseq from 28 patients on a set of melanoma patients treated with anti-PD-1. He found an interesting innate anti-PD-1 resistance (IPRES) signature in 9 of the 13 non-responding patients, (Hugo et al. 2016). New prospective trials with large cohorts could validate many of these findings. **Although unfortunately, most research groups have different evaluation criteria.**

Epigenomic changes associated with melanoma progression and growth are poorly understood and should be targeted by multi-omics approaches. Through systematic transcriptomic analysis chromatin state changes associated with non-tumourigenic versus tumourigenic states have been identified (Fiziev et al. 2017). Functionally relevant chromatin states associated with melanoma progression have been identified. Systematic analysis of gene sets clearly suggests that the set of genes that had lower acetylations and lower gene expression was linked to pathways with known roles in tumour progression. These data are showing the importance of chromatin-associated gene expression changes in cancer progression.

The next frontier appears to be an extensive characterization of the phenotypic diversity of the tumour in real-time using **single-cell technologies**. These approaches will provide a description of the variability of tumoural cells at a given stage and given individuals and allow a dynamic understanding of the evolution of this diversity with time, along with the accumulation of mutations.

The single-cell analyses using *omics* technologies now provide the opportunity to catalogue all human cells in health and disease. Single-cell technologies can dissect intra- and intertumour heterogeneity, and identify rare cells that play a role in cancer progression and invasion, like CTCs and cancer stem cells. In addition, these

technologies offer the opportunity to discover new immune cell subpopulations (Villani et al. 2017) and unlock the potential of precision immuno-oncology.

In conclusion:
Omics analyses can identify disease markers that can monitor the efficacy of therapies (Dumitru et al. 2017). It can also identify new chromosomal alterations and discover new deregulated genes that can be further used as therapeutic targets. Overall, there are 3 main aspects to consider in the future potential developments linked to omics:

- Large standardized datasets and databases will provide new opportunities to find useful correlations (*ie* new biomarkers) to produce integrated multiparametric classifications of tumours.
- Such classifications based on comprehensive knowledge at the genomic, transcriptomic, epigenomic, phenotypic **and clinical** levels should allow safe predictions of the characteristics of the tumour based on a reasonable set of markers, hence providing guidance for therapy design.
- Finally, omics data should allow a better understanding of the biology of the tumour and the mechanisms of antitumoural response. The quality of the predictions based on biomarkers would be greatly increased by the understanding of their functional involvement in the pathology.

1.7 Immune response against melanoma

The immune system is a dominant force in cancer control but it also responsible for the poor responses to cancer immunotherapy. An immune response can be an innate or adaptive type, depending on the cells and systems involved in it. The innate immune system relates to anatomical and biochemical barriers, unspecific cellular responses that are mediated by monocytes, natural killer (NK) and dendritic cells (DC). The adaptive immune system relates to the response to specific antigens that are mediated by B and T lymphocytes. The adaptive immune system depends on the generation of a diverse repertoire of antigen receptors on T and B lymphocytes and subsequent activation and clonal expansion. The induction of adaptive immunity depends on the recognition of the particular antigen receptor and is in most cases activated by the innate immune system (Schenten et al. 2011). The primary function of B lymphocytes is the production of antibodies and T helper (Th) cell the production of cytokines in response to infection from a pathogen (Gitlin et al. 2015, Passarelli et al. 2017). Cytotoxic T (Tc) cells circulate in the body and control the expression of peptides in MHCI. When the peptides are from foreign origin or mutated cytotoxic T cell can kill the cells expressing those peptides. T helper cells will decide the type and direction of the immune response by expressing certain cytokines, by which they can either activate or inhibit Tc and/or B cell. Cytokines provided by Th cell also activates B cell helps produce the right isotype of antibody.

Melanoma is an immunogenic tumour whose relationship with immune cells influences cancer cell proliferation, progression, and metastasis. Immunogenicity of a tumour means its capacity to induce adaptive immune responses that can prevent tumour growth (Passarelli et al. 2017). Melanoma cells express on the surface specific antigens that are capable to induce immune activation instead of immune tolerance. Immunogenicity of the tumour depends not only on the antigenicity of cancer cells but also on the dynamic interplay between melanoma and the immune system. This provides a suitable model to investigate the molecular crosstalk of cancer cells with cells of the immune system.

1.7.1 The Triple-E and Immune System-Melanoma Interaction: Elimination, Equilibrium, and Escape

During melanoma development, both cell proliferation and apoptosis are associated with the immune editing that means basically the *elimination* of all tumour cells that the immune system can recognize. During this phase, the tumour cells are not yet clinically detectable. The ***elimination*** *of tumour* is based on the anti-melanoma cytotoxicity by natural killer (NK), γ/δ T cells, and dendritic cells (DCs), T-cells, B-cells either within the tumour microenvironment or in the closest lymph node (Dunn et al. 2004). Immune effector cells are responsible for TME remodelling (Daley et al. 2016, Tucci et al. 2019) and are stimulated by interleukins (ILs) produced by melanoma cells, tumour-associated macrophages (TAMs), and stromal cells. Effector T cells are primed against melanoma cells also through the endogenous production of interferon-gamma (IFN-γ). Enhanced interferon (IFN)-γ release by NK and T cells and the induction of apoptosis of melanoma cells (through perforin, TRAIL, and Fas-L), is leading to the release of new antigens that stimulate the adaptive immune response (Tucci et al. 2019). Tumour-associated tissue eosinophilia has also been reported since eosinophils can directly fight with tumours (Varricchi et al. 2018).

By the time, cancer cells become resistant to effector immune cells via the selection of clones with limited immunogenicity. The **equilibrium** phase consists of a prolonged period during which the tumour cells are constantly suppressed. That is a slow process, lasting tens of years during which takes place the education of tumour not to be visible for the immune system. The equilibrium phase, therefore, is characterized by a ‘quiescence’ during which any proliferation or cancer cells are counterbalanced by the adaptive immune system. Effector memory T-cells are the main activators of the equilibrium phase, although tumour cell variants progressively lose major histocompatibility complex (MHC) class-I and -II molecules on the surface that makes them resistant to T cells. During equilibrium phase melanoma cells harbour a number of somatic mutations that enhance their genetic instability, thereby supporting the development of less immunogenic clones with a strong propensity to expand within the TME (Alexandrov et al. 2014).

The majority of malignant cells are eliminated during the equilibrium phase unless they acquire new mutations that increase the resistance to immune system control (Schreiber et al. 2011).

Melanoma cells may acquire up to more than one hundred mutations per megabase that is much higher mutational load compared to other malignant populations. These mutations may generate a high number of novel epitopes that can cause T cell exhaustion (Passarelli et al. 2017).

The immune **escape phase** depends on the exhaustion of the immunosurveillance. The functional exhaustion of the immune system depends on the continuous presence of antigen on melanoma cells and induces the activation of inhibitory receptors on immune cells, which results in negative feedback for the cytotoxic T-cells (Jacobs et al. 2012, Pardoll 2012).

In addition, the enrichment of the melanoma environment in tumour-associated macrophages (TAMs), regulatory T cells (T reg) and myeloid-derived suppressor cells (MDSCs) represents a parallel mechanism that directly inhibits the normal immune response by effector cytotoxic T-cells (Polak et al. 2007). The inefficient killing of melanoma cells is mostly due to the direct effect of inhibitory signals.

Also, melanoma cells through the over-production of negative modulators inhibit immune cell cytotoxicity (Munn et al. 2016).

Those interactions among immune cells as well as on immunomodulatory factors released by the tumour cells may result in the final escape of the tumour.

1.7.2 Activation of the immune system in melanoma

Several studies have been shown, that the defective immune system allows melanoma cell proliferation (Dunn et al. 2004). This has been shown in murine melanoma models that acquire a rapid increase of tumour growth once depleted of $CD8^+$ cells and in patients whose treatment response is better when to a higher amount of T cells will infiltrate to the tumour microenvironment (Muul et al. 1987, Mahmoud et al. 2011, Dang et al. 2019).

An efficient anti-melanoma immune response requires first a proper activation of the innate immune response. It has been shown recently, that low MHC-I expression allows for disseminated pulmonary melanoma metastasis post i.v. inoculation of low MHCI expressing tumour cells (Dang et al. 2019), and correlates with a delayed anti-cancer adaptive humoral and cellular immune responses. The formation of the tumour microenvironment (TME) is typically characterized by increased Th2/Th1 profiles, which makes metastatic tumour resistant to the adaptive immune response. Using either active or passive immunization that activates adaptive immune response helps reverse the Th2/Th1 profile in a time-sensitive manner (Dang et al. 2019). TME is not irreversible and adequate adaptive immunity is central in anti-tumour immunotherapy.

Those T effector cells are primed against melanoma cells through the endogenous production of interferon-gamma (IFN-γ).

1.7.3 Immune escape of melanoma

The immune escape of melanoma cells is regulated by the 'immune editing' that is mostly based on immune reaction against melanoma and selection of melanoma cells with limited immunogenicity (Dunn et al. 2002). Immune editing also involves the colonization of distant tissues by melanoma cells and includes the 'immune escape' phase (Bhatia et al. 2011). It is driven by the constant stimulation of the immune system and by the reaction of malignant cells to respond to the immune-mediated antigenic recognition (Shankaran et al. 2001).

During an immune editing process, the progressive exhaustion of the immune system takes place (Pardoll 2012). Immune cells are not able over a long period to counterattack highly proliferating malignant cells. The escape of melanoma cells also depends on the defective immune recognition as well as on a high mutation rate that causes increased resistance to apoptosis of melanoma cells. All the above mentioned factors development of an immunosuppressive microenvironment that inhibits the immune system attack and helps the tumour to escape (Dunn et al. 2002).

1.7.4 Neoatigenic mutations generate immunogenically silent peptides

The immune system is thought to play a dual role in the generation of malignant melanoma. At the beginning of cancerogenesis, the immune system is capable of eliminating arising cancer cells. In contrast immune systems also initiating signalling of wound healing pathways that in later steps on melanoma development will on the contrary induce and aid tumourigenesis.

The MHCI molecules present in the thymus all nucleated cell's proteomes on the cell surface, to educate developing T cells to recognize their own normal healthy proteome. This peptide presentation covers mainly peptides derived from proteins that are highly represented across the population. Unfortunately, some peptides are purely or not at all presented by common MHCIs (Fritsch et al 2014). Those "unprotected regions" that are immunogenically silent, coinciding with frequently mutated hotspots in cancer. For example, common cancer mutations including BRAF-V600E and KRAS-G12D are predicted to bind none of the common MHCI alleles and are thus "immunogenically silent" in the human population (Fritsch et al. 2014). This is explaining why the BRAF-V600E mutation carrying melanomas are so frequent.

1.8 Treatments and their outcome

1.8.1 List of treatments

Based on the melanoma location, genetic profile and stage, there are several therapeutic strategies including surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapies including cytotoxic drugs, BRAF/MEK inhibitors, drugs targeting mutations in melanoma cells or intratumoural oncolytic viral therapy.

The most common and effective treatment is early excision of melanoma, with or without lymph node management. This treatment is applied during melanoma stage 0 up to stage II. For melanoma stage III intralesional therapy, immunotherapy, signal transduction inhibitors, chemotherapy and/or palliative local therapy are used in addition to surgery (NCCN guideline).

1.8.2 Surgery

Proper surgical management is always a critical step for the primary diagnosis, staging, and optimal treatment of primary cutaneous melanoma. Depending on the melanoma type, narrow or wide margin excision is used.

Narrow-margin local excision

A full-thickness excisional biopsy of suspicious lesions with a 1 to 3 mm margin of normal skin including part of the subcutaneous fat should be performed. This technique (narrow-margin excision) allows the assessment of the entire lesion without compromising subsequent wider surgery or potential staging with the sentinel lymph node biopsy technique.

A partial incisional biopsy is also acceptable if the excision of the entire lesion is not feasible (or for example, if there are large lesions or if the melanoma is located in certain complicated sites of the body) (Swetter et al. 2019). Excisional biopsy may include different techniques, that includes to a depth below the anticipated plane of the lesion, usually extending to the deep reticular dermis with a narrow peripheral: 1–3 mm (Swetter et al. 2019).

Wide local excision

For primary cutaneous melanoma, a definitive surgical excision is used with a wide local margin down to the deep fascia. The recommended width of the normal tissue around the lesion has decreased as a result of multiple clinical trials that have examined the impact of the optimal surgical margin of 2, 3, or 5 cm on the local recurrence rate.

Based on this data, the use of certain margins is currently recommended for patients depending on melanoma thickness (Gillgren et al. 2011).

Melanoma thickness (according to pathohistology) is the main factor that is determining the stage of the lesion and also recommended margin of normal tissue to be resected.

Melanomas lesions thickness and excision margin:
Tumour *in situ* melanoma (Tis): For patients with *in situ* (Figure 10) melanomas 0,5–1 cm margin of normal tissue surgical excision is recommended. Margins larger than 0.5 cm may be necessary for melanoma *in situ*, lentigo maligna type.

- T1 ≤1 mm thick: to resect with a 1 cm margin of normal tissue
- T2 >1 to 2 mm thick melanomas: use a 2 cm margin of normal tissue if this is feasible without the need for a skin graft.
- T3 2.01 to 4 mm melanomas: a 2 cm margin is suggested.
- T4 > 4mm thick melanomas.

Clinical trials have not demonstrated a benefit of excision margins >2 cm for T4 lesion (Gillgren et al. 2011). On the contrary, a less invasive, narrow excision margin (2 cm vs 4 cm) did not affect melanoma-specific nor OS (Utjes et al. 2019). However, margins may also be modified for functional considerations or anatomic location.

Most world guidelines (NCCN etc) recommend surgery as first-line therapy for eradication of melanoma *in situ*. In case a patient refuses surgery, second-line treatment on a case-by-case basis is used. Alternatives, such as imiquimod or RT can be considered as second-line treatment after full discussion of the associated risks, benefits, and uncertainties.

Excision of regional lymph nodes
The decision of whether or not to surgically stage regional lymph nodes is based upon the risk of recurrence and staging suggestions.

For patients with clinically negative nodes and a primary melanoma at intermediate or high risk for lymph node metastasis, lymphatic mapping with sentinel lymph node biopsy is recommended to perform. This includes generally patients with melanomas >0.8 mm thick. I concern melanomas <0.8 mm thick only with the dangerous aspect as ulceration. Those recommendations should be individualized based upon the immunological state of a patient, age, comorbidities, and discussion with the patient. In low-risk patients, the doctor should discuss the pros and cons of sentinel node biopsy considering the risk of additional surgery and cost.

Sentinel lymph node biopsy (SLNB), should be performed before wide excision of the primary tumour. Whenever it is possible to minimize disruption of the lymphatic channels and optimize the accuracy of lymphatic mapping and identification of the correct SLN(s). SLNB is not recommended for T1a patients (Swetter et al. 2019) because during this stage melanoma metastasis are very rear. <5% SLNB provides the most reliable and accurate means of staging for appropriate patients with primary CM (Current National Comprehensive Cancer Network (NCCN)).

In preoperative lymphatic mapping (lymphoscintigraphy), intraoperative vital blue dye injection around the primary cutaneous melanoma or biopsy scar, and gamma probe localization with technetium-99 sulfur colloid are used to identify and remove the SLN(s). The SLNs are then examined histologically for the presence of tumour involvement by using both routine histology and immunohistochemistry.

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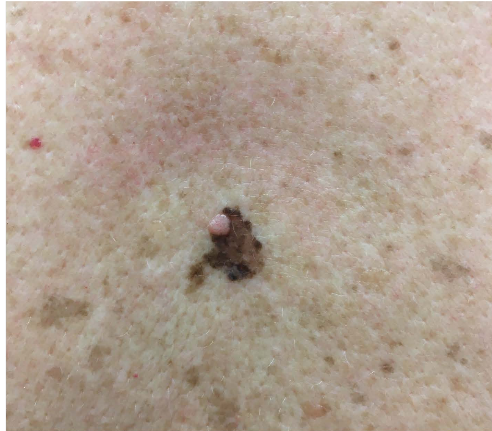

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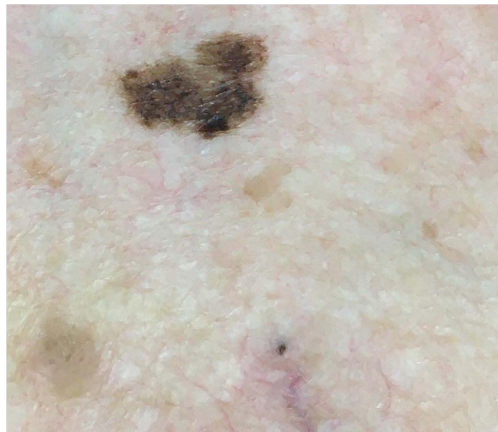

Figure 10. Typical in situ melanomas. A and B: Typical in situ melanomas. Photos: Dr. Marina Teras personal collection.

Completion LN dissection (CLND) is also traditionally recommended and performed following a positive SLNB because approximately 8% to 20% of patients will harbour nonsentinel nodal metastases.

Retrospective studies raise the question of whether CLND is indicated following a positive SLNB. Current NCCN guidelines recommend that CLND versus active nodal basin surveillance with ultrasound be discussed and offered in the setting of a positive SLNB.

Melanoma in-transit metastases (ITM) are typically located between the primary site and the regional nodes and are the manifestation of intralymphatic melanoma cells dissemination. A mouse model indicates that tumour-associated blood vessels and peritumoural lymphatic vessels in the primary tumour site are increased of the lymphedema (LE) mice and this is comparable to the unfavourable clinical behaviour of melanoma ITM in humans (Oashi et al. 2013, Oashi et al. 2013)

Those metastases migrate via dermal or subdermal intralymphatic pathways and occur close to the primary lesion (>2 cm). Typically ITMs are not beyond the regional

lymph node basin. Lesions occurring within 2 cm of the primary tumour are classified as satellite metastases. The diagnosis of ITM is usually clinical. ITMs are different from distant cutaneous metastases, due to its haematogenous dissemination (Testori et al. 2017). It has been reported that after treatment of the primary melanoma up to 6% of patients develop in-transit metastases (ITMs), (Bagge et al. 2016). This rates of ITMs exceeds 20% in patients with a more advanced stage of primary melanomas. However, in some patients ITM remain confined to a region of the body for many years in isolation without progression to other sites.

Without treatment, the quality of life of these patients is poor, due to bleeding, ulcerated and painful lesions as well as limited limb functionality (Bagge et al. 2016). For patients with ITM a careful clinical and imaging assessment is needed to determine whether a disseminated disease is also present (Koops et al. 1998, Teras et al. 2019).

There are many treatment options for in transit and satellite metastases:

1. Local therapy- to reduce exact in-transit lesions but low impact to the appearance of new lesions (excision to clear margin, intralesional injections: IL-2, GM-CSF, T-VEC, BCG, Rose Bengal, carbon dioxide or pulsed dye laser ablation, topical immunotherapy with imiquimod and diphencyprone; radiation, electrochemotherapy).
2. Regional therapy- for treatment the entire lymphatics basin to eliminate/treat visible tumours and also prevent outgrowth of new lesions in that region: (ILI/ILP- isolated Limb Infusion or perfusion).
3. Systemic therapy- anti-tumour effects on existing ITM and may help delay further regional or systemic recurrence.

The choice of every individual therapy depends on the number of lesions, their anatomic location, whether or not these are dermal or subcutaneous, the size and the presence of extra-regional disease (Testori et al. 2017).

The current algorithm to treat ITM melanoma begins with resection. If melanoma surgery is unsuccessful or not feasible a reasonable cosmetic and functional outcome, other options, for example, intralesional treatment is recommended. **Intralesional treatment** with IL-2 has an overall response rate of 82% (CR 51% and PR 31%) with a relatively simple regime and low toxicity (IL2 ref). **If intralesional treatment fails**, ILI/ILP is the treatment to be used. If all of these treatments fail, systemic therapy is used and any successful treatment can be repeated, including ILI.

As systemic therapies have improved, the number of patients with regionally confined in-transit disease of the extremities is decreased and multidisciplinary discussion of these patients is strongly encouraged (Chin-Lenn et al. 2015).

1.8.3 Isolated limb infusion (ILI): Regional chemotherapy

When other treatments are not possible or nor preferred, regional chemotherapy with ILI or isolated limb perfusion (ILP) may be considered and indicated.

During ILI, the extremities of the body are hypoxic which leads to marked acidosis. This procedure potentiates the cytotoxic effect of melphalan.

When surgical resection is not possible in ITM, regional chemotherapy with ILI or isolated limb perfusion (ILP) may be considered and indicated with the hope of improved life expectancy and life quality. These techniques are based on the

knowledge that under vascular isolation of the limbs the dose of the locally administered chemotherapy would be tolerated in higher concentrations without causing damage of important organs (Figure 11).

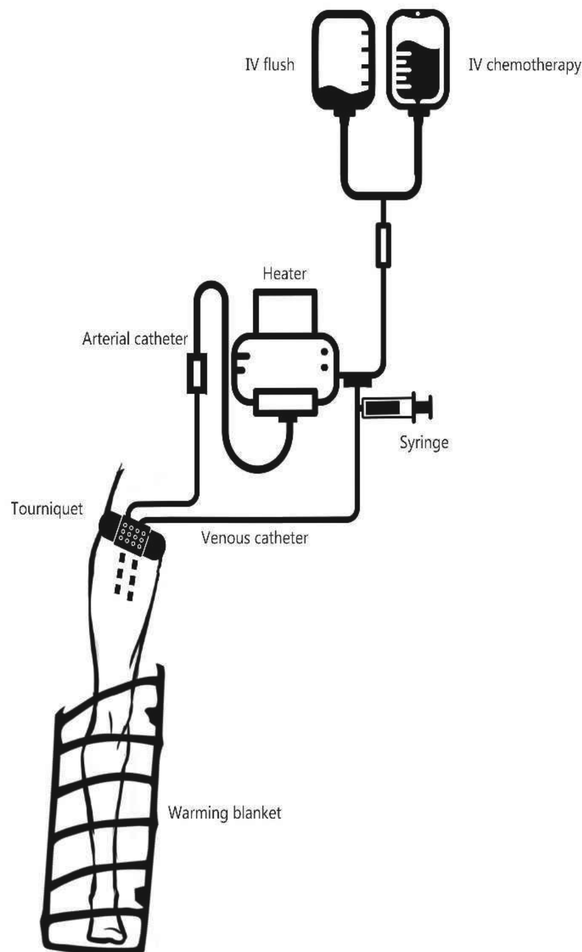

Figure 11. Schematic representation of Isolated limb infusion method. In an Isolated Limb Infusion, a tourniquet is used to stop the blood circulation in the affected limb. A catheter is inserted into both the artery and vein and used to circulate a high dose pre heated cytotoxic drug into the limb for around 60 minutes. When the session is over, the drugs are flushed from the limb, and normal blood flow is returned. Author Dr. Jüri Teras.

ILI is now a widely accepted procedure in the world as-localised chemotherapy to treat melanoma in-transit metastases.

Since the 1950s, hyperthermic isolated limb perfusion (HILP) has been applied to administer chemotherapy only to one isolated limb and with this method, up to 69% complete response was obtained (achieved?). But due to its technical complexity, the procedure has not been widely used, despite quite good complete response and favourable 5-year overall survival rate. Hyperthermic (40ˇ) ILP is effective, but it is

technically complex and time- and resource-consuming, as well as locally toxic and can result in high morbidity rates (Krementz et al. 1994).

In 1994, in the Sydney Melanoma Unit was developed a less-invasive alternative to it, called percutaneous isolated limb infusion. This method is associated also with marked improved overall survival (Koops, Vaglini, et al. 1998, Teras, Magi, et al. 2019). ILI was developed as a simpler and less toxic technique to achieve high regional drug concentrations.

Technically:

With ILI, a pneumatic tourniquet is used proximally to isolate the extremity (that is a less invasive alternative for regional chemotherapy, circulates blood in an isolated extremity at a much slower rate for only 30 minutes).

ILI is most commonly performed via cannulation of the external iliac vessels, femoral or axillary artery, and vein under fluoroscopic guidance. Special venous and arterial catheters are inserted in the melanoma affected limb. A preheated chemotherapy drug (mostly a combination of 7.5mg/L melphalan and 75ug/L actinomycin-D) is infused into the limb via the catheter. The infuscate of the cytotoxic drug is then typically circulated for 60-90 minutes. After that, the limb is flushed with Hartmann solution to eliminate the remaining drug from the isolated limb. Warming of the limb is essential to improve the efficacy of the cytotoxic drugs. The entire procedure usually takes about an hour. During ILI, the extremities of the body are hypoxic which leads to marked acidosis.

ILI is mainly using melphalan that is an alkylating agent which is a derivative of mechlorethamine and inhibits DNA and RNA synthesis and consequently acts on both resting and rapidly dividing tumour cells.

Other agents that have been used include actinomycin-D (Act D), tumour necrosis factor (TNF), cisplatin, dacarbazine, and temozolomide. Unfortunately, none of these combinations have shown results superior to melphalan (with/without TNF) but did result in greater limb toxicity.

ILI could be regarded as a palliative procedure to control limb disease that decreased the incidence of local recurrences. Responses to ILI regional chemotherapy occur gradually, and maximal tumour regression is usually present within three months (Jiang, Beasley, et al. 2014).

ILI has proved to be an attractive treatment modality to achieve regional disease control and limb preservation in melanoma patients with ITMs confined to a limb (Teras et al. 2019).

1.8.4 Surgical management of distant metastatic melanoma. Oligometastatic disease

Most cases of melanoma are usually diagnosed at an early stage when surgical excision can be still curative. Complete surgical excision of limited metastatic disease can result in prolonged overall relapse-free survival in selected patients (Sosman et al. 2011, Tyrell et al. 2017).

