Investigation of predictive markers in patients with metastatic melanoma treated with bevacizumab

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Scientific environment

This PhD project has been performed within the Tumor Biology Research Group at the Centre for Cancer Biomarkers CCBIO (Norwegian Centre of Excellence) directed by Professor Lars A. Akslen, Department of Clinical Medicine, Section for Pathology, University of Bergen, Norway.

Associate Professor Oddbjørn Straume has been my main supervisor and Professor Lars A. Akslen has been my co-supervisor.

The research group headed by Lars A. Akslen was established in 1995 and counts around 25 members including research fellows, postdocs, senior researchers, students and technicians. The focus is on assessment and validation of cancer biomarkers and investigation of anti-angiogenic treatment in clinical trials. Oddbjørn Straume is the Principle Investigator in several of the clinical studies. Translational cancer research is performed at a high level with national and international collaboration networks. Preclinical models, patient samples and clinical as well as registry data are available to study prognostic and predictive markers. The aim is to establish validated and robust biomarkers for personalized treatment of cancer patients.

One main goal is to learn more about the role of the tumor microenvironment and angiogenesis in cancer progression and metastasis to identify key molecules that may serve as treatment targets. Another approach is to conduct clinical studies and then go back to the bench to identify predictive protein markers and analyze complex interactions. In addition, analyses of DNA and mRNA are performed to explore signatures that may predict prognosis and to identify tumors with more aggressive behavior that may need more extensive treatment.

Acknowledgements

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The study has used data from the Cancer Registry of Norway. The interpretation and reporting of these data are the sole responsibility of the authors, and no endorsement by the Cancer Registry of Norway is intended nor should be inferred.

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Bergen, June 2016

Cornelia Schuster

Errata

Published in **Paper I**: Number of patients treated 1st line: 20 and 2nd line: 15 (page 3).

Correct: Number of patients treated 1st line with bevacizumab: 21 and 2nd line: 14.

Published in **Paper I**: from April 2005 until August 2009 (page 2).

Correct: The time of inclusion was from April 2005 until March 2010.

These corrections do not affect the published results.

Abstract

Background: The incidence of malignant melanoma is still rising among fair-skinned people worldwide, and it is among the three major cancer types in young adults in Norway. Progression free survival and overall survival have improved for metastatic melanoma, but there is still a need to improve treatment options. Only about 50% of the melanomas are *BRAF* mutated and not all patients are eligible to immunotherapy or respond to this kind of treatment. Therefore, further treatment options and validated predictive markers needs to be investigated.

Purpose: This PhD project is based on a clinical Phase II trial that investigated efficacy and safety of bevacizumab monotherapy in patients with metastatic melanoma. Based on the encouraging results of a disease control rate of 31%, we wanted to investigate predictive markers in tissues and blood samples.

Material and Methods: 35 patients with metastatic melanoma in progression were included in this clinical Phase II trial conducted at Haukeland University Hospital. Bevacizumab was given in a dosage of 10mg/kg every second week until disease progression or unacceptable toxicity. Blood pressure was monitored at every treatment cycle. The primary endpoint was objective response (OR) including patients with complete response (CR) or partial response (PR) according to RECIST guidelines as well as disease control (DC) including patients with stable disease (SD) for more than 6 months in addition. Tissues from primary tumors and metastases as well as blood samples were collected. *BRAF* and *NRAS* mutation status was assessed (**Paper I**).

In this work, we focused on investigations in serum and plasma samples taken before treatment with bevacizumab was started. We performed immunohistochemical staining of whole tissue sections from primary melanomas and metastases. Based on pre-specified hypotheses, we investigated the angiogenesis-related markers VEGF-A, its splicing variant VEGF-A_{165b}, bFGF and HSP27 (**Paper II**). Microvessel density, the number of proliferating vessels and the presence of glomeruloid proliferations

(GMPs) were also assessed in tissue samples. In addition, we analyzed serum concentrations of VEGF-A, bFGF and HSP27 by ELISA (**Paper II**).

Furthermore, we investigated 60 angiogenesis-related proteins in serum samples by a multiplex array. Unsupervised hierarchical clustering as well as supervised analysis for particular proteins were performed. Candidate proteins that were associated with response to treatment were further validated by immunohistochemistry and ELISA (**Paper III**).

