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ASSESSMENT OF PROGNOSTIC MARKERS IN BENIGN AND MALIGNANT MELANOCYTIC TUMOURS

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TO MY FAMILY

ABSTRACT

Cutaneous melanoma is a malignant tumour that arises from pigmented skin cells – melanocytes, represents the most rapidly increasing cancer worldwide which is causing significant public health problem. During the past decade a significant progress has been made in the understanding of genesis and progression of melanocytic lesions. However treatment of malignant melanomas is in a majority of cases limited to surgical excision of the primary tumours or metastases. In advanced metastatic disease there are few treatment options. The present thesis reviews current knowledge on epidemiology, ethiology, pathology and treatment options of the benign and malignant melanocytic tumours and concentrates on the analysis of several molecules that are thought to play a role in the processes of cell growth, apoptosis, division, motility and adhesion as well as regulation of cytoskeletal components, protein phosphorylation, cell-cycle and cell survival. Archival material from fine needle aspirates as well as formalin-fixed tumour tissue was used in the study. Primary benign and malignant melanocytic tumours as well as metastatic melanomas were included in the study. The expression of the following proteins in malignant and benign melanocytic tumours was analyzed: S100, CD40, CD44, Bcl-2, Ki-67, COX-2 and HSP90. The expression patterns of the proteins were correlated to pathomorphological properties of the tumours as well as clinical parameters. Moreover, a presence of the BRAF gene mutation V600 has been analyzed in metastases from malignant melanomas. We found that melanocytic tumours are heterogeneous in respect to expression of the analyzed proteins as well as mutational status. Analysis of biomarkers and genetic aberrations might therefore be of importance for predicting the biological behaviour of the tumours. The results of the study are discussed in the light of eventual prognostic, diagnostic and therapeutic application of the analyzed proteins and mutational status.

LIST OF PUBLICATIONS

I. **Sviatoha V**, Rundgren Å, Tani E, Kleina R, Skoog L.

Expression of CD40, CD44, Bcl-2 antigens and rate of cell proliferation on fine needle aspirates from metastatic melanoma.

Cytopathology. 2002 Feb;13(1):11-12.

II. **Sviatoha V**, Kleina R, Tani E, Skoog L.

Expression of the CD117, COX-2 and HSP90 antigens and cell proliferation in fine-needle aspirated cells from metastatic melanomas.

Anticancer Research. 2009 Nov;29(11):4345-52.

III. **Sviatoha V**, Tani E, Kleina R, Sperga M, Skoog L.

Immunohistochemical analysis of the S100A1, S100B, CD44 and Bcl-2 antigens and the rate of cell proliferation assessed by Ki-67 antibody in benign and malignant melanocytic tumours.

Melanoma Research. 2010 Apr;20(2):118-25.

IV. **Sviatoha V**, Tani E, Ghaderi M, Kleina R, Skoog L.

Assessment of V600E mutation of BRAF gene and rate of cell proliferation using fine-needle aspirates from metastatic melanomas.

Anticancer Research 2010 Sep;30(9):3267-72.

CONTENTS

LIST OF ABBREVIATIONS

MC1R	Melanocortin-1 receptor
α-MSH	Alpha-Melanocyte stimulating hormone
CALM	Café-au-lait macula
JN	Junctional nevus
CN	Compound nevus
IN	Intradermal nevus
SSM	Superficial spreading melanoma
NM	Nodular melanoma
LMM	Lentigo malignant melanoma
ALM	Acral lentiginous melanoma
MM	Mucosal melanoma
RGP	Radial growth phase
VGP	Vertical growth phase
ASR	Age standardized rate
CDKN2A	Cyclin-dependent kinase inhibitor 2A
ACT	Adoptive cell therapy
CDK	Cyclin-dependent kinase
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol 3-kinase
MITF	Microphtalmia-associated transcriptional factor
AJCC	American Joint Committee on Cancer
FNAB	Fine needle aspiration biopsy
RAGE	Receptor for advanced glycation end products
HA	Hyaluronic acid
TNF-R	Tumour necrosis factor-R
SCF	Stem-cell factor
ICC	Interstitial cells of Cajal
GIST	Gastro-intestinal stromal tumours
COX	Cyclooxygenase
PGHS	Prostaglandin H2 synthase
EGF	Epidermal growth factor
NSAIDs	Non-steroid anti-inflammatory drugs
HSP90	Heat shock protein 90
ATP	Adenosine triphosphate
RTK	Receptor tyrosine kinases
LSAB	Labelled strepravidin-biotin
PCR	Polymerase chain reaction
SD	Standard deviation
EGFR	Erythrocyte growth factor receptor
MOMP	Mitochondrial outer membrane permeabilization

1 BACKGROUND.

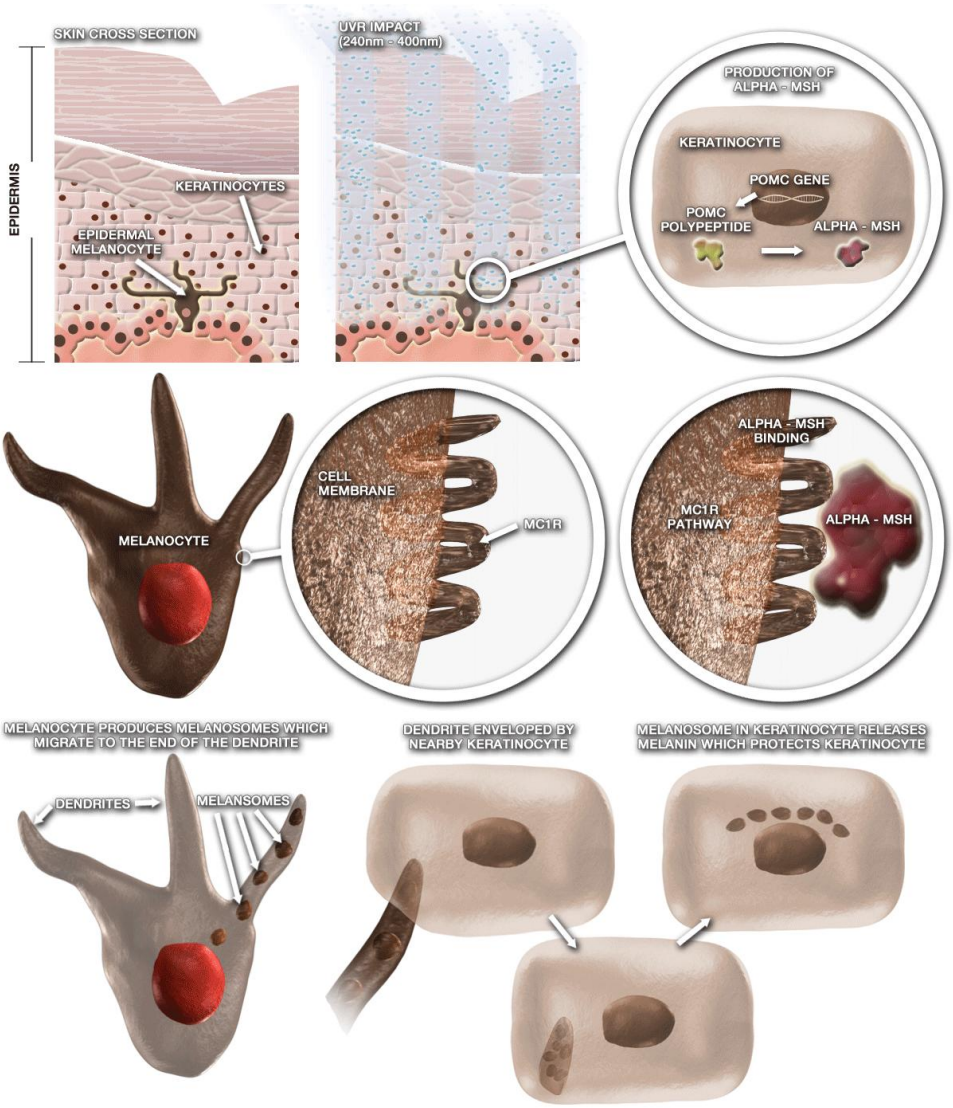
1.1 Melanocytes and normal skin.

Melanocytes are dendritic cells that derive from the neural crest and migrate into the epidermis as well as other epithelia (Kanitakis 2002). Depending on localization and the control mechanism regulating specific differentiation and melanin synthesis the melanocytes are divided in three major groups: cutaneous melanocytes, which continuously synthesize small melanosomes to be transferred to keratinocytes; the uveal melanocytes, which synthesize larger melanosomes for only a short while to be retained by this melanogenically dormant cell; and the hair melanocyte, which intermittently produces melanin either in a cyclic manner or as a periodic supply from a stem population(Boissy 1988). In the epidermis, the melanocytes are localized in the basal layer and their dendritic processes extend in all directions. The melanocytes are evenly distributed among basal keratinocytes at a ratio of 1 melanocyte per 4-10 basal keratinocytes. Their density reaches 500-2,000 cells per mm^2 of cutaneous surface, with regional variations reaching maximal density on genital skin. Through the enzymatic activity of tyrosinase from the substrate tyrosine, melanocytes produce pigment melanin. To produce melanin, the MC1R (melanocortin-1 receptor) pathway is activated by the ligand alpha-Melanocyte Stimulating Hormone (α-MSH) binding to and stimulating the MC1R on the outside surface of the melanocyte (Figure)(Luger et al. 1997). Approximately 1000 of MC1R receptors exist on each normal melanocyte.

Two types of melanin are known: pheomelanin (red) and eumelanin (yellow-black)(Quevedo 1979). The synthesis of melanin takes place in melanosomes, which at the early stages of development are membrane-limited vesicles located in the Golgi-associated endoplasmic reticulum. The developing melanosomes, with their content of

melanin, are transferred to the neighbouring basal cells and hair follicular cells. The melanin transfers to keratinocytes by phagocytosis of the tips of the melanocytic dendrites in a process called pigment donation(Wolff 1973). In the adjacent keratinocytes the melanin pigment forms an umbrella-like cap over the nucleus, protecting it from injurious effects of UV light.

Function on melanocytes in the normal skin.



1.2 Benign melanocytic tumours.

In analogy with tumours of other origins, melanocytic neoplasias are grossly divided in two major groups: benign and malignant. Benign tumours, in turn, are divided in several subgroups depending on:

1) Extension of tumour growth:
   - Intraepidermal/Superficial type, confined to basal membrane and epidermis (pigmented macules/freckles, Café-au-lait macules (CALMs), solar lentigo, Becker's melanosis).
   - Junctional nevi (JN), the cells are confined to the dermoepidermal junction but in contrast to superficial type where cells are mostly organized in a single layer at the basal membrane, JN have a tendency to build nests of proliferating melanocytes, sometimes extending to the upper half of reticular dermis.
   - Compound nevi – melanocytic benign tumours where both epidermal and dermal components can be appreciated.
   - Intradermal nevi – tumours confined to the dermis.
2) Genesis: congenital and acquired nevi.
3) Cell morphology: small round cells, epithelioid, spindle cells, clear cells, balloon cells, polymorphic, dysplastic nevi, and nevi of mixed cellularity.
4) Presence of stromal response, e.g. sclerosis and desmoplastic reaction (Spitz nevus, blue nevus)
5) Anatomical site: skin, mucosal, acral, genital nevi.
6) Presence of pigment: pigmented or amelanotic.

Melanocytic nevi, in general, have an overall histologic symmetry and usually are well-circumscribed(Ackerman 1982). The surface profile of a nevus may vary from being almost flat to dome shaped, polypoid or papillomatous(Rao et al. 2001). Existing classifications of benign melanocytic lesions are based on clinical and

histomorphological findings which not always reflect a complex nature of these lesions. The progress in our understanding of genetics, molecular events and pathways which lead to development of melanocytic lesions has stimulated attempts to extend the existing classification including evaluation of genes and molecules that are involved in melanogenesis(Bittner et al. 2000). It has been shown that gene signatures established using DNA microarray gene expression profiling can distinguish melanomas from nevi, indicating the feasibility of using molecular classification as a supplement to standard histology (Koh et al. 2009).

1.3 Malignant melanoma

Cutaneous melanoma is a malignant tumour that arises from skin pigmented cells – melanocytes. In most of the cases melanoma arise either via de novo proliferation of basal epidermal melanocytes, (Ackerman 1980), or as transformation of pre-existed benign nevi (Gruber et al. 1989; Holman et al. 1983). Depending on clinico-histopathological properties of the tumour malignant melanomas are divided into four main types(Barnhill and Mihm 1993):

1- Superficial spreading malignant melanoma (pagetoid melanoma)
2- Nodular malignant melanoma
3- Lentigo maligna melanoma
4- Acral lentiginous malignant melanoma.

Superficial spreading melanoma (SSM) is the most common type of cutaneous melanomas and accounts for approximately 70% of all primary tumours (MacKie 2000). This type of melanoma usually originates in a pre-existing nevus and has a tendency for slow growth. The common sites for superficial spreading melanoma are the female leg and the male back which suggest that both intermittent and chronic UV exposure may be important initiating and/or promoting events. Patients are often in the

fourth or fifth decade at presentation(MacKie 2000). A remarkable morphologic feature of SSM is the presence of pagetoid spread – trans/intradermal migration of melanoma cells(Clark et al. 1984). Typical melanoma cell in SSM have an epithelioid appearance, with abundant, usually granular/eosinophilic cytoplasm, containing finely divided melanin.

Nodular melanomas (NM) is the second most common type of melanoma, accounting for approximately 10-15% of all melanomas (Langley and Sober 1998). The main feature of the NM is a rapidly expanding nodular lesion often without history of a pre-existing nevus(Barnhill and Mihm 1993). It is commonly seen on trunk and head /neck region(Lens 2008). Nodular melanomas present as rapidly growing protuberant or polypoid blue-black lesions, with much of the pigmentation being due to blood rather than to melanin. Ulceration is frequently present. A subset of NM lacks pigment and presents as a pink (amelanotic) tumour. The tumour cells most frequently are of epithelioid type, but other cell types may predominate, such as spindle cells multinucleate cells or even a mixture of the various types (Clark et al. 1986). The surrounding stroma often demonstrates variable degree of mononuclear cell infiltrates and accumulation of macrophages.