However, it is rarely curative since the majority of patients with distant metastases have still a widespread micrometastatic disease.

Resection should be reserved for the relief or prevention of morbidity due to local tumour growth and for patients in whom a longer survival might be expected with surgical rather than medical treatment.

If metastatic disease is identified, then the imaging is recommended to determine the full extent of disease prior to any surgical therapy. Imaging can help distinguish malignant and nonmalignant tissue and reveal additional sites. Magnetic resonance imaging and diagnostic PET/CT with F-18-fluorodeoxyglucose are the most common of them.

Surgical resection of the **oligometastatic disease** may be useful in some carefully selected patients after systemic therapy. Although this approach has not been proven to prolong survival, it is still prolonging the pain-free period and the general good condition of the patient (Yang et al. 2006).

Since melanoma is capable to involve other organs in the body more than any other cancers, the metastatic disease in melanoma patients maybe **even** underestimated by imaging procedure and also by physical examination (Sampson et al. 1998).

Adjuvant immune therapy

Adjuvant immunotherapy is recommended for those patients who are at high risk for recurrence after their initial treatment. For patients, treatment options include alternative active systemic therapy.

For example, adjuvant immunotherapy with high-dose interferon alfa (IFNα) or nivolumab is recommended for patients with lymph node involvement in appropriately selected patients.

Adjuvant therapy is not generally recommended because of the associated toxicity and the relatively favourable prognosis compared with those with lymph node involvement. In addition, adjuvant therapy with checkpoint inhibitors or targeted agents has not been evaluated in this patient population, as high-risk node-negative patients were excluded from the phase III clinical trials evaluating nivolumab, ipilimumab, and dabrafenib plus trametinib (Long et al. 2017, Weber et al. 2017).

For patients who have stage IIIB and IIIC melanoma, immunotherapy using any programmed cell death protein 1 (PD-1) inhibitor, for example Nivolumab, is generally recommended. (See 'Nivolumab' below) (Long et al. 2017, Weber et al. 2017).

For patients whose tumour contains a BRAF V600 mutation, dabrafenib and trametinib could be an alternative, particularly for patients with stage IIIB or IIIC disease who are unable to take adjuvant immunotherapy due to active autoimmune disease. (See 'Dabrafenib plus trametinib' below (Long et al. 2017).

Adjuvant radiation therapy (RT)

1. RT is used to treat the primary lesion in rare cases in unresectable places (for example paranasal sinuses lesion or nasal cavity); some mucosal lesion or *in situ* melanoma cases (Gilligan et al. 1991, Lopez Pereira et al. 1992).
2. RT is used for situations in which the risk of and morbidity from local recurrence may be substantial. This type of therapy is used mainly for patients with head and neck melanomas if satisfactory margins cannot be achieved without significant morbidity. Desmoplastic and neurotropic melanomas have a higher risk of local recurrence, making postoperative local radiation an important consideration in patient management (Oliver et al. 2016). However, in some cases, the adjuvant RT does not significantly impact the overall survival but shows an improved local control (Strom et al. 2014).
3. RT has also been used in rare cases as RT after therapeutic lymphadenectomy to reduce the rate of local or nodal recurrence for certain types of melanoma.

In spite of that the adjuvant radiation therapy decreases the likelihood of local recurrence after complete resection, it does not impact survival (Henderson et al. 2015).

4. Adjuvant radiation therapy is applied efficiently in some cases reserved for palliation, for example for brain metastasis, painful bony metastasis; spinal cord compression, or soft tissue metastasis (Chou et al. 2013).
5. In case of special need, the RT can be applied as consolidation in patients not achieving a major response to systemic treatment. Stereotactic body radiation therapy (SBRT) and stereotactic radiosurgery (SRS) can be effective in ablating oligometastatic disease in cases with hepatic metastases or brain metastases, also it does not impact significantly the survival (Powell et al. 2008, Rusthoven et al. 2014, Choong et al. 2017).
6. Abscopal effect: It has been observed, that the tumour regression of a secondary site following RT may be mediated through an adaptive T-cell immune response. This kind of RT treatment is used in combination with systemic immunotherapy. RT can induce the expression of checkpoints receptors PD-L1, PD-L2, and CTLA-4 and promotes tumour cell death, releasing tumour debris and tumour antigens. Removing the immune inhibition by checkpoints therapy leads to enhanced tumour control effect (Postow et al. 2012, Derer et al. 2016).

1.8.5 Cytotoxic drugs

Chemotherapies can be divided into the following categories:

1. alkylating agents that bind covalently to DNA via their alkyl group and exert an antitumour effect, such as melphalan, dacarbazine, cyclophosphamide (CPA), carboplatin and cisplatin.
2. anti-metabolites that inhibit DNA and RNA synthesis, such as fluorouracil and actinomycin-D.
3. anti-microtubule agents that interfere with the microtubule function and inhibit cell division, such as docetaxel and paclitaxel.
4. topoisomerase inhibitors that prevent DNA replication and translation, such as aclarubicin and novobiocin.
5. cytotoxic antibiotics that interrupt cell division, such as doxorubicin and mitomycin C.

The most widely used chemotherapy agents historically for the treatment of advanced melanoma have been dacarbazine and its prodrug temozolomide. Other agents with some activity include nitrosoureas, platinum compounds, **vinca alkaloids, and taxanes.**

Dacarbazine DTIC – is an alkylating agent that is converted to the active alkylating metabolite MTIC. The cytotoxic effects of MTIC are obtained through alkylation or methylation of DNA which lead to DNA double-strand breaks and apoptosis of quickly dividing cell. Dacarbazine was the first drug approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma. In the initial studies, the overall response rate with dacarbazine was 22%, with no impact on survival. Although dacarbazine was the most active single agent in patients with metastatic melanoma with a response rate of 8 to 20 percent and the median response duration

of four to six months. However, the long-term follow-up of patients treated with dacarbazine indicates that less than 2 percent of patients can be anticipated to survive six years (Hill et al. 1984) and single-agent dacarbazine (DTIC) intravenously for five days yields only a 10–15% response rate (Vuoristo et al. 2005).

Temozolomide: is an orally absorbed analogue of dacarbazine, that can cross the blood-brain barrier and have some activity in brain melanoma metastases. However, there was no difference in overall or progression-free survival compared with DTIC.

Fotemustine: The nitrosoureas fotemustine, carmustine (BCNU), and lomustine (CCNU) all have produced slightly higher overall response rates of 13 to 18 percent in patients with metastatic melanoma. Although the median time to progression and median survival were not significantly different with DTIC.

Platinum compounds: cisplatin and carboplatin show modest activity in patients with metastatic melanoma. Tumour responses were noted in 53 percent of patients, but the median response duration was only four months.

Nanoparticle albumin-bound paclitaxel: that works as a microtubule-stabilizing drug during cell division which induces mitotic arrest and leads to cell death. Paclitaxel has shown response rates of 22 percent; however, in chemotherapy -naïve and previously treated patients only 3 percent (Wiernik et al. 1993, Falzone et al. 2018).

Melphalan: is an alkylating agent widely used in cancer chemotherapy. It has a short half-life (75 minutes) *in vivo* and is excreted mostly through urine. Melphalan is a strong immunomodulator that activates endogenous cytotoxic T cells (Bocian, 1984) and increases release of IL-1β, IL-6, and IL-8 (Dudek-Peric et al. 2015) which in turn contributes to the antitumour efficacy of chemotherapy.

Actinomycin D (Act D) is an antibiotic isolated from the fungi genus Streptomyces. Act D is the first antibiotic used for treating cancer. It is a transcription inhibitor that is preventing the progression of RNA polymerases. The more precise mechanism by which Act D causes tumour cell death is not established but it may activate both the intrinsic and extrinsic apoptotic pathways (Praveen et al. 2009).

Cytotoxic Drugs Combination therapy.

For patients with advanced-stage melanoma are: dacarbazine or temozolomide combinations or the Dartmouth regimen, which consists of cisplatin, DTIC, carmustine, and tamoxifen; both IL-2 and IFNα have been combined with single-agent and combination chemotherapy regimens. Unfortunately, there is no effect compared with single-agent dacarbazine or temozolomide in patients with metastatic melanoma**.** Due to increased toxicity, it is not used anymore (Pasquali et al. 2018).

1.8.6 Targeted therapy: BRAF/MEK inhibitors

Targeted therapy

Response to targeted treatment in melanoma depends on each tumour's mutational status. According to the Cancer Genome Atlas network, the overall mutation rate in melanoma is the highest among cancers (about >10 mutations/Mb) which leads to a considerable variation inside of each tumour and between every patient. Melanoma growth and progression can occur through constitutive activation of the mitogen-activated protein kinase (MAPK) and subsequent signalling through the RAS-RAF-MEK-ERK pathway. MAPK pathway regulates both nuclear and cytoplasmic activities. Membrane receptor stimulation activates RAS GTPase through phosphorylation and activates RAF⟶MEK⟶ERK. BRAF thereafter forms dimers with

other RAF-family proteins (ARAF or CRAF) leading to MEK activation. BRAFV600E is constitutively active and phosphorylates MEK independent of RAS activation and dimerization (Figures 12 and 13).

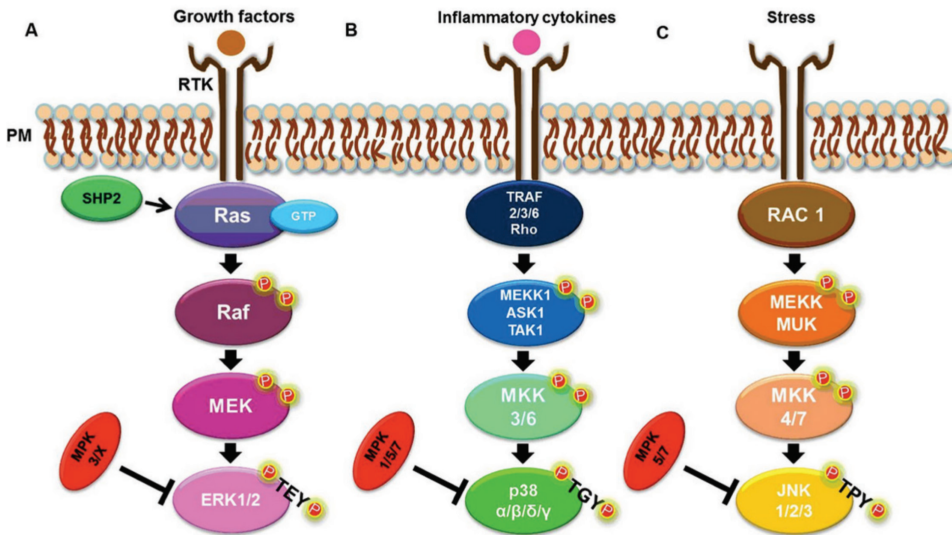

Figure 12. Simplified MAPK signalling pathways. (A) ERK1/2 pathway. (B) p38 α, β, δ, and γ pathways. (C) JNK 1, 2, and 3 pathways. Mitogen-activated protein kinases (MAPK) are protein kinases that phosphorylate their own dual serine and threonine residues by autophosphorylation. MAPK phosphorylation events can be inactivated by MAPK protein phosphatases (MKPs) that dephosphorylate residues phosphothreonine and phosphotyrosine MAPKs. There are three well-known MAPK pathways in mammalian cells. The Extracellular-signal-regulated kinase 1/2 (ERK1/2), the c-Jun N-terminal kinase (c-JUN) N-terminal kinase 1, 2 and 3 (JNK1/2/3), and the p38 MAPK α, β, δ, and γ pathways. ERK, JNK, and p38 isoforms are grouped according to their activation motif, structure and function. ERK1/2 is activated in response to several stimuli (as growth factors, hormones and proinflammatory stimuli) while JNK1/2/3 and p38 MAPK α, β, δ, and γ are also activated by cellular and environmental stresses, in addition to proinflammatory stimuli. From (Soares-Silva et al. 2016)

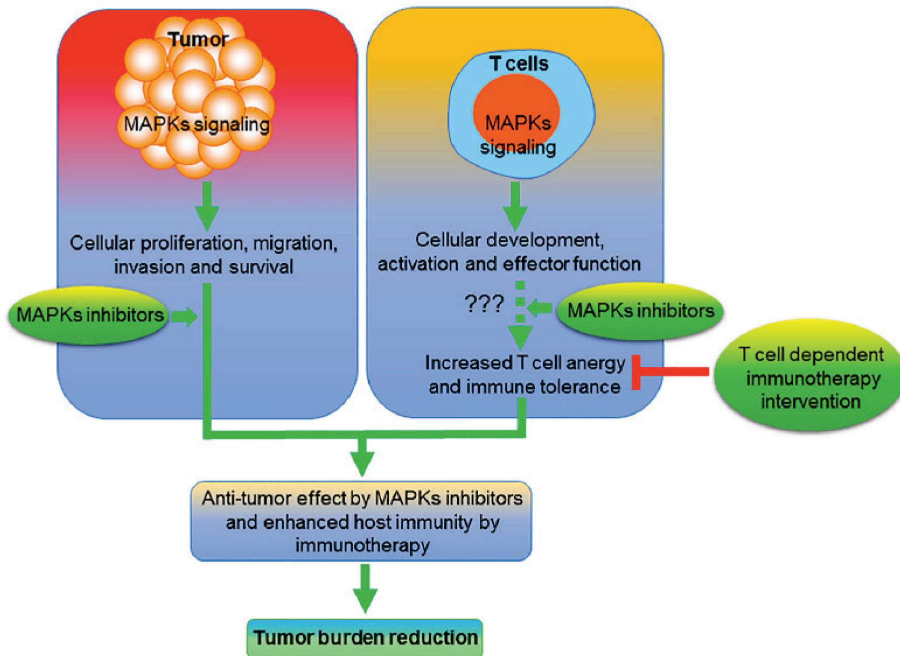

Figure 13. Schema describing the potential interaction between MAPK inhibitors and cancer immunotherapy. The proposed model is suggesting that MAPK inhibition may function through two distinct mechanisms. While blockade of various MAPKs limits the proliferation of tumour cells and promotes apoptosis, they may also precipitate T-cell exhaustion and/or anergy, which may potentially be reversed through the use of selective immunotherapies (Kumar et al. 2020).

Mutations along melanoma's downstream signalling pathway, such as with RAS, BRAF, or MEK activating mutations, are often the culprits of this cancer's pathogenesis and are thus also potential treatment targets. At the diagnosis, patients with advanced melanoma undergo analyses to assess the presence or absence of a mutation in the gene *BRAF* at codon V600 prior to treatment initiation. Although genetic sequencing for NRAS and KIT mutational status is also available.

BRAF mutation treatment

Although the BRAF V600 mutation is an oncogenic mutation involved in cancer cell proliferation, it is not enough to induce melanoma in the absence of other cytogenetic abnormalities. It has been shown that BRAF V600 mutations are frequently found in benign and dysplastic melanocytic nevi, indicating that additional cellular changes are needed for transformation. Other changes that drive cancerogenesis include loss of tumour suppressors, activation of the TERT promoter, inactivation of genes involved in DNA repair, or activation of other protein kinases and signalling cascades allowing cellular proliferation to occur (Shain et al. 2015).

Common tumour suppressor genes in which loss-of-function mutations can occur are CDKN2A, PTEN, and BAP1. Mutation of CDKN2A removes a key element of the p53 tumour suppressor pathway. It eliminates a mechanism that would normally consign precancerous to apoptosis or senescence. Consequently, cells that have a melanoma

driver mutation and they lose a tumour suppressor at the same time, those cells are primed to undergo a transformation and develop into melanoma.

Once the transformation has occurred, discordance in the BRAF mutational status between primary and metastatic lesions is known to occur. It is suggested that primary melanomas may contain mixed populations of BRAF-mutant and BRAF WT cells, both able to metastasize (Chiappetta et al. 2015) and that resistance to therapies is mediated by some genetically distinct tumour subclones. Therefore, tumour heterogeneity and the development of resistance have important implications for molecular testing and treatment of patients with advanced-stage melanoma (Cheng et al. 2018).

BRAF/WT melanoma treatment

BRAF-mutated melanoma patients are more often younger and have tumours with superficial spreading or nodular histology and/or in body regions without the chronic sun damage. Interestingly, BRAF-mutant tumours are more likely to metastasize to the brain and they are linked to shorter OS. However, concerning in-transit metastasis, the BRAF V600E mutation is found only in 8–15% while BRAF WT in about 22% of cases (Adler et al. 2017).

BRAF inhibition has a specific anti-melanoma effect. Targeted anti BRAF therapy may restore immune system activity. Early studies proved that inhibition of BRAF cascade raises the number of CD8+ T cells nearby tumour cells and increases the exposition of TAAs synthesized by melanoma cells.

Moreover, after exposure to BRAF mutations inhibitors, the increased intratumoural CD8+ lymphocyte infiltration is apparently correlated with the reduction of tumour size and enlarged necrosis in biopsies. Furthermore, reduced secretion of IL-10 and IL-6, and VEGF by melanoma cells has also been reported within the tumour microenvironment. A mechanism of resistance to targeted agents may include the high expression of PD-1 and PD-L1 by infiltrating T cells in the tumour microenvironment.

BRAF/MEK drugs

For patients with a BRAF mutation, the NCCN is recommending targeted combination therapy using either **vemurafenib/cobimetinib or dabrafenib/trametinib**. Targeted therapy is preferred if the early and quick response is clinically needed. All targeted therapies that are currently in use can slow tumour growth (eg, BRAF inhibition) or block effector T cell inhibition resulting in T cell reactivation and tumour lysis.

In patients with WT BRAF (no mutation) current guidelines from the National Comprehensive Cancer Network (NCCN) recommend single-agent immunotherapy with the PD-1 inhibitor pembrolizumab or nivolumab or combination therapy with nivolumab plus ipilimumab (Coit et al. 2019).

Trametinib (Mekinist) is a mitogen-activated, extracellular signal-regulated kinase **(MEK) inhibitor** that was approved by the FDA in 2013 for metastatic melanoma with BRAF V600E or V600K mutations. MEK inhibitors work by acting downstream of the mutant BRAF and inhibiting cellular proliferation. Trametinib, selumetinib, and cobimetinub are several MEK inhibitors approved for treating BRAF positive metastatic melanoma. Similar to BRAF inhibitors, MEK inhibitors encounter problems with resistance, and combination treatments with two or more agents may improve treatment outcomes (Coit et al. 2019).

Interestingly, BRAF inhibitor was found to have radiosensitization effect (Xu et al. 2017) and outcomes of patients treated with SRS alone compared to SRS with BRAF inhibitor showed the benefit of combination therapy (Xu et al. 2017). BRAF inhibitor shows also an increased risk of skin (and sometimes other organs) toxicities with radiation. A good practice for combination therapy includes treating with BRAF inhibitor for at least 3 days before and after fractionated RT and at least 1 day before and after SRS (Anker et al. 2016).

Activating mutations in RAS oncogenes are present in 30% of all human cancers and **NRAS** mutations are found in 15%–20% at all non-uveal sites of melanoma. The NRAS-mutant melanoma is more aggressive and associated with poorer outcomes. NRAS mutations occur at sun-exposed and sun-unexposed body sites. NRAS mutations occur in both CSD and non-CSD skin although a predisposition for the upper extremities has been observed in many cases. NRAS mutations are usually linked more often to in congenital nevi and the associated congenital nevus-derived melanoma (Ellerhorst et al. 2011).

Unfortunately, current therapies for NRAS-mutant melanoma remain limited and this has led to a search for alternative treatment options.

There is an urgent medical need for new targeted therapy opportunities in metastatic patients whose tumours harbour an NRAS mutation. Binimetinib, a mitogen-activated protein kinase (MEK) inhibitor, has shown clinical activity in this group of patients. Nevertheless, binimetinib represents another promising treatment option for advanced melanoma and this treatment is the first molecularly targeted therapy for the NRAS-mutant population. Binimetinib may also aid in treating NRAS-mutated melanoma patients after failure of immunotherapy (Johnson et al. 2015, Sarkisian et al. 2018).

1.8.7 Immunotherapy

Immune therapies are playing an increasing role in the treatment of metastatic melanoma, regardless of its NRAS or BRAF mutation status. Immunotherapy is increasing T cell-mediated antitumoural immunity. An important aspect of the cellular immune response is the activation response of immune cells.

Both CTLA4 and PD1 receptors function as coinhibitory receptors on T-cells that suppress T cell activation. Novel therapies target these coinhibitory molecules which serve to dampen the immune response.

PD-1 and its ligands 1 PD-L1 and PD-L2 are expressed on the surface of activated T cells under normal conditions. The PD-L1/PD-1 interaction inhibits immune cell activation and reduces the cytotoxic activity of T cell. This negative feedback is essential for immunoregulation and limits T-cell activity to protect normal cells during chronic inflammation.

Tumour cells may circumvent T cell mediated cytotoxicity by expressing PD-L1 on the tumour cell membrane or tumour-infiltrating immune cells, resulting in the inhibition of immune-mediated killing of tumour cells by T cells and poorer prognosis (Wu et al. 2019).

Antibodies targeting PD-1 (nivolumab, pembrolizumab) or PD-L1 (MEDI4736, MSB0010718C- both still in trials) thus may restore an antitumour immune response and produce tumour regressions in cancer patients (Tsai et al. 2014).

Immunotherapy using anti PD1 antibodies demonstrated long-term progression-free survival (PFS) in a significant number of patients.

Ipilimumab is an anti-CTLA-4 inhibitor, known also as a checkpoint inhibitor. CTLA-4, a protein that prevents T cells from attacking body cells but also cancer cells. The normal function of CTLA-4 is to prevent T cells from attacking the body in autoimmune diseases (Tai et al. 2018). An anti-CTLA-4 antibody promotes the cytotoxic function T cell. Monoclonal antibody Ipilimumab blocks the activity of CTLA-4, by blocking CTLA-4, ipilimumab activates T cells so that they can attack melanoma cells anywhere in your body. However, all patients do not respond favourably to anti-PD-1, and prescreening is needed for it.

Unfortunately, both checkpoint inhibitors anti PD1 and anti CTLA4 antibodies also induce autoimmune disease because they interfere with the regulatory function of T regs.

1.8.8 Intratumoural oncolytic viral therapy

The oncolytic viral therapy appeared as a complement to new systemic drugs that sometimes showed too little effect (Zhang et al. 2017). Proliferative diseases such as melanoma and other cancers may be treatable by apoptotic proteins encoded by viruses and targeted to the multiplying cells.

Many natural or natural and genetically modified viruses (for example adeno, herpes simplex virus, vaccinia virus, reovirus, coxsackie virus, influenza virus) show oncolytic activity (Zhang et al. 2017). For example, COX-2 and CXCR-4 promoters incorporation in adenovirus has been shown to significantly enhance the virus-mediated gene expression in the malignant melanoma instead of non-malignant primary melanocytes. Incorporation of tyrosinase promoter to drive the expression of the viral genes of adenovirus leads to an obvious melanoma-selective viral cytotoxicity (Zhang et al. 2017). Many more viral genes have been targeted in order to facilitate the efficient infection to the targeted tumour cells.

Viruses often possess an innate tropism for cancer cells where they can replicate since many viruses prefer to attach rapidly dividing cells. If oncolytic virus kill established tumour cells the dying tumour cells can serve as a target for cross-priming tumour-specific immune responses generating systemic anti-tumour immunity. This is important since tumour cells are recognized and targeted for elimination by the immune system. While most oncolytic viruses as for example circovirus (Teras et al. 2018), are given by **direct injection** into established tumours. Several viruses can be delivered also by the intravenous route without the need for tumour localization, although this application appears to be more toxic.

To date, the virus that has gained the most attention is an attenuated herpes simplex virus, type 1 (HSV-1) engineered to express human granulocyte-macrophage colony-stimulating factor (GM-CSF), termed Talimogene laherparepvec (T-VEC) is used to treat melanoma (Rehman et al. 2016).

T-VEC is available for the treatment of advanced melanoma. VEC is an *in vitro* modified herpes simplex virus, type 1 (HSV-1) that has selective adaptation to replicate in tumour cells. T-VEC replicates within neoplastic cells, and accumulation of the virions leads to lysis and death of the cancer cell, which may release tumour-associated antigens, help tumour antigen presentation and prime anti-tumour T cell responses.

The preferential replication within tumour cells is enhanced by the deletion of the herpes virus ICP34.5 genes that also eliminated the neuropathogenic HSV-1 virus.

T-VEC can be mounted to patients' melanoma lesion with an ultrasound-guided injection and this technique is especially useful for older patients. Further studies of T-VEC combinations, with checkpoint inhibitors, will be also interesting.

The oncolytic VV is engineered by addition of GM-CSF gene and deletion of viral thymidine kinase gene. The expression of GM-CSF induces the recruitment and activation of APCs and therefore the tumour-specific T cell responses (Kaufman et al. 2015).

Combination of oncolytic viruses (OVs) and other therapies:

1. Oncolysis in combination with radiotherapy resulted in a synergistic therapeutic effect that significantly inhibits the tumour growth. Radiation exposure significantly increased virus replication in a variety of cancer cell lines (Dilley et al. 2005, Zhang et al. 2017).
2. Combination of oncolysis with chemotherapy (cisplatin or paclitaxel) showed also a cumulative effect which resulted in a more pronounced tumour-killing effect in malignant melanoma than chemotherapeutic agents alone. Neutralizing antibodies preventing the virus to cause toxicities in systemic organs have a strong positive impact (Pandha et al. 2009).
3. Oncolysis in combination with Immunotherapy shows antagonistic results. The current immunotherapy for melanoma treatment include cytokine agents (IL-2 and IFN-α) and antibodies against CTLA-4 and PD-1. However, cotreatment of immunotherapy and OVs provides another appealing option but more studies in this field need to be done (Rajani et al. 2016).