Results: 6/35 patients had objective response (OR) to treatment with bevacizumab monotherapy and 5 more patients had stable disease, resulting in a disease control (DC) rate of 31%. Responses were observed independently of *BRAF* and *NRAS* mutation status. Development of early hypertension was associated with a better DC-rate and may serve as a clinical predictive marker (**Paper I**).

Strong expression of HSP27 in metastases was associated with objective response to bevacizumab monotherapy. Strong expression of VEGF-A was associated with a higher number of proliferating vessels in primary melanomas and with a higher number of microvessels in metastases. However, microvessel density, the number of proliferating vessels or presence of GMPs in metastases were not associated with response to treatment. Serum concentration of VEGF-A, bFGF and HSP27 could not predict treatment response **(Paper II**).

Unsupervised hierarchical clustering of serum concentrations assessed by the multiplex array did not show any specific pattern. Proteins with a defined fold change difference between responders and non-responders were further investigated by specific analysis. Low serum concentration of Activin A as well as high serum concentrations of IL1b, uPAR and VEGF-A were associated with objective response to bevacizumab monotherapy. Single protein ELISA was performed for these candidate proteins but could not confirm the results from the multiplex array. However, strong expression by immunohistochemistry of Activin A, IL1b and uPAR in tumor cells from metastases was associated with objective response to bevacizumab monotherapy (**Paper III**).

Conclusions: Treatment with bevacizumab monotherapy showed promising efficacy in patients with metastatic malignant melanoma in progression. Development of early hypertension may be a potential clinical predictor. We identified strong expression of HSP27, Activin A, IL1b and uPAR in metastases as possible predictive markers. Since our results are based on a single-arm trial with limited sample size, they have to be interpreted carefully. Validation in a larger randomized trial has to be performed.

Abbreviations

AJCC: American Joint Committee on Cancer

AKT: v-akt murine thymoma viral oncogene homolog

ALM: Acral lentiginous melanoma

bFGF: Basic fibroblast growth factor

BAP1: BRCA1 associated protein-1

BRAF: V-RAF murine sarcoma viral oncogene homolog B1

BRCA 1/2: Breast cancer 1 and 2

CAE: Critical adverse events

CDK: Cyclin-dependent kinase

CDKN: Cyclin-dependent kinase inhibitor

CLND: Complete lymph node dissection

CMM: Cutaneous malignant melanoma

CNS: Central nervous system

CSC: Cancer stem cells

CSD: Chronic sun damaged

CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4

DC: Disease control

DFS: Disease free survival

DNA: Deoxyribonucleic acid

DNS: Dysplastic nevi syndrome

EC: Endothelial cell

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EH: Early Hypertension

ELISA: Enzyme linked immunosorbent assay

EORTC: European Organization for Research and Treatment of Cancer

ERK: Extracellular signal-regulated kinase

FAMMM: Familial atypical multiple-mole melanoma

FDA: Food and Drug Administration

GMP: Glomeruloid microvascular proliferation

GPI: Glycophosphatidylinositol

HILP: Hyperthermic isolated limb perfusion

HLA: Human leukocyte antigen

HPF: High power fields

HSP27: Heat Shock protein 27

IL1b: Interleukin 1b

IFN α: Interferon alfa

INR: International normalized ratio

KIT: Viral oncogene *v-KIT*

LDH: Lactate dehydrogenase

LLM: Lentigo maligna melanoma

MAPK: Mitogen-activated protein kinase

MCR1: Melanocortin-1 receptor

MDD: Minimal detection dose

MDSC: Myeloid-derived suppressor cells

MEK: Mitogen-activated protein kinase kinase

MITF: Microphthalmnia-associated transcription factor

MMIC: Malignant melanoma initiating cells

MVD: Microvessel density

MW: Microwave

NF1:	Neurofibromin 1
NIH:	National Institutes of Health
NM:	Nodular melanoma
NRAS:	Neuroblastoma RAS viral (v-ras) oncogene homolog
OR:	Objective response
OS:	Overall survival
PCR:	Polymerase chain reaction
PD:	Progressive disease
PD-L1:	Programmed death-ligand 1
PFS:	Progression free survival
PI3K:	Phosphatidylinositol 3-kinase
PTEN:	Phosphatase and tensin homolog
RB:	Retinoblastoma
RECIST:	Response evaluation criteria in solid tumors
RT:	Room temperature
RTK:	Receptor tyrosine kinase
SD:	Stable disease
SI:	Staining index
SLNB:	Sentinel node biopsy
SSM:	Superficial spreading melanoma
TNM:	Tumor-node-metastases
uPAR:	Urokinase