Lentigo malignant melanoma (LMM) accounts for approximately 5-10% of all melanomas (MacKie 2000). It is mostly diagnosed in elderly people, with a peak incidence in the seventh decade (Langley and Sober 1998). The risk for development of LMM is strongly associated with a long-term cumulative sun exposure (Cohen 1995) which reflects that typical sites of development of LMM are the face (particularly cheeks and nose) and the neck. Lentigo maligna melanomas have a well identified slowly evolving radial growth phase known as lentigo maligna or Hutchinson's melanotic freckle. Over time a proportion of LMM will progress to invasive lentigo maligna melanoma, characterized by an invasive nodule developing within the pre-

existing lentigo maligna(MacKie 2000). Histologically, LMM is characterized by increased number of basal melanocytes with a variable, low-grade cytological atypia. A contiguous and often nested intraepidermal proliferation of pleomorfic melanocytes may occur. In addition, LMM is characterized by atrophy, effacement of the epidermis, involvement of skin appendages and solar elastosis(Clark and Mihm 1969).

Acral lentiginous melanoma (ALM) represents approximately 2-8% of all melanomas(Barnhill and Mihm 1993). However, this type is the most common among dark-skinned population with a relatively low incidence of other melanoma types (Africans, Asians and Hispanics) where it may account for 30-70% of all melanomas (Cress and Holly 1997). Acral melanoma usually occurs in older patients on palms and soles with feet/hand ratio being approximately 8:1 (MacKie 2000). Acral melanoma presents with features very similar to those of superficial spreading melanoma, but modified due to the thicker epidermis and stratum corneum of palmar or plantar skin. The lesions are usually heavily pigmented within an irregular outer margin and variegated central pigmentation and are often ulcerated at the time of diagnosis. Amelanotic variants of acral lentiginous melanoma do occur. A distinct variant of ALM that arises in the nail bed is named subunqual melanoma. The peak incidence of this type of acral lentiginous melanoma is in the seventh decade. These lesions are more commonly seen on the big toe or the thumb than on the other digits, and present with irregular pigmentation under and around the nail. A striking and strongly suggestive clinical feature is the presence of Hutchinson's sign: the presence at the proximal nail fold margin of an area of visible melanin pigmentation which is not seen in association with other causes of subunqual pigmentation such as a traumatic haemorrhage. The tumour is most often composed by spindle type melanocytes and may exhibit bone invasion and a prominent fibrovascular stromal response(Patterson and Helwig 1980).

Mucosal melanomas are rare melanomas comprising 2.2% of all melanoma cases(Patrick et al. 2007). Mucosal melanoma is most common in the head and neck region (hard palate, tongue and nasal mucosa) where it comprises 55.4% of all mucosal melanomas. The female genital tract, anorectal region and urinary tract are responsible for 18.0, 23.8% and 2.8% of mucosal melanoma cases, respectively (Chang et al. 1998). The peak age for diagnosis of mucosal melanoma is between 70 and 79 years.

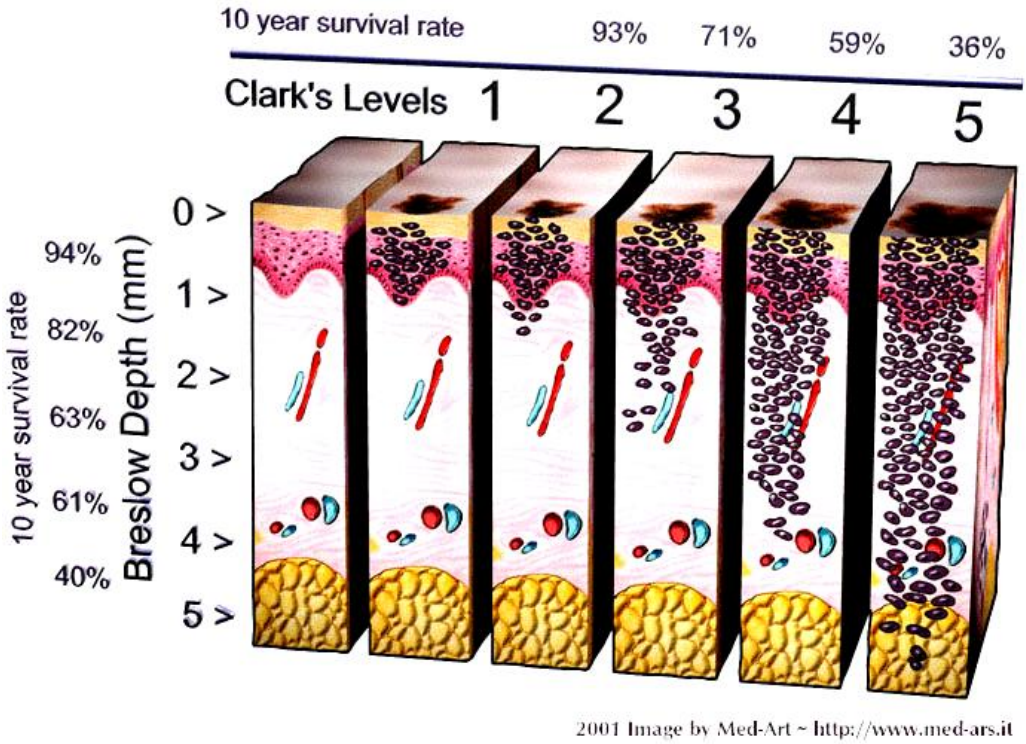
Like other cancers, most malignant melanomas evolve through several stages of tumour progression. Clinically, many melanomas begin as a pigmented patch of skin which evolves to become a palpable plaque with varying degree of pigmentation. This stage of melanoma progression has been termed the "radial growth phase" (RGP) which is divided in intraepidermal melanoma (melanoma in situ) and microinvasive melanoma. The duration of the intraepidermal phase may vary from several months to many years. A set of characteristic cytological and architectural changes is observed during this phase: proliferation of basal melanocytes with foci of confluence and upward migration of melanocytes expressing increasing cytological atypia (pagetoid spread). Commonly, there is extension of these proliferations along zones of skin appendages. After a period of intraepidermal proliferation melanomas in situ invade the papillary dermis(Clark et al. 1984). There is evidence that at this stage melanocytes lack capacity for proliferative nodular growth and metastasis(Clark et al. 1989). Microinvasive melanoma features a host response in the papillary dermis in form of a dense cellular infiltrate of lymphocytes and monocytes in a perivascular or bandlike pattern. Subsequently, malignant melanoma progresses into a vertical growth phase (VPG) which is characterized by the focal proliferation of melanoma cells as cohesive aggregates in the dermis. It has been postulated that progression of melanoma in to a vertical growth phase signals the onset of the metastatic phenotype(Clark et al. 1989).

Depending on invasion depth, melanomas are categorized into five groups named as Clark's levels(Clark et al. 1969):

1- Level I: confined to the epidermis (top-most layer of skin); called "in situ" melanoma
2- Level II: invasion of the papillary (upper) dermis
3- Level III: filling of the papillary dermis, but no extension in to the reticular (lower) dermis
4- Level IV: invasion of the reticular dermis
5- Level V: invasion of the deep, subcutaneous tissue

Invasion depth in millimetres reported as Breslow's invasion depth (Breslow 1970).

Clark's invasion level and tumour thickness in relation to survival rate in melanomas.



1.3.1 Epidemiology of malignant melanoma

Cutaneous malignant melanoma is one of the most rapidly increasing cancers in the white population worldwide (Diepgen and Mahler 2002; Lens and Dawes 2004) and as a consequence the increase in incidence of melanomas represents a significant and growing public health problem. The frequency of malignant melanoma is closely associated with the constitutive colour of the skin and also depends on the geographical zone. The highest incidence of melanoma in the world has been reported from New Zealand, Auckland, with an incidence of 77.7 per 100 000 and an age standardized annual rate of 56.2 per 100 000 with no statistical differences between males and females (Jones et al. 1999). High incidence rates have been reported also from Australia with 56 new cases per year per 100,000 for men and 43 for women in 1992 (MacLennan et al. 1992). The rates of malignant melanomas in other countries are depicted in Table. The incidence of malignant melanoma in Sweden was 17.1 per 100 000 in males and 15.5 per 100 000 in females during period 1990-1999 (Lindholm et al. 2004). It is noteworthy that incidence of cutaneous melanomas in Sweden in patients aged between 12-19 years doubled in 1983-1992 as compared to 1973-1982 (Karlsson et al. 1998). A 5-fold increase of the melanoma incidence in Sweden was observed between 1960 and 2000. The estimated lifetime melanoma risk in Sweden for 2006 was 1 in 50 at age 85 years (Karlsson and Fredrikson 2007). A 2.5-fold increase in melanoma incidence between 1970-1997 was observed in Finland and 3.6-fold increase in White Americans(Parkin and Iscovich 1997). From 1979 until 1998, a 2.4-fold increase was observed in Scotland (MacKie et al. 2002) and from 1980 until 2000, 2.8-fold increase was reported from France(Remontet et al. 2003). A study evaluating the incidence of malignant melanoma in situ in Sweden in 1968-1992 demonstrated that among men, the age-standardized rates increased from 0.1 per 100 000 in 1968 to

2.9 per 100 000 in 1992 with a mean annual increase of 15%(Thorn et al. 1992). The rates among women increased from 0.3 to 3.7 per 100 000 with mean annual increase of 12.8%.

The number of deaths caused by cutaneous malignant melanoma has been increasing in most fair-skinned populations throughout the world(Diepgen and Mahler 2002). Between the mid-1950s and mid-1980s, the mortality rates from malignant melanomas have been increasing in both young (20-44years) and middle-aged (45-64 years) adults in most European countries(Franceschi and Cristofolini 1992) as well as North America, Australia and New Zealand with annual increase of 2-4% (La Vecchia et al. 1999). Cohort analysis of age-specific rates shows a difference between older and younger cohorts in same countries. In cohorts aged 60 years and over the mortality rates have continued to increase in paste decades, while younger cohorts, particularly those born in 1960s and 1970s, the mortality rates have stabilized or even begun to decrease in North America, Scotland, Australia and several European countries(Marks 2000). However, in France and Italy the mortality rate is still increasing (Severi et al. 2000). The 5-year survival rate for males with malignant melanoma in Sweden increased from 54.5% in 1960-1964 to 73.1% in 1980-1982. The corresponding rates for females were 65.8% and 84.9%, respectively(Thorn et al. 1989). A further increase in relative survival rate in patients with malignant melanomas in Sweden to 88% in males and to 94% in females was reported between 1990 and 1999 (Lindholm et al. 2004).

Summarizing the above it can be concluded that malignant melanomas represent a major health problem in most western countries and it should be of high concern for both public and professional educational programmes to control it.

Incidence of cutaneous malignant melanoma (per 100 000)

Country	Male		Female	
	Crude	ASR	Crude	ASR
Australia	51·6	40·5	40·7	31·8
New Zealand	45·2	36·7	44·4	34·9
Sweden	19·8	12·6	19·9	13·3
U.S.A.	16·4	13·3	12·9	9·4
Denmark	14·8	10·6	17·6	13·0
Switzerland	12·5	9·3	15·0	11·1
The Netherlands	12·2	9·4	16·7	12·9
Austria	11·5	8·8	15·4	10·4
Canada	10·6	8·2	10·6	8·0
Hungary	10·3	7·6	10·3	6·8
Israel	9·7	9·4	11·0	9·8
Germany	9·3	6·5	11·4	7·1
France	8·6	6·8	11·1	7·9
U.K.	8·3	6·1	11·3	7·7
Poland	6·6	5·6	8·6	6·7
Italy	6·5	4·6	8·2	5·5
Russian Federation	6·3	5·4	6·4	4·7
Spain	4·0	2·8	6·8	4·5
South Africa	3·8	6·4	3·6	4·8
Brazil	2·9	3·5	2·0	2·2
Greece	2·5	1·9	3·2	2·0
Japan	0·63	0·40	0·49	0·29
China	0·21	0·22	0·17	0·17

ASR- age standardized rate.

1.3.2 Etiology and risk factors in malignant melanoma.

There are no proven causes of melanoma so far, however approximately 5% of all invasive cutaneous melanomas occur in a familial setting with two or more close relatives affected. This observation indicates that, in a small minority of melanoma patients, low prevalence/high penetrance genes are involved. In addition, the typical phenotype of the melanoma patient, with pale Caucasian skin, red or blond hair and blue eyes indicates that high prevalence/low penetrance genes such as MC1R (melanocortin 1 receptor), CDKN2A and HERC2/OCA2 may interact with environmental factors (Ibarrola-Villava et al. 2010). Association of pigmentation gene variants ASIP and TYR with malignant melanoma has been demonstrated [2]. The major constitutional risk factor for melanoma is skin colour and type. Depending on skin type as proposed by Thomas Fitzpatrick (Table)(Astner and Anderson 2004), the skin types I and II are most likely to develop malignant melanomas, while incidence of this tumour in skin types V and IV is considerably lower(Evans et al. 1988) with more than 100-fold difference in theirs sensitivity to the harmful effects of ultraviolet radiation(Rees 2004).

The presence of a large number of both common acquired and dysplastic naevi is a second major constitutional risk factor in fair-skinned people (Bliss et al. 1995). Moreover, data from both prospective clinical and cohort studies are also consistent with greatly increased risk of melanoma in individuals with dysplastic nevi. Xeroderma pigmentosum, a skin disorder of impaired ability to repair DNA following UV exposure/damage is associated with substantially increased risk of developing melanoma, particularly at young age(Jen et al. 2009). Among the major risk factors, intense episodes of burning sun exposure appear to be most significant for the development of malignant melanoma (Elwood and Gallagher 1998). However, a cumulative exposure to UV-radiation over the years may also contribute to the risk.

Studies of place of birth and residence during first decade of life report an increasing risk of melanoma in individuals in high-UV environment (Fears et al. 2002). There are data which show an interaction between chronic sun exposure and the type of melanoma that may subsequently develop. The lentigo maligna variety of melanoma, found most commonly on constantly exposed body sites as the face, is associated more with chronic occupational UV exposure than intermittent burning UV exposure episodes (Whiteman et al. 2003). The potential risk factor of using artificial UV sources has been investigated by several studies. A recent large meta-analysis study of sunbed use and melanoma has concluded that overall sunbed exposure does add to risk of developing melanoma(MacKie et al. 2002). Different melanoma incidence between males and females with prevalence of the latter has been reported (Brochez and Naeyaert 2000). This has led to a number of studies investigating the role of pregnancy, oral contraceptives and hormone replacement therapy as risk factors for malignant melanoma. Cumulative data from publications on these topics provide no evidence that pregnancy(Karagas et al. 2006), the use of oral contraceptives (Lea et al. 2007) or hormone replacement therapy (MacKie and Bray 2004) contribute to the risk of developing melanoma. However, it was proposed that patterns of behaviour may explain the higher rates of melanoma among young women than men. Adolescent girls are more likely to sunbathe, to get sunburned, to want to tan, and to use tanning beds. The rate of sunburns and tanning bed use are of great concern, and may contribute to the rising incidence rates in young women(Tucker and Goldstein 2003). Several studies have shown the melanoma prevalence in the wealthier strata of society. It has been shown that incidence of melanoma is higher in a large income groups and other measures of affluence (Kirkpatrick et al. 1990; Lee and Strickland 1980). The greater opportunity for the recreational sun exposure and sunny holidays during winter time were suggested as a possible explanation to this relationship.