1.8.9 Vaccines against melanoma

Melanoma vaccines can be either systemic or local therapy. Unlike vaccines for infectious diseases (flu, measles, etc), melanoma vaccines do not prevent melanoma but instead, they aid to remove tumours from the cancer patients who have already had surgery.

Autologous tumour vaccines are derived from tumours surgically resected from patients. These tumour cells are manipulated *in vitro* (irradiated or weakened in some fashion to prevent proliferation) and thereafter reinjected into patients (sometimes with medications that modify the immune reaction). The main limitation of this type of vaccination is that a tumour sample must be taken from each patient and this vaccine usually does not work when used allogeneically.

Allogeneic cellular vaccines for melanoma are based on stable cultured cell lines derived from tumours obtained from other patients. Those shared antigens can enhance the host's immune system. These vaccines are more readily applicable to a greater number of patients. Good examples of used allogenic vaccines:

Canvaxin® (Cancer Vax Corp, Carlsbad, CA, USA) is comprising of three viable irradiated whole melanoma cell lines and were chosen for their high immunogenicity. They contained about 11 known tumour-associated antigens such as MAGE-1 (melanoma-associated antigen 1), MAGE-3, tyrosinase, gp100, gp75, and Mart-1 (melanoma-associated antigen recognised by T cells)/Melan-A.

Antigen vaccines. Autologous and allogeneic melanoma vaccines contain several antigens but the most important antigens remain unknown. The use of defined antigens as melanoma vaccines has permitted the measurement of immune responses specific to those antigens. Those tumour antigens may be created synthetically or they can be cell-surface molecules on melanoma cells that may be targeted by antibodies. For example, GM2, a cell-surface ganglioside- is inducing B-cell responses to the melanoma antigen.

An allogeneic, gene-modified, therapeutic vaccine (AGI-101H) that after genetic modification shows melanoma stem cell-like phenotype is a promising approach that maintains antigen-specific T cell response by up-regulating BCL6 (Czerwinska et al. 2020). AGI-101H immunization shows clearly that that whole melanoma stem cell immunization activated the TNF-α and TGF-β signalling pathways and dampened IL2-STAT5 signalling in T cells. The IL2-STAT signalling pathway is critical for regulatory T cell development, homeostasis, and function.

AGI-101H immunization also up-regulates a BCL6 transcriptional repressor. High levels of BCL6 transcripts negatively correlated with the expression of exhaustion markers CTLA4, PTGER2, etc. Therefore, Bcl6 is promoting a progenitor fate for cancer-experienced T cells by repressing the exhaustion markers (Czerwinska et al. 2020).

Conclusion: New treatments are needed because there is still no good treatment that solves all the problems.

2 AIMS OF THE THESIS

Question of the thesis: How the development of novel biomarkers and principles of treatment can assist in the understanding of melanoma mechanisms, and lead to improvements in therapy and outcomes?

The first aim of this thesis was to evaluate the presence of naturally occurring antibodies against melanoma antigens (MAGE4 and MAGE10) in the blood samples of melanoma patients with different stages of the disease.

The second and third aim of this thesis concerned the treatment of melanoma. We aimed to investigate the oncosuppressive mechanism of viral apoptin and its success in a mouse model. Finally, we aimed to test and analyze a new treatment option that is rarely used in Europe, but which has proven to be cost-effective and safe.

3 MATERIALS AND METHODS

The main method in publication 1:
The MAGEA4 and MAGEA10 proteins were expressed in bacteria, purified and used in the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies. We measured the anti-MAGEA4 and anti-MAGEA10 antibody levels using ELISA from 185 stage 0 (*in situ*) to stage IV melanoma patients and also from 43 healthy individuals, who had donated their blood to the Estonian blood bank. The ELISA was performed using MAGEA4 and MAGEA10 recombinant proteins, which were purified from E. coli, immobilized on microtiter plates, and thereafter probed with 1:200 to 1:800 human sera dilutions.

The main method in publication 2:
In the current study, the PCV2 ORF3 was expressed and assessed for apoptotic activity in mouse and human melanoma, human leukemia cell lines, and in mouse primary splenocytes. We used a mouse model of melanoma graft. When grafted melanomas were 5mm in diameter, pcDNA-ORF3 was injected into the basis of the tumour.

The main method in publication 3:
Special venous and arterial catheters were inserted in the limb that was affected with melanoma. A preheated chemotherapy drug (a combination of 7.5mg/L melphalan and 75µg/L actinomycin-D) was infused into the limb for 5 min to 10 min. via the catheter. Thereafter the infusate was circulated manually for 20–25 min. The entire procedure usually takes about an hour. Postoperatively, the patients were monitored for three months to assess limb pain and potential limb muscle toxicity and the OS rate in the follow-up period of up to 70 months was analysed.

4 RESULTS AND DISCUSSION

4.1 Publication 1: Predictive value of markers, comparison with literature, new strategies to get markers

Õunap K, Kurg K, Võsa L, Maiväli Ü, **Teras, M**, Planken A, Ustav M, Kurg R. Antibody response against cancertestis antigens MAGEA4 and MAGEA10 in patients with melanoma. Oncology Letters; 2018, 16(1): 211-218

Introduction to the subject
Melanoma-associated antigen A (MAGEA) represents a class of tumour antigens that are expressed in a variety of malignant tumours (bladder, lung, skin and breast malignancies), however, their expression in healthy normal tissues is restricted to germ cells of the testis, fetal ovary, and placenta. The restricted expression and immunogenicity of these antigens make them ideal targets for immunotherapy in human cancer. The MAGE-A genes were initially identified as tumour antigens that can be recognized by cytotoxic T-lymphocytes in melanoma patients. MAGEA expression is observed mainly in cancers that have acquired malignant phenotypes, invasiveness or metastasis, and the expression of MAGE-A family proteins has been linked to poor prognosis in cancer patients. MAGE-A family proteins have oncogenic functions: they support growth, metastasis and general survival, contributing actively to malignancy. At the molecular level, MAGEA proteins are involved in the regulation of the tumour suppressor protein p53 pathway (through direct and indirect mechanisms). MAGEA proteins can also activate specific RING finger-type E3 ubiquitin ligases, thereby regulating the ubiquitin signalling in malignant cells. Expression of MAGEA antigens is highly heterogeneous in a variety of tumours of different histological origin, with percentages of positive cells ranging between 5 and 60%

In the present study the presence of naturally occurring antibodies against two MAGEA subfamily proteins, MAGEA4 and MAGEA10, was analyzed in patients with melanoma at different stages of the disease.

The aim of this study was to evaluate the presence of naturally occurring antibodies against two MAGEA proteins in the blood samples of melanoma patients with different stages of the disease.
We were curious to know 1) whether the melanoma patients have antibodies against these proteins, and 2) whether these antibodies can be considered/used as a potential prognostic marker.

Results indicated that the anti-MAGEA4/MAGEA10 antibody level in melanoma patients was heterogeneous, with only ~8% of patients having a strong response.

Comparing the number of strongly responding patients between different stages of disease revealed that the highest number of strong responses was detected among stage II melanoma patients. These findings support the model proposing that the immune system is involved in the control of melanoma in the early stages of the disease. **Our data showed that the sera of 15 patients out of 185 (8%) had a strong antibody response against the MAGEA4 and/or MAGEA10 proteins.** When we grouped patients according to the level of the disease, then stage II patients had more antibodies than others, reaching 16% in the case of MAGEA10.

The existence of strongly responding patients suggests that their immune system has been activated and has started to generate antibodies against the primary tumour. Thus, our data support the hypothesis that the immune system is involved in the control of melanoma, at least in the early stages. Several studies have shown spontaneous regression of primary melanomas, but the regression of metastatic tumours is very rare. A good antibody response at early stages can stop the growth of the primary tumour and halt further spreading to the lymph nodes and other organs. Among the 15 patients of our study with the positive status for MAGEA antibodies, only one patient has died, and one has disease progression during the 2-year post-study follow-up period.

Importance of the findings

The present study is the first report following the antibody response against MAGEA-s and comparing it with the disease progression.

Prevalence of MAGEA in different studies: Scultz-Thater et al (Schultz-Thater et al. 2011) have studied the prevalence of MAGEA10 in different cancers and shown that it is expressed in 38% of malignant melanomas. Several studies have shown that MAGEA proteins are associated with or contribute to solid malignancies, MAGE-A expression is considered to be an important predictor of malignant transformation. For instance, MAGEA4 is expressed in 9% of primary tumours, but reaching to 44% in distant metastasis and MAGEA1 expression has been found in 16% of primary melanomas and 48% of metastatic melanomas. However, in another study, no correlation was observed, and MAGEA3/4 protein was present in 25% of primary invasive and metastatic tumours, but not *in situ* melanomas. In our study, the prevalence of MAGEA antibodies was highest in stage II and lowest in stage 0 and stage IV patients.

Discussion

We have analyzed the presence of naturally occurring antibodies against two MAGEA family proteins, MAGEA4 and MAGEA10, in melanoma patients with different stages of the disease. MAGEA proteins are cancer-testis antigens (CTAs), which are normally expressed both in germ cells and in the testis. Male germ cells do not express MHCI molecules and cannot present antigens to T cells. Therefore, MAGEA is considered as neoantigens when it is expressed in the surface of cancer cells.

MAGEA4 and MAGEA10 are good immunogens, inducing both B and T cell responses. Previous studies have shown that MAGEA4specific immune responses correlated well with the prognosis of patients and that antibody response could be a marker for a good prognosis (Saito et al. 2014).

In our first paper, we could show that 8% of analyzed patients had strong antibody responses against the MAGEA4 or/and MAGEA10 protein. In our current study, we focused on naturally occurring B cell responses against MAGEA proteins. The existence of strongly responding patients suggests that their immune system has been activated and has started to generate antibodies against the primary tumour. The prevalence of MAGEA antibodies was highest in stage II and lowest in stage 0 and stage IV patients. Stage II melanoma patients have a stronger melanoma-specific immune response compared to patients with more advanced stages of the disease. Stage II melanoma has spread to the lower part of the dermis, but not yet into the tissue below or into the closest lymph nodes. The dermis contains many antigen-presenting cells, which can

recognize neoantigens and activate the immune response. Our data demonstrate clearly, that the immune system is involved in the control of melanoma, in the early stages (II) of melanoma.

One of the limitations of the current study is the fact that we do not have biopsies and we were not able to perform neither immunochemical analysis nor quantitative PCR (qPCR) to exclude possible cross-reaction with other MAGE proteins. We cannot rule out the possibility that antibodies detected in our assay are formed against some other member of the family. MAGEA proteins are highly similar to each other.

The MAGEA subfamily consists of 11 MAGEA proteins and in addition, MAGEB, MAGEC, MAGED, etc. families all share the MHG (MAGE homology) domain. All these proteins are to some extent similar to each other (MHG domains have similarities from 25 to 80%) and may give some cross-reactivity. However, simultaneous expression of several MAGE proteins /expression occurs in 60–70% of melanomas.

Another limitation of our study is that we have used only ELISA assay for screening of the sera. We choose ELISA, because it is sensitive analysis, but have not analyzed the sera by *western blotting.*

The presence of strongly responding patients in our study suggests that their immune system has been activated and has started to generate antibodies against the primary tumour. A good antibody response at early stages can stop the growth of the primary tumour and further spread to the lymph nodes and other organs. Among the 15 patients of our study with the positive status for MAGEA antibodies, only one has died and one has disease progression during the 2 years follow-up period, which indicates that disease of the majority of patients with strong antibody response is under control.

Anti-MAGEA antibody response can be treated as a potential diagnostic biomarker. Unfortunately, another limitation is that MAGEA proteins are expressed only up to 50% of melanoma cells. When we assume that only 50% of these people have a strong immune response, then the expected % of well-responding patients will be 12 to 25%. Of course, it is not enough for clinical diagnostics, but on the other hand, these antibodies are early markers and there is a great need for early melanoma markers.

Conclusion

Our current study was the first report following the antibody response against MAGEAs and comparing it with the disease progression. We can conclude that a healthy immune system enables to create antibodies against cancer antigens that are expressed specifically by tumour cells.

We compared the number of strongly responding patients between different stages of the disease and found that the highest number of strong responses was detected among stage II melanoma patients.

- Our study supports the role of the host immune response in the progression of melanoma. The strong antibody response could be a marker for a good prognosis as well as an early marker that can be used as cancer diagnostics from a liquid biopsy.
- The disease of the majority of patients with strong antibody response is under control.
- The highest antibody response was detected in stage II melanoma patients.

4.2 Publication 2: How viral apoptin can inhibit melanoma?

Teras M, Viisileht E, Pahtma/Hall M, Rump A, Paalme V, Pata P, Pata I, Langevin C, Rüütel Boudinot S. Porcine Circovirus Type 2 ORF3 protein induces apoptosis in melanoma cells. BMC Cancer; 2018, 18: 1237

In our second paper, we have explored the impact of the Porcine circovirus type 2 (PCV2), pro-apoptotic protein ORF3 and its apoptotic activity *in vitro* and *in vivo* models of melanoma.

Introduction to the subject

Porcine circovirus type 2 (PCV2) infection in swine is resulting in several clinical syndromes all of them related to immunosuppression. PCV2 infection is involving mainly lymph nodes and thymus, the places where rapidly dividing cells are located. PCV2 infection decreases the number of $CD8^+$ and $CD4^+CD8^+$double-positive cell subsets compared with PCV2-negative pigs (Darwich et al. 2002). Previous studies in our lab have indicated that PCV2 protein ORF3 is the interacting regulator of G protein 16. This protein PCV2ORF3 is related to apoptin and it is causing apoptosis in rapidly dividing cells including cancer cells.

The aim of our second publication was to investigate the oncosuppressive mechanism of viral apoptin and its success to reduce tumour growth in a mouse model.

Result

Quantitative analysis of the apoptotic cells by flow cytometry showed that apoptotic cell death was significantly increased in PCV2 ORF3-expressing malignant cells, compared with mock control (cells transfected with empty vector).

Intriguingly, the apoptotic activity of ORF3 was not observed in mouse primary cells, indicating that apoptosis induced by PCV2 ORF3 is facilitated in rapidly dividing cancer cells.

Apoptosis maintains a healthy balance between cell survival and death in an organism. Apoptosis contributes to normal tissue homeostasis, while a defect in apoptosis control may promote carcinogenesis. Many animal viruses encode pro- or anti-apoptotic proteins (Zhang et al. 2017). Understanding the apoptotic function of PCV2 ORF3 has been the subject of research for preventing the proliferation of transformed cells.

Therefore, our results suggest that PCV2 ORF3 may cause apoptosis in cells through the induction of abnormal mitoses by interacting with centrosomes during cell division and thereby disrupting mitotic spindle formation. However, further studies are needed to elucidate the exact molecular mechanisms of ORF3-induced apoptosis.

We also showed that ORF3 can inhibit tumour growth in *vivo*, in a mouse model of melanoma graft. Histocompatible melanoma was transplanted to mice and when it was 5mm in diameter, mice were treated with pcDNA-ORF3 or empty vector. In mice injected with pcDNA-ORF3, the development of melanoma was delayed, the tumour growth was slower, and the final weight of the tumour was significantly smaller compared to mice injected with the empty pcDNA3.1 vector.

Importance of the findings

Ability of circovirus to induces the apoptosis of melanoma cells was demonstrated before using PCV1-ORF3 and chicken anemia virus apoptin, but not with PCV2 ORF3. While ORF3 binds RGS16 that can interfere with tumour growth since the tumour was much larger in RGS16KO mice, we also show that the effect of ORF3 does not require RGS16. Using pan-caspase inhibitor Q-VD-OPh and an antibody that binds to active Caspase 3 we have added to the revised version the evidence that PCV2-ORF3 induces apoptosis in melanoma cells via a caspase-independent pathway, while in the porcine kidney cell line (PK15) the induction of apoptosis involved caspase 3.

We show that PCV2 ORF3 causes non-bipolar mitosis in rapidly dividing cells and increases the apoptosis of cancer cells and the anti-tumour effect in mice. A better understanding of the mechanism of PCV2 ORF3-induced apoptosis would help determine its potential as possible cancer therapeutic.

Discussion

Porcine circovirus type 2 (PCV2) ORF3, is a homolog of the apoptin of the circovirus chicken anemia, which has been reported to induce cell death in cancer cells. The rate of apoptosis in tested human and mouse melanoma cell lines overexpressing ORF3 was significantly higher than in control cells (ORF3-negative). This result was indicating that PCV2 ORF3 is able to induce apoptosis in different melanoma cell lines. Usually, caspases play an important role in the induction of apoptosis and are responsible for the morphological changes characteristic of apoptosis. Surprisingly, our results indicate that PCV2 ORF3 induces apoptosis in melanoma cells probably in a caspase-independent manner. One explanation for that could be that viruses simply can also encode caspase inhibitors that may prevent cell death induced by various stimuli. For example, the expression of p35 during viral infection may result in the inhibition of several caspases and make cells resistant to cell death. Knowing that tumour development relies largely on irregular cell apoptosis, a lot of attention has been paid to the apoptotic mechanism. Our current study demonstrated clearly that PCV2-ORF3 had a significant anti-tumour effect which was mediated through induction of apoptosis. Studying the underlying molecular mechanism, we show that induction of apoptosis in melanoma cells was induced via a **caspase-independent pathway**. In addition, our mouse model indicates s that this compound could serve as a promising candidate in melanoma therapy, further in-depth research in the clinical application is still needed.

Conclusion

Our results bring new information about the potential antitumoural activity of circovirus proteins. Previously an impact of the PCV2 ORF3 system in cell apoptosis has been observed but not to clarify mechanistically in their model systems.

4.3 Publication 3. Perspectives for local chemotherapy (Isolated limb infusion as anti-cancer chemotherapy)

Teras J, Kroon HM, John F. Thompson JF, **Teras M**, Pata P, Mägi A, Teras RM, Rüütel Boudinot S. First Eastern European Experience of Isolated Limb Infusion for In-Transit Metastatic Melanoma Confined to the Limb: Is it still an Effective Treatment Option in the Modern Era? European Journal of Surgical Oncology; 2020, 46(2): 272-276

The third publication was obtained in cooperation between the research group of the North Estonian Medical Centre Foundation, Australian doctors (from the Sydney Royal Prince Alfred Hospital; the Royal Adelaide Hospital) and TalTech immunologists.

Introduction to the subject

Nowadays, the most advanced methods, applied in cancer treatment involve activation or re-activation of immune cells that can kill melanoma cells. This is also called immunomodulatory methods, that block the immune-inhibitory pathways in the cell to cell communication. These are the most effective but very costly treatments that are unfortunately not available to all patients.

These treatment options have also serious side effects, which are especially exhausting to elderly patients. However, chemotherapy offered so far as a less expensive alternative is unfortunately difficult for the patient's organism to tolerate, extremely devastating to the whole body and, what is most important, this method is effective only in a small number of patients.

To mitigate the severe overall effects of chemotherapy, a new method has been developed known as isolated limb infusion (ILI). This means that patients with melanoma metastases confined to an extremity can be treated, instead of systemic whole-body chemotherapy, by less invasive chemotherapy targeted at the concrete affected limb i.e. isolated limb infusion (ILI). ILI was introduced in the 1990s by one of the members of our research group Dr. John F. Thompson in Australia at the Sydney Royal Prince Alfred Hospital.

Isolated limb infusion as an anti-cancer treatment is an effective method for in-transit metastatic melanoma. So far, this method has been seldom used in Europe, but it is already available in major cancer treatment centres in Australia and the USA. The North Estonia Medical Centre is the only medical centre in the Baltic region where patients are cured with this method.

The aim of the third publication was to test and analyze a new treatment option that is rarely used in Europe, but which has proven to be cost-effective and safe.

The main results

In the current study, we analysed the data of 21 patients from Estonia, who underwent treatment with the drug combination of melphalan and actinomycin-D. Drug circulation times were 20–30 min. at 38–39°C. In spite of all the patients that were tested, who underwent an ILI treatment, had a quite poor prognosis (thick primary melanoma, ulceration, high mitotic rate), the overall response rate was 76% and the overall long-term limb salvage rate was 90%.

Importance of the findings

In our third publication, we showed that the cytostatic infusion treatment of the limbs is safe and cost-efficient also in our hospital. Exploiting this niche opportunities, especially in the context of treatment, efficacy is very promising.

Discussion

ITM of malignant melanoma is a relatively rear but potentially devastatingly resulting disease when left untreated. All treatment modalities should be taken into account when attempting limb salvage without compromising OS. Recurrence of melanoma in a limb presents usually either cutaneously, subcutaneously or in a combination of both. Involvement of limb bones or muscle is rare but can occur, usually together with lymph node metastases. Also, the bulky disease may occur in superficial deposits. Usually, in-transit metastases can easily be treated, often repeatedly, with surgical resection. However, sometimes ITMs demand challenging skills in plastic reconstruction. There are still numerous cases, where ITM presentation is far beyond even the most capable surgical skills and a multidisciplinary approach may be needed to effectively treat the patient. There are cases or even countries where modern targeted therapies together or not with immunomodulating treatment is not an option, therefore other treatment modalities should be sought.

Regional chemotherapy, with well-known agents like melphalan and actinomycin D, for ITM distal to axilla or groin, offers a good and effective alternative in experienced hospitals. ILPs and infusion have been gaining popularity also in many other countries outside its original Australia. ILI, as technically much simpler procedure than ILP, can be performed repeatedly with low morbidity and mortality but acceptable efficacy. Short operation time, low overall costs and good response rates offer patients nice treatment alternative, sometimes being the only acceptable option. Our overall toxicity data show low morbidity rates.

Conclusion

The outcome obtained in the publication 3 shows ILI as a reliable, fast and effective procedure which provides benefits in terms of curative and palliative treatment for unresectable cutaneous lesions without seriously impacting the quality of life of patients. ILI trials for melanoma ITMs showed that ILI can be safely and effectively implemented in our hospital.

5 GENERAL DISCUSSION AND CONCLUSIONS

1. Importance of training of family doctors and dermatologists to identify melanomas

Malignant melanoma is an aggressive tumour with initial lesion usually on the skin. Hence, it is theoretically detectable at an early stage, but in practice, very often late diagnosed. Moreover, superficial malignant melanoma foci also in rare cases metastasize.

It is important to note here that besides biomarkers, it is essential for an efficient treatment approach of melanomas in Estonia that family doctors and dermatologists are well trained to identify both primary tumours and metastases. This is especially important for melanomas since they are visible at the surface of the body. While it is not always intuitive to discriminate nevi and more evolved tumours, training and the use of dermoscope would improve initial diagnostic to a very significant extent. The vasculature and pigment structures are different between normal skin lesions and melanoma, and this is a sign relatively easy to identify, so the patient can be redirected to an onco-dermatologist for verification.

Too often, family doctors and dermatologists do not examine the full body of their patients, hence missing opportunities to detect tumours or potentially dangerous evolutions of pre-existing nevi. Besides, looking for skin tumours on a systematic basis would itself constitute an efficient training for the physician.

At the moment, it is offered widely by different training programs (including the Estonian dermatological society ENSAS). It is especially important to spread widely in the population the notion that exposure to UV is dangerous for skin, and has to be carefully dosed. Also, everyone should keep an eye of his/her own nevi. Old people with declining vision should be followed by their doctors.

It is obviously critical to identify melanomas as early as possible, to improve the chances of recovery and also to reduce the costs associated with treatments. Across all stages of melanoma, the average five-year survival rate in the U.S. is 92 percent. The estimated five-year survival rate for patients whose melanoma is detected early is about 99 percent. The survival rate falls to 65 percent when the disease reaches the lymph nodes and 25 percent when the disease metastasizes to distant organs (from Cancer-facts-and-figures-2020)

Also, people who present particular risk factors for melanoma development should be fully informed, and reminded by their family doctor to do regular checking with a specialist. In particular, patients with other cancers in their family should be examined with special attention. Also, elderly people – especially those living alone – should be examined by their doctors since they usually do not check themselves easily.

It has to be reminded that obvious prevention measures should be promoted and explained: reduction of exposure to sun and other risk factors (sunbeds and other tanning devices emitting artificial ultraviolet radiation (UVR), laser treatments in pigmented areas,) remains of primary importance. The role of family doctors and dermatologists, but also general media is important in this matter.

Those considerations are very obvious, but their application has drastic impacts on the early treatments of melanomas in the general population, hence on the success of

therapies. The costs associated to late and complex treatments of melanomas are also critical to consider.

2. Why develop new markers of melanomas? What would be the most relevant markers?

By definition, a melanoma biomarker should allow the detection of the tumour and/or help to characterize its properties to help to select the best therapy.

Biomarkers of melanomas may be expressed by the tumour, or by other cells reacting to the tumoural transformation (mainly cells of the immune system). Optimally, markers or combinations of markers should inform about the stage and the potential evolution of the tumour and orientate the decisions of oncologists. When a tumour has not been detected at its early stage, it is very important to understand its characteristics and its metastatic potential. Thus, elaborated classifications of tumours produced by advanced technologies such as transcriptome (more and more single-cell) sequencing have to be translated into clinical choices by the selection of the best marker genes/combinations.

Especially important is to search for markers of organ metastasis. Usually, the patient does not feel or sense it until it is too late. For example, a reliable blood marker for it would be the best. The quest for new markers is, therefore, more active and important than ever to refine our understanding of the complexity and heterogeneity of melanomas, as shown by the present work.

When the number of markers will increase, their cost should be considered to decide if they can be all tested, or if hierarchical decisions should be made to apply them to produce the most relevant predictions in the higher number of situations.