Several studies have focused on studying occupational risks for developing of melanomas. A higher incidence in airline crews, particularly pilots has been reported (Pukkala et al. 2002). In addition, a relationship between immunosuppression and risk of developing melanomas has been of concern in several studies. While a 1.6 to 2.5-fold increased risk of the melanoma was found in one study(Dinh and Chong 2007), these results could not be confirmed by another study(Lindelof et al. 2000). Finally, a recent case-control study on use of non-steroidal anti-inflammatory agents showed a reduced risk of developing malignant melanoma(MacKie et al. 2009).

Fitzpatrick's Skin phototypes.

Phototype	**Sunburn & Tanning history (defines the phototype)**	**Immediate pigment darkening**	**Delayed tanning**	**Constitutive colour (unexposed buttock skin)**	**UV-A MED (mJ/cm^2)**	**UV-B MED (mJ/cm^2)**
I	Burns easily, never tans	None (–)	None (–)	Ivory White	20–35	15–30
II	Burns easily, tans minimally with difficulty	Weak ± (± to +)	Minimal to weak (± to +)	White	30–45	25–40
III	Burns moderately, tans moderately and uniformly	Definite +	Low +	White	40–55	30–50
IV	Burns minimally, tans moderately and easily	Moderate ++	Moderate ++	Beige-Olive, Lightly tanned	50–80	40–60
V	Rarely burns, tans profusely	Intense (brown) +++	Strong, intense brown +++	Moderate brown or tanned	70–100	60–90
VI	Never burns, tans profusely	Intense (dark brown) +++	Strong, intense brown +++	Dark brown or black	100	90–150

1.3.3 Therapy options and clinical course of malignant melanomas.

In his lecture from 1939 which was published by British Medical Journal (1939) professor Grey Turner described a case of a malignant melanoma in a 45 years old man who "*had a black patch on the back, just below the angle of the scapula on the right side. It began to grow rapidly, and, two months before he himself saw the patient, was removed by his medical man. He was then discovered to have a melanotic tumour and a large mass of glands in the right axilla. The tumour was removed, with a reasonable amount of skin, and glands dissected out. A month later the black patch has recurred; there was a nodule at a little distance, and a mass of glands in the left axilla. Again the patch and the glands were removed, but in a very short time another nodule appeared and grew rapidly, enlarging in a few days from size of a sixpence to that of shilling. The patient died within seven months of the first interference with his melanotic grow*". Professor Grey had reported several clinical cases of malignant melanoma and in all patients "*the story of treatment was on whole a melancholy one*" which shows a highly malignant nature of this tumour. The situation described for almost a century ago is still highly actual. Despite the survival experience in stage IV melanoma patients has improved since 1985, the use of varied approaches to treat this disease has not produced a favourable long-term prognosis with estimated survival to be as low as 13.6%, 9.7% and 2.3% for 2, 3 and 5 years, respectively(Lee et al. 2000). While approximately 75% of patients with stage I melanoma can be cured with surgery, stage IV melanomas are considered incurable by currently available therapies(Wagner et al. 2000). Adjuvant therapy has not been particularly useful in such patients. Single-agent chemotherapy (Dacarbazine (DTIC)) and/or immunotherapies (either interleukin or interferon) remain the therapy of choice with a response rate of 16%. Patients with metastatic melanoma at stage III and IV generally do poorly and have a median

survival of 4 to 6 months(Atallah and Flaherty 2005). Such patients are treated with systemic therapy unless they have a surgically resectable single site of metastasis, in which case the surgical resection is rather of a palliative character. Generally, survival worsens from non-visceral to visceral sites. Medial survival drops from 15 to 4 months as metastases involve skin/subcutaneous tissue, lung, liver/spleen/adrenal and brain(Essner et al. 2004). Survival is also related to the number of metastases. Median survival for involvement of a single site is approximately 7 months and decreases to less than 4 months for metastases with two or more sites(Young et al. 2006). This illustrates the need of development of new therapeutic modalities in patients with advanced melanomas.

For the melanoma patients with disease at stage I and stage II the surgical excision remain the treatment of choice where tumour thickness dictates the appropriate excisional margins. Wide excision of primary tumours with a normal skin margin of 0.5 cm for *in situ* melanomas, of 1 cm for tumours with a Breslow thickness up to 2 mm and 2 cm for thicker tumours is recommended (Thompson et al. 2005). Sentinel lymph node biopsy in melanoma with a tumour thickness of >1 mm provides more accurate staging information, particularly for lesions of intermediate thickness (1–4 mm) but has no therapeutic value as it did not result in overall survival benefits. It may be followed by complete clearance of regional lymph nodes if the sentinel node was positive for micro metastases. However, this procedure has no proven effect on overall survival (Morton et al. 2006). Adjuvant immunotherapy with interferon-α at this stage might lead to a prolongation of disease-free survival. A study has demonstrated a positive effect on disease-free and distant metastases-free survival in patients with micrometastases (Eggermont et al. 2008).

At the stage of locoregional (stage III) tumour, surgery remains a main therapeutical option. Removal of the primary tumour, and regional metastases including the

surrounding lymph node region, is indicated (Dummer et al. 2009). Adjuvant therapy with interferon and interleukin might be used at this stage.

The current options in therapy of the stage IV advanced melanoma is of a palliative type. The use of tolerated single-agent cytotoxic chemotherapy such as dacarbazine, temozolomide or in combination with cytokines is used. However, such therapies do not result in survival prolongation and is used for symptom palliation only (Huncharek et al. 2001; Verma et al. 2008). In the absence of active biologic and chemotherapeutic agent for systemic melanoma, a surgery removal of visceral metastases may be appropriate for selected cases (Young et al. 2006). Surgery of advanced melanoma is most effective in patients exhibiting tumour which is limited to a few sites. Several retrospective studies on such patients have reported long-term survival, in some cases beyond 5 or 10 years, despite being initially considered incurable (Allen and Coit 2002; Allen et al. 2002; Essner 2003). Adjuvant radiotherapy has been shown to have a good regional control in some cases of metastatic melanomas and is highly depending on anatomical and pathological features of the tumour(Bibault et al. 2011).

An adoptive cell therapy in patients with advanced melanoma is currently being evaluated. Adoptive T cell therapy (ACT) consists of isolating tumour reactive lymphocytes from a patient, growing and activating them in the laboratory, and infusing them back into the autologous patient(Rosenberg et al. 2008). Effectiveness of the ACT therapy in patients with metastatic refractory melanoma has been recently demonstrated in one study (Dudley 2011).

Advances in our understanding of the genetic and molecular events that control genesis, cell division, invasion, metastasis, evasion of immune surveillance and resistance to chemotherapy in malignant melanomas have led to production of antagonists for critical molecules which are involved in these processes (table). These substances are currently under estimation for specific targeted therapy in several studies (reviewed by

C. Garbe (Garbe et al. 2011)) giving us a sense of optimism for improvement in the outcome for patients with advanced melanomas.

Targeted therapies under evaluation

Drug	Target
Sorafenib	RAF, VEGF-R, PDGR-R
Bortezomib	Proteasome inhibitor
MS-275	Histone deacetylase inhibition
Thalidomide	Anti-angiogenic, immunomodulatory
Revemid (CC5013)	Anti-angiogenic, immunomodulatory
Vitaxin (MEDI 522)	αvβ3 integrin mAb
CNTO95	αv integrin mAb
Oblimersen	bcl-2 antisense
XIAP	XIAP antisense
Semaxanib (SU5416)	VEGF-R2, kit
Bevacizumab	VEGF-R mAb

1.3.4 The genes and molecular genetics of malignant melanoma.

During the past decade a significant progress has been made in understanding of the genetics in melanocytic lesions. A family history of malignant melanoma is one of the most significant risk factors for developing this tumour(Tucker and Goldstein 2003). Understanding of genotype correlations in these patients may lead to improved diagnostic and prognostic information for early detection and application of tailored

therapies for all melanoma patients. Three high-penetrance and one low-penetrance melanoma-predisposing genes have been identified and characterized.

Melanoma-predisposing genes.

Gene nomenclature	Chromosomal locus	Protein	Risk of melanoma
CDKN2A, p16, p16(INK4), p16(INK4A)	9p21 (exons 1α, 2, 3)	p16, p16INK4a, INK4a	35-fold to 70-fold relative risk
ARF, p14(ARF)	9p21 (exons 1β, 2, 3)	p14, p14ARF	Unknown
CDK4	12q13	Cell division kinase 4	Unknown
MC1R	16q24.3	Melanocortin 1 receptor	2-fold to 3.6-fold risk

CDK4, cyclin-dependent kinase 4; *CDKN2A*, inhibitor of cyclin-dependent kinase 4A; INK4A, inhibitor of cyclin-dependent kinase 4A; ARF, alternate reading frame; *MC1R*, melanocortin 1 receptor gene.

The best-characterized high-penetrance gene locus is mapped to chromosome 9p21 (Cannon-Albright et al. 1992) which encodes two distinct melanoma predisposition genes, cyclin-dependent kinase inhibitor 2A (CDKN2A, also called p16, or inhibitor of cyclin-dependent kinase 4A, INK4a) and ARF or p14. Germline mutations in one or both of these genes are associated with familial melanoma. The principal action of p16/ CDKN2A is to regulate G1-S passage by inhibiting CDK-4/6 kinases phosphorylation of the retinoblastoma protein. Overexpression of the p16/ CDKN2A results in G1 arrest in cells with inactive retinoblastoma protein(Serrano et al. 1996). P14/ARF acts via the p53-pathway to induce cell cycle arrest in response to hyperproliferative oncogenic signals (Gallagher et al. 2005). The third high-penetrance melanoma predisposition gene, cyclin-dependent kinase 4 (CDK4), is located on 12q13(Zuo et al. 1996). Mutation of this gene abrogate the capacity for p16/ CDKN2A to associate with and inactivate CDK4, resulting in a similar effect which is seen in cases with p16/ CDKN2A mutation(Hayward 2003). Since the activities of CDK4 and p16 both affect the same downstream effector, the retinoblastoma protein, it has been shown that clinical characteristics such as mean age of diagnosis of melanoma, mean number of melanomas and number of nevi are indistinguishable between *CDKN2A* mutation-positive and *CDK4* mutation-positive families(Goldstein et al. 2000). The likelihood of

finding mutation in CDKNA2 is dependent on the number of affected family members overall, rising from 5% in families with two affected members, to 20-40% in families with three or more affected, to 100% in families with 13 or more affected (Stahl et al. 2004).

In contrast to above mentioned high-penetrance genes, the MC1R, melanocortin receptor gene, is a low-penetrance gene located on chromosome 16q24.3 (Palmer et al. 2000). Melanocortin-1 receptor plays a critical role in determining the type of melanin produced by melanocytes: eumelanin and pheomelanin (Scherer and Kumar 2010). The balance of red/yellow pheomelanin and black/brown eumelanin produced by activation of the receptor provides the basis for variation in skin pigmentation among humans(Rouzaud and Hearing 2005). The molecular mechanism contributing to the increased risk is that non-functional MC1R reduces the capacity of melanocytes to repair DNA damage from ultraviolet radiation (Rouzaud et al. 2005). More than 65 MC1R variants have been identified and confer an increased (2.2-fold) risk for melanoma, making MC1R a low-penetrance predisposition gene [9]. In p16/ARF mutation carriers who inherit an MC1R variant, penetrance increased from 50% to 84% and the mean age of melanoma onset decreased from 58.1 to 37.8 years. Thus, MC1R appears to be both an independent low-penetrance predisposition melanoma gene and also a modifier gene in p16/ARF pedigrees. Moreover, the association of MC1R mutations and somatic BRAF/NRAS mutations results in a crucial for melanomas development mitogen-activated protein kinase (MAPK) (Scherer et al. 2010).

Mutations of several oncogenic involved in essential pathways for melanoma development have been shown in several studies. Mutations of NRAS and BRAF genes which are involved in mitogen-activated protein kinase (MAPK) pathway have been identified in about 15% and 66% of malignant melanomas, respectively [15, 16]. NRAS and BRAF mutations are mutually exclusive, thus accounting for the MAPK

pathway activation in more that 80% of melanomas. Another pathway which is often activated in malignant melanomas is a Phosphatidylinositol 3-kinase (PI3K) pathway. PTEN (phosphatase and tensin homolog deleted in from chromosome ten) is a protein and lipid phosphatase that inhibits Akt activations by PI3K. Akt, in turn, is a serine/threonine kinase that leads to stimulation of cell cycle progression, cell proliferation, and inhibition of apoptosis (Dai et al. 2005). The mutations in the region of chromosome 10q24 which harbours the PTEN and impair its function locus was demonstrated in up to 50% of melanomas(Takata and Saida 2006). Moreover, the overexpression of Akt protein increases dramatically with melanoma invasion and metastasis and is inversely correlated with patient survival (Dai et al. 2005). Recently, amplification of the microphtalmia-associated transcriptional factor (MITF) gene was demonstrated in 10% of primary melanomas and 20% of metastatic melanomas, suggesting that MITF is a melanoma oncogene(Levy et al. 2006). MITF acts as a master regulator of melanocyte development, function and survival by modulating various differentiation and cell-cycle progression genes (Widlund and Fisher 2003).

Thus, assessment of genetic status of malignant melanomas might dramatically contribute to our knowledge of melanoma genesis and progression and insight in development of novel therapeutic tactics pointed at critical events in the cascades of melanoma pathways.

1.3.5 Pathology of malignant melanoma.

Patients with primary cutaneous melanocytic lesions rely not only on the knowledge, skill and experience of their physician but also on the important input of the pathologist who is responsible for an accurate diagnosis which is the basis for appropriate therapeutical management. However, the patient's prognosis and further therapy are dependent not only on the pathologic diagnosis alone but also on other crucial pathologically assessed parameters: tumour thickness, Clark's level of invasion, ulcerative state, inflammatory response and proximity to the resection margins. However, it is not always possible to accurately predict the melanoma prognosis purely from the histopathologic assessment which makes an inclusion of the molecular and genetic status of the analysed tumour a desirable option.

In 1998, the American Joint Committee on Cancer (AJCC) Melanoma Staging Committee developed the AJCC melanoma staging database, an international integrated compilation of prospectively accumulated melanoma outcome data from several centres and clinical trial cooperative groups. Analysis of this database resulted in major revisions to the TNM staging system reflected in the sixth edition of the AJCC Cancer Staging Manual published in 2002(Kim et al. 2002). More recently, the committee's analysis of an updated melanoma staging database, including prospective data on more than 50,000 patients, led to staging revisions adopted in the seventh edition of the AJCC Cancer Staging Manual published in 2009(Balch et al. 2009). Generally, the classification and staging manual implicates the use of pathohistological and clinical criteria to stratify the melanoma patients to groups which differ in terms of tumour stage, thus demanding an individualized therapeutic approach.

TNM Staging Categories for Cutaneous Melanoma

Classification	Thickness (mm)	Ulceration Status/Mitoses
T		
Tis	NA	NA
T1	≤ 1.00	a: Without ulceration and mitosis < 1/mm^2 b: With ulceration or mitoses ≥ 1/mm^2
T2	1.01-2.00	a: Without ulceration
		b: With ulceration
T3	2.01-4.00	a: Without ulceration
		b: With ulceration
T4	> 4.00	a: Without ulceration
		b: With ulceration

N	No. of Metastatic Nodes	Nodal Metastatic Burden
N0	0	NA
N1	1	a: Micrometastasis[*] b: Macrometastasis[†]
N2	2-3	a: Micrometastasis[*] b: Macrometastasis[†] c: In transit metastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes	

M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Abbreviations: NA, not applicable; LDH, lactate dehydrogenase.
[*]Micrometastases are diagnosed after sentinel lymph node biopsy.
[†]Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically.

Anatomic Stage Groupings for Cutaneous Melanoma.

	Clinical Staging*				Pathologic Staging†		
	T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Any T	N > N0	M0	IIIA	T1-4a	N1a	M0
					T1-4a	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4a	N1b	M0
					T1-4a	N2b	M0
					T1-4a	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0
					T1-4b	N2c	M0
					Any T	N3	M0
IV	Any T	Any N	M1	IV	Any T	Any N	M1

*Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

†Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial (ie, sentinel node biopsy) or complete lymphadenectomy. Pathologic stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes.

The pathology report on a primary cutaneous melanoma serves as ground source for melanoma staging and classification. The following histopathological features that affect prognosis and management of the melanoma patient are recommended for inclusion in the pathology report(Scolyer et al. 2004):

1) Site of the tumour
2) Diagnosis
3) Histologic type
4) Breslow Thickness
5) Ulceration
6) Dermal mitotic rate (per mm2)
7) Clark's level
8) Vascular or lymphatic invasion
9) Neurotropism
10) Desmoplasia
11) Satellites
12) Features of regression
13) Predominant cell type
14) Associated nevus
15) Nearest lateral and deep margin

As immunohistochemistry and molecular biology has become a widely available and integral part of a modern pathology practice, the result of adjunctive immunohistochemical analyses as well as molecular analyses should preferably accompany the final pathology report.

Another challenge in pathology practice is evaluation of sentinel nodes in patients with malignant melanomas. The sentinel lymphadenectomy procedure allows the regional lymph nodes of melanoma patients to be histopathologically staged without the need to

perform an elective complete lymph node dissection (Balch et al. 2001; Davids et al. 2003). In this way only those patients with definite occult nodal involvement are exposed to a selective completion lymph node dissection and adjuvant therapy(Reintgen et al. 1997). Those patients who have no sentinel node metastases can be spared from undergoing unnecessary further surgery and/or adjuvant therapy and can also be reassured of a better prognosis. The use of histological, immunochemical and molecular techniques in evaluation of the sentinel nodes are constantly improving which may reduce the false negative pathology reports.

Fine needle aspiration biopsy (FNAB) became a useful diagnostic modality for the assessment of palpable(Zajicek 1977) and non-palpable masses(Perry et al. 1986). It is particularly useful for visible or palpable masses occurring in melanoma patients that are clinically suspicious of recurrence of metastasis(Nasiell et al. 1991). CT- or ultrasound guidance allows fine needle aspiration and cytological diagnosis of non-palpable melanoma metastases in a cost effective and time saving manner.

Despite the fact that the clinical and pathological criteria distinguish most benign lesions from malignant ones are well recognized, occasionally difficulties in diagnosis occur, resulting in benign lesions being diagnosed as malignant and vice versa. The consequences of misdiagnosis may be disastrous. Benign nevi that are treated as melanomas may cause unnecessary disfigurement and morbidity, while melanomas misdiagnosed as nevi may result in delayed treatment and death of the patient. Therefore, the pathological diagnosis of malignant melanoma requires a thorough knowledge of its diagnostic features, pathogenesis and natural history as well as a keen awareness of potential diagnostic pitfalls. It is likely that future advances in understanding of the genetic and molecular basis of melanoma improve diagnostic accuracy and prognostic ability, which in turn will lead to the development of precisely targeted therapies.

1.3.6 Prognostic markers in malignant melanomas.

1.3.6.1 S100

The first member of the S100 protein family was originally isolated by Moore (Moore 1965) as a protein fraction from bovine brain tissue. This protein fraction was named S100 due to its solubility in 100% saturated ammonium sulphate solution at neutral pH. S100 was purified from bovine brain and at that time believed to be brain specific. Since then expression of S100 proteins has been demonstrated in a diverse spectrum of tissues and a total of 25 forms of this protein have been isolated and described (Santamaria-Kisiel et al. 2006). Generally, an S-100 family is found exclusively in vertebrates and are small acidic proteins with molecular weight ranging between 9 and 13 kDa.(Schafer and Heizmann 1996). Twenty one S-100 family members (S100A1-S100A18, trichhylin, filaggirin and repetin) have genes clustered at chromosome locus 1q21, while other S-100 proteins are found at chromosome loci 4p16 (S100P), 5q14 (S100Z), 21q22 (S100B) and Xp22 (S100G). All S-100 proteins, with the exception of S100G, exist as a symmetric dimmer, with each monomer containing two EF-hand motifs. The two EF hands are interconnected by an intermediate region, referred to as the hinge region. Each of EF-hand motifs characterized by presence of two Ca2+ binding sites of different affinity. S 100 members differ from one another mostly for the length and sequence of the hinge region and the C-terminal extension, which are therefore suggested to specify the biological activity of individual proteins (Donato 2003) Three members of the S-100 family (profilaggrin, trychohyalin and repetin) are large proteins that exhibit an S100 motif along their primary sequence. S100 proteins have no known enzymatic function and exert their intracellular effects by interacting with and modulating the activity of other proteins. S100 proteins are proposed to have intracellular and extracellular roles in the regulation of many diverse processes such as regulation of cytoskeletal components, protein phosphorylation, cell growth and

motility, cell-cycle regulation, transcription differentiation and cell survival (Donato 2001) Upon Ca2+ binding, each of S100 monomer opens up to accommodate a target protein. In this way, an S100 dimer functionally cross-bridges two homologous or heterologous target proteins. Specification of S100 protein interaction with target proteins depends on the amino acid sequence of the individual S100 regions participating in the formation of the binding site, differences in the orientation of helices in individual s100 proteins and structural characteristics of target proteins (Donato 2003) Currently more than 90 target proteins for activated S100 forms have been identified. The most significant number of S100 proteins has been shown to affect components of the cytoskeleton (S100B, S100A1, S100A2, S100A4, S100A6 and S100A11). However several of S100 have been also implicated in a variety of signal-transduction pathways activating an intracellular signal cascade that contributes to cell proliferation and survival (S100B, S100A1, S10012 and WS100P) (Santamaria-Kisiel et al. 2006) Some S100 members have been shown to regulate the activity of the same target molecules, often with different affinity. Several S100 family proteins have been demonstrated to play a role in development and progression of malignant tumours including those of melanocytic origin. The present theses have been focused on expression of the S100A1 and S100B in melanocytic tumours. The first one, S100A1 protein, has a molecular weight of 10.4kDa and exists as a homodimer of two S100A1 molecules (Rohde et al. 2010) Dimerization of S100A1 occurs independently of Ca^{2+} binding in a symmetric and antiparallel manner and is stabilized through hydrophobic bonds between helices. At present, more than twenty target proteins for S100A1 have been discovered (Wright et al. 2009). S100A1 exerts its function mainly as regulator of the dynamics of cytoskeleton constituents. However, having p53 and RAGE (receptor for advanced glycation end products) as target proteins S100A1 takes a part in inhibition of the cell repair as well as acts as growth factor. Involvement of S100A1 in

neoplastic disorders has been showed in several studies. Increasing levels of S100A1 in endometrial and ovarian cancers have been revealed in one study (DeRycke et al. 2009). Another study conducted by on renal neoplasms showed that S100A1 protein is expressed in oncocytomas, clear cell and papillary renal cell carcinomas but not in chromophobe renal cell carcinomas showing potential use of S100A1 staining for the differential diagnosis between chromophobe renal cell carcinoma and oncocytoma. (Rocca et al. 2007) Similar observation was reported by Li G and colleagues in study conducted on renal cancers (Li et al. 2007) Moreover, it has been shown that S100A1 has a modulation effect on metastasis promoting activity of S100A4 protein in mammary cancer (Wang et al. 2005) Several studies have been focused on expression of S100A1 proteins in malignant melanocytic tumours. In one study the researchers found that S100A1 protein in not expressed in melanocytic lesions of benign and malignant character (Boni et al. 1997). However, a different expression pattern has been shown in a study conducted on conjuctival melanocytic lesions showed high expression levels of S100A1 in malignant melanocytic lesions compared to benign (Keijser et al. 2006) which was confirmed by another study conducted on different subtypes of malignant melanomas (Nonaka et al. 2008)

The second protein the present which theses have been focused on is a S100B protein. It has a mass of 21.5-kDa and shows structure of a symmetric homodimer. Among other target proteins S100B has been shown to modulate the activity of p53 in different ways. By protecting of p53 from thermal denaturation and aggregation S100B stimulates p53-dependent cell growth arrest and apoptosis, thereby exerting tumour-suppressing activity. On contrary, by inhibiting of p53 transcription activation via disruption of the p53 tetramer the S100B protein exerts its tumour-promoting activity (Donato 2003) Several studies have been focused on analysis of the S100B expression in neoplastic disorders of different origin. A decreased expression of S100B protein

has been showed in bladder cancers (Yao et al. 2007). However, another study concluded that there no difference in S100B expression between renal cells cancer and normal kidney tissue, (Yang et al. 2004) Immunohistochemical analysis of S100B expression in colon cancer showed that this protein might be an independent predictor of early tumour relapse in II and III postoperative colon cancer patients stage (Hwang et al. 2011). Increased expression of S100B protein has been demonstrated in highly metastatic cell lines derived from lung cancer (Hu et al. 2010). A relation between increase of S100B protein expression and grade of malignancy in tumours of central nervous system has been reported (Camby et al. 1999). A number of studies have been focused on analysis of S100B expression in melanocytic tumours. Serum levels of S100B in patients with malignant melanomas have been extensively studied while, to our knowledge, only a few number of papers report expression of this protein in tumour cells. A variable expression pattern of this protein has been demonstrated. In a study conducted on conjunctival and uveal melanomas the authors reported an increased level of S100B in conjunctival melanomas while tumours of uveal origin showed a decrease of expression (Keijser et al. 2006). An expression analysis of S100B in cutaneous melanomas as well as normal melanocytes showed equally high levels in melanocytes, primary melanomas as well as metastatic tumours (Boni et al. 1997).]. It has been shown that analysis of S100B in serum may be useful in identifying high-risk cases and monitoring the response to therapy in patients with malignant melanoma (Hansson et al. 1997) as well as used as a independent prognostic factor (Bouwhuis et al. 2011).

1.3.6.2 CD44

CD44 is a ubiquitous multistructural cells surface adhesion molecule involved in cell-cell and cell-matrix interactions (Naor et al. 1997) and exerts its function as a principal receptor for hyaluronate (Aruffo et al. 1990). The CD44 gene has been mapped to the chromosomal locus 11p13 and is composed of two groups of exons. One group, comprising exons 1-5 and 16-20 are spliced together to form standard isoforms (CD44s). Another group, containing variable exons 6-15 (v1-10) can be alternatively spliced and inserted within the standard exons generating variants of CD44 (CD44v)(Tolg et al. 1993). The standard CD44 protein isoform consists of three regions, a 72AA C-terminal cytoplasmic domain, a 21AA transmembrane domain and a 270AA extracellular domain (FIG) (Goodison et al. 1999). The variable region at which alternatively spliced variant are inserted is located in extracellular domain. The principal ligand for CD44 is hyaluronic acid (HA) which is a linear, polymeric glycosaminoglycan with at least three binding sites to CD44 molecule (Yang et al. 1994). All CD44 isoforms contain the HA recognition sites, however, not all cells expressing CD44 bind the HA ligand constitutively. Cells can express CD44 protein in active, inducible and inactive state in respect to HA binding capability. Besides HA a number of extracellular ligangds such as osteopontin, collagen, fibronectin and laminin can bind to CD44 molecule (Goodison et al. 1999). The CD44s protein has multiple functions which explained by diversity of the CD44 variants. Primarily the standard variant of the CD44 is involved in the maintenance of three dimensional organ/tissue structure(Lesley et al. 1993). It has been shown that CD44 plays a critical role in angiogenesis, stimulating proliferation of endothelial cells (Trochon et al. 1996), wound healing (Jain et al. 1996), cell migration (Trochon et al. 1996), cells adhesion and aggregation (Green et al. 1988). Moreover, it has been shown that by interacting with HER2 cell surface protein the CD44 potentates invasiveness of tumour cells (Xu

et al. 1997). Involvement of the CD44 protein in neoplastic processes by diverse ways has been reported. Recently it has been found that the CD44 expression correlated with tumorigenicity in oesophageal squamous cell carcinomas (Zhao et al. 2011). Another study has summarized evidence of the CD44 involvement in ovarian cancer initiation, progression and intra-peritoneal spreading as well as distant metastasizing (Ween et al. 2011). It has been reported that the CD44 protein involved in progression of prostate cancer (Ghatak et al. 2010; Iczkowski 2010), breast cancer (Bagnoli et al. 2010), neurofibroma (Riddle et al. 2010), glioblastomas (Xu et al. 2010) and colorectal cancers (Wan et al. 2009). Several studies confirmed the role of the CD44 molecule in progression and proliferation of malignant melanomas (Ahrens et al. 2001; Dietrich et al. 1997) High level of expression of CD44 protein in primary malignant melanomas have been associated with increased metastatic risk and reduced survival. Highly metastatic melanoma cell lines show increased expression of the CD44 compared to melanocytes and melanoma cell lines of low metastatic activity(Goebeler et al. 1996). Besides its prognostic importance, the CD44 protein may have a role in therapeutic strategies in malignant tumours including that of melanocytic origin. There is evidence that blocking of HA by soluble CD44s decrease proliferation of melanoma cells [22]. Another study shows that binding of the CD44 protein by anti-CD44 antibody inhibits melanoma cells proliferation (Ahrens et al. 2001). The expression of the CD44 protein in metastatic melanomas might have therefore a prognostic as well as therapeutic importance.