An interesting recent development in the field is the single-cell transcriptome approach.

A recent research paper describing the single-cell analyses of 19 melanoma patients concluded (Tirosh et al. 2019) that malignant cells within the same tumour displayed huge transcriptional heterogeneity. As these analyses included also tumour-infiltrating cells, the overall survey was quite complete. Interestingly, every individual tumour contained malignant cells from distinct transcriptional cell states: harbouring cells for example with a high and low MITF transcription factor, with distinct tumour microenvironmental patterns, including cell-to-cell interactions and immune cells with several activational or exhaustion state.

This intra- and interindividual genomic heterogeneity of malignant melanoma indicated the urgent necessity to clarify the interplay between these cell types and functional states in space and time to find the objective combination of markers that may aid in the future in selecting patients for immune checkpoint blockade.

The development of such approaches raises many issues. When single cell technologies will become more efficient (and will allow sequencing a large part of the transcriptome of each cell, which is not the case now), it will be important to design new approaches based on these data to understand better the evolution and physiology of the tumour, and to get useful predictions.

Single cell technologies will let us design new approaches:

- large-scale analyses on single-cell datasets should allow identification of a manageable number of combinations of markers, which would be very informative and would draw a detailed typology of tumours. A key question

will be to use their full predictive potential, to help to choose the best treatments.

How will it be possible to reduce and optimize these lists/combinations to get something economically usable in clinics?

- how such an arsenal of markers should be used also during treatment, to tune its progression and adaptation to the development of the tumour?
- finally, it will be important to see what they predict and teach us about the metastasis and the biology of metastasis.

Omics analyses can identify disease markers that monitor the efficacy of therapies (Dumitru, Constantin, et al. 2017). It can also identify new chromosomal alterations and discover new deregulated genes that can be further used as therapeutic targets. Overall, there are 3 main aspects to consider in the future potential developments linked to omics:

1. Large standardized datasets and databases will provide new opportunities to find useful correlations (*i.e.* new biomarkers) to produce integrated multiparametric classifications of tumours.
2. Such classifications based on comprehensive knowledge at the genomic, transcriptomic, epigenomic, phenotypic and clinical levels should allow safe predictions of the characteristics of the tumour based on a reasonable set of markers, hence guiding therapy design.
3. Finally, omics data should allow a better understanding of the biology of the tumour and the mechanisms of antitumoural response. The quality of the predictions based on biomarkers would be greatly increased by the understanding of their functional involvement in the pathology (Tirosh et al. 2019).

Also, microbiota studies clearly represent a key component of future personalized medicine. The number and diversity of phenotypes linked to the composition of the microbiota are immense: obesity, inflammatory bowel disease, diabetes, allergies, autism, cardiac function, fibromyalgia, various cancers (including melanoma), anti-cancer therapy, and depression have all been reported to correlate to the composition of the microbiome. The microbiome is plastic; it changes metagenomically within hours and metatranscriptomically within minutes, in response to antibiotics or certain food intake. For any phenotype to which the microbiome is causally linked, opens the possibility of pharmaceutical, prebiotic, or probiotic treatments (Goodman and Gardner 2018).

Circulating tumour DNA can predict relapse and correlates with survival in high-risk resected melanoma and could aid in the selection of patients for adjuvant therapy. The detection of CTCs in circulation is an important prognostic marker, unfortunately, their number is very small, thus these cells are not easily detected.

Importantly any marker that can be evaluated simply from the blood can be extremely useful and often affordable, to perform early detection of the tumour. **More important for melanoma would be such circulating markers associatedwith the early metastatic activity.**

Melanoma metastasizes predominantly lymphatically or hematogenically. Melanoma distant metastases may occur virtually every side of the body, however, each patient goes through this process differently. The treatment of primary MM foci is surgical.

Individual metastases are also operated and sometimes irradiated. However, extensive metastases require systemic treatment, to extend the survival and tumour-free period or reduced tumour size as well as patient discomfort. Systemic therapy has been developing in recent years, with an expanded list of available drugs including CTLA-4 and PD-1 inhibitors, and combinations of other drugs. Unfortunately, these treatments still have poor efficacy.

1. Importance of combined therapies to cope with the heterogeneity of melanoma cells within the tumour, in a patient (or: Biomarkers of the antitumoural response: importance and perspectives)

New systemic treatments offer better efficacy than classical chemotherapy, including recent treatments, such as small-molecule targeted drugs, which are used to treat melanoma with specific gene mutations. Compared with chemotherapy, biochemotherapy (where chemotherapy is combined with both IFNα and IL2 therapies) and BRAF (for BRAF-mutated melanoma) inhibitorsimproved significantly progression-free survival. Both BRAF inhibitors and anti-PD1 monoclonal antibodies also improved OS. For example, anti-PD1 monoclonal antibodies, alone or especially with anti-CTLA4, improved progression-free survival compared with anti-CTLA4 monoclonal antibodies alone. Anti-PD1 treatment improved OS more compared to anti-CTLA4 monoclonal antibodies. Anti-PD1 treatment in combination with BRAF plus MEK inhibitors was associated with a better OS for BRAF-mutated melanoma, compared to BRAF inhibitors alone (Pasquali, Hadjinicolaou, et al. 2018). Future research should include the longer-term effect of new therapeutic agents (*i.e*. immune checkpoint inhibitors and targeted therapies) on OS.

New therapeutic epitopes and strategies are constantly being sought and there are many opportunities for this kind of approaches. Indeed, it takes a lot of time and is very expensive, but it may be worth it in the future. Oncoviral therapy alone and in combination with other systemic therapies has been a trend in recent years, as the ideal and universal treatment regimen that has a lot of potential.

Even the occasional cytostatic infusion treatment of the limbs can be as efficient as modern systemic drugs. Exploiting and integrating new and niche opportunities, especially in the context of treatment efficacy and cost savings, is very promising. We demonstrated clearly in our third publication, that the results achieved in ILI treatment in Eastern Europe are comparable to those reported by high-volume centres and hospitals in the USA and Australia and this method is still an important treatment option in the current era of immunotherapy. ILI has the advantage of being a relatively cheap and simple treatment modality in the era of effective targeted and immune therapies for selected patients.

It is important to point out that our findings suggest that a **2-cm resection margin is sufficient** and safe for patients with cutaneous melanoma thicker than 2 mm involving patients with stage II A-C. While the larger margin is unnecessarily too invasive by causing bigger trauma and reducing healing.

I would like to bring attention to the fact that **melanoma often appears later in the location of previous trauma or chronic pain.** This is my personal observation and consistent with the literature, showing the importance of the inflammatory factor in melanoma development.

In order to maximize therapeutic efficacy and avoid unnecessary costs, the research is now focusing on identifying biomarkers which could predict a patient's response to treatment prior treatment initiation or sometimes very early during the treatment course. This pre-treatment analyses can also avoid undesirable heavy side effects for the patient.

Regarding melanomas markers, such as the BRAF mutation, there the predictive value is often hampered by the heterogeneity of the tumour. For example, metastases from BRAF positive tumours are often losing this marker after therapy, indicating that other tumoural cells, which are less sensitive to the treatment, have expanded. This underscores the need for new markers, which can be combined for a better and more comprehensive description of the whole tumour. Single cell technologies are to play a key role in the definition of such markers and combinations of markers. We can hope that using combined markers will allow a better prediction of the most relevant treatment, then leading to efficient systemic therapies that may cure metastases.

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Abstract

Melanoma markers and new treatment perspectives

The situation of melanoma in the world and in the Estonian context is largely similar: the frequency of cases does not decrease but has been on a constant upward trend so far, despite the fact that melanoma is a very old and well-studied cancer. Melanoma is an aggressive tumour based on pigment cells, which are mainly localized in the skin and to a lesser extent in other tissues, depending on the location of the source cells and the contributing factors.

Melanocyte number and location in the skin is very stable, but very easily affected by both internal and external factors. With the development of melanoma, there is a progressive accumulation of genetic and epigenetic changes, which results in the development of uncontrolled tumour tissue, especially with increasing age of patients.

Melanoma cells have extremely different morphologies in different localizations, and regions of the body, where they are affected by different growth and gene expression factors in the local environment. Many gene mutations in melanoma are known, but it is still thought that an even greater number of mutations are unknown, the largest of which are still acquired and with the type of UV mutation. Melanoma, unlike other tumours, is characterized by the need for several different gene mutations to coexist for the disease to occur. On the other hand, the state of the reserve and reactivity of the surrounding immune system is also important, which unfortunately decreases and dysfunctions with age or due to concomitant pathologies. The immune system has a very important role to play here, and it intriguing and interesting to follow developments in these searches.

It can be stated that melanoma has a very multifactorial mechanism of origin, which is, however, different from individual to individual. Melanoma has rather definite external features, especially in the case of cutaneous melanoma and is therefore theoretically easy to diagnose with sufficient prior information and training, as well as in the presence of auxiliary diagnostic apparatus at an early stage of development. Early detection is very important, especially knowing that the lifelong progression of the disease is very low in this case. However, despite the continuous improvement of this link, metastatic cases often still remain for very different reasons and are the most worrying in the future.

The **first goal of this dissertation** was to detect melanoma with antibodies to the blood markers MAGE4 and 10 – this possibility is very important in a situation where melanoma is not visible to the naked eye. Using an ELISA method and recombinant MAGE4 and 10 proteins, we showed that a strong antibody response against MAGE4 and 10 is both a good prognostic indicator and an early marker to help diagnose melanoma. What then influences the further course of melanoma? Both local barrier abilities and systemic immune mechanisms, but also further mutations and interactions of the tumour cells themselves with the immune system that is constantly attacking it. In practice, it is a type of tumour that can metastasize to any region of the body. However, the ability of immune reserves is important for the spread and progression of melanoma, and is largely determined by an individual's own genetic background and age-related loss of immunity, which generally results in more frequent tumours and other diseases in old age. To date, a major step has been taken to investigate what may affect our immune system, and one of them is intestinal lymphoid tissue, one of the major components of the immune system that affects both local and systemic immune responses and the microflora acting as a microbiome. Also in the organization of

anti-tumour responses through modulation of the tumour microenvironment is one of the objects of novel personalized medicine.

Cancer stem cells, which are involved in the spread and progression of melanoma from the original site to other tissues, also accumulate genetic transformation and escape immune attack and are functionally good targets for both disease spread and treatment strategy planning. The number of circulating tumour cells, as still inactive melanoma cells, is one of the key components in the development of metastatic melanoma and is usually correlated with the risk of disease progression and is, of course, constantly under the control of the immune system and usually a successful self-limiting process. These are tumour cells that could also be used as a prognostic marker in research and as such they are again in great clinical need. A melanoma biomarker, by definition, is capable of detecting a tumour or being helpful in nature in selecting the best treatment at a given time. Current melanoma markers are subjective in their non-specificity and do not reliably reflect the current status of melanoma consumption and are open to various interpretations. The need for more robust and accurate markers has long been prevalent in the clinical context, where patients do not yet have any definite complaints and the disease would be easier to eliminate at an early onset. Unfortunately, there is no universal or very specific candidate from those currently in progress. There are also major shortcomings in evaluating treatment efficacy in the same area, which certainly complicates the work of clinicians and does not reflect the potential that could be achieved with them. Molecular markers expressed by melanoma cells are very important in this context, especially tumour antigens, which are very specific to melanoma. However, antibodies, with their ability to recognize some melanoma antigens, would be a good and early marker that can be used in tumour diagnosis.

Because melanoma cells have extremely different morphologies, they are affected by very different growth factors and gene expression in different regions of the body. All of these components can be involved in the development of melanoma treatment and diagnosis strategies.

The second and third objectives of this dissertation both concern the treatment of melanoma. Our aim was to investigate the oncosuppressive mechanism of viral apoptin and its success in a mouse model. Viral apoptin significantly inhibited the growth of tumour cells without affecting native cells, thus having cancer therapeutic potential. The third article aimed to test and analyze a new treatment option that is rarely used in Europe, but which has proven itself in both Australia and the United States. This method is less invasive for the patient, and if the melanoma metastasis is located on the limb, it is possible to perform chemotherapy only within the limb. This treatment proved to be successful in our hospital, showing up to 90% long-term survival, and our results show that it can also be used safely and effectively.

Existing treatment options are now much richer and increasingly take into account individual differences and personalization, but are still far from being complete. In this context, the use of new as well as old methods in a new perspective is relevant and needs to be further developed, and sometimes also in terms of cost. The effect of the oncoviruses we studied on melanoma cell growth was inhibitory and it would be a promising research trend. Even the occasional cytostatic infusion treatment of the limbs does not lag far behind of more modern systemic drugs. Exploiting and integrating new and niche opportunities, especially in the context of treatment efficacy and cost savings, is very promising.

Lühikokkuvõte

Melanoomi markerid ja uued raviperspektiivid

Melanoomi situatsioon maailmas ja Eesti kontekstis on suures plaanis sarnane: juhtude sagedus ei lange vaid on olnud siiani pidevas tõusutendentsis, vaatamata sellele, et tegemist on väga vana haigusega.

Melanoom on agressiivne pigmentrakkudest lähtunud kasvaja, mis peamiselt lokaliseerub nahas ja vähem mujal kudedes sõltuvalt lähterakkude asukohast ja soodustavatest faktoritest.

Melanotsüütide arv ja lokalisatsioon nahas on väga stabiilne ning seeläbi on need rakud väga kergesti mõjutatavad nii sise- kui välisfaktorite poolt. Melanoomi tekke korral toimub progressiivne geneetiliste ja epigeneetilise muutuste akumuleerumine, mille tulemuseks on kontrollimatu tuumorikoe teke ja seda eriti vanuse kasvades. Melanoomi rakkudel on eri lokalisatsioonides erakordselt erinev morfoloogia ja erinevates keha regioonides on nad mõjutatud väga erinevatest kasvufaktoritest, geeni ekspressioonist ja kasvukeskkonda mõjutavatest teguritest.

Geenimutatsioone melanoomi osas on teada palju, kuid ikkagi arvatakse, et teadmata on veelgi suurem hulk, neist suurim hulk on siiski omandatud ja UV mutatsiooni tüübiga. Melanoomile on iseloomulik, eristuvalt teistest tuumoritest, mitme erineva geenimutatsiooni kooseksisteerimise vajadus, et haigus realiseeruks. Teiselt poolt on oluline ka ümbritseva immuunsüsteemi reservi seisund ja reaktiivsus, mis paraku eaga või kaasuvate patoloogiate tõttu väheneb ja düsfunktsioneerub. Immuunsüsteemil on siin kanda väga suur roll ja seda intrigeerivam ja huvitavam on jälgida arenguid nendes otsingutes ja seoste loomisel.

Võib konstateerida, et melanoomil on väga multifaktoriaalne tekkemehhanism, mis on indiviiditi siiski eristuv, kuid lõppresultaadina sarnane.

Diagnostika eripärad: haigusel on üsna kindlad välised tunnused, eriti just nahamelanoomi korral, ja on seetõttu teoreetiliselt lihtsalt diagnoositav piisava eelinformatsiooni ja treenituse korral ning ka abistava dignostilise aparatuuri olemasolul varases arengu etapis. Varajane tuvastamine on väga oluline eelkõige teadaoleva info foonil, et haiguse elupuhune progressioon on vastasel juhul väga madal. Vaatamata varajase diagnoosimise kasvule jäävad ikkagi metastaseeruvad juhud väga erinevatel põhjustel ja nende puhul ongi kõige rohkem muresid edaspidi.

Käesoleva doktoritöö **esimene eesmärk** oli melanoomi tuvastamine veremarkerite MAGE4 ja 10 vastaste antikehade abil – see võimalus on väga tähtis olukorras, kus melanoom ei ole silmaga nähtav. Kasutades ELISA meetodit ja rekombinantseid MAGE4 ja 10 valke näitasime, et tugev MAGE4 ja 10 vastane antikehaline vastus on ühtlasi nii hea prognostiline näitaja kui ka üks varajane marker, mis aitab melanoomi diagnoosida.

Mis siis mõjutavad edasist melanoomi kulgu: ühelt poolt nii lokaalsed immuunbarjäärid kui ka süsteemsed immuunmehhanismid, kuid teisalt ka kasvajarakkude enda edasised mutatsioonid ja suhestumine teda igati pidevalt rünnata püüdva immuunsüsteemiga. Praktikas on see kasvajatüüp, mis võib metastaseeruda igasse organismi regiooni.

Melanoomi levikuks ja progressiooni edukuseks on aga oluline immuunreservide võimekus, mis paljuski on määratud indiviidi enda geneetilise tausta ja immuunsenestsentsi ehk immuunvõimekuse kaoga vanusega seotuna, mille tulemusel sagedasemad tuumorid ja ka paljud teised haigused esinevad vanemaealiselt, kuid teatud geneetiliste muutuste korral ka juba varasemas eas.

Praeguseks on astutud suur samm edasi uurimaks, mille poolt võiks olla mõjutatud meie immuunsüsteem. Üks selline tegur on näiteks soolestikuga seotud lümfoidkude kui üks suurimaid immuunsüsteemi komponente, mis mõjutab nii lokaalset kui ka süsteemset immuunvastust.Teda mõjutav mikrofloora mikrobioomina mängib suurt rolli nii kogu keha immuunstaatuses kui ka kasvajavastase ravivastuse organiseerimisel läbi tuumori mikrokeskonna moduleerimise ja on uudse personaliseeritud meditsiini üheks huviobjektiks.

Ka kasvajate multipotentsed tüvirakud, millega on seotud melanoomi levik ja progressioon algkoldest teistesse kudedesse, akumuleerivad geneetilist transformatsiooni. Nende kaudu toimub samuti põgenemine immuunrünnaku eest ja oma funktsionaalsuses oleksid nad heaks märklauaks nii haiguse leviku kui ravi strateegia planeerimisel.

Tsirkuleerivate tuumorirakkude ehk veel inaktiivsete melanoomirakkude hulk on üks võtmekomponente metastaseeruva melanoomi juhtude tekkimises. See on tavaliselt korrelatsioonis haiguse progressiooni riskiga ning on muidugi pidevalt immuunsüsteemi kontrolli all ja enamasti ka edukalt iselimiteeruv protsess. Need on kasvajarakud, mida uuringute alusel saaks samuti kasutada prognostilise markerina ja mille järele on kliiniliselt taas väga suur vajadus.

Melanoomi biomarker on oma definitsiooni järgi võimeline tuvastama kasvaja või võib olla abistav valimaks parimat ravi antud ajahetkel.

Kasutuselolevad melanoomi markerid on oma mittespetsiifilisuses subjektiivsed ja ei kajasta kindlalt melanoomi kulu hetkestaatust ja on mitmeti interpreteeritavad. Vajadus kindlamate ja täpsemate markerite osas on olnud pikka aega, eriti kliinilises kontekstis, kus haigetel veel mingeid kindlaid kaebusi pole ja haigus oleks oma varase alguse puhul lihtsamalt elimineeritav. Paraku universaalset ega ka väga spetsiifilist kandidaati hetkel veel kasutusel pole. Ka raviefektiivsuse hindamisel on samas vallas suured puudujäägid, mis raskendab kindlasti klinitsistide tööd ja ei kajasta seda potentsiaali, mis nendega võiks saavutada.

Melanoomi rakkude poolt ekspresseeritud molekulaarsed markerid on selles kontekstis väga olulised, eriti tuumori antigeenid, mis on väga iseloomulikud kindlale kasvajale, antud kontekstis melanoomile. Tsütotoksilised antikehad aga oma võimega neid ära tunda oleks heaks ja varaseks markeriks, mida saab kasutada kasvaja diagnostikas.

Kuna melanoomi rakkudel on erakordselt erinev morfoloogia ja erinevates keha regioonides on nad mõjutatud väga erinevatest kasvufaktoritest, geeni ekspressioonist ja kasvukeskkonda mõjutavatest teguritest, siis neid kõiki komponente on võimalik teoreetiliselt ja juba osaliselt ka praktikas kaasata melanoomi ravi- ja diagnostika strateegia arengusse.

Käesoleva doktoritöö **teine ja kolmas eesmärk** puudutavad mõlemad melanoomi ravi. Meie eesmärk oli uurida viraalse apoptiini onkosupressiivset mehhanismi ja selle edukust hiirmudelis. Viiruse apoptiin pidurdas oluliselt kasvajarakkude kasvu, avaldamata mõju natiivsetele rakkudele, omades seega vähiteraapilist potentsiaali. Kolmanda artikli eesmärk oli katsetada ja analüüsida uusi ravivõimalusi, mida Euroopas on harva kasutatud, kuid mis on ennast nii Austraalias kui ka Ameerikas tõestanud. See meetod (ILI) on patsiendile vähem invasiivne ja kui melanoomi metastaasid paiknevad jäsemel, on selle meetodi abil võimalik kemoteraapiat teostada ainult jäseme siseselt. See ravimeetod osutus meie haiglas edukaks näidates kuni 90% pikaajalist elulemust ning meie tulemused näitavad, et seda saab ka meil ohutult ja tõhusalt rakendada.

Olemasolevad ravivõimalused on praeguseks tunduvalt rikkalikumad ja arvestavad üha enam individuaalsete erisuste ja personaliseeritusega, kuid on endiselt veel kaugel täiusest.

Selles kontekstis on uute ja ka vanade meetodite uues rakursis kasutamine aktuaalne ja edasiarendamise vajadusega ja teinekord ka kulukuse poole pealt mõistlik. Meie poolt uuritud onkoviiruste mõju melanoomirakkude kasvule oli pärssiv. See on korrelatsioonis ning paljulubav võrreldes ka teiste uurimistendentsidega selles vallas teistes riikides ja arvestades, millist üldimmuunvastust need mikroorganismid võivad põhjustada oma hävitavas kontekstis teinekord. Ka aeg-ajalt vajaminev jäsemete infusioonravi tsütostaatilise ravimiga ei jää oma kaugtulemustelt alla moodsamatele süsteemravimitele.

Uute ja nišivõimaluste kasutamine ja integreerimine, eriti raviefektiivsuse ja kulukuse kokkuhoiu kontekstis, on väga paljulubav.

Appendix

Publication I

Õunap K, Kurg K, Võsa L, Maiväli Ü, **Teras, M**, Planken A, Ustav M, Kurg R. Antibody response against cancertestis antigens MAGEA4 and MAGEA10 in patients with melanoma. Oncology Letters; 2018, 16(1): 211-218

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Antibody response against cancer-testis antigens MAGEA4 and MAGEA10 in patients with melanoma

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Abstract. Melanoma-associated antigen A (MAGEA) represent a class of tumor antigens that are expressed in a variety of malignant tumors, however, their expression in healthy normal tissues is restricted to germ cells of testis, fetal ovary and placenta. The restricted expression and immunogenicity of these antigens make them ideal targets for immunotherapy in human cancer. In the present study the presence of naturally occurring antibodies against two MAGEA subfamily proteins, MAGEA4 and MAGEA10, was analyzed in patients with melanoma at different stages of disease. Results indicated that the anti-MAGEA4/MAGEA10 immune response in melanoma patients was heterogeneous, with only ~8% of patients having a strong response. Comparing the number of strongly responding patients between different stages of disease revealed that the highest number of strong responses was detected among stage II melanoma patients. These findings support the model that the immune system is involved in the control of melanoma in the early stages of disease.

Introduction

Melanoma-associated antigen A (MAGEA) subfamily proteins are members of cancer/testis antigens (CTAs), whose normal expression is limited to germ cells, but ectopic expression can be observed in tumor cells of different origins (1). The *MAGEA* genes were initially identified as tumor antigens that can be recognized by cytotoxic T-lymphocytes in melanoma patients (2). The MAGEA subgroup of CTA family comprises eleven genes that show striking homology with each other and are encoded as a cluster at the Xq28 region (3). Their normal expression is restricted to the testis, trophoblast and placenta (3,4). MAGEA expression in somatic cells is silenced by promoter DNA methylation (5), but in tumor cells genome-wide epigenetic reprogramming can result in promoter hypo-methylation, leading to aberrant expression of one or more of these genes (1,6).

MAGEA expression is observed mainly in cancers that have acquired malignant phenotypes, invasiveness or metastasis, and the expression of MAGEA family proteins has been linked to poor prognosis in cancer patients. MAGEA family proteins have oncogenic functions, including support of growth, survival and metastasis, and are thought to contribute actively to malignancy (7). At the molecular level, MAGEA proteins are involved, through direct and indirect mechanisms, in the regulation of the tumor suppressor protein p53 pathway (8-12). MAGEA proteins can also activate specific RING finger type E3 ubiquitin ligases (13,14), thereby regulating the ubiquitin signaling in cancer cells.

MAGEA proteins are known to be highly expressed in a wide range on cancers including bladder, lung, skin and breast malignancies (6,15-18). Expression of these antigens may be highly heterogeneous in a variety of tumors of different histological origin, with percentages of positive cells ranging between 5 and 60% (18). MAGEA subfamily proteins are highly conserved and it is very difficult to get antibodies that recognize only one member of the family specifically. For example, MAGEA4 and MAGEA10 proteins share more than 50% sequence identity on the amino acid level, but have different sizes and cellular localizations (19). Several antibodies used in immunohistochemical studies cross-react with many MAGEA proteins and have been seen in multiple cancer types to localize both in the cytoplasm and in the nucleus (20-22). This has complicated the immunohistochemical analysis of cancer tissues and limited the analysis of specific subfamily members, which may have different expression patterns, subcellular localizations and impacts on the malignancy.