1.3.6.3 CD40

CD40 is a cell surface receptor that belongs to the tumour necrosis factor-R (TNF-R) family and was first identified and functionally characterized on B lymphocytes (van Kooten and Banchereau 2000). It is a 45-50 kDa transmembrane glycoprotein and found on the surface of B lymphocytes, dendritic cells, follicular dendritic cells, haematopoietic progenitor cells, epithelial cells, and carcinomas (Banchereau et al. 1994) The CD40 gene maps to human chromosome 20q11-2-q13-2. The CD40 molecule composed of 277AA with 193AA extracellular domain, a 22AA transmembrane domain and a 62AA intracellular tail. The ligand for CD40 (CD40L, CD154, TRAP) is a type II membrane protein with significant sequence homology to TNF is primarily expressed on activated CD4+ T but is also found on stimulated CD8+ T cells, mast cells, eosinophils, B cells and dendritic cells(Grewal and Flavell 1998). Contact dependent interaction of the CD40 with its ligand at the surface of helper T-cells triggers the activation of protein tyrosine kinases, phosphoinositide-3 kinase and phospholipase resulting in rescue from apoptosis and growth and differentiation of B-cells (Splawski et al. 1993) The demonstration of the critical role of CD40/CD40L interactions in vivo came from discovery that the hyper IgM syndrome, an X-linked immunodeficiency, is due to a genetic alteration of the CD40L gene located at the X chromosome(Notarangelo et al. 1996). On non-haematopoietic non-neoplastic cells, the CD40 is involved in the amplification and regulation of inflammatory responses (van Kooten and Banchereau 2000). A number of studies have shown that CD40 is also expressed in a majority of carcinomas including ovarian, nasopharyngeal, bladder and breast (Eliopoulos et al. 2000) as well as melanoma (von Leoprechting et al. 1999) Interestingly that CD40 ligation on the surface of many malignant tumours including that of melanocytic origin have an opposite effect to what was reported on normal haematopoietic cells and results in inhibition of proliferation, induction of apoptosis

and direct cytotoxic effect (Vonderheide 2007) Therefore, treatment of tumour cells with CD40L or homologous substances might have an important therapeutical implications. A number of studies have showed positive effect of CD40L treatment inhibiting growth of breast cancer (Hirano et al. 1999), B-lymphomas (Funakoshi et al. 1994), ovarial cancer (Ghamande et al. 2001), myeloma (Tai et al. 2004), cervical cancer (Hill et al. 2005), mesothelioma (Friedlander et al. 2003) and melanomas (von Leoprechting et al. 1999). Moreover, expression of CD40 as well as CD40L by melanoma cells has been shown to be an independent prognostic marker(van den Oord et al. 1996). Thus, detection of CD40 molecule of the surface on metastatic melanoma cells might have therapeutic as well as prognostic implication.

1.3.6.4 Bcl-2

Bcl-2 is an acronym for the B-cell lymphoma/leukaemia-2 gene. The bcl-2 gene was first discovered because of its involvement in B-cell malignancies (Tsujimoto et al. 1985) , where chromosomal translocation from its normal position at 18q21 into juxtaposition at 14q32 results in overproduction of bcl-2 mRNAs and their encoded proteins (Nowell et al. 1985) Bcl-2 is novel among proto-oncogenes in that its coded protein localizes in inner mitochondrial membrane (Hockenbery et al. 1990). It has been discovered that stable expression of bcl-2 permitted prolonged survival of immature pre-B-cells without concomitant cell proliferation (Vaux et al. 1988). Thus, it was suggested that bcl-2 plays a central role in apoptosis – an autonomous pathway of programmed cell death that helps restrict cells numbers, which is crucial during embryonic development, organogenesis and homeostasis (Wyllie 1987a; Wyllie 1987b). A number of proteins which share structural and functional homology with its predecessor, bcl-2, have been discovered during last decades. The proteins comprise a bcl-2 family characterized by presence of at least one homology domain (BH). These

proteins are further divided into three subfamilies based on their function and structure. The anti-apoptotic members include Bcl-2, Bcl-X_L, Mcl-1, Bcl-w and A1/Bfl1. The second subfamily has a pro-apoptotic function and includes Bax and Bak. The third subfamily is the BH3-only group, so named because their sequence homology with the rest of the family is restricted to the BH3 domain (FIG)(Leibowitz and Yu 2010). The BH3-only proteins are largely responsible for sensing the apoptotic signals and then transmitting them to other Bcl-2 family members (Huang and Strasser 2000). This degree of functional diversity allows for cell and tissue-specific regulation of apoptosis in response to a broad range of stress stimuli. Regardless of the pathway to activation of proapoptotic Bax and/or Bak, the end result is mitochondrial outer membrane permeabilization, leading to the release of several pro-apoptotic proteins, including cytochrome *c*, SMAC/DIABLO and AIF (FIG) The release of these proteins into the cytosol leads to activation of caspases that carry out the controlled demise of the cell, through cleavage of structural proteins, kinases, transcriptional proteins and others, all leading to cell death(Li and Yuan 2008). The Bcl-2 family of proteins is a common target for deregulation in cancer, with several anti-apoptotic members being overexpressed and pro-apoptotic members being mutated or silenced in a variety of tumour types (Yip and Reed 2008). A number of studies have focused on bcl-2 expression in human malignancies showing different, sometimes controversial expression pattern of this protein. A increased expression of the bcl-2 has been associated with carcinogenesis and progression of prostate cancer (Catz and Johnson 2003), while overexpression of this protein in breast cancer marks a good prognosis (Schorr et al. 1999). However another study concerning expression of bcl-2 in breast cancer reported that there is no significant association of bcl-2 expression with either overall survival or disease free survival (Cecka et al. 2008). Longer survival rates were reported in colon cancers overexpressing bcl-2 (Kouraklis et al. 2003; Meterissian et al.

2001). Further, association of the increased expression of bcl-2 and better prognosis was reported in renal carcinomas (Itoi et al. 2004), squamous carcinomas of head and neck region(Lo Muzio et al. 2005) and cervical cancer (Crawford et al. 1998). However, expression of bcl-2 has no or minimal prognostic impact in gastric (Muller et al. 1998) and ovarian(Berker et al. 2002) carcinomas. Expression of the bcl-2 in melanocytic tumour of benign as well as malignant type has been extensively studied. While normal melanocytes and benign pigmented nevi show strong bcl-2 expression, a decrease in bcl-2 expression in malignant melanomas has been reported (Radhi 1999; Saenz-Santamaria et al. 1994). A significantly shorter survival was documented in melanoma patients with increased expression of bcl-2 which suggesting the bcl-2 measurement as a useful prognostic marker (Grover and Wilson 1996). However, other studies failed to show such a relationship and discovered that high bcl-2 expression in primary melanomas did not correlate with a worsening of prognosis and even reduced levels were found in melanoma metastases (Zhuang et al. 2007). Besides its possible prognostic value, detection of bcl-2 in cancers might have a therapeutic application. In the first place, a silencing method using antisense technology that in principle inhibits bcl-2 expression level by oligonucleotide G3130 showed promising results in several clinical trials of different cancers including malignant melanoma (Di Cresce and Koropatnick 2010; Moulder et al. 2008). Another approach is to block the activity of bcl-2 using antibodies directed against this protein. An intracellular anti-bcl-2 single chain antibody has been shown to increase drug-induced cytotoxicity in the breast cancer cell lines(Piche et al. 1998). Other approaches which use a blockade of bcl-2 includes use of ribozymes and syntetic Bak HB3 peptide that binds to bcl-2 (Wang et al. 2000). Since a limited success has been showed using the blockage approach, different strategies, mainly focused on antagonizing the function of Bcl-2 rather inhibiting it are currently studied and might have application in therapy of malignant

melanoma (Azmi et al. 2011). It is therefore of high interest to estimate expression of Bcl-2 in metastatic malignant melanoma since both prognostic and therapeutic application of that knowledge may be of importance.

1.3.6.5 Ki-67

The Ki-67 was initially described as a monoclonal antibody which recognizes a nuclear antigen present in proliferating cells but absent in resting cells (Gerdes et al. 1983). These findings suggested that Ki-67 is directed against a nuclear antigen associated with cell proliferation. The gene encoding Ki-67 protein is localized in chromosome 10 (Schonk et al. 1989)and consists of 15 exons which are transcribed to several splice variants including two forms detectable at protein level(Gerdes et al. 1991). It has been demonstrated that regulation of the Ki-67 protein during the cell cycle is associated with mitosis-specific phosphorylation (Endl and Gerdes 2000). Phosphorylation and dephosphorylation of the Ki-67 protein are controlled by key regulatory structures of the cell cycle and occur at two hallmark events within the cell cycle: the breakdown and the reorganization of the nucleus during mitosis(Endl and Gerdes 2000). This feature is extensively used in tumour diagnostics to estimate the growth fraction of a given cell population. The antibody against Ki-67 was used to assess the rate of proliferation virtually in all human malignancies providing valuable prognostic information as well as monitoring tumour response to established and trial therapies (Brown and Gatter 1990) Assessment of Ki-67 expression in melanocytic tumours of benign and malignant type is well documented. While benign melanocytic tumours show a low growth fraction, malignant melanomas and its metastases express high rates of Ki-67 protein expression (Bergman et al. 2001; Soyer 1991). Moreover, immunohistochemical assessment of Ki-67 may be used as a diagnostic tool and helps

distinguishing compound nevi of Spitz type from malignant melanomas(Kanter-Lewensohn et al. 1997).

1.3.6.6 CD117

CD117 (KIT) is a 145-kDa transmembrane glycoprotein that is structurally related to the receptor for macrophage growth factor (CSF-1) and the receptor for platelet-deriver growth factor and belongs to group of tyrosine kinase receptors (Yarden et al. 1987). The CD117 protein is encoded by a KIT (in the literature and here the name KIT is used instead of the name c-kit for simplicity for both the protein and gene name; the context tells which one is meant) proto-oncogene which is widely expressed as a single transcript and has been mapped to chromosome 4q12. The genomic DNA of KIT gene contains 21 exons (Vandenbark et al. 1992). The KIT protein consists of a extracellular, a transmembrane, a juxtamembrane and a intracellular split tyrosine kinase domains(FIG) (Pawson 2002). The Extracellular domain of the KIT protein contains five immunoglobulin-like loops which are encoded by exons 1-9. The transmembrane and the juxtamembrane domains are encoded by exons 10 and 11, respectively. Finally, the intracellular split tyrosine kinase domain is encoded by exons 13-21. The second immunoglobulin like loop of the extracellular portion of the CD117 protein binds to a ligand known as a stem-cell factor (SCF) (Williams et al. 1990) and is essential for development of melanocytes, erythrocytes, germ cells, mast cells and interstitial cells of Cajal (ICCs) (Huizinga et al. 1995; Maeda et al. 1992). SCF binding induces KIT dimerization and phosphorylation on tyrosines in the intracellular domain of the receptor. This then activates downstream protein targets such as mitogen-activated protein kinase (MAPK) in a cascade manner. The end results of KIT activation include

regulation of apoptosis, cell proliferation, differentiation, adhesion and motility (Miettinen and Lasota 2005; Ullrich and Schlessinger 1990). The significance of KIT in pathology of non-neoplastic diseases is illustrated by the consequences of its inactivating mutations leading to impairment of dependent cell types with macrocytic anaemia(Munugalavadla and Kapur 2005), mastocytosis(Longley et al. 1999), sterility(Huynh et al. 2002), and loss of skin pigmentation-piebaldism (Huynh et al. 2002) and albinism(Giebel and Spritz 1992). On the contrary, gain-of-function KIT mutations lead to tumours of KIT-dependent cell types(Kitamura and Hirotab 2004). Activating mutations typically confer constitutional KIT phosphorylation and downstream activation independent of ligand binding. Detection of the KIT protein in human malignancies have been of great interest in respect to therapeutical, aetiological, diagnostic and prognostic prospective. A lot of studies have been focused on use of a tyrosine kinase blocking agent, imatinib mesylate, in gastro-intestinal stromal tumours (GIST). It was found to be highly effective against metastatic and unresectable tumours (Demetri et al. 2002). The response is best in tumours with exon 11 mutations. The response is moderate with exon 9 mutation and poor with exon 17 mutations (Heinrich et al. 2003). Encouraged by the therapeutic success in GISTs, several researchers have been studying the expression of KIT proteins in various human malignancies. Increased expression of KIT have been documented in mesenchymal tumours of uterus and ovary (Erdogan et al. 2007), ovarian cancer(O'Neill et al. 2005), small cell lung carcinoma(Lopez-Martin et al. 2007), colon cancer(Toyota et al. 1993), breast cancer(Eroglu and Sari 2007), pancreatic cancer(Yasuda et al. 2006) and acute leukaemia(Valverde et al. 1996). Expression of KIT protein in melanocytic lesions of benign and malignant type has been extensively studied. It has been shown that whereas benign melanocytic tumours frequently express KIT at high levels, a decrease of KIT expression is found in malignant melanomas(Ohashi et al. 1996). This

relationship was later confirmed by several studies (Huang et al. 1998; Montone et al. 1997). However, some subtypes of malignant melanomas constitutively show high levels of KIT protein (All-Ericsson et al. 2004; Potti et al. 2003). It therefore can be speculated that malignant melanomas are heterogeneous tumour group in respect to expression of the KIT protein and detection of KIT-positive tumours might have diagnostic, prognostic and therapeutic importance.