Melanoma is the most serious type of skin cancer and its incidence has risen over the years. The etiology of melanoma is multi-factorial, resulting from gene-environment interactions, with the main environmental factor for melanoma development being exposure to sunlight and UV radiation (23). The importance of the immune system in the etiology of human skin cancer has been long recognized, based primarily upon

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the increased incidence of skin cancers in organ transplant recipients and mechanisms of ultraviolet (UV) radiation-mediated immunomodulation (24). Although the rate of melanoma incidence is rising, especially within young females, there is no direct correlation with the increase of mortality. Histological regression in primary cutaneous melanoma has been shown to occur in 10-35% of cases (25). Thus, it can be hypothesized that the immune system is involved in controlling the melanoma progression, especially in younger individuals.

The aim of this study was to evaluate the presence of naturally occurring antibodies against two MAGEA proteins in the blood samples of melanoma patients with different stages of disease. MAGEA proteins have oncogenic functions contributing to malignancy, and they are known to be immunogenic proteins. The MAGEA4 and MAGEA10 proteins were expressed in bacteria, purified and used in the enzyme-linked immunosorbent assay (ELISA) for detection of antibodies. We were curious to know i) whether the melanoma patients have antibodies against these proteins, and ii) whether these antibodies can be treated as a potential prognostic marker.

Materials and methods

Patients and sera. Human sera were obtained from 185 patients with melanoma attending the North Estonian Medical Centre (Tallinn, Estonia) within two years (2013-2014). The melanoma stage was assigned based on tumor thickness, ulceration and the involvement of lymph nodes or organs. The characteristics of patients are shown in Table I. As a control, we included 43 sera of healthy blood donors from the Estonian Blood Bank. We have no data about the gender nor age of blood bank controls. All samples were handled by standard procedures and stored at -80˚C. Approval for the use of blood samples for the study was obtained from the Tallinn Medical Research Ethics Committee (Tallinn, Estonia).

Proteins. MAGEA4 and MAGEA10 coding sequences from pQMCF-MAGEA4 and pQMCF-MAGEA10 vectors (19) were cloned in frame into pET28a vector using NheI restriction enzyme to fuse the coding sequence with His-tag. Recombinant His-tagged MAGEA4 and MAGEA10 proteins were transformed into *Escherichia coli* (*E coli*) cells BL-CodonPlus™ RP (Invitrogen; USA); transformed bacteria were grown at 37˚C to the spectrophotometric density 0.6 (OD 600 nm; Ultraspec 7000; GE Healthcare Life Sciences, Little Chalfont, UK) and induced with 1 mM IPTG for 2 h at room temperature. Then the cells were collected by centrifugation (at 8,000 x g for 3 min at 4˚C; Centrifuge 5810R; Eppendorf, Hamburg, Germany) and resuspended in buffer containing 50 mM Tris (pH 8.0) and 500 mM NaCl. Proteins were purified with Ni-Sepharose™ 6 Fast Flow beads (GE Healthcare Life Sciences) under standard native conditions following manufacturer's recommendations; 20 mM imidazole was added to the buffer for binding reactions, 25 mM for wash buffers and 250 mM for elution of proteins from the beads. Both proteins were purified using the same protocol. After purification, the buffer was exchanged to PBS with Amicon® Ultra centrifugal filters (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and the concentration of proteins was determined by the Bradford assay using bovine serum albumin (BSA) as a standard.

ELISA. Recombinant MAGEA4 or MAGEA10 protein (2 μg/ml) in phosphate-buffered saline (PBS) containing 0.1% of Tween-20 was adsorbed onto 96-well MaxiSorp NUNC-immunoplates (Sigma-Aldrich; Merck KGaA) and incubated overnight at 4ºC. Plates were washed with PBS/0.1% Tween-20 and blocked with 2% BSA in PBS/0.1% Tween-20. Serial dilutions of human serum in 100 μl of 0.4% BSA/PBS/0.1% Tween-20 were added to each well and incubated for one hour at room temperature on the shaker (Titertek-Berthold; Berthold Detection Systems GmbH, Pforzheim, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Zymax/Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used as a secondary antibody for 45 min. After washing four times, the reaction was developed with the TMB Peroxidase E1A substrate kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 10 min. and stopped with H_2SO_4. The absorbance at 450 nm was measured spectrophotometrically using the ELISA plate reader Sunrise™ (Tecan, Männedorf, Switzerland). For quality control, we included three reference sera which were analyzed on every ELISA plate. The CVs of their ODs did not exceed 20%.

Statistical analysis. The data were analyzed in R (version 3.3.0). Parameter estimates and corresponding CI (credible intervals) were calculated using the BayesFirstAid package (26). Analysis of variance (ANOVA) with the Tukey post-test was also done in R.

The patients with positive antibody response were defined as follows: pooled MAGEA4 and MAGEA10 response values obtained from the blood bank donors were log-transformed to ensure normality, after which the mean and the standard deviation was calculated from the control subjects only. Then the melanoma patients, whose log-response value > mean + 2* SD, were redefined as having a strong response. To classify subjects based on their MAGEA protein levels a logistic regression model, including both MAGEA proteins, sex, and age as additive predictors, was trained on the subset of data containing stage 0, I, and II patients. The pROC package was used to calculate the receiver operating characteristic (ROC) curve (27).

Results

Antibody response against MAGEA4 and MAGEA10 proteins in melanoma patients. We measured the anti-MAGEA4 and anti-MAGEA10 antibody levels by ELISA from 185 stage 0 (*in situ*) to stage IV melanoma patients and from 43 healthy individuals, who had donated their blood to the Estonian blood bank. The ELISA was performed using MAGEA4 and MAGEA10 proteins, which were purified from *E. coli*, immobilized on microtiter plates, and subsequently probed with 1:200 to 1:800 human sera dilutions. The serums that exhibited high OD values, indicating the presence of anti-MAGEA antibodies, were tested at least three times on separate ELISA plates and the mean OD value was used in further analysis. The OD values obtained from 1:400 diluted serums were used in statistical analysis.

We first compared the OD values of the controls with the melanoma patients separately for anti-MAGEA4 and anti-MAGEA10 response (Fig. 1). In Fig. 1, the Tukey box

Table I. Characteristics of the melanoma patients.

Number	Stage 0 24	Stage I 67	Stage II 43	Stage III 30	Stage IV 21	Total 185
Sex						
Male (%)	4 (16.7%)	17 (25.4%)	14 (32.6%)	12 (40%)	9 (42.9%)	56 (30.3%)
Female (%)	20 (83.3%)	50 (74.6%)	29 (67.4%)	18 (60%)	12 (57.1%)	129 (69.7%)
Disease duration						
<5 years	21 (87.5%)	47 (70.1%)	29 (67.4%)	20 (66.7%)	18 (85.7%)	135 (73%)
≥5 years	3 (12.5%)	20 (29.9%)	14 (32.6%)	10 (33.3%)	3 (14.3%)	50 (27%)
Mean (range)	2.0 (0-13)	4.5 (0-26)	3.7 (0-18)	4.5 (0-19)	2.8 (0-25)	3.8 (0-26)
Median	1	2	3	3	1	2
Age						
Mean (range)	51.9 (18-87)	61.3 (28-87)	64.3 (33-90)	63.4 (43-82)	73.1 (35-92)	62.5 (18-92)
Median	51	65	66	65	78	65

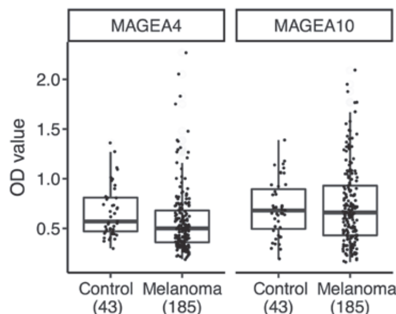

Figure 1. Antibody response against MAGEA4 and MAGEA10 proteins in melanoma patients and controls. A comparison of the magnitude of the anti-MAGEA4 and anti-MAGEA10 immune responses of blood bank controls vs. melanoma patients (stages 0-IV combined) by Tukey box plots showing median and interquartile ranges. Dots correspond to individual blood bank controls and patients. The Y-axis denotes optical density values obtained from the ELISA assay. The number of sera is shown in the parentheses. MAGEA, melanoma-associated antigen A.

blots were used to show the median and interquartile ranges, as well as dots corresponding to individual patients and blood bank controls. The mean immune responses of patients were not elevated as compared with the blood bank controls. The mean OD value of the sera of melanoma patients was 0.59 (SD 0.31) for MAGEA4 and 0.73 (0.38) for MAGEA10. For blood bank controls, the mean OD value was 0.67 (0.26) for MAGEA4 and 0.70 (0.28) for MAGEA10. We could not find evidence for elevated mean effects of melanoma patients over blood bank controls, the probability that the patients mean value was higher than the controls was 0.3% for MAGEA4 (two-sided P-value=0.004) and 62% for MAGEA10 (P=0.87). On the other hand, the patients had higher variability at their anti-MAGEA4 and anti-MAGEA10 responses than controls; the probability that the controls have higher standard deviations was 2 and 6% for MAGEA4 and MAGEA10, respectively. We suggest that the higher variability of the immune response in patients could mean that in some patients the antibody response is induced, while in others is not. The failure of the patients to exhibit aggregate effects over the controls is likely due to the voluntary blood donors, who make up the control group. We have no data about their age and gender, but they are probably younger than the melanoma patients. This limits the usefulness of the blood donors as controls. Therefore, in the subsequent analysis we omitted the blood bank controls and looked at melanoma stages 0 to IV as distinct groups.

To follow the antibody response from limited to advanced disease, we divided the patients into subgroups, depending on the status of their disease. In Fig. 2, the Tukey box blots were used to show the median and interquartile ranges, as well as dots corresponding to individual patients; the number of patients is shown in parenthesis under the stage number. As shown in Fig. 2, some stage I, II and III patients exhibited elevated anti-MAGEA4 and/or anti-MAGEA10 immune responses. The one-way ANOVA P-value was 0.10 for MAGEA4 and 0.043 for MAGEA10, indicating that there are statistically significant contrast(s) in the MAGEA10 data. We used the Tukey HSD post-test to find groups that significantly differ from each other. This showed that in the case of MAGEA10, there was a single contrast, stage 0 vs. stage II patients, which had a significant difference in mean OD values (P=0.047). The mean OD values between stage II and stage IV patients were slightly different (P=0.10), but no difference was observed between stage II and III patients (P=0.78). In the case of MAGEA4, we did not observe statistically significant differences in mean values between patients with different stages of disease (P=0.74 for stage 0 vs. stage II; P=0.18 for stage II vs. stage IV and P=0.47 for stage II vs. stage III). These data show that there is no strong difference in mean OD values between stage II and III, but is a slight difference between stage II and IV for MAGEA10. Although, we found only a single statistically significant contrast, due to the limited sample size this does not necessarily mean that there are no real differences between stage II and III&IV. We sought to clarify this point further by polynomial regression modelling. We predicted anti-MAGEA4/A10 response levels (as measured by OD) from the stage of melanoma modelled as a continuous ordinal variable. These models indicate that for both proteins there

Table II. Patients with strong antibody response.

Patient	Gender	Age (years)	Disease duration (years)	Stage	Protein	OD value
M35	F	61	2	IIB	MAGEA4	2.05
M38[a]	F	73	18	II	MAGEA4	2.27
M111[b]	M	67	1	IIIC	MAGEA4	1.49
M123	F	65	0	IB	MAGEA4	1.48
M162	M	64	3	IA	MAGEA4	1.75
M3	F	57	1	IB	MAGEA10	1.49
M38[a]	F	73	18	II	MAGEA10	1.67
M47	F	71	4	IIB	MAGEA10	2.09
M63	F	80	3	IIIA	MAGEA10	1.48
M70	F	64	9	IIB	MAGEA10	1.43
M76	F	61	6	IIB	MAGEA10	1.77
M99	F	66	5	IIB	MAGEA10	1.88
M111[b]	M	67	1	IIIC	MAGEA10	1.55
M115	M	72	1	IIB	MAGEA10	1.95
M119	F	76	11	IB	MAGEA10	1.45
M137	F	72	0	IIB	MAGEA10	1.58
M144	F	52	1	IIIA	MAGEA10	1.77

[a,b]These patients have a strong antibody response against MAGEA4 and MAGEA10 protein. MAGEA, melanoma-associated antigen A.

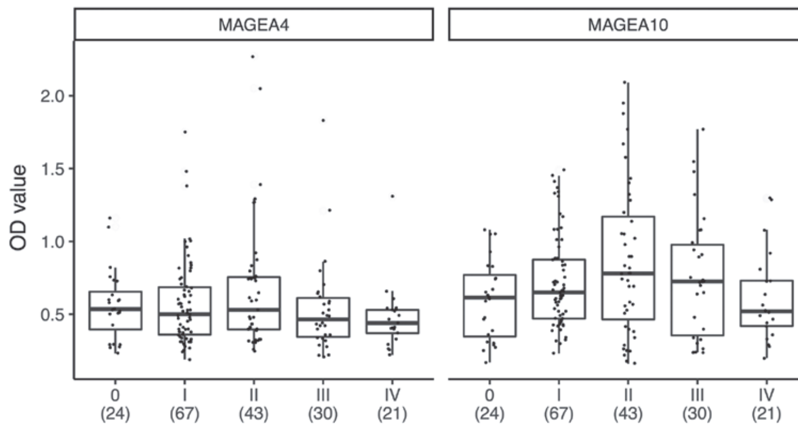

Figure 2. A subgroup analysis of the magnitude of the anti-MAGEA4 and anti-MAGEA10 immune responses of melanoma patients (stages 0-IV). Tukey box plots with median and interquartile ranges are shown, as well as dots corresponding to individual blood bank controls and patients. The Y-axis denotes optical density values at 495 nm. The number of sera is shown in the parenthesis. MAGEA, melanoma-associated antigen A.

is an initial rise in optical density that peaks at stage II, and thereafter falls again (data not shown).

As our samples were not balanced for age and sex (Table I), we also looked for associations between these variables and anti-MAGE response. By applying linear regression towards our stage 0 to IV melanoma samples, we could find no significant association between the age of the subjects and anti-MAGEA4 or anti-MAGEA10 response (data not shown). However, we found a weak association between anti-MAGEA10 levels and sex (r^2=0.025; female melanoma patients have on average 0.15 OD units higher anti-MAGEA10 response than male patients, 95% CI: 0.04, 0.26). But the width of the CI indicates that our sample size is not large enough to decide whether this effect is scientifically relevant.

Patients with strong antibody response. Next, we focused on patients with strong anti-MAGEA4 and/or anti-MAGEA10 immune responses. Here we included patients whose OD values were higher than the mean OD of the healthy blood bank donors plus 2 SD-s (28). Table II shows the patients with strong antibody response. The sera of 15 patients from 185 (8.2; 95% CI: 4.7, 13%) had a strong antibody response against the MAGEA4 and/or MAGEA10 protein (Table II). Two patients (M38 and M111) had a strong response against both proteins,

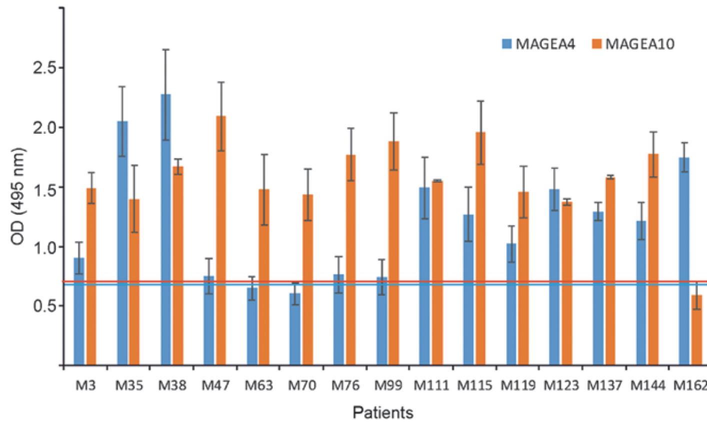

Figure 3. Comparison of OD values of MAGEA4 and MAGEA10 among the strongly responding patients. Lines correspond to mean values of ELISA assay for MAGEA4 (blue) and MAGEA10 (orange), respectively. Error bars show the SD of at least three different experiments performed on separate ELISA plates. MAGEA, melanoma-associated antigen A.

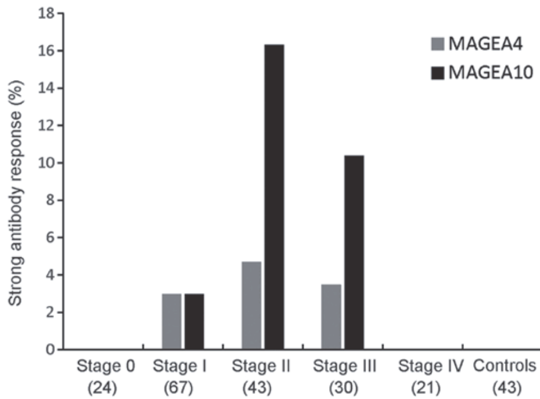

Figure 4. The fraction of strongly responding patients in relation to the melanoma stage. Patients with OD values higher than the mean OD of the healthy blood bank donors plus 2 SD-s are included. The number of sera is shown in the parenthesis. MAGEA, melanoma-associated antigen A.

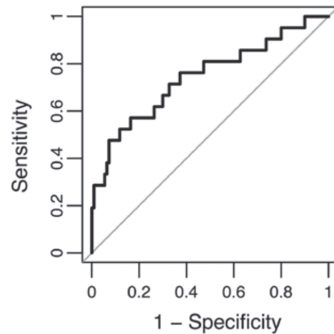

Figure 5. ROC curve for anti-MAGE antibody detection. Antibody levels among 185 melanoma patients and 43 blood bank controls were determined by ELISA. The AUC value was 0.74. ROC, receiver operating characteristic; MAGE, melanoma-associated antigen; AUC, area under the curve.

that is why there are 17 patients listed in Table II. The mean age of strongly responding patients was 67 years (median 67 years) and they were first diagnosed from 0 to 18 years (mean 5 years, median 3 years) before this analysis was performed. Most of them are women, there are only 3 men (20; 95% CI: 5.4, 43%) among the patients with strong antibody response, while the whole cohort consists of 30.3% of men. Altogether, 5.6% of men and 9.3% of women had strong antibody response against one or two of the MAGEA proteins.

MAGEA proteins are highly similar to each other with half of the amino acids identical between MAGEA4 and MAGEA10 proteins. We have analyzed the sera separately for MAGEA4 and MAGEA10 response, and the statistics was performed and cut-off values calculated independently of each other. Interestingly, there were 12 anti-MAGEA10 responses and 5 anti-MAGEA4 responses, out of the total of 17 strong responses (estimated relative frequency of MAGEA4 is 0.31; 95% CI: 0.13, 0.52; 5% probability of relative frequency >0.5) (Table II). Consequently, only two patients out of 15 (13.3, 95% CI: 2.7, 35%) had strong and statistically significant responses against both, MAGEA4 and MAGEA10 proteins. In Fig. 3, we compare the antibody response against two different antigens among the same patients. Some patients (M38, M111, but also M35, M123 and M137) had antibodies against both MAGE-A proteins, the others (M47, M63, M70, M76, M99 and M162) against only one of the two proteins, either MAGEA4 or MAGEA10. Five patients of 15 (33%) had a statistically significant higher OD value ($P<0.01$) against MAGEA10 than MAGEA4, while only one patient, M162, had a better immune response against MAGEA4 (Fig. 3). These data show that among strongly responding patients, there are more anti-MAGEA10 than anti-MAGE4 responses.

Comparing the number of strongly responding patients between different stages of disease revealed that the highest number of strong responses was detected among stage II melanoma patients (Fig. 4). In the case of MAGEA10, 7 of 43 (16.3, 95% CI: 7.3, 29%) sera were positive among stage II patients, and 3 of 29 (10.3, 95% CI: 2.4, 24%) in stage III patients. In

the case of MAGEA4, there was no clear preference to any stage, strongly responding sera belonged to patients of stages I, II and III. We could not detect any strong response from the blood samples of melanoma patients with stage 0 and IV.

Predictive modelling of anti-MAGE-A responses. To explore the potential diagnostic value of anti-MAGEA antibodies, we classified all stage 0 vs. pooled stage I and II patients using an additive logistic regression model that includes both MAGEA proteins, age, and sex. We summarized the model performance in a ROC curve where we plotted the sensitivity (true positive rate) values against 1-specificity (false positive rate) values for each possible cut-point (Fig. 5). The area under the curve (AUC) is 0.74, suggesting that anti-MAGEA antibodies can be treated as potential diagnostic biomarkers.

Discussion

MAGEA proteins are cancer-testis antigens (CTAs), which elicit both cellular and humoral responses. In this study, we have analyzed the presence of naturally occurring antibodies against two MAGEA family proteins, MAGEA4 and MAGEA10, in melanoma patients with different stages of disease. Our data showed that sera of 15 patients out of 185 (8%) had a strong antibody response against the MAGEA4 and/or MAGEA10 protein. The highest antibody response was detected in stage II melanoma patients.

CTAs are named after their typical pattern of expression in a variety of malignant tumors. Their expression in normal tissues is restricted to germ cells of the testis. Male germ cells are devoid of HLA-class I molecules and cannot present antigens to T cells. Therefore, MAGEA antigens can be considered neo-antigens when expressed in cancer cells (29). Recent studies have shown that the induction of MAGEA4-specific immune responses correlated well with the prognosis of patients vaccinated with MAGEA4 protein and that antibody response could be a marker for a good prognosis (28).

Our study revealed that 8% of patients had strong antibody responses against the MAGEA4 or/and MAGEA10 protein. When we grouped patients according to the level of the disease, then stage II patients had more antibodies than others, reaching to 16% in case of MAGEA10. Scultz-Thater *et al* have studied the prevalence of MAGEA10 in different cancers and shown that it is expressed in 38% of malignant melanomas (18). Several studies have shown that MAGEA proteins are associated with or contribute to solid malignancies, MAGE-A expression is considered to be an important predictor of malignant transformation (21). For instance, MAGEA4 is expressed in 9% of primary tumors, but reaching to 44% in distant metastasis (30) and MAGEA1 expression has been found in 16% of primary melanomas and 48% of metastatic melanomas (15). However, in another study no correlation was observed, and MAGEA3/4 protein was present in 25% of primary invasive and metastatic tumors, but not in *in situ* melanomas (31). In our study, the prevalence of MAGEA antibodies was highest in stage II and lowest in stage 0 and stage IV patients. We have not determined the expression of MAGEA proteins in the tissue samples of our patients, but it is very unlikely that MAGEA expression declines in advanced stages. Previous studies have shown that MAGEA4 is rarely lost when once acquired (30). We favor the explanation that stage II melanoma patients have a better immune response than patients with more advanced stages of disease. This is consistent with the immune evasion seen in metastatic cancers (32). Interestingly, there were also very few responses amongst *in situ* and stage I melanoma patients. This can be explained by the localization of the primary tumor. Stage 0 or *in situ* and stage I melanoma are found mostly on the outer layer of the skin, in epidermis. Stage II melanoma has spread to the lower part of the inner layer of skin (dermis), but not yet into the tissue below the dermis or into nearby lymph nodes. The dermis contains many antigen presenting cells, which may help to boost the immune response.

In our study, some patients had strong antibody response against both MAGE-A proteins, the others exhibited antibodies against either MAGEA4 or MAGEA10 protein. One of the limitations of this study is that we do not have biopsies of patients and we were not able to perform neither qPCR nor immunochemical analysis to confirm that the antibodies are specific to MAGEA4 or MAGEA10. However, tumor cells very often express more than one MAGEA protein. Simultaneous expression of five or more MAGEA proteins occurs in more than half of oral squamous cell carcinomas (33) and simultaneous expression of MAGEA1 and MAGEA4 expression occurs in 60-70% of melanomas (30). We favor the explanation that the two MAGEA proteins used in our study have different immunogenic properties, so that the MAGEA10 protein is a better antigen than MAGEA4. Thus, our work is consistent with the studies, which have suggested that MAGEA10 is the most immunogenic antigen of the MAGEA family (34-36). It is well known that obtaining antibodies against one specific MAGEA protein may be challenging; we cannot rule out the possibility that antibodies detected in our assay are formed against some other member of the family. MAGEA proteins are highly similar to each other, with half of the amino acids identical between MAGEA4 and MAGEA10. The MAGEA subfamily consists of 11 MAGE-A proteins and in addition, there are MAGE-B, MAGE-C, MAGE-D etc. families which all share the MHG (MAGE homology) domain (37). All these proteins are to some extent similar to each other (MHG domains has similarities from 25 to 80%) and may give some cross-reactivity. This may also explain the immune response against MAGEA proteins in some healthy donors seen in our study. The other limitation of our study is that we have used only ELISA assay for screening of the sera. We choose ELISA, because it is suitable for high-throughput analysis, but have not analyzed the sera by western blotting. However, by doing so we might have missed some antibody responses generated against linear and/or denatured epitopes of MAGEA proteins.

The existence of strongly responding patients suggests that their immune system has been activated and has started to generate antibodies against the primary tumor. So, our data support the hypothesis that the immune system is involved in the control of melanoma, at least in the early stages. Several studies have shown spontaneous regression of primary melanomas, but regression of metastatic tumors is very rare. A good antibody response at early stages can stop the growth of primary tumor and further spreading to the lymph nodes and other organs. Among the 15 patients of our study with the positive status for MAGEA antibodies, only one has died

and one has disease progression during the 2-year post-study follow-up period (data not shown). So, the disease of the majority of patients with strong antibody response is under control. However, this cohort is too small to make long-term conclusions about the prognosis. Longitudinal time-course studies on larger cohorts are needed to establish the prognostic significance of the presence of MAGEA antibodies in patients. We plan to follow the patients and their antibody response for at least five more years and perform then the survival analysis.

The sensitivity and specificity calculations suggest that the anti-MAGEA antibody response can be treated as a potential diagnostic biomarker. One of the limitations for use in clinics is that MAGE-A proteins are expressed only in a portion of cancer cells; different works have shown that the amount of expressing cells is between 25 and 50% (30-31). When we assume that only half of these people have a strong immune response, then the expected % of strongly responding patients will be 12 to 25%. Is this enough for clinical diagnostics? On the other hand, these antibodies are so-called early markers and there is a great need for early cancer markers. So, when the presence of strong antibody response correlates with good prognosis, then they are/will be useful for clinics.

In addition, from the clinical aspect, the longitudinal detection of MAGEA antibody levels could be utilized for profiling of disease status or of effectiveness of novel immunotherapies, as there exists a great need for biomarkers which could assist in discrimination of patients suitable for immunotherapy or for monitoring the therapy effectiveness of these expensive drugs. For example, it has been shown that during immunotherapy with ipilimumab the MAGEA protein levels declined and elevation correlated with either treatment response or failure, respectively (38). However, the anti-MAGEA antibody status of patients prior to and after checkpoint therapy has never been evaluated.

In summary, our study supports the role of the host immune response in the progression of melanoma. To the best of our knowledge, the present study is the first report on following the antibody response against MAGEA-s and comparing it with the disease progression. A healthy immune system enables to create antibodies against cancer antigens that are expressed specifically by tumor cells. The link between MAGEA antigens and cancer is widely known and accepted; several works have shown a good cellular and humoral response against MAGEAs (1,6,37). Due to their relatively high tumor specificity, they represent attractive targets for active specific and adoptive cancer immunotherapies (39). In the current study, we are not interested in antigens, but we focus on the antibody response against the antigens. There are some studies who have analyzed the antibodies against melanoma antigens (tyrosinase, and TRPs) in melanoma patients, but not against MAGE-A proteins (40,41). In the current study, we focused on naturally occurring antibody response against MAGEA proteins. We compared the number of strongly responding patients between different stages of disease and found that the highest number of strong responses was detected among stage II melanoma patients. The strong antibody response could be a marker for a good prognosis (28) as well as an early marker which can be used for cancer diagnostics from liquid biopsy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KÕ, KK and RK designed the experiments; KÕ, KK and LV conducted the experiments; MT and AP collected the clinical data and were responsible for ethics approvals and consent of patients to participate in the study; ÜM performed the statistical analysis; MU was responsible for overall the design and funding of the project and KÕ, ÜM, AP and RK prepared the figures and wrote the manuscript. All authors read and have approved the final manuscript.

Ethics approval and consent to participate

Approval no. 2781 (from June 21, 2012) for the use of blood samples of melanoma patients, and no. 254 (from December 13, 2012) for controls were obtained from the Tallinn Medical Research Ethics Committee of Estonian National Institute for Health Development. All the patients, whose blood samples have been used, had signed the consent to participate in the study.

Consent for publication

The patients have provided written informed consent that the results of the study are published.

Competing interests

The authors declare that they have no competing interests.

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Publication II

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Porcine circovirus type 2 ORF3 protein induces apoptosis in melanoma cells

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Abstract

Background: The current treatment of malignant melanoma is limited by the lack of effective therapeutic approaches, and alternative treatments are needed. Proliferative diseases such as melanoma and other cancers may be treatable by virally-encoded apoptotic proteins that are targeted to rapidly multiplying cells. Caspase-dependent apoptosis, that is frequently used in chemotherapy, can boost the cell proliferation that caspase-independent cell death does not.

Methods: In the current study, the porcine circovirus type 2 (PCV2), proapoptotic protein ORF3 was expressed in mouse and human cancer cell lines, and its apoptotic activity was assessed.

Results: Quantitative assessment of the apoptotic cells by flow cytometry showed that apoptotic cell death was significantly increased in ORF3-expressing malignant cells, compared to ORF3 non-expressing cells. Our data show that PCV2 ORF3 induces apoptosis in a caspase-3 and -8 independent manner. ORF3 expression seems to cause an increase in abnormal mitosis in B16F10 melanoma cells by interacting with centrosomes and thereby disrupting the formation of the mitotic spindle. In addition, we show that ORF3 of PCV2 also exhibits significant anti-tumor effects in vivo. Although the expression of Regulator of G protein Signaling (RGS)-16 by recipient mice inhibited the development of grafted melanoma in vivo, it was not required for the antitumoral activity of ORF3.

Conclusion: PCV2 ORF3 causes abnormal mitosis in rapidly dividing cells and increases the apoptosis of cancer cells. Apoptin might, therefore, be considered to develop future antitumoral strategies.

Keywords: Melanoma, Porcine circovirus type 2 (PCV2) ORF3, Apoptosis

Background

Apoptosis - also known as programmed cell death - maintains a healthy balance between cell survival and cell death in an organism. Apoptosis is triggered by the action of caspases, cysteine proteases that cleave key cellular structural components and activate degradation enzymes. Apoptosis actually has a critical impact effect on the malignant phenotype. Oncogenic mutations can lead to defects in apoptosis, and tumor initiation, progression or metastasis. Furthermore, mutations that disrupt apoptosis during cancer development may also decrease the sensitivity to cytotoxic antitumoral drugs. Cell death by apoptosis can also trigger immune responses, which can be useful in cancer treatment. The control of apoptosis, therefore, appears as an important approach to developing new cancer strategies.

The role of apoptosis in host-virus interaction is complex. During viral infection, apoptosis induces elimination of infected host cells to limit viral replication [1, 2]; in contrast, apoptosis of infected cells at the end of the viral life cycle may contribute to an efficient viral spread in the organism [3]. Interestingly, viral apoptins can mediate apoptosis selectively in oncogenically transformed cells with minimal toxicity for the normal cells of the host [4]. In tumor cells, such "apoptins" are phosphorylated and located mainly in the nucleus, whereas in normal cells they are not phosphorylated, reside in the cytoplasm, and can be readily neutralized [5]. Multiple studies have demonstrated the therapeutic potential of oncolytic viruses [6–9].

Several members of the Circoviridae family encode proteins with pro-apoptotic activity in tumor cells

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(*ie*, "apoptins"). The best-studied model for such oncolytic activity is probably the Chicken anemia virus (CAV) whose apoptin (VP3) can selectively induce apoptosis in H1299 non-small lung adenocarcinoma and chicken lymphoblast MDCC-MSB1 cells upon phosphorylation and translocation to the nucleus [10]. Porcine circovirus type 1 and 2 (PCV1 and 2) apoptin ORF3 also induces apoptosis in transformed cell [11]. An apoptotic activity has been reported for PCV2 ORF3 [2, 12], but its oncolytic activity remains unknown. It has been shown that PCV2 ORF3 induces apoptosis of infected porcine cells in a caspase-dependent manner, involving significant activation of both caspase-3 and caspase-8 [2]. However, in the porcine kidney (PK)-15 cell line PCV2 ORF3 protein induces apoptosis in cells by competitive binding to E3 ubiquitin-protein ligase Pirh2, thereby inhibiting its interaction with p53, leading to nuclear accumulation and activation of p53, and an increase of apoptosis [13].

In this study, we describe a pro-apoptotic activity of PCV2 ORF3 in human and mouse melanoma cell lines. ORF3 of PCV2 induced apoptosis in melanoma cells in vitro and in vivo. We also examined whether Regulator of G protein Signalling 16 (RGS16), which binds specifically to PCV2 ORF3 [14] and may be involved in oncogenic pathways [15, 16], modulates the effect of apoptin on melanoma growth. While the control of autologous tumor growth was impaired in RGS16 KO mice, our data suggest that the effect of ORF3 does not implicate RGS16.

Materials and methods

Mice

Two mouse strains were used in this study. Wild type (WT) *C57BL/6* J mice were initially obtained from Dr. Sulev Kuuse, Institute of Molecular and Cell Biology, University of Tartu, (Estonia). RGS16 knockout (KO) mice generated on C57BL/6 genetic background were obtained from Pr. Kirk Druey, NIAID, Bethesda (USA). All mice used in this study were grown in the fish facilities if Tallinn Technical university. Before experiments RGS16 KO mice were backcrossed 6 generation to our *C57Bl6*/J from Tartu University, to ensure an homogeneous background in WT and RGS16KO. The total number of the mice used in this study was 44 (20 for the comparison of tumor growth in WT and RGS16 KO mice, and 24 for the comparison of tumor growth in WT and RGS16−/− mice treated with PCV2ORF3 or control plasmid).

Animal experiments

Animal handling and maintenance were performed according to the interdisciplinary principles and guidelines for the use of animals in research, testing and education (FELASA) prepared by the Ad Hoc Committee on Animal Research (The New York Academy of Sciences, New York, NY, USA). The animal experiments described in this study were authorized by the Ethical and Animal Welfare Committee of Estonia (University of Tartu, ERC PERMIT nr. 181 T-1). Mice were euthanized by cervical dislocation, according to our approved protocols.

Cultivation of cell lines

B16F10 mouse melanoma cells and A375M human metastatic melanoma cells were cultured in DMEM (Dulbecco's Modified Eagle Medium; PAA). THP-1 human acute monocytic leukemia cells were cultured in RPMI 1640 (Roswell Park Memorial Institute medium 1640; PAA). All media were supplemented with 12% fetal calf serum (FCS; Gibco) and 1% penicillin-streptomycin (Sigma). M2 human filamin-A-deficient melanoma cell line was cultured in MEM (Minimal Essential Medium; PAA), supplemented with 8% newborn calf serum (NBCS; Gibco), 2% FCS and 1% penicillin-streptomycin (Sigma). After transfection, cells were cultured in 1/1 mix of new medium and conditioned medium (culture supernatant collected from cell plates), greatly enhancing cell survival. The cells were maintained at 37 °C in a humidified incubator of 5% CO_2 in 95% air and subcultured twice per week. B16F10, A375, and M2 cells were provided by Dr. Teet Velling (Department of Gene Technology, Tallinn University of Technology, Estonia), THP-1 cells were provided by Prof Jean-Marc Cavaillon (Institute Pasteur, Paris, France).

Generation of the stable B16F10-luc cell line

B16F10 cell line expressing stably firefly luciferase (B16F10-luc) was generated using HIV1-based self-inactivating lentiviral vector system as described in [17]. Sequence encoding luciferase from the plasmid pGL3 (Promega) was cloned under the control of the strong constitutive human elongation factor 1-α promoter in a transfer vector. The virus was produced by transient transfection of the transfer vector with accessory plasmids in HEK293T cells and pseudotyped with VSV-G envelope protein. Viral supernatant was used to infect B16F10 cells from which single cell-derived positive clone was isolated.

Isolation and cultivation of primary cells

RGS16 knockout (KO) and wild-type (WT) C57BL/6 mice were anesthetized using carbon dioxide and euthanized by cervical dislocation. Spleens from mice were removed and cell suspensions were obtained by pressing the spleens through a cell strainer in DMEM (PAA). Erythrocytes were lysed with ACK (Ammonium-Chloride-Potassium) lysis buffer (Lonza) for 10 min at room temperature. Then, cells were pelleted by centrifugation, washed twice with PBS and cultured in DMEM supplemented with 10%

FCS (Gibco) at 37 °C in a humidified incubator of 5% CO_2 in 95% air.

Construction of expression plasmids

To generate the pcDNA-ORF3 expression construct, the ORF3 of PCV2 was PCR amplified from the purified PCV2 genome (GenBank, AF055392). The PCR products were cloned into mammalian expression vector pcDNA3.1 (Invitrogen) using the *Kpn*I/*Xho*I sites. Additionally, ORF3 was also cloned into the *Kpn*I/*Xho*I sites of pcDNA3.1-His plasmid (Invitrogen) and the coding region of mCherry, excised from the pBSK-mCherry E3 vector, was added using the *Xho*I/*Xba*I sites, thus giving rise the expression plasmid pcDNA3-his-ORF3-mCherry. To obtain the pCEP-GFP-RGS16 expression construct, the full-length cDNA of porcine RGS16 (GenBank accession EU271873) was RT-PCR-amplified from LPS-activated poPBMCs. The PCR products were cloned into the *Xho*I/*Eco*RI sites of the pCEP-GFP plasmid (BD Biosciences). All of the used PCR primers were designed on the basis of ORF3- and RGS16-encoding sequences and the appropriate plasmid sequences.

Transfection of cells

The plasmid pcDNA-ORF3 was transiently transfected into B16F10, A375M and M2 melanoma cells using Lipofectamine ™ LTX (Invitrogen) in accordance with the manufacturer's instructions. For comparison, pcDNA-ORF3 was also transiently transfected into THP-1 human acute monocytic leukemia cells. As a negative control, parallel transfections with empty pcDNA3.1 plasmid were carried out. The cells were seeded in six-well plates at a density of 5×10^5 cells per well. For each well of cells, 2.5 μg of plasmid DNA was diluted in 500 μl Opti-MEM®I Reduced Serum Medium, 2.5 μl of PLUS™ Reagent was added, and the mixture was incubated at room temperature for 10 min. 10 μl of Lipofectamine™ LTX was then added into the DNA solution and DNA: Lipofectamine complex was allowed to form by incubating at room temperature for 30 min. 500 μl of the DNA-Lipofectamine™ LTX complex was added to each well and the cells were then maintained at 37 °C in a humidified incubator with 5% CO_2 in 95% air. After 24–48 h of transfection, cells were harvested, transfection efficiency was determined (Additional file 1: Figure S1) and cells were used for various assays described below.

Investigation of apoptosis by AnnexinV/PI staining flow cytometry assay

The extent of apoptosis was determined by flow cytometry using FITC-labelled annexin V and propidium iodide (PI). The cells that were transfected with either a pcDNA-ORF3 or an empty pcDNA3 plasmid were analyzed for apoptosis using annexin-V-FITC/PI double staining and subsequent flow cytometry at 24 and 48 h after transfection according to the manufacturer's protocol. Approximately 1×10^6 cells were double stained with annexin-V-FITC and propidium iodide using the annexin-V-FITC Apoptosis Detection Kit (Sigma). Transfected cells were washed with cold PBS, resuspended in 1× binding buffer and combined with annexin-V-FITC and propidium iodide. The cells were incubated at room temperature in the dark for 15 min. Fluorescence was detected using a fluorescence-activated cell sorter (FACS Calibur, BD Biosciences). A total of 30,000 cells were collected for each sample and analyzed using CellQuest software (Becton Dickinson). A homogenous population of malignant cells or primary cells was chosen according to their size and granularity; debris and duplicates of cells were eliminated by gating. All treatments were performed at least in triplicate and all experiments were carried out three times.

Measurement of caspase activities

1. Caspase-3 and caspase-8 activities were measured first using a Caspase Colorimetric Assay Kit (Enzo) following the manufacturer's instructions. In brief, ORF3-transfected B16F10 melanoma cells and WT mouse primary splenocytes were collected and lysed in chilled lysis buffer at 24 and 48 h post-transfection. The supernatant was removed, and the total protein concentration of each sample was quantified using Nanodrop™ spectrophotometer. Then, 150 μg of protein was diluted to 50 μl cell lysis buffer for each assay. 50 μl of 2× reaction buffer (containing 10 mM DTT) and 5 μl of the 4 mM IETD-*p*NA or DEVD-*p*NA (200 μM final concentration) substrate were added to each sample and incubated at 37 °C for 2 h. The optical densities were measured at 405 nm with an ELISA microtiter plate reader (Thermo Scientific Multiskan FC Microplate Photometer). The cells transfected with the empty pcDNA3 plasmid were used as negative control.
2. Caspase-3 activity was measured thereafter using intracellular staining in flow cytometry and an antibody that recognizes only cleaved and active form of Caspase-3. Since caspase-8 can be activated by autocleavage and can then trigger effector pro caspase-3 we determined the presence of a cleaved and active form of caspase-3 inside of B16F10 melanoma and PK15 cells.

Cell death in the presence of caspase inhibitors

To reveal any underlying caspase-independent pathways induced by PCV2 ORF3 Q-VD-Oph, an irreversible pan-caspase inhibitor was added to the cells prior to treatment. Mouse melanoma B16F10 or porcine kidney 15 (PK15) cells were pretreated with 5 μM Q-VD-Oph

(quinolyl-valyl-O-methylaspartyl-[– 2, 6-difluorophenoxy]-methyl ketone) (Sigma) for 30 min to inhibit caspases and then transfected with PCV2-ORF3 or control plasmid as described before. Transfection efficiency, cell viability, and morphology were investigated as previously described. After 48 h cells were harvested and apoptosis was investigated by AnnexinV/PI staining as described above (in Flow Cytometry assay section).

Nuclear staining with 4′,6-diamidino-2-phenylindole

ORF3-transfected B16F10 cells grown on glass coverslips were washed 24 and 48 h post-transfection with PBS and subsequently fixed with 4% paraformaldehyde in PBS for 15 min. Fixed cells were then washed with PBS and incubated with 100 ng/ml DAPI (4′,6-diamidino-2-phenylindole; Sigma) in water for 30 min at room temperature in the dark. Stained slides were mounted using Fluoroshield Mounting Media (Sigma). The cells that were transfected with the empty plasmid were used as negative control. The nuclear morphology of the cells was examined by fluorescence microscopy. One hundred ORF3-positive and ORF3-negative control cells in mitotic phase were scored for normal/abnormal mitotic spindle formation.

Indirect immunofluorescence assay

For the indirect immunofluorescence assay, previously transfected and paraformaldehyde-fixed B16F10 cells were washed with PBS containing 0.1% saponin (PBS-S). Non-specific immunoreactions were blocked with PBS-S containing 1% bovine serum albumin (BSA; PAA) at room temperature for 40 min. The cells were then washed three times with PBS-S. Primary antibody, mouse anti-α-tubulin monoclonal antibody IgG (Dako) were diluted 1:1000 in PBS-S and incubated with cells at room temperature for 2 h. After washing with PBS-S, the cells were incubated with FITC-conjugated anti-mouse IgG (Dako) diluted 1:1000 in PBS-S at room temperature and protected from light for 1 h. After three further washes, nuclei were stained with DAPI as described above. The cells were visualized by fluorescence microscopy.

Co-expression of ORF3 and RGS16 in porcine peripheral blood mononuclear cells

Porcine peripheral blood mononuclear cells (poPBMC) were isolated from conventionally reared Yorkshire pig blood by density-gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech) and cultivated directly in coverslip that was placed in the bottom of 24 well plates. Porcine PBMCs were cultured in RPMI 1640 medium (Bio-Whittaker) supplemented with 20 mM HEPES, 2 mM L-glutamine, 200 IU penicillin ml^{-1}, 100 μg streptomycin ml^{-1}, 0.5 μM 2-Mercaptoethanol and 5% FCS at 37 °C in a humidified incubator with 5% CO_2 in 95% air. The cells were activated with LPS (lipopolysaccharide) from *Escherichia coli* serotype 0111: B6 (2,5 μg ml^{-1}; Sigma).

LPS-activated poPBMCs were then transiently transfected with pcDNA3.1-His-ORF3-mCherry in combination with pCEP-GFP-RGS16. The cells were seeded on glass coverslips placed in the bottom of six-well plates and transfected using FuGene® 6 reagent (Roche), following the manufacturer's instructions. The cells were harvested 48 h after transfection.

The endogenous expression of RGS16 and ORF3 in poPBMCs was determined by indirect immunofluorescence assay using a rabbit-human RGS16-specific polyclonal antibody (Aviva Systems Biology) and a mouse monoclonal antibody recognizing the 6× His (Clontech) tag of the histidine-tagged ORF3 construct, respectively. Porcine PBMCs were fixed in 4% paraformaldehyde and non-specific immunoreactions were blocked by using PBS-S containing 1% BSA. After incubation with the primary antibody, the cells were stained with FITC-labeled antibody to rabbit Ig (Dako) or with monoclonal anti-mouse Igκ coupled to Texas red (Serotech). The cells' nuclei were stained with the fluorescent dye Hoechst 33258 (Sigma). The cells were then visualized by fluorescence microscopy.

Fluorescence microscopy

All fluorescence microscopy was performed using an Axioplan II Imaging fluorescence microscope equipped with appropriate filter sets, an Axiocam charge-coupled device camera and Axiovision software (Carl Zeiss Light Microscopy). Digital images were processed using Adobe Photoshop version CC software.

Subcutaneous grafts of B16F10-luc melanoma cells in RGS16 knockout mouse model

Exponentially growing B16F10-luc cells expressing stably firefly luciferase were harvested and injected subcutaneously into the right flank of 6–7-week-old female WT and RGS16 KO mice (1×10^6 cells per implant). Tumor development was monitored by luciferase activity using IVIS Lumina II imaging system (Caliper Life Sciences, now PerkinElmer). Before the scan, mice were shaved, injected intraperitoneally with D-luciferin (Regis Technologies, Illinois, USA) at 150 mg/kg body weight and dozed with 2.5% isoflurane. Sequential images were taken at 2 min intervals to determine the peak value of the luciferase signal. After 18 days mice were sacrificed, and the tumors were isolated and weighed.

In vivo induction and treatment of melanoma

For in vivo induction of melanoma, 8–10-week-old WT and RGS16 KO C57BL/6 mice were injected subcutaneously into the derma of the right side of the back with 5×10^4 B16F10 cells in 50 μl PBS. After tumor transplantation,

its growth was followed at least every second day. After 18 days or with the appearance of either a dark pigmentation or a solid tumor on the melanoma inoculation site, 10 μg of pcDNA3-ORF3 expression plasmid (dissolved in 10 μl PBS) was injected into the tumor site. The control mice were injected with the same volume of the empty pcDNA3.1 plasmid. The length of the tumor was measured along the imaginary longitude of the back and its width was measured in the direction of the latitude with an accuracy of 0.5 mm using a digital caliper. Subcutaneous tumor volume was calculated using the following formula: $V = \pi/6 \cdot f \cdot (\text{length} \cdot \text{width})^{3/2}$, where $f = 1.69$ (+/− 0.3) for male mice and $f = 1.58$ (+/− 0.1) for female mice [18]. Mice were sacrificed at day 28 after B16F10 melanoma cells injection, and all primary tumors were excised and weighed.

Statistical analysis

The statistical significance was determined using Student's t-test. The significance level was set at a p-value of 0.05.

Results

PCV2 ORF3 induces apoptosis in tumor cell lines

In order to assess the pro-apoptotic activity of PCV2 ORF3 in cancer cells, several tumor cell lines were transiently transfected with an expression plasmid encoding ORF3 and were observed during the following 3 days. We selected B16F10 mouse melanoma cells, metastatic A375M human melanoma cells, filamin-A-deficient M2 human melanoma cells, and THP-1 human acute monocytic leukemia cells.

The fraction of apoptotic cells was measured by flow cytometry 48 h after transfection using annexin-V-FITC and propidium iodide double staining. Annexin-V single-positive cells (annexin+/PI-) were considered as early apoptotic, double-positive cells (annexin+/PI+) as late apoptotic, while double-negative cells (annexin-/PI-) and PI single-positive cells (annexin-/PI+) representing non-apoptotic and necrotic cells, respectively. Cells transfected with empty pcDNA3 were used as a negative control. The rate of apoptosis in three melanoma cell lines and monocytic leukemia cell line all overexpressing ORF3 was significantly higher than in ORF3-negative cells, transfected with empty plasmid (Fig. 1).

These results show that PCV2 ORF3 induces apoptosis in cancer cells, and therefore appears to function similarly to its homologs in other circoviruses.

B16F10 mouse melanoma cells were selected for further studies on the basis of their higher transfection efficiency and sensibility to ORF3-induced apoptosis.

PCV2 ORF3 expression elicits nuclear morphological changes characteristic of apoptosis in B16F10 cells

We also examined nuclear morphology in B16F10 mouse melanoma cells that were transfected with the ORF3 expression plasmid or with an empty pcDNA3 vector as a control. Nuclear morphology was analyzed by fluorescence microscopy of cells stained by the fluorescent DNA-binding agent DAPI at 24 and 48 h post-transfection.

No obvious nuclear morphological changes were observed in ORF3-negative control cells (Fig. 2Aa), showing low fluorescence intensity and homogeneous nuclear chromatin. In contrast, ORF3-transfected B16F10 cells displayed intense blue fluorescence and typical apoptotic morphological features, including nuclear condensation and nuclear fragmentation (Fig. 2Ab). In addition, cell proliferation of ORF3-transfected cells was reduced compared with control cells at 48 h post-transfection (Fig. 2B).

DAPI staining demonstrated that ORF3 expression elicited nuclear morphological changes characteristic of apoptosis in B16F10 cells, confirming that PCV2 ORF3 indeed induces cell death through apoptosis.

ORF3 of PCV2 causes abnormal mitosis in B16F10 cells

The impact of PCV2 ORF3 overexpression on mitosis of B16F10 mouse melanoma cells was also studied. Endogenous tubulin was stained 48 h post-transfection with a FITC-labeled anti-α-tubulin antibody and DNA was stained with DAPI. One hundred ORF3-positive and ORF3-negative control cells in mitotic phase were scored for normal/abnormal mitotic spindle formation by fluorescence microscopy.

Most ORF3-negative B16F10 cells in the mitotic phase displayed normal, i.e. symmetric and bipolar mitotic spindle formation with only 6% of abnormality (Fig. 3, Additional file 2: Movie). B16F10 cells expressing ORF3 seems to display chromosome misalignment, disrupted mitotic spindles and abnormal mitosis at increased frequently (18%) These data would suggest that ORF3 expression causes an increase in abnormal mitosis formation in B16F10 cells that was not observed in ORF3-negative control cells.