1.3.6.7 COX-2

The enzyme cyclooxygenase (COX) also designated as prostaglandin H2 synthase (PGHS) catalyzes the first step of the synthesis of prostanoids, including g prostaglandins, prostacyclin and tromboxane(Hinz and Brune 2002). It has been demonstrated that COX exist as two distinct isoforms: COX-1 and COX-2 (Fu et al. 1990). COX-1 is located on chromosome regions 9q32-q33.3 and COX-2 maps to 1q25.2-1q25.3 (Vane et al. 1998). COX-1 is constitutively expressed in most tissues as a "housekeeping" enzyme and appears to be responsible for the production of prostaglandins that mediates regulation of homeostatic functions throughout the body(Adelizzi 1999), such as the maintenance of the integrity of the gastric mucosa(Sakamoto 1998), normal platelet function and the regulation of the blood flow(Crofford 1997). In contrast, COX-2 is undetectable in most normal tissues but expressed by cells that are involved in chronic and acute inflammation (e.g., macrophages, monocytes, synoviocytes)(Hinz and Brune 2002). The existence of a third COX isoform, COX-3, with proposed anti-inflammatory function is currently discussed(Willoughby et al. 2000).

The COX-2 may be induced by cytokines (interleukin), growth factors (TGF-B, transforming growth factor; EGF, epidermal growth factor), oncogenes and tumour promoters thereby contributing to the synthesis of prostaglandins in inflamed tissues

and malignant processes(Fosslien 2000). Most of the transcriptional signalling pathways require activation of the mitogen-activated protein kinase (MAPK) cascade. Moreover, MAPK signalling pathways are involved in regulating COX-2 gene expression at the post-transcriptional level(Tanabe and Tohnai 2002). Interestingly, the cyclooxygenase is irreversibly self-deactivating during the oxygenation of its substrate and therefore must be replaced to sustain production of prostaglandins(Fosslien 2000). In tumours, prostaglandins synthesised by COX-2 signal via receptors to stimulate cancer cell proliferation[10], inhibit apoptosis[9] and stimulate cancer cell motility(Singh et al. 2005), enhance cancer-induced angiogenesis(Toomey et al. 2009) and invasiveness(Chen et al. 2001). Moreover, it has been demonstrated that COX-2 may play a critical role in carcinogenesis of colorectal cancer(Wu et al. 2003), oesophageal squamous cells carcinomas[15], hepatocellular carcinoma(Ogunwobi and Liu 2011)and gastric carcinomas (Forones et al. 2008). Expression of COX-2 enzyme in melanocytic neoplasms of benign and malignant type has been demonstrated in several studies. The levels of COX-2 expression are significantly higher in melanomas compared to benign naevi (Chwirot and Kuzbicki 2007) which may have a diagnostic importance in distinguishing cutaneous melanomas from benign melanocytic lesions. Moreover, an escalation of the expression of the COX-2 enzyme during malignant melanoma progression has been demonstrated.

A selective blockage of COX-2 by non-steroid anti-inflammatory drugs (NSAIDs) is widely used in anti-inflammatory and analgesic therapeutic strategies. However, involvement of COX-2 in carcinogenesis and cancer progression has made this enzyme attractive for anti-neoplastic therapy. It has been showed that regular and prolonged intake of NSAIDs is associated with 40-50% reduction in colorectal cancer incidence(Sinicrope and Gill 2004). Positive results of combination therapy with meloxicam, a selective COX-2 inhibitor, and interferon-alpha have been documented in

a multicenter phase II trial for cases of metastatic renal cell carcinoma suggesting this regimen as a first-line treatment(Shinohara et al. 2009). Another study demonstrated that use of a selective COX-2 inhibitor, celecoxib, shows beneficial effects in gliomas which are expressing high levels of the enzyme(Cherukuri and Nelson 2004). An enhancing effect of the celecoxib to preoperative treatment of non-small cell lung cancer has been documented [20]. A study on melanoma cell lines showed both cytostatic and cytotoxic effect of cyclooxygenase inhibitors (Chiu et al. 2005)
It is therefore might be of high interest to identify cases of malignant melanomas expressing high levels of COX-2 enzyme since those cases may potentially benefit from the treatment with selective COX-2 inhibitors.

1.3.6.8 HSP90

Shortly after exposure to a significant increase over basal temperature, the cells in most tissues dramatically increase the production of a restricted class of proteins collectively termed heat shock or stress proteins which serve as central integrators of protein homeostasis within cells(Bagatell and Whitesell 2004). Increased expression of the heat shock proteins enhances cell survival in tissues damaged by a variety of stressors including heat, heavy metals, hypoxia, or acidosis. One of such proteins, heat shock protein 90 (Hsp90), is required for the stability and function of a number of conditionally activated and/or expressed signalling proteins, as well as multiple mutated, chimeric, or overexpressed signalling proteins, which promote cancer cell growth and survival(Neckers and Ivy 2003). Despite the name “heat shock protein” most chaperones are produced under basal conditions at substantial levels and even under non-stressed conditions the Hsp90 comprises as much as 1% to 2% of total cellular protein. HSP90 (90kDa) is a highly conserved and essential stress protein that is expressed in all eukaryotic cells (Pratt and Toft 2003). The complete HSP90 protein is

a homodimer of two identical subunits. Each monomer has four distinct domains; a highly conserved N-terminal (NTD) domain (25 kDa); a "charged linker" region that connects the N-terminus with the middle domain; a middle domain (MD) with molecular mass of 40 kDa and a 12 kDa C-terminal domain (CTD)(Prodromou and Pearl 2003). The N-terminal domain contains the ATP-binding site and is responsible for intrinsic ATPase activity of HSP90. Structural alterations driven by the hydrolysis of ATP to ADP in this domain play an essential role in the chaperoning activity of the HSP90 protein. This domain is also the binding site of the structurally unrelated natural products geldanamycin and radicicol as well as the growing number of semisynthetic compound which are studied as potential anti-neoplastic agents. The middle domain is a major site of client protein binding [5]. The c-terminal serves as a dimerization site. Increased expression of chaperone protein HSP90 has been demonstrated in several human neoplasias which may reflect the ability of malignant cells to maintain homeostasis in a hostile environment(Picard 2002). In addition to facilitating cell survival the HSP90 also allows tumour cells to tolerate alterations from within, including mutations of critical signalling molecules that would otherwise be lethal(Takayama et al. 2003).

Increased expression of HSP90 has been demonstrated in several human cancers including those of salivary gland (Vanmuylder et al. 2000), bladder (Lebret et al. 2003), endometrium (Nanbu et al. 1998), liver (Lim et al. 2005), oesophagus (Faried et al. 2004), breast (Pick et al. 2007) as well as melanoma (McCarthy et al. 2008). Generally, HSP90 protein overexpression indicates a poor prognosis in terms of survival and response to therapy of neoplastic processes. Elevated HSP90 expression in malignant tumours cells plays a key role in protection from spontaneous apoptosis associated with malignancy as well as the apoptosis generated by therapy. Besides its prognostic importance, the HSP90 has received much attention as a potential target for blocking

anti-cancer therapy development. Since the client proteins of the chaperoning function of the HSP90 include many oncogenic proteins such as Her2/ErbB2, Akt, Raf-1, Hif-1a, hormone receptors, mutant p53 and hTERT, it is plausible to speculate that the inhibition of HSP90 might lead to the simultaneous degradation of these oncogenic proteins(Hahn 2009). The use of currently available selective HSP90 inhibitors, geldanamycin and radicicol, as well as theirs synthetic analogs is currently studied(Taldone et al. 2008). Studies on melanoma cell lines showed a positive effect with heat shock proteins inhibitors providing a base for the clinical use of this therapeutic strategy in malignant melanomas(Babchia et al. 2008; Beloueche-Babari et al. 2010; Burger et al. 2004). As such, the estimation of the HSP90 protein expression in malignant melanomas may provide both prognostic and therapeutic information.

1.3.6.9 BRAF gene mutation V600E

The responses that cells make to their environment are controlled by a series of highly conserved signalling pathways which transmit signals from the cell surface to the nucleus through a series of protein cascades. One of such cascades is the RAS/RAF/MEK/ERK pathway(FIG) (Marais and Marshall 1996). In this pathway growth factors bind to and activate receptor tyrosine kinases (RTKs) which stimulate activation of RAS-proteins: K-RAS, H-RAS and N-RAS. RAS proteins are attached to the inner surface of the plasma membrane. In their active form, they bind to and recruit RAF proteins from the cytosol to the plasma membrane where RAF is activated. RAF is a group of serine/threonine protein kinases which includes A-RAF, B-RAF, and C-RAF. In the signal cascade, RAF phosphorylates and activates a second protein kinase called MEK, which in turn phosphorylates and activates a third protein kinase - ERK. The ERK phosphorylates proteins in the cytosol, and it also translocates to the

nucleus, where it phosphorylates proteins such as transcription factors(Ballif and Blenis 2001). Classically, this pathway is associated with proliferation and also regulates differentiation, apoptosis and survival(Garnett and Marais 2004). Alterations which activate the members of this pathway are implicated in development of many of the human malignancies(Davies et al. 2002; Mercer and Pritchard 2003). The b-raf gene mapped to chromosome 7q34 which encodes one of the RAF kinases, BRAF, often shows activating mutations in human neoplasias. Mutation of this gene have been reported in gliomas(Dougherty et al. 2011), colorectal cancers(Baldus et al. 2010), adrenocortical carcinomas[8], prostate cancer(Jeong et al. 2008), germ cell tumours(McIntyre et al. 2005), papillary thyroid cancers(McIntyre et al. 2005) as well as melanomas(Gray-Schopfer et al. 2005). It has been shown that approximately 42% of melanomas (Rubinstein et al. 2010) and 80% of benign nevi (Pollock et al. 2003) harbour activating BRAF mutations. Most commonly, the valine at amino acid 600 is replaced by glutamate (V600E) through mutation of a single nucleotide: GTG to GAG. It has been shown that incidence of BRAF gene mutation in malignant melanoma correlates with its progression which implicates prognostic importance (Dong et al. 2003). The incidence of BRAF mutation is increasing towards the advanced stage of disease and is maximal in metastatic malignant melanomas(Shinozaki et al. 2004). The detection of the activating BRAF mutation in malignant melanomas may also have a therapeutical importance. Specific BRAF kinase inhibitors, such as sorafenib, are undergoing rapid clinical development and promising data on efficacy have been demonstrated. It has been shown that use of sorafenib enhances the response of melanoma to regional chemotherapy (Augustine et al. 2010). Anti-cancer activity of sorafenib in malignant melanomas has been demonstrated in several studies (Eisen et al. 2011; Escudier et al. 2007). Among the latest inhibitors of the aberrant BRAF activity is a synthetic compound PLX4032 (vemurafenib). Recent clinical data from

clinical trials indicate that PLX4032 causes tumour regression and prolongs survival in patient with advanced malignant melanoma (Chapman et al. 2011; Lee et al. 2010). It therefore seems reasonable to assume that the detection of a V600E BRAF mutation in malignant melanomas may have prognostic as well as clinical importance.

2. AIMS OF THE STUDY.

- To analyze the expression of proteins CD40, CD44, Bcl-2, CD117, COX-2 and HSP90 in fine needle aspirates from metastatic malignant melanomas and correlate the expression to histopathological parameters of the primary tumours as well as rate of cell proliferation estimated by Ki-67 expression. (Papers I and II).

- To analyze the expression of proteins S100A, S100B, CD44 and Bcl-2 in formalin-fixed paraffin-embedded sections from primary benign and malignant melanocytic tumours as well as metastases of malignant melanomas and compare the expression to histopathological parameters and the rate of cell proliferation estimated by Ki-67 expression in the primary tumours. (Paper III).

- To analyze frequency of BRAF V600E mutation in fine needle aspirates from malignant melanomas and compare and compare the results to the clinical and histopathological parameters of the primary tumours as well as rate of cell proliferation estimated by Ki-67 expression (Paper IV).

3. MATERIAL AND METHODS.

3.1 Protein expression

A series of archived non-fixed frozen cytospin preparation slides from fine needle aspirates of melanoma metastases were used for immunocytochemical detection of proteins CD40, CD44, Bcl-2, CD117, COX-2, HSP90 and Ki-67 (Papers I, II and III). Ninety cases (57 males and 33 females, age ranging from 25 to 89 years, mean 64 years) were used in Paper I, thirty cases (18 males and 12 females, aged between 34 and 90 years, mean 67 years) were used in Paper II and forty-four cases (25 males and 19 females, age range 28-90 years, mean 66 years) were used in Paper III. Initially, the fine needle aspirates were obtained using an aspiration procedure as described by J. Zajicek (Zajicek 1974). The leftover material after initial diagnostic work-up was suspended in isotonic phosphate buffered saline and centrifuged on three-well cytospine slides using approximately 10^5 cell concentration. The cytospine slides were then air dried, placed in plastic containers and stored at -20C^0. After retrieval from the archive the containers were allowed to reach the environmental temperature in order to avoid cell lysis. The slides were then taken from the containers and placed on slideholders. A three step alkaline phosphatase immunocytostaining technique as described earlier(Tani et al. 1988) was used for the detection of the CD40, CD44, Bcl-2, CD117, COX-2 and HSP90 proteins in cell cytoplasm and cell membrane. A three step peroxidase-avidin-biotin immunocytostaining technique for detection of the nuclear Ki-67 antigen was applied as previously described (Skoog et al. 1990). The slides were counterstained with Giemsa staining and mounted using water-based mounting media. In all immunoassaying series, reactive lymphatic nodules were used as positive control and omitting the step of application of the primary antibody served as a negative control.

A series of archived formalin-fixed, paraffin-embedded material from benign nevi, primary and metastatic melanomas were used for detection of proteins S100A1, S100B, Bcl-2 and Ki-67 (Paper III). One hundred and twenty-six patients (32 males and 94 females with range 11-94 years, mean 43 years) were included in this retrospective study. After retrieval from the archive, four-micrometer thick sections on preparation slides were made. The preparations were subsequently deparaffinized and rehydrated and used for immunohistochemical staining procedure utilizing a high-sensitivity labelled strepravidin-biotin (LSAB) technique (LSAB+ it, Dakopatts AB, Sweden) (Giorno 1984). A commercially available antibodies to cytoplasmatic and membranous proteins S100A1 (clone DAK-100A1/1), S100B (clone DAK S100B/2), CD44 (clone DF 1485), Bcl-2 (clone 124) and nuclear protein Ki-67 (clone MIB-1) were used in the study. A technique for heat-induced microwave antigen retrieval was used as described earlier(Shi et al. 1991). Omitting application of primary antibody was used as a negative control. Slides, known to express the analyzed proteins, containing brain tissue, squamous carcinoma and breast carcinoma were used as positive controls for S100A1 and S100B, Bcl-2 and CD44 markers, respectively. The slides were counterstained with Haematoxylin-Eosin stain and mounted using water-based mounting media. Three pathologists independently reviewed the archived primary slides from respective cases. Then a consensus was reached among the pathologists in all cases regarding the type of melanocytic tumour using criteria as proposed by world health organization classification of skin tumours. All cases were stratified to the following morphological groups: intradermal nevi, compound nevi, dysplastic nevi, malignant melanoma and metastatic melanoma.