PCV2 ORF3 induces apoptosis in B16F10 cells through a Caspase-8 and Caspase-3 independent pathway

In order to characterize the apoptotic cell signaling pathway induced by PCV2 ORF3, activation of initiator caspase-8 and effector caspase-3 were examined in ORF3-transfected B16F10 mouse melanoma cells and WT mice primary splenocytes. Cells transfected with the empty pcDNA3.1 were used as negative control. Cell lysates were analyzed 24 and 48 h post-transfection for IETDase and DEVDase activity using specific colorimetric substrate IETD-*p*NA (caspase-8) or DEVD-*p*NA (caspase-3). B16F10 cells expressing ORF3 did not show any significant increase of caspase-8 or caspase-3 activity, compared with those transfected with an empty plasmid (Additional file 3: Figure S2).

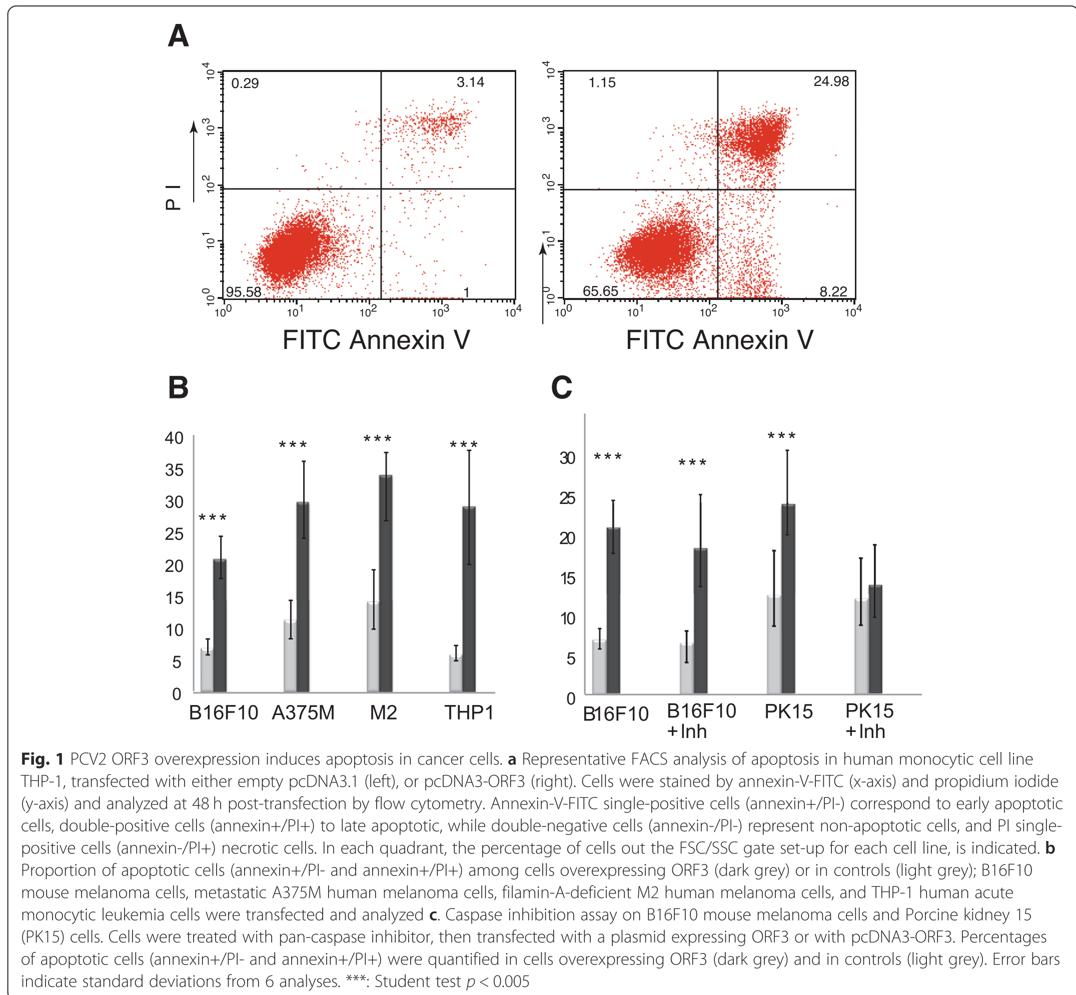

Fig. 1 PCV2 ORF3 overexpression induces apoptosis in cancer cells. **a** Representative FACS analysis of apoptosis in human monocytic cell line THP-1, transfected with either empty pcDNA3.1 (left), or pcDNA3-ORF3 (right). Cells were stained by annexin-V-FITC (x-axis) and propidium iodide (y-axis) and analyzed at 48 h post-transfection by flow cytometry. Annexin-V-FITC single-positive cells (annexin+/PI-) correspond to early apoptotic cells, double-positive cells (annexin+/PI+) to late apoptotic, while double-negative cells (annexin-/PI-) represent non-apoptotic cells, and PI single-positive cells (annexin-/PI+) necrotic cells. In each quadrant, the percentage of cells out the FSC/SSC gate set-up for each cell line, is indicated. **b** Proportion of apoptotic cells (annexin+/PI- and annexin+/PI+) among cells overexpressing ORF3 (dark grey) or in controls (light grey); B16F10 mouse melanoma cells, metastatic A375M human melanoma cells, filamin-A-deficient M2 human melanoma cells, and THP-1 human acute monocytic leukemia cells were transfected and analyzed **c**. Caspase inhibition assay on B16F10 mouse melanoma cells and Porcine kidney 15 (PK15) cells. Cells were treated with pan-caspase inhibitor, then transfected with a plasmid expressing ORF3 or with pcDNA3-ORF3. Percentages of apoptotic cells (annexin+/PI- and annexin+/PI+) were quantified in cells overexpressing ORF3 (dark grey) and in controls (light grey). Error bars indicate standard deviations from 6 analyses. ***: Student test $p < 0.005$

To reveal any underlying caspase-independent pathways induced by PCV2 ORF3 Q-VD-Oph, an irreversible pan-caspase inhibitor was added to mouse melanoma (B16F10) and porcine kidney 15 (PK15) cells 30 min prior transfection with PCV2-ORF3 encoding or empty plasmid. The presence of caspase-3 activities was measured thereafter using intracellular staining in flow cytometry and an antibody that recognizes only cleaved and active form of caspase-3. Since caspase-8 can be activated by autocleavage and it can then trigger effector procaspase-3, we determined the presence of a cleaved and active form of caspase-3 inside B16F10 and PK15 cells. Using intracellular staining and flow cytometry we could detect caspase 3 activity in PK15 cells and not in B16F10 melanoma cells (Fig. 4).

These results indicate that PCV2 ORF3 induces apoptosis in B16F10 and in mouse primary splenocytes in a caspase-8- and – 3-independent manner (Additional file 3: Figure S2) while in porcine kidney cell line PCV2 ORF3 induces apoptosis at least in caspase-3 dependent way.

PCV2 ORF3 exhibits anti-tumor effects in vivo

Melanoma growth was assessed in 7–8-week-old mice after subcutaneous injection of B16F10-luc melanoma cells expressing stably and constitutively firefly luciferase. A clonal B16F10-luc cell line had been generated by lentiviral transduction and subsequent subcloning [17]. We took advantage of the histocompatibility of this cell line and regulator of G protein 16 (RGS16) knock out mice to test the impact of RGS16 on the tumor growth.

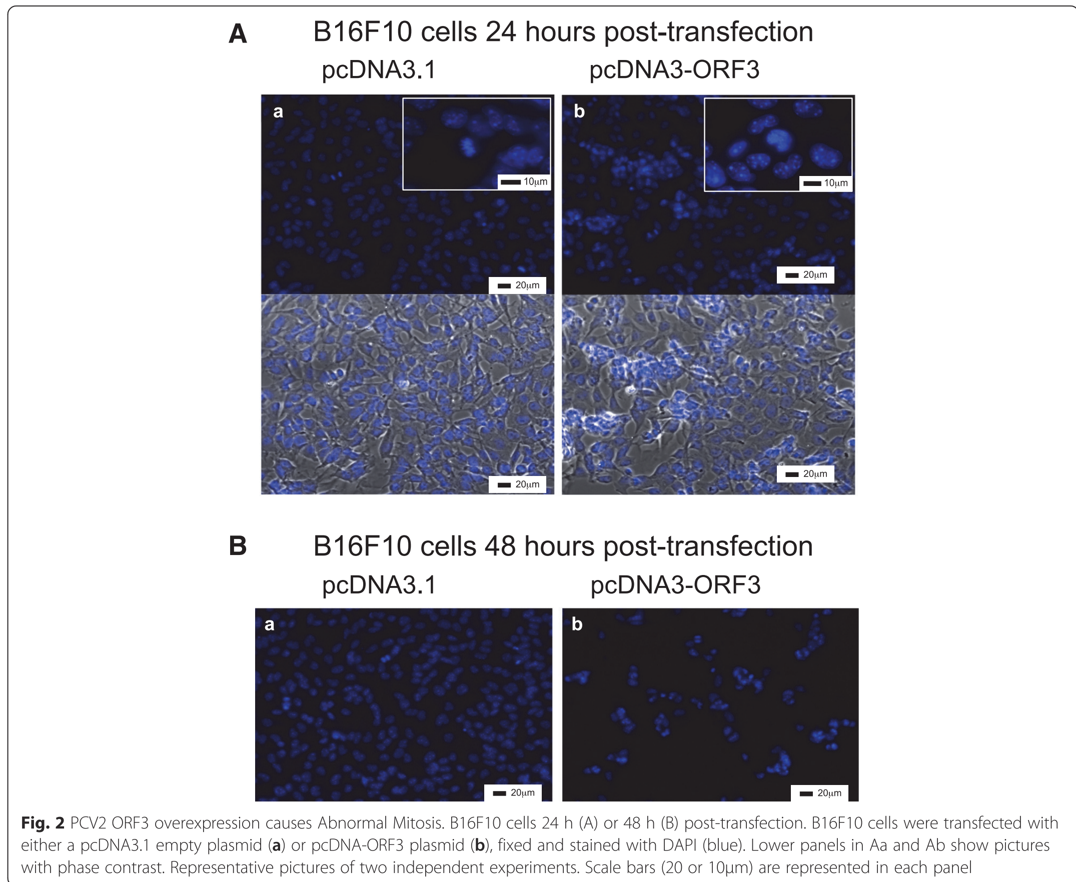

Fig. 2 PCV2 ORF3 overexpression causes Abnormal Mitosis. B16F10 cells 24 h (A) or 48 h (B) post-transfection. B16F10 cells were transfected with either a pcDNA3.1 empty plasmid (**a**) or pcDNA-ORF3 plasmid (**b**), fixed and stained with DAPI (blue). Lower panels in Aa and Ab show pictures with phase contrast. Representative pictures of two independent experiments. Scale bars (20 or 10μm) are represented in each panel

Tumor progression was monitored for 28 days (tumor transplantation set-up as day 0), then mice were sacrificed, and the tumors weighed. The effect of ORF3 on tumor development was tested by injection of ORF3 expression plasmid or a control empty pcDNA3.1 plasmid directly into the tumor region as soon as an area of dark pigmentation or a solid nodular tumor appeared at the site of injection. Pigmentation became first visible at day 15 after tumor implantation and by day 18 it was possible to measure the tumor by caliper. In literature, several methods have been proposed for the calculation of tumor size, most often based on the measurement of the three dimensions of the tumor. We followed the growth of tumors by measuring two dimensions - the height and width of the tumor using an empirical formula developed by Feldman [18]. The final tumor weight is in good correlation with tumor volume calculated according to this empirical formula ($R = 0.88$).

A significant difference in tumor growth between pcDNA-ORF3 and pcDNA3.1 treated groups was observed in the following weeks (compare Fig. 5a and b): the visible appearance of tumors occurred in average at day 18 and 25 in control and ORF3 treated mice, respectively. Additionally, the end weight of tumors was much lower when the ORF3 expression plasmid was injected (Fig. 5a and b).

Hence, overexpression of ORF3 in the tumor reduced the growth of the melanoma in vivo.

The expression of RGS16 by the tissues surrounding the tumor controls its growth but is not required for the anti-tumoral effect of ORF3 in vivo

We have previously shown that PCV2 ORF3 interacts with the Regulator of G protein Signalling (RGS)-16 [14]. RGS16 is involved in the regulation of signaling via

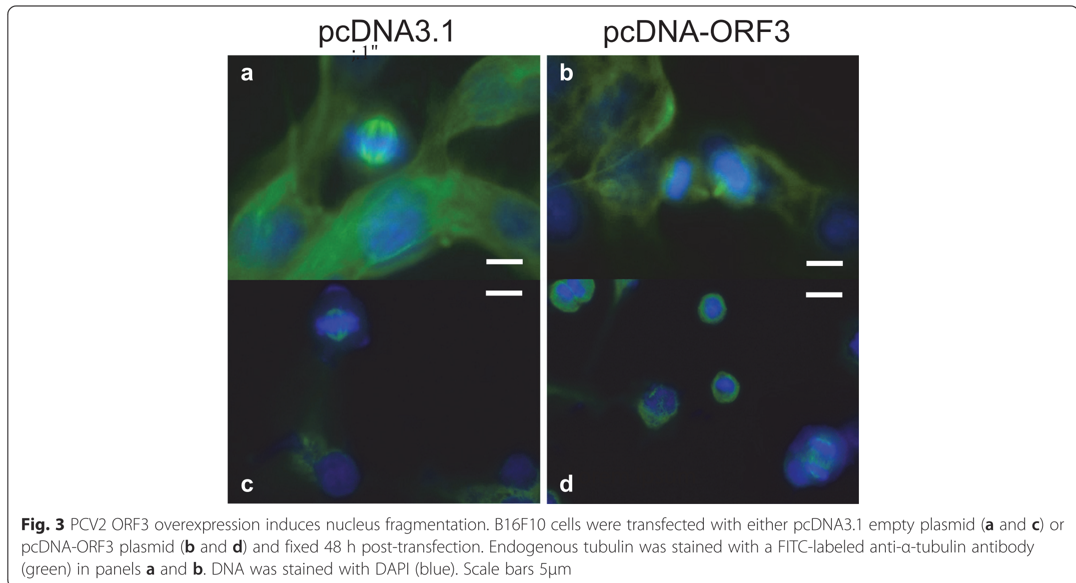

Fig. 3 PCV2 ORF3 overexpression induces nucleus fragmentation. B16F10 cells were transfected with either pcDNA3.1 empty plasmid (**a** and **c**) or pcDNA-ORF3 plasmid (**b** and **d**) and fixed 48 h post-transfection. Endogenous tubulin was stained with a FITC-labeled anti-α-tubulin antibody (green) in panels **a** and **b**. DNA was stained with DAPI (blue). Scale bars 5μm

several GPCRs, including chemokine receptors, and plays an important role in the activation of the antiviral response [19]. RGS16 also contributed to the translocation of ORF3 to the cell nucleus in activated cells co-transfected with RGS16 and ORF3 [14]. We, therefore, tested the idea that RGS16 might be involved in the induction of apoptosis by PCV2 ORF3. For this purpose, we used the in vivo melanoma model described above.

B16F10-luc melanoma cells expressing stably luciferase were injected subcutaneously into WT and RGS16 KO mice, and tumor progression was monitored for 28 days. Mice were then sacrificed and tumors weighed.

Strikingly, RGS16 expressed by the recipient mouse controlled tumor development since larger primary tumors developed in RGS16 KO mice compared to WT (Fig. 6a). The difference in tumor weight between WT

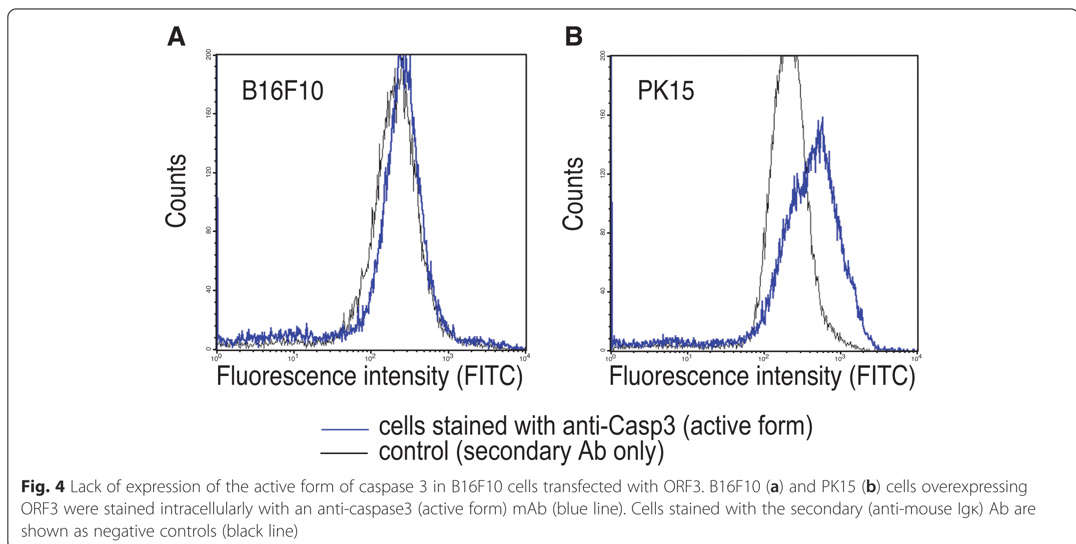

Fig. 4 Lack of expression of the active form of caspase 3 in B16F10 cells transfected with ORF3. B16F10 (**a**) and PK15 (**b**) cells overexpressing ORF3 were stained intracellularly with an anti-caspase3 (active form) mAb (blue line). Cells stained with the secondary (anti-mouse Igκ) Ab are shown as negative controls (black line)

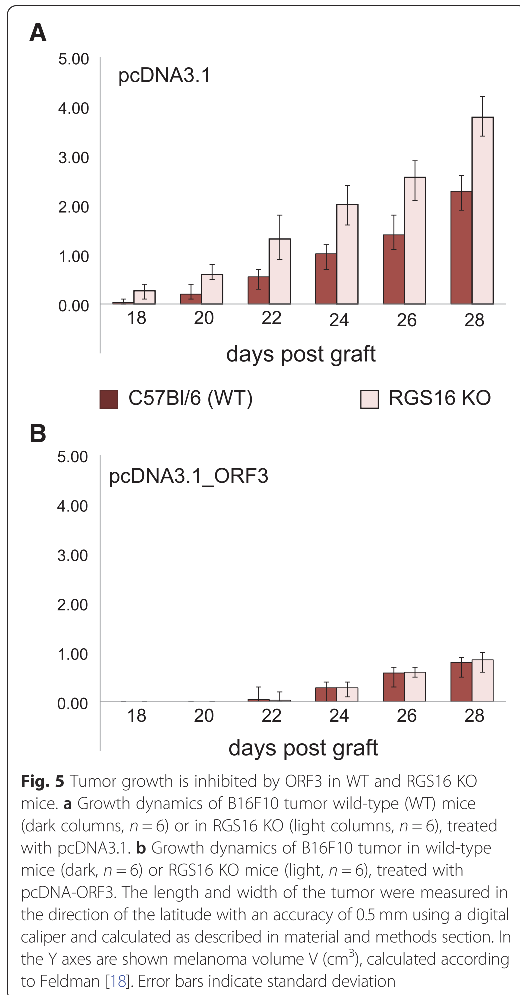

Fig. 5 Tumor growth is inhibited by ORF3 in WT and RGS16 KO mice. **a** Growth dynamics of B16F10 tumor wild-type (WT) mice (dark columns, $n = 6$) or in RGS16 KO (light columns, $n = 6$), treated with pcDNA3.1. **b** Growth dynamics of B16F10 tumor in wild-type mice (dark, $n = 6$) or RGS16 KO mice (light, $n = 6$), treated with pcDNA-ORF3. The length and width of the tumor were measured in the direction of the latitude with an accuracy of 0.5 mm using a digital caliper and calculated as described in material and methods section. In the Y axes are shown melanoma volume V (cm^3), calculated according to Feldman [18]. Error bars indicate standard deviation

Fig. 6 Tumor growth in enhanced in RGS KO mice. **a** Bioluminescence imaging of B16F10-luc subcutaneous grafts in WT and RGS16 KO mice at 8 days post-injection. Total flux (radiance) of the ROI (region of interest) of WT = $4229 \cdot 10^5$ (p/s, photons per second). Total flux of the ROI of RGS16 KO = $2111 \cdot 10^7$ p/s. Exposure time 3 min. **b** Melanoma graft tumor weight (g), isolated from female C57BL6 WT mice ($n = 10$) and RGS16 KO mice ($n = 10$) 21 days after cell engraftment. The difference in tumor weight between WT and RGS16 KO mice was statistically significant ($p < 0.05$) regardless of vast individual variability

and RGS16 KO mice was statistically significant ($p < 0.05$) in spite of high individual variability (Fig. 6b). Thus, RGS16 is involved in the immune response against tumors, possibly by affecting immune cell activation and infiltration into the site of the tumor or influencing the local milieu in the tissue. However, the tumor development started at the same time both in WT and RGS16 KO mice.

Importantly, we observed that the decrease in tumor growth induced by the overexpression of ORF3 was still observed in RGS16 KO mice, demonstrating that an RGS16 expression around the tumor is not required for ORF3 antitumoral activity. It also suggests that the mechanisms through which ORF3 control the tumors are predominant over the impact of the RGS16 $^+/_+$ cellular environment of the tumor (Fig. 6a and b).

Altogether, our data show that ORF3 mediates an anti-tumoral effect in vivo, most likely by inducing apoptosis of cancer cells through mechanisms independent of the expression of RGS16 in the host tissues.

Discussion

We report here a pro-apoptotic activity of the PCV2 ORF3, a homolog of the apoptin of the circovirus chicken anemia, which has been previously reported to induce cell death in human cancer cells [20, 21]. Our results are consistent with another study describing a pro-apoptotic capacity of PCV2 ORF3 in porcine kidney cells [2, 22].

The rate of apoptosis in different melanoma cell lines overexpressing ORF3 was significantly higher than in ORF3-negative control cells, indicating that PCV2 ORF3 is able to induce apoptosis in malignant melanoma cells at least in vitro. THP-1 cells, which divide faster than melanoma cells, when overexpressing ORF3, also show a higher rate of apoptosis, suggesting that PCV2 ORF3-induced apoptosis might be generally facilitated in rapidly

dividing cancer cells. Similar results have been observed earlier with CAV apoptin, which can induce programmed cell death in human transformed and malignant cells but fails to induce cell death in normal cells [23]. In normal cells, CAV apoptin is predominantly localized to the cytoplasm where it is efficiently degraded by the proteasome, whereas in transformed cells it localizes to the nucleus [24, 25]. Similarly, PCV2 ORF3 contains a putative nuclear localization signal (NLS) and a nuclear export signal (NES) and shows expected localization pattern.

Caspases play a critical role in the induction of apoptosis and are responsible for the morphological changes characteristic of apoptosis [26]. Previous studies have demonstrated that PCV2 ORF3 induces apoptosis in PK-15 cells and poPBMCs through the extrinsic pathway via sequential activation of caspase-8 and-3 [2]. In the current work, no significant differences in caspase-8 and-3 activity could be detected between ORF3-expressing and non-expressing B16F10 cells using a well-established assay, which suggests that apoptosis might not be induced here via the same pathway. These results indicate that PCV2 ORF3 induces apoptosis in cancer cells most probably in a caspase-independent manner. Caspase-independent cell death has been described in cervical cancer, where apoptosis was induced by oxidative stress-mediated activation of p53 and p38 with selenium [27].

Q-VD-OPh is a potent, cell-permeable inhibitor of caspase activity that has increased selectivity for the caspases over other cysteine proteases. The addition of Q-VD-OPh prevents the maturation of caspase-3 subunit p19 into the fully active p17, which explains the lack of PARP1 cleavage and inhibition of apoptosis under these conditions.

In fact, several studies suggest that PCV2 ORF3 protein-induced apoptosis may occur through a caspase-independent, p53-dependent pathway. The ORF3 protein has been shown to induce apoptosis in PK-15 cells by competitive binding to the E3 ubiquitin-protein ligase Pirh2, over at the expense of p53 binding. The ORF3-mediated inhibition of the p53-Pirh2 interaction has been proposed to lead to nuclear accumulation and activation of p53 and may increase the propensity of the cells to undergo apoptosis [13]. Apoptotic dysregulation is a hallmark of melanoma pathogenesis and chemoresistance. Mutations in p53 are seldom observed in melanoma and they are not critical for tumor development. Moreover, the presence of intact p53 can even increase the rate of apoptotic and autophagic cells [28]. Since p53 is often structurally preserved, but functionally defective due to loss of CDKN2A in melanoma, restoration of p53 function in melanoma could be a potential therapeutic strategy [29]. Therefore, the ORF3-mediated inhibition of the p53-Pirh2 interaction might represent an attractive mechanism for activating wild-type p53 in tumors by an anticancer agent. Since approximately half of all human tumors carry a mutation in the p53 gene, it is important to note that p53-dependent treatment strategies are useless against those cancer types.

Viruses can also encode caspase inhibitors as for example p35 (a baculovirus gene product) that may prevent cell death induced by various stimuli. Expression of p35 during viral infection may result in the inhibition of several caspases and make cells resistance to cell death [30]. PCV2 genome also encodes a protein with an anti-apoptotic activity called ORF4 that is smaller and overlaps with ORF3 in the same direction. Previous results have indicated that the ORF4 protein may play an important role by restricting ORF3 transcription thereby preventing virus-induced apoptosis [31]. It may be possible that in some host cells - PCV2 virus is able to infect only limited species and cell lines included PK15 - both proteins ORF3 and ORF4 are expressed to regulate cell death in the host.

Induction of apoptosis by PCV2 ORF3 in B16F10 cells was further confirmed by typical apoptotic morphological features, such as nuclear shrinkage and nuclear fragmentation. We also observed that cells expressing ORF3 show abnormal division figures. Interestingly, the localization of ORF3 in porcine PBMC transfected with an overexpression plasmid for an ORF3-mCherry fusion protein suggests that it might be coupled to centrosomes (Additional file 4: Figure S3), which could explain its impact on mitosis. Centrosomes have a crucial role in the formation of bipolar mitotic spindles, which are essential for accurate chromosome segregation into two daughter cells during cytokinesis. Cells with aberrant mitotic spindle formation fail to undergo cytokinesis, that, in turn, triggers the checkpoint response involving the p53, eventually leading to cell death [32]. Therefore, results presented here suggest that PCV2 ORF3 may cause apoptosis in cells through induction of abnormal mitoses by interacting with centrosomes and thereby disrupting mitotic spindle formation. However, further studies are required to elucidate the exact molecular mechanisms of ORF3-induced apoptosis.

We also showed that ORF3 can inhibit tumor growth in vivo, in a mouse model of melanoma graft. In mice injected with pcDNA-ORF3, the development of melanoma was delayed, the tumor growth was lower, and the final weight was significantly smaller compared to mice injected with the empty pcDNA3.1 vector.

Since ORF3 of PCV2 interacts directly with the RGS16 [14] and a number of studies have shown a role for RGS16 in the pathogenesis of several cancers [33–35], we tested whether RGS16 might be involved in the ORF3-mediated inhibition of melanoma growth. RGS16 KO mice developed larger primary tumors than WT mice after injection of melanoma cells, indicating that RGS16 expressed in the tissues surrounding the tumor has a protective effect [36]. However, our experiments

show clearly that this expression of RGS16 is not necessary for the antitumoral activity of ORF3, indicating that the mechanisms are distinct.

Conclusion

Taken together, our results show that PCV2 ORF3 causes non-bipolar mitosis in rapidly dividing cells and induces apoptosis. This antitumoral activity was observed in vitro and in vivo and is apparently independent of caspase 3 and caspase 8 and uses distinct mechanisms from those triggered by the host RGS16. We hope that the development of antitumor therapies might benefit from the understanding of the apoptin-dependent antitumoral activity.

Additional files

Additional file 1: Figure S1. Transfection efficiency. To find out the most effective way for overexpression of recombinant proteins DNA was introduced into all cell lines by transfection with Lipofectamine (LTX) or polyethyleneimine (PEI). Consequently, the DNA: LTX and DNA: PEI complex penetrate cell membranes and recombinant proteins are produced. After 24–48 h of transfection, cells were harvested, and transfection efficiency of every experiment was determined using parallel wells that were transfected only with the plasmid encoding green flourescent protein (GFP). Since LTX was more effective transfection agent compared to PEI in all used cell lines, it was used in subsequent experiments. (PDF 417 kb)

Additional file 2: Movie. ORF3 expression causes an increase in abnormal mitosis formation in B16F10 cells. The movie shows that B16F10 cells expressing ORF3 can display chromosome misalignment, disrupted mitotic spindles and abnormal mitosis. (MOV 139 kb)

Additional file 3: Figure S2. PCV2 ORF3 Induces Apoptosis in B16F10 Cells through a Caspase-8 and Caspase-3 Independent Pathway. Analysis of caspase-8 and -3 activities of pcDNA3-ORF3 or empty pcDNA3.1 plasmid transfected B16F10 cells at 24 and 48 h post-transfection. pcDNA3-ORF3 24 h (1st bar); pcDNA3-ctr 24 h (2nd bar); pcDNA3-ORF3 48 h (3rd bar); pcDNA3-ctr 48 h (4th bar). Error bars are representative of the standard deviation of triplicates. B: Analysis of caspase-8 and -3 activities of pcDNA3-ORF3 or empty pcDNA3.1 plasmid transfected c57/bl6 mice primary splenocytes at 24 h post-transfection. pcDNA3-ORF3 24 h (1st bar); pcDNA3-ctr 24 h (2nd bar); Non-treated mouse primary splenocytes were used as control (3rd bar); pcDNA3-ORF3 24 h blue bars; pcDNA3-ctr 24 h red bars; Non-treated mouse primary splenocytes - green bars; Error bars are representative of the standard deviation of triplicates. (PDF 496 kb)

Additional file 4: Figure S3. PCV2 ORF3 intracellular expression pattern in porcine PBMC. The intracellular localization of PCV2 ORF3 (red) and RGS16 (green, here a counterstaining) was examined in LPS-activated poPBMCs co-transfected with pcDNA3.1-His-ORF3-mCherry and pCEP-GFP-RGS16, then stained with Texas red and FITC 48 h post-transfection. The cells nuclei were stained with the Hoechst 33258 (blue). The cytoplasmic dot-like staining pattern of PCV2 ORF3 is indicated by arrows in all panels. (PDF 1021 kb)

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Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MT, EV and SRB: conceived and designed this study. EV, VP, CL, MP-H, AR, IP and PP developed the methodology. EV, VP, MP-H, AR, CL, SRB were responsible for data acquisition. MT, EV, AR, PP, IP, MP-H, VP, CL and SRB analysed the data. MT and SRB supervised this study. All authors contributed to interpretation of the data. MT, SRB and EV were major contributors in writing the manuscript, and all the other authors were involved in drafting the manuscript and revised it. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal handling and maintenance were performed according to the interdisciplinary principles and guidelines for the use of animals in research, testing and education (FELASA) prepared by the Ad Hoc Committee on Animal Research (The New York Academy of Sciences, New York, NY, USA). The animal experiments described in this study were authorized by the Ethical and Animal Welfare Committee of Estonia (University of Tartu, ERC PERMIT nr 181 T-1).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publication III

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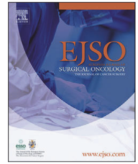

First Eastern European experience of isolated limb infusion for in-transit metastatic melanoma confined to the limb: Is it still an effective treatment option in the modern era?

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ABSTRACT

Background: Isolated limb infusion (ILI) with cytotoxic agents is a simple and effective treatment option for patients with melanoma in-transit metastases (ITMs) confined to an extremity. Data for ILIs performed in Europe are sparse and to date no Eastern European ILI experience has been reported. The aim of the current study was to evaluate the efficacy of ILI in Estonia.
Patients and methods: Data for twenty-one patients were collected and analysed. All patients had melanoma ITMs and underwent an ILI between January 2012 and May 2018. The cytotoxic drug combination of melphalan and actinomycin-D was used. Drug circulation times were 20–30 min under mildly hyperthermic conditions (38–39 °C). Primary outcome measures were treatment response and overall survival.
Results: Nineteen lower limb and two upper limb ILIs were performed. The female to male ratio was 18:3. The overall response rate (complete + partial response) was 76% (n = 16), with a complete response in 38% (n = 8). The overall long-term limb salvage rate was 90% (n = 19). During follow-up, eight patients (38%) died, two due to metastatic melanoma. Five-year overall survival was 57%.
Conclusion: This first Eastern European report of ILI for melanoma ITMs shows results comparable to those from other parts of the world. In this era of effective targeted and immune therapies, ILI remains a useful treatment option, with a high overall response rate and durable responses in patients with melanoma ITMs confined to a limb.

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Introduction

The incidence of skin cancers, mostly basal cell carcinoma, squamous cell carcinoma and melanoma, continues to rise in the Western world [1]. Of these three, melanoma has the worst prognosis due to its metastatic potential. After treatment of the primary melanoma, it has been reported that 4.3–6.6% of patients develop in-transit metastases (ITMs), defined as metastatic lesions between the primary tumour site and the nearest lymph node basin with reported rates of ITMs exceeding 20% in patients with more advanced stage primary melanomas [2,3]. Without treatment, the quality of life of these patients is often poor, due to ulcerated, bleeding and sometimes painful lesions as well as limited limb functionality [4]. Furthermore, achieving a favourable response following treatment of ITMs is associated with improved overall survival [5,6].

In recent years, new immune-modulating and targeted

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therapies have dramatically changed the treatment of patients with metastatic melanoma. However, there are large variations in the availability of these new and costly therapies around the world, and according to a recent study from Europe a large proportion of melanoma patients have restricted access to them [7]. In these circumstances, clinicians mostly continue to use less costly locoregional treatment options.

Traditionally, ITMs confined to a limb have often been treated by hyperthermic isolated limb perfusion (ILP). However, due to its technical complexity, the procedure has not been widely used, despite complete response rates of 25–89% and favourable five-year overall survival rates of 32–50% [6]. Isolated limb infusion (ILI) was introduced in the early 1990's by Thompson et al. at the Sydney Melanoma Unit in Australia (now Melanoma Institute Australia) as a minimally-invasive alternative to ILP [8]. Since then, ILI has proved to be an attractive treatment modality to achieve regional disease control and limb preservation in melanoma patients with ITMs confined to a limb [9]. However, there are few ILI reports available from centres outside Australia and North America, with only two publications originating from Europe and none from Eastern Europe [10,11]. Therefore, the present study was conducted to analyse the Eastern European ILI experience in Estonia, to evaluate its feasibility and its efficacy outside of high-volume centres elsewhere in the world.

Patients and Methods

The data for all patients with melanoma ITMs treated by ILI between January 2012 and May 2018 at the Centre for Surgical Oncology and General Surgery in the North Estonia Medical Centre Foundation, Tallinn were collected retrospectively. All patients provided informed consent for data collection and usage for clinical research.

The North Estonia Medical Centre Foundation is the only institution in the region with an ILI program and functions as a tertiary referral centre for melanoma. The decision for either radical surgical resection or an ILI if the ITMs were deemed unresectable was made during a melanoma multidisciplinary team meeting in which all patients were discussed and evaluated by an expert group. Burden of disease (BOD) of the affected limb was defined as low ($\leq$10 lesions with no lesion >2 cm) or high (>10 lesions or any one lesion >2 cm) [9].

All patients underwent ^{18F}-FDG PET/CT imaging preoperatively to identify the presence and extent of active disease in the affected limb and elsewhere.

A schematic overview of the ILI procedure is shown in Fig. 1. The ILI procedure was performed in accordance to the Memorial Sloan Kettering Cancer Center (MSKCC) protocol [12,13]. In brief: high-flow 6F or 7F arterial and venous catheters (Bernstein Occlusion Catheter, Boston Scientific®, MA, USA) were inserted via the femoral or axillary artery and vein under fluoroscopic guidance with their tips placed at the level of the knee or elbow joint. Catheters were placed on the day of the procedure or the day before in case of an early ILI. No systemic heparin was administered during catheter placement. In eight patients, the catheters were inserted via the contra-lateral femoral vessels and in 13 patients they were inserted via the ipsilateral femoral or axillary vessels. After insertion of the catheters, low-dose heparin infusion through them was initiated and continued until full systemic heparinization immediately before the ILI procedure.

Limb volume was determined by taking circumferential limb measurements at 1.5 cm intervals as described by Beasley et al. [14]. A standard dose of 7.5 mg/L of melphalan and 75μg/L of actinomycin-D was used for both lower limb and upper limb ILIs, with dosage modification made according to the patient's ideal body weight (IBW) [15,16]. The cytotoxic agents were admixed with 400 mL of heparinized normal saline. A proximal pneumatic tourniquet was applied once subcutaneous (SC) temperatures reached 37.0 °C, after which the chemotherapeutic drugs were infused into the limb over 5–10 min via the arterial catheter through a heating coil. Heating of the limb started before chemotherapeutic drug infusion and continued throughout the study with patient-warming systems. The infusate was then circulated manually for 20–25 min using a 60 mL syringe and 3-way stopcock. Needle probes were used to monitor the SC temperatures continuously, aiming for 38.5 °C by the end of the procedure. Upon completion of drug circulation, the limb was flushed with 1 L of Ringer's solution via the arterial catheter, while as much venous blood as possible was extracted from the limb via the venous catheter using suction attached to it, with the effluent discarded as cytotoxic waste. The tourniquet was then deflated and removed and heparin fully reversed with protamine 3 mg/kg.

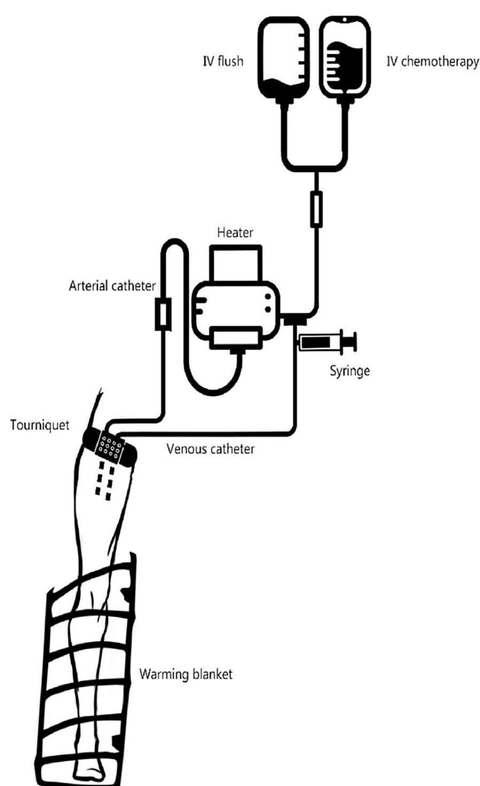

Fig. 1. Schematic diagram of the ILI circuit. Chemotherapy is rapidly infused using a pressurised circuit incorporating a blood-warmer with bubble excluder. Venous blood is manually extracted from the limb using a 60 ml syringe and reinjected into the isolated circuit.

Postoperatively, patients were monitored in the recovery unit for 2–3 h and subsequently transferred to the surgical ward. Limb toxicity was assessed by physical examination daily during the hospital stay and after 3 months using the scale proposed by Wieberdink et al. [17]. As a prophylactic measure, for the duration of their hospital stay, all patients received low-molecular-weight heparin (LMWH), 4000IU enoxaparin sodium SC, starting directly after the procedure until discharge. Post-procedure limb pain was

assessed using the visual analogue scale (VAS); myoglobin levels and limb circumference changes were monitored, to assess limb muscle toxicity and limb swelling.

Follow-up consisted of an outpatient clinic visit every 3 months, with assessment of response using the RECIST 1.1 guidelines for solid tumors [18]. The response to ILI was evaluated at 6 months. All patients underwent ^{18F}-FDG PET/CT imaging twice during the first year after ILI, and once yearly thereafter.

Data were analysed using descriptive statistics and the Mann Whitney *U* test. Kaplan–Meier curves were used to display overall survival. Data analysis was performed using JMP 10.0 (SAS®) and Microsoft Excel software (Microsoft ®).

Results

Patient details, tumour characteristics and perioperative parameters are listed in Table 1. A total of 21 patients underwent an ILI procedure, 18 of whom were female. The median age was 72 years (range 30–88 years). There were 19 lower limb ILIs performed and 2 upper limb ILIs. BOD was low in 7 patients (33%), and high in 14 patients (67%). The mean Breslow thickness of the primary melanoma was 7.5 mm (range 2.01–14.0 mm) and ulceration of the primary melanoma was present in 8 patients (42%) and 14 patients (67%) had a high mitotic rate.

In all but one patient a single ILI procedure was performed, while one female patient underwent a repeat ILI for recurrent upper limb ITMs two years after an initial ILI. On average, removal of the primary melanoma was performed 24 months (range 4–60 months) prior to the ILI procedure. In all patients, at least one attempt at surgical removal of one or more ITMs had been carried out before ILI was considered. Three patients (14%) underwent multiple surgical resections of ITMs with a median time to recurrence of 5 months (range 2–8 months).

Median limb volume for the lower limb was 10.7 L (range 5.7–12.9 L) and for the upper limb 2.6 L (range 2.5–2.7 L). The median melphalan dose for lower limb procedures was 46.6 mg (range 13.6–66 mg), and 16.3 mg (range 13.6–19.1 mg) for upper limb ILIs. For actinomycin-D the median dose was 490.1 μg (range 427.5–500 μg) and 237 μg (range 195–280 μg), respectively. Median hospital stay was 16 days (range 9–24 days). Limb toxicity was mild (Wieberdink grade II) in 13 patients (60%) and moderate (grade III) in six patients (30%). Two patients (10%) experienced grade IV limb toxicity: One patient underwent a fasciotomy and recovered fully. The second patient developed muscle necrosis of the superficial posterior tibial compartment, which was surgically debrided at the time of a fasciotomy.

Limb swelling, assessed by the change in circumference, reached its maximum on postoperative day 4. The median post-procedure peak myoglobin level was 1366 μg/L (range 67–6937 μg/L; normal range 19–72 μg/L). Patients with Wieberdink grade II toxicity had a significantly lower peak myoglobin (319 μg/L, range 67–2193 μg/L) compared to patients with who had Wieberdink grade III/IV toxicity (2202 μg/L, range 1430–6937 μg/L; $p = 0.001$). Myoglobin levels rose on post-procedure day 3 and peaked on day 5. Thirty-day mortality was 5%: one patient died on day 20 post-ILI due to pulmonary embolism.

Sixteen patients (76%) experienced an overall response (complete + partial response). A complete response was achieved in 8 patients (38%). Four of these patients (19%) developed new ITMs after a median of 18 months (range 6–30 months). A partial response was achieved in 8 patients (38%) and was sustained in one patient (5%), however this patient developed distant metastases and died 12 months after the ILI procedure. Five patients (24%) had either stable disease or progressive disease following ILI. Long-term limb salvage was achieved in 19 patients (90%). In two patients an amputation was carried out: In one a lower limb amputation was

Table 1
Patient and tumor characteristics and perioperative parameters.

Characteristic	Value		
Gender, n (%)			
Male	3 (14)		
Female	18 (86)		
Age in years, median (range)	72 (30–88)		
Involved limb, n (%)			
Lower	19 (90)		
Upper	2 (10)		
Limb volume in litres, median (range)	8.8 (2.6–13.0)		
Burden of disease, n (%)			
Low (<10 lesions and all < 2 cm)	7 (33)		
High (≥10 lesions or any ≥ 2 cm)	14 (67)		
Melphalan dose in mg, median (range)	46.6 (13.6–66)		
Actinomycin-D dose in μg, median (range)	450 (195–500)		
Drug circulation time in minutes, median (range)	28.5 (20–30)		
Tourniquet time in minutes, median (range)	58.4 (50–65)		
Length of hospital stay in days, median (range)	15.8 (9–24)		
Postoperative myoglobin peak value in μg/L, median (range)	1366 (67–6937)		
Myoglobin peak postoperative day, median (range)	4 (2–6)		
Perfusate blood gas, after 30 min of ischaemia	Overall (n = 21)	Upper Limb (n = 2)	Lower Limb (n = 19)
pH, median (range)	7.12 (7.03–7.32)	7.22 (7.12–7.32)	7.18 (7.03–7.32)
Base excess, median (range)	−10.39 (−5,3–20.60)	−6,4 (−4,4–8.4)	−10.77 (−5,3–20.60)
PaO_2 in mmHg, median (range)	19.69 (7.7–41.3)	18.0 (16–20)	19.76 (7.7–41.3)
Lactate mmol/L, median (range)	3.28 (1.88–8.29)	2.65 (2.56–2.73)	3.35 (1.88–8.29)
Wieberdink toxicity grade, n (%)			
I no visible effect	0		
II slight erythema/oedema	13 (60)		
III considerable erythema/oedema with blistering	6 (30)		
IV extensive epidermolysis/obvious damage to deep tissues with threatened or actual compartment syndrome	2 (10)		
V severe tissue damage necessitating amputation	0		

performed due to uncontrollable local recurrence causing pain 13 months after ILI, and in the other an amputation of the lower limb was carried out 24 months after ILI due to limb ischaemia. Median follow-up was 31.0 months (range 1–70 months). In total, four patients (19%) developed distant metastases during follow-up, two of whom were still alive at the time of data analysis. Five-year overall survival was 57% (Fig. 2). Thirteen patients (62%) were still alive at the time of analysis, 11 of them without evidence of active disease in the treated limb.

Discussion

This study demonstrates that ILI for melanoma ITMs can safely and effectively be performed in a tertiary referral centre in Eastern Europe. The results achieved were comparable to those reported by high-volume centres in Australia and the USA, with a long-term limb salvage rate of 90% and acceptable locoregional toxicity [5,19].

Multiple treatment options are available for melanoma ITMs. The US National Comprehensive Cancer Network guidelines list surgical excision, ILI, ILP, intra-lesional therapies, local ablation therapies, radiotherapy and systemic treatments all as appropriate treatment options for these patients [20]. Surgical excision is a reasonable procedure, and can be performed repeatedly when lesions are relatively small and limited in number. If surgical excision can provide satisfactory disease control, often no further treatment is necessary. However, it has been reported that over 30% of patients have another local recurrence and 23% develop distant metastases [21]. Options for local treatment include intra-lesional injection of Bacille Calmette-Guerin (BCG), interleukin 2 (IL-2), talimogene laherparepvec (T-VEC), Darleukin (L19IL2), Daromun, PV-10 (rose bengal), and electrochemotherapy (ECT) [22,23].

Patient selection is important when starting a ILI program. In previous studies, for instance, it has been shown that on average ILI is applied to older patients than ILP [24,25]. In this regard, the median age of 72 years in our series is comparable to that reported in previous ILI studies, reflecting the fact that we selected patients similarly to high-volume centres in Australia and the USA [9]. Furthermore, our patients had a Breslow thickness of the primary melanoma (7.5 mm), ulceration (42%), high mitotic rate (67%), and

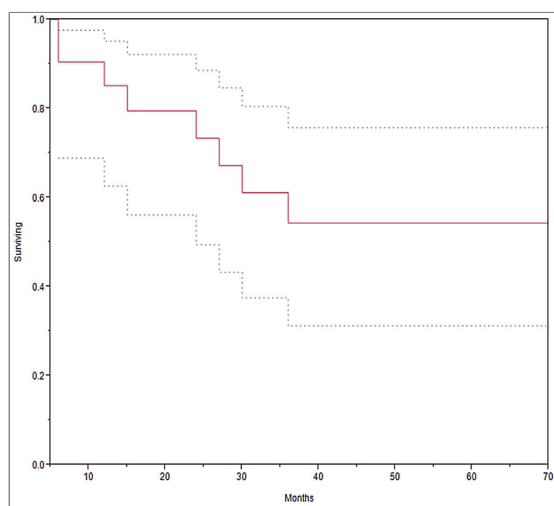

Fig. 2. Overall survival (months) following isolated limb infusion for the complete cohort.

high BOD (67%) also comparable to previous ILI series [5,9,26]. Since the abovementioned melanoma features are prognostic factors for a worse outcome following ILI, our complete response rate of 38% and overall response rate of 76% are comparable to those reported by larger ILI series, as they relate to similar patients [5,9].

According to the MSKCC ILI protocol, we used the same dosages of melphalan (7.5 mg/L) and actinomycin-D (75µg/L) for upper and lower limb procedures, and corrected both for IBW. Although compared to Australian ILI series this resulted in substantially reduced cytotoxic drug doses in the upper limb procedures, a complete response was achieved in both patients in our series [9,27]. However, we will need more experience in order to draw any firm conclusions.

In our experience, the ILI procedure was well-tolerated, with mild to moderate limb toxicity in most patients (grade II and III). However, two patients suffered grade IV limb toxicity, both requiring a fasciotomy, one of whom also requiring surgical debridement of necrotic muscle in the superficial posterior tibial compartment. As in previous reports from high-volume ILI centres, no patient experienced limb toxicity necessitating amputation, however, one patient died following ILI due to a pulmonary embolism despite the use of LMWH prophylaxis [9,28]. In comparison, following ILP, toxicity-induced amputations have been reported, and systemic side-effects are regularly observed due to leakage into the systemic circulation because of the high vascular pressure in the isolated limb circuit [6,29].

In recent years, immunotherapy and checkpoint inhibitor therapy have dramatically changed the treatment landscape for metastatic melanoma. Their role in patients with melanoma ITMs, however, remains unclear. Most trials of these therapies have included only small numbers of patients with ITMs or none at all [30–33]. Therefore, to date none of these new agents has been shown to be as effective for ITMs as ILI, especially in patients with bulky disease or when numerous lesions are present [26,34,35]. Furthermore, the systemic therapies can sometimes result in severe systemic side-effects, something not experienced by patients after ILI. Finally, around the world, there are large discrepancies in access to these new and costly drugs, largely due to economic differences between countries and available healthcare funds [7]. In contrast, the drugs for ILI, melphalan and actinomycin-D, are readily available and not unduly expensive. Therefore, there is still a place for ILI in the treatment of ITMs in this modern era.

Some limitations of this single-centre retrospective analysis have to be addressed. The small number of patients does not allow us to make conclusions on response rates and survival outcomes. Also, we followed the MSKCC ILI protocol, resulting in lower melphalan and actinomycin-D dosage and shorter drug circulation times compared to the Australian centres [36]. This could potentially have negatively impacted response to ILI. In previous ILI studies, serum creatine phosphokinase levels have been measured to indicate the degree of limb toxicity [9,14]. For practical reasons, we used serum myoglobin levels in the current study for the same purpose and found, in contrast to a recent ILP publication, a significant correlation between a high post-ILI serum myoglobin and increased Wieberdink toxicity grades [37,38]. Lastly, the current series includes the learning curve of mastering the ILI procedure at our institution. Despite these limitations, however, we feel that the current study does provide a realistic overview of the possibility of ILI treatment in Eastern Europe.

In conclusion: In this first series of ILI for melanoma ITMs from an Eastern European country, we have shown that ILI can be safely and effectively implemented outside of high-volume centres in the USA and Australia. In the Estonian healthcare system, ILI offers the advantage of being a relatively cheap and simple method with low morbidity.

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Declaration of competing interest

None.

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Curriculum vitae

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Language competence

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