All cells with preserved structure showing clear cytoplasmatic/membrane staining (CD40, CD44, Bcl-2, CD117, COX-2 and HSP90) or nuclear staining (Ki-67) were scored as positive. The number of positive cells was registered using electronic cells

counter (CelloDiff III, Analysis Instrument AB, Sweden). Cells with damaged cell membrane or artefacts were not included in scoring results. The results were given as percentage of positive cells divided by the total cell number in the analyzed area. The scores were documented using Microsoft ® Excel ® software. Data related to the histopathological features of the primary tumours as well as evaluated clinical parameters were available either from a computerized file archive system at Karolinska Hospital (SymPathy, TietoEnator AB, Sweden, Papers I and II) or archived recordings at Latvian Centre of Pathology (Paper III). Statistical analysis was performed using Statistica ™ software (Statsoft AB, Uppsala, Sweden).

3.2 BRAF gene mutation V600E

A series of forty-four cases of metastatic malignant melanomas (25 males and 19 females, age range 28-90 years, mean 66 years) was used for detection of BRAF V600E mutation (Paper IV). All patients were previously surgically treated for primary malignant melanomas and the diagnosis was histologically verified. The patients were referred to the cytology department at Karolinska Hospital between 2006 and 2007 for fine-needle aspiration of lesions suspicious for metastatic disease. The aspirations were performed as described earlier (Zajicek 1974) and one part of aspirates was used for diagnostic work-up including microscopy and immunocytostaining, and the other part of the aspirates was suspended in phosphate-buffered saline (pH 7,4). The saline-suspended material was then centrifuged, the supernatant was removed and the pellet was frozen at -20^0C. The material was retrieved from the archive and used for genotyping of BRAF V600E mutation. Extraction of the genomic DNA was performed using Wizard ® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The concentration of DNA was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). A 5’ nuclease assay for allelic

discrimination method was used for genotyping of the exon 15 V600E mutation of the BRAF gene. A Tag-Man MGB biallelic discrimination system was used as previously described(Livak et al. 1995). Probes and nucleotides were synthesized in 40 concentrations by Applied Biosystems (Foster City, CA, USA) using the Assay-by-Design ordering system. PCR's were performed on ABI prism Sequence Detection System 7000 (Applied Biosystems. The 25µl PCR contained 1x of the TagMan Universal PCR Master Mix, No AmpErase UNG, 1x of the assay probe and primer mix (forward primer CATGAAGACCTCACAGTAAAAATAGGTGAT, reverse primer GGATCCAGACAACTGTTCAAACTGA, VIC MGB probe CCATCGAGATTTCACTGTAG, FAM MGB probe CCATCGAGATTTCTCTGTAG), and 25-50 ng of the sample genomic DNA and was performed on 96-well optical PCR plates. Each genotyping plate contained no DNA template controls, and random samples were run as duplicates to confirm the successful genotyping process. SDS version 2.0 software was used to analyze real-time PCR data and end-point fluorescence. Genotyping data were exported from SDS software into Microsoft ® Excel ® programme data sheets for further analysis.

3.3 Statistical analysis.

Statistica ® software (Statsoft ™ AB, Uppsala, Sweden) was used for performing statistical calculations. Differences in means were analyzed using paired and unpaired t-test when appropriate. ANOVA test was used for overall comparison of immunoreactivity and diagnostic categories. A Pearson r2-correlation test was used for analysis of explanatory variables. The p-value levels of significance were set at ≤ 0.05.

4. RESULTS AND DISCUSSION.

4.1 Protein expression.

The immunostaining for antigens CD40, CD44, CD117, COX-2, HSP90 and Bcl-2 resulted in a cytoplasmic and membranous staining pattern with dominance of the later in staining pattern for CD44. The staining of the Ki-67 protein was strictly nuclear with varying intensity. All cells showing weak to strong were scored as positive.

4.1.1 S100.

S100 is a dimeric protein composed of α and β subunits, which regulates cellular cytoskeletal system as well as plays a role in cell cycle regulation, cell division and cell-to-cell communication (Zimmer et al. 1995). Since a majority of malignant melanomas express S100, immunochemical detection of this protein is used for purpose of differential diagnose. At present a total of 25 S100 isoforms are identified and several studies have been analyzing expression of different S100 variants in melanocytic tumours. Whereas a lot of studies have been analyzing level of the S100 variants in serum, only a few studies have been focused on analysis of S100 expression in primary and metastatic melanomas (Keijser et al. 2006; Nonaka et al. 2008). In paper III we have analyzed expression of S100A1 and S100B in benign melanocytic tumours, primary melanomas as well as melanoma metastases using monoclonal antibodies against these proteins. The fraction of S100A1 positive cells was lower in the group of benign melanocytic lesions with means of 41.1%, 55.7% and 74% for intradermal, compound and dysplastic nevi, respectively, as compared to that of primary melanomas and melanoma metastases with mean of 85% and 86.0, respectively. The difference in the S100A1 expression in benign and malignant melanocytic tumours reached

statistical significance (r=0.61; r^2=0.376). In contrary, the fraction of S100B positive cells was higher in the group of benign melanocytic tumours as compared to that of malignant tumours with means of 86.3%, 88.4%, 75.6%, 47.7% and 52.5% for intradermal, compound and dysplastic nevi, and primary and metastatic melanomas, respectively. A statistically significant difference in respect to S100B in groups of benign and malignant melanocytic tumours was observed in our study (r=-0.627; r^2=0.393). This observation allows us to conclude that simultaneous analysis of S100A1 and S100B proteins in melanocytic lesion may be used in purpose of differential diagnose between benign and malignant tumours. Interestingly, the staining of S100A1 in malignant melanomas was more intensive in areas with increased proliferative activity of the tumour cells as estimated by Ki-67. This observation allow us to assume that the activity of the S100A1 might be related to cell cycle regulation, which is in concordance with study on cells cultures (Zimmer et al. 1995). Further studies are needed to explain the discrepancy between elevated serum level of S100B and its decreased expression in tumour cells.

4.1.2 CD40.

CD40 was originally described as a type I transmembrane glycoprotein on B lymphocytes It plays an important role in the immune response mediating maturation of B-cells as well as regulating the response of regulatory T cells both during their development (Foy et al. 1995) and their encounter with the antigen (Cayabyab et al. 1994). CD40 molecule is expressed in non-haematopoietic tissues including tumours and has been suggested to have a critical role in the processes of cell proliferation, migration and apoptosis (Pirozzi et al. 2000). Expression of CD40 and its prognostic significance in primary cutaneous melanomas has been described (van den Oord et al.

1996). Thus, it was of high interest to analyze the expression of CD40 protein in metastatic malignant melanomas and evaluate if the expression levels might be correlated to well known parameters of melanoma progression and prognosis such as survival rate and histomorphological properties of corresponding primary tumours. In Paper I we demonstrated that metastatic melanomas are a heterogeneous tumour in respect to CD40 expression. This observation is in agreement with previous studies showing variable frequencies of CD40 expression in melanoma cells (Pirozzi et al. 2000; Thomas et al. 1996; van den Oord et al. 1996). In our series, the fraction of the CD40-positive cells in metastatic melanomas varied from negative to maximal value of 58.9% with mean 8.5% and median 3.0%. Moreover, the fraction of CD40 positive cells in metastatic melanomas was higher in patients with clinical stage III, median 22.8%, as compared to that of clinical stage II with median number of 3.2%. However, no significant correlation was found between expression of the CD40 and histopathological parameters of the corresponding primary tumours. Median percentage of CD40 positive cells in melanoma metastases originating from melanomas with Clark's invasion levels 2, 3, 4 and 5 were 1.3%, 2.1%, 2.4% and 4.2% in, respectively. Median percentages of CD40 positive cells were 3.4%, 2.6% and 0.4% for metastases from primary tumours with thickness equal or less than 1mm, 1mm to 4mm, and more than 4mm, respectively. No correlation between survival after diagnosis of first metastasis and expression of CD40 was found in our study. It is therefore plausible to speculate that other pathways than those regulated by CD40 are involved in progression of malignant melanomas. However, the expression of CD40 might reflect unique features of an individual tumour rather than whole tumour group. A study on melanoma cell lines has demonstrated that the CD40 molecule may play a role in the complex interactions underlying recruitment and/or expansion of tumour recognizing' immune effectors, thus identifying CD40 as one of the molecules involved in melanoma cell-

mediated co-stimulation of anti-CD3-induced human T cell proliferation(Pirozzi et al. 2000). Thus, we speculate that the expression of the CD40 protein in malignant melanomas reflects ability of melanocytes act as "non-professional" antigen-presenting cells and reflects individual properties of the tumour and the patient.

4.1.3 CD44.

Several recent studies on melanoma cell cultures showed that CD44 protein plays an important role in regulating adhesion and migration of melanoma cells, thus contributing to theirs high metastatic potential(Hernandez et al. 2011; Kozlowska et al. 2004; Weimann et al. 2003). However, only a few studies have been focused on role of CD44 molecule in malignant melanomas *in vivo*. One study reported an increased metastatic risk and reduced survival in tumours with high expression of CD44 (Dietrich et al. 1997). In the present study we have analyzed the expression of CD44 in benign melanocytic tumours (Paper III), primary melanomas (Paper III) as well as metastatic melanomas (Papers I and III) and compared the expression levels to histopathological parameters of the corresponding primary tumours as well as survival. Immunostainig of the CD44 antigen in all melanocytic tumours in all our studies resulted in membranous staining pattern obviously reflecting the nature of CD44 as a transmembrane glycoprotein. In the group of benign melanocytic lesions the mean percentage of CD44 expression was 60.4%, 62.1%, and 52.1%, with standard deviations (SD) of 33.5, 33.5 and 35.1 for groups of intradermal nevi, compound nevi and dysplastic nevi, respectively (Paper III). The mean percentage of the CD44 expression in primary melanomas was 59.4% (SD=33.7) (Paper III). Metastatic melanomas showed mean percentage of 76.1% (SD=22.5) (Paper I) and 55.7% (SD=42.0) (Paper III). The percentage of CD44 positive cells in metastatic melanomas showed a tendency to be lower in patients with long survival after the first metastasis which is in concordance

with a previous study(Dietrich et al. 1997). However, this tendency did not reach statistical significance in our series (r-value= 0.12). No significant correlation was found between the expression of the CD44 protein in metastatic melanomas and histomorphological properties of the corresponding primary tumours. Benign and malignant melanocytic tumours had similar expression of CD44. On the basis of our results we may conclude that melanocytic neoplasias of benign as well as malignant character constitutively express the CD44 antigen which suggests functional and/or structural involvement of the CD44 molecule in these tumours. However, we could not demonstrate a statistically significant difference in CD44 expression in various groups of melanocytic lesions. It should be pointed out that in our studies a monoclonal antibody which is reactive with all more than 20 known isoforms of CD44 protein was used. Thus, we were not able to estimate the expression of individual variants of CD44 in melanocytic tumours. A heterogeneous type of expression of CD44 in human cancers has been reported (Goodison and Tarin 1998) which allow us to hypothesise that progression of melanocytic tumours, in analogy with other neoplastic processes, involves a specific isoform variant of CD44 protein rather than the entire class of these proteins. Further studies are needed to confirm this hypothesis.

4.1.4 Bcl-2.

The mitochondrial pathway of apoptosis constitutes one of the main safeguards against tumorigenesis. The Bcl-2 family includes the central members of this pathway that regulate cell fate through the control of mitochondrial outer membrane permeabilization (MOMP). Bcl-2 proteins are often dysregulated in cancer, leading to increased survival of abnormal cells (Llambi and Green 2011). It has been shown that dysregulation of Bcl-2 proteins appears of critical importance for melanoma cell survival and drug resistance (Eberle and Hossini 2008). Elevated levels of Bcl-2 protein

have been demonstrated in conjuctival nevi as compared to conjuctival melanoma, indicating the diagnostic application of the Bcl-2 analysis in conjuctival melanocytic lesions (Westekemper et al. 2011). In one study, an increase of Bcl-2 protein levels has been demonstrated during melanoma progression from localized to metastatic disease (Zhang and Rosdahl 2006). Several studies showed that overexpression Bcl-2 in melanomas is associated with tumour progression, growth, decreased survival as well as malignant phenotype of melanomas (Trisciuoglio et al. 2005; Utikal et al. 2002; Vlaykova et al. 2002). However, another study showed that there is no association between Bcl-2 expression and survival in cutaneous melanomas (Espindola and Corleta 2008). Besides its possible prognostic importance, the analysis of Bcl-2 in malignant melanomas may have therapeutical importance. Moreover, targeting survival pathways which control the Bcl-2 protein expression is suggested as one of possible therapeutic strategies in malignant melanomas (Eberle and Hossini 2008). Oblimersen, an antisense oligonucleotide directed against Bcl-2, decreases expression of Bcl-2 by blocking the translation from mRNA. One study showed that a progression-free survival as well as overall survival is improved in patients with metastatic melanoma who receive oblimersen and dacarbazine as compared to those received only dacarbazine (Bedikian et al. 2006). Thus, it was of high interest for us to analyze expression of Bcl-2 in benign melanocytic tumours, primary melanomas and melanoma metastases. In Paper I we analyzed expression of Bcl-2 in fine-needle aspirates from melanoma metastases in ninety patients. In Paper II, the expression of Bcl-2 was analyzed on formalin fixed material from 45 intradermal (IN), 27 compound (CN) and 8 dysplastic nevi (DN) as well as 32 primary and 14 metastatic melanomas. The expression of Bcl-2 was higher in the group of benign melanocytic tumours with means of 46.6%, 34.7% and 38.6% for IN, CN and DN, respectively, as compared to that of primary and metastatic melanomas with means of 3.0% and 4.0%, respectively (Paper III). This trend was also

demonstrated in Paper I where mean of Bcl-2 expression in metastatic melanomas consisted 8.5%. Interestingly, the range of Bcl-2 expression was more prominent in groups of benign melanocytic tumours with variation of the mean percentage between 36.0%-57.1, 20.7%-48.6% and 4.1-73.1 in IN, CN and DN, respectively, as compared to that of 0.2%-5.8% and 1.2%-9.2% in primary and metastatic melanomas, respectively. Staining for Bcl-2 in melanomas had more intensive character in areas with increased proliferative activity as assessed by Ki-67 antibody. A significant correlation between Bcl-2 expression and shorter survival after first melanoma metastasis was demonstrated in Paper I. No correlation was found between Bcl-2 expression in metastases and Clark's invasion level as well as thickness of the corresponding primary tumours. The results allow us conclude that melanomas have decreased rates of Bcl-2 expression as compared to that of benign melanocytic tumours. However, the tumours with overexpressed Bcl-2 show shorter survival which allow to select melanoma cases with more aggressive clinical course. Malignant melanomas are heterogeneous in respect to bcl-2 expression which may be used in patient selection for individual treatment approach with specific bcl-2-blocking therapy.

4.1.5 Ki-67

Since its discovery by Gerdes (Gerdes et al. 1983) in 1983, assessment of Ki-67 became an important method for the evaluation of proliferating cell fraction in benign as well as malignant tumours. The strict association of Ki-67 expression with G1, S, and G2, phases of the cell cycle and mitosis and absence in resting cells (G0-phase) as well as high reproducibility made it an excellent tool for determining the so-called cell growth fraction. Estimation of growth fraction by analysis of Ki-67 has independent prognostic value for survival, tumour progression and recurrence, which repeatedly been proven in uni- and multivariate analyses of several human malignancies (Scholzen

and Gerdes 2000). Many studies have focused on analysis of growth fraction in melanocytic tumours of benign type as well as melanomas. Increased rates of proliferating cell fractions have shown significant association with shorter survival in patients with uveal (Karlsson et al. 1996), primary cutaneous (Henrique et al. 2000; Ladstein et al. 2010) and disseminated melanomas (Vlaykova et al. 1999). Moreover, analysis of proliferation rate by Ki-67 helps to discriminate Spitz nevi from malignant melanomas (Kanter-Lewensohn et al. 1997; Vollmer 2004) which makes it a useful tool in diagnostic work-up of melanocytic tumours. In our studies we have focused on analysis of proliferating rate as estimated by Ki-67 in fine-needle aspirates from metastatic malignant melanomas (Paper I, II and IV) as well as formalin-fixed, paraffin-embedded benign melanocytic tumours, primary melanomas and metastatic melanomas. In all series, immunostaining of Ki-67 antigen by monoclonal antibody MIB-1 resulted in strict nuclear staining of varying intensity. Expression levels of Ki-67 antigen varied in benign melanocytic tumour, primary melanomas and metastatic melanomas (Table). It can be seen from the table that growth fraction in melanocytic tumours was lower than that of malignant tumours. There was no overlap in proliferating fraction range of benign tumours (min=0.48%, max=1.84%) and malignant melanomas (min=5.9%, max=64.6%). This observation reached statistical significance (Paper III, r=0.814, r^2=0.663) and is in concordance with previous studies that showed the use of the growth fraction assessment in melanocytic lesion for discerning benign and malignant tumours. A significant correlation between growth fraction in metastatic melanomas and shorter survival rate was observed in Paper I (Pearson r= -0.317). The rate of cell proliferation was higher in primary melanomas which were 3mm or thicker compared to those less than 3mm (Paper III). However, this tendency was not statistically significant which was in line with similar observations in Paper I and IV. The mean percentage of growth fraction was lower

(20.7%) in melanomas without BRAF V600E mutation as compared to that of mutated tumours (26.2%), Paper IV. No difference was observed between growth fraction in malignant melanomas and time of developing metastatic disease. Summarizing the above we conclude that assessment of proliferating rate by detection of Ki-67 antigen in melanocytic tumours may have an important role in diagnostic work-up helping to discern benign and malignant tumours as well as contribute as a being a prognostic marker.

4.1.6 CD117.

Detection of the CD117 (KIT) protein in human malignancies has been of great interest from therapeutical, aetiological, diagnostic and prognostic perspective. Expression of CD117 in colorectal cancers, breast cancers, sarcomas, renal cell cancers and ovarian cancers has been associated with worse prognosis (Medinger et al. 2010). Detection of CD117 protein plays a diagnostic role in GISTs (de Silva and Reid 2003). Moreover, detection of an activating mutation in the KIT gene is used to identify individual tumours which may be sensitive to a specific kinase-blocking therapy with PLX4032, imatinib mesylate or other blocking agents in malignancies which are depending on MAPK (Mitogen Activated Protein Kinase) pathway (Miettinen and Lasota 2005). Several studies have been focused on expression of CD117 in primary melanomas. In one study the authors showed that expression of CD117 in melanocytic tumour may help to differentiate melanomas from benign nevi, but it did not correlate with prognosis (Oba et al. 2011). However, in a recent large-scale study on more than 500 cases of malignant melanomas, both overexpression of CD117 and presence of mutated KIT gene have been showed to correlate with a significantly shorter survival(Kong et al. 2011). In Paper II we analyzed the expression of CD117 in fine-needle aspirates

from metastatic melanomas. Immunostaining with a monoclonal CD117 antibody resulted in cytoplasmic staining pattern of varying intensity. Fourteen out of total thirty cases (46%) of metastatic melanomas showed CD117 expression. The fraction of CD117 positive cells ranged from 0% to 93% with mean of 12.2%. The expression of CD117 was similar in cases of monometastatic and multimetastatic melanomas with mean of 12.8 and 11.0, respectively. We have compared the expression of the CD117 in melanoma metastases with histopathological properties of corresponding primary tumours as well as rate of cell proliferation. No significant correlation was found between CD117 expression and Clark's invasion level, tumour thickness or rate of cell proliferation as estimated by Ki-67. We therefore conclude that metastatic melanomas are heterogeneous in respect to expression of CD117 and a small proportion of metastatic melanomas (46%) express this antigen. Only 16% of the tumours in our study showed expression of CD117 in more than 30% of tumour cells. This observation allows us to speculate that the MAPK pathway which has been shown to play an important role in of malignant melanomas is more often activated via other signalling pathways than KIT cascade. Studies on specific therapy with KIT blockers on unselected melanoma patients showed lack of clinical efficacy of this treatment (Kim et al. 2008; Ugurel et al. 2005). However, an increased frequency of KIT aberrations has been demonstrated in mucosal and acral melanomas but not in melanomas in areas from intermittent sun exposure (Curtin et al. 2006; Satzger et al. 2008). This shows that there is a subset of melanomas which still may represent targets for specific therapy based on KIT blockade, but an individual approach is needed for selection of patients. Thus, analysis of CD117 overexpression and KIT gene mutation may comprise a basis for individual approach in treatment of malignant melanoma.

1.4.7 COX-2

A range of experimental and clinical evidence has showed the importance of COX-2 protein in cancer development (Coussens and Werb 2002; Greenhough et al. 2009). Increased expression of COX-2 protein has been demonstrated in most human malignancies and it may have both prognostic and therapeutic implication. (Ghosh et al. 2010). It has been suggested that upregulation of COX-2 may result from activation of Ras and mitogen-activated protein kinase (MAPK) pathway(Sheng et al. 1998) – the molecular signalling which has been demonstrated to have an important role in melanoma progression(Fecher et al. 2008). Thus, specific blocking treatment directed against the COX-2 protein may have a therapeutic importance in malignant melanomas. Several studies have analyzed COX-2 expression in melanocytic tumours. An increased expression of COX-2 in malignant melanomas compared to benign melanocytic lesions has been shown in one study suggesting its diagnostic implication (Minami et al. 2011). Another study demonstrated that overexpression of COX-2 in malignant melanomas has significant correlation with disease-free survival (Becker et al. 2009). However, collective analysis of immunohistochemical data on the COX-2 expression in melanomas, presented by different researchers, shows lack of consistency and different expression patterns of COX-2 were reported (Kuzbicki et al. 2009). In Paper II focused on immunocytochemical analysis of COX-2 expression in fine-needle aspirates from malignant melanomas. In our series all melanoma cases showed expression of COX-2 protein with positive cell fraction ranging from 5% to 93% (mean=49.2%, median=49.0). In cases with multiple melanoma metastases, the median number of COX-2 positive melanoma cells was higher as compared to that of tumours with a single metastasis. A median of 62% and 41% was found in multimetastatic and monometastatic tumours, respectively. We have compared the expression of the COX-2 with histopathological prognostic parameters such as Clark's invasion level, tumour

thickness in millimetres as well as proliferation rate as detected by Ki-67. Although there was a variation in tumours with different invasion levels and thickness, this relationship did not reach a statistical significance. Our observations suggest that expression of the COX-2 protein in metastatic melanomas lacks prognostic importance. However, in our study we have shown that all melanomas express the COX-2 which suggests involvement of this protein in signalling pathways responsible for melanoma development or/and progression. Thus, cases overexpressing this COX-2 may represent candidates of a selective targeted therapy of malignant melanomas with COX-2 inhibitors (e.g. celecoxib) which is currently being evaluated (Bhatt et al. 2010; Wilson 2006).

4.1.8 HSP90

The Hsp90 chaperone is a main regulator of the stability and activity of more than 200 proteins including oncoproteins such as Her2, Akt, Bcr-Abl, c-Kit, EGFR and mutant BRAF (Porter et al. 2010). It has been demonstrated that cancer cell use the HSP90 chaperone machinery to protect an array of mutated and overexpressed oncoproteins from missfolding and degradation (Trepel et al. 2010). Therefore, HSP90 is recognized as a crucial facilitator of cancer cell survival making it an attractive target for specific blocking anti-cancer therapy. Increased expression of HSP90 in malignant melanomas has been observed in several studies suggesting it as a marker of tumour progression (Becker et al. 2004; McCarthy et al. 2008). In Paper II we analyzed the expression of HSP90 protein in tumour aspirates from malignant metastatic melanomas. All tumours in our series expressed HSP90 antigen. The fraction of HSP90 positive cells ranged from 9% to 98% with median of 84.0% and mean of 68.1%. Different expression levels of HSP90 were observed in patients with single and multiple melanoma metastases. In monometastatic tumours the median value of HSP90 expression was 64% whereas in

multimetastatic disease it was 89%. We also compared the rate of HSP90 expression in metastatic melanomas and histopathological properties of the corresponding primary tumours such as Clark's invasion level, tumour thickness as well as proliferating fraction as estimated by Ki-67. Although some differences in HSP90 expression in relation to these variables were observed the results did not reach statistical significance. Moreover, no statistical significant correlation was observed between expression of HSP90 and time of developing of metastatic disease. Therefore, our results show that despite lack of possible independent prognostic importance, expression of the HSP90 molecule can be demonstrated at high rates in metastatic melanomas, especially in multimetastatic disease. Thus, metastatic melanomas may have the molecular prerequisites to respond to HSP90-blocking therapy. A number of studies are currently evaluating anti-tumour activity of a novel class of specific HSP90 inhibitors *in vitro* and *in vivo*, which may present a new therapeutic possibility for metastatic melanoma (Mehta et al. 2011).

4.2 BRAF gene mutation V600E

The mitogen activated protein kinase (MAPK) pathway plays a key role in melanoma development making it an important therapeutic target. In normal melanocytes, the tightly regulated pathway conducts extracellular signals from cell membrane to nucleus via a cascade of phosphorylation events. In melanomas, dysregulation of the MAPK pathway occurs frequently due to activating mutations in the B-RAF and RAS genes or other genetic or epigenetic modifications, leading to increased signalling activity promoting cell proliferation, invasion, metastasis, migration, survival and angiogenesis. Approximately 70 – 80% of acquired melanocytic nevi and 50 – 70% of melanomas contain a B-Raf mutation, largely confined to a specific point within the genome, designated V600E (B-Raf^{v600e}) (Davies et al. 2002; Pollock et al. 2003). High

frequency and its presence in both benign and tumours confirms the important role of the V600E BRAF gene mutation an early and common genetic alteration in melanocytic tumours. Several studies have been focused on analysis of the presence of V600E BRAF mutation and melanoma progression. It has been shown that V600E BRAF mutation induces melanoma cells to metastasise (Dankort et al. 2009), promotes melanoma cell survival (Cartlidge et al. 2008) and is involved in a cascade of molecular events in early melanoma development (Cheung et al. 2008). Involvement of this mutation in such important steps of melanoma progression has made mutated BRAF oncoprotein an attractive target for a specific blocking therapy. Thus, detection of BRAF V600E mutation may represent an important step in diagnostic work-up of melanomas, providing useful information for individual treatment approach. In Paper IV study we analyzed frequency of V600E BRAF gene mutation in fine-needle aspirates from metastatic melanomas. We showed that the presence of V600E BRAF gene mutation was not restricted to any cytological type of melanomas and seventeen out of thirty-nine melanoma metastases (38.6%) showed presence this mutation. Interestingly, in the group of melanomas arising from the skin with chronic sun exposure (7 cases), only 1 case (14.28%) had this mutation, whereas 15 cases (40.5%) were mutated in the group of tumours arising in the skin without chronic sun exposure. This observation is in agreement with previous studies which showed that a subset of primary melanomas with primary tumours without chronic sun exposure are more likely to harbour the BRAF gene mutation (Si et al. 2011). In Paper IV we showed that the group of metastatic melanomas harbouring V600E BRAF mutation had higher rate of proliferating melanoma cells (26.2%) as compared to that of non-mutated tumours (20.7%). Mutated and non-mutated melanomas in our study showed different time of development of metastatic disease. While median of 14.4 months was observed in the group of mutated tumours, a median of 25.0 months was demonstrated in the group of

mutated melanomas. Moreover, correlation to histopathological properties of the corresponding primary tumours showed that both tumour thickness and Clark's invasion level were lower in primary tumours with BRAF V600E mutation compared to those without mutation. Summarizing the above, we may conclude that malignant melanomas are heterogeneous in respect to the presence of BRAF gene mutation and a subset of tumours arising from skin without chronic sun exposure are more likely to have this type of aberration. Detection of the BRAF V600E gene mutation in metastatic melanomas provides additional prognostic information, indicating tumours which may have more aggressive biological behaviour and providing a tool for patient selection in an individual treatment approach.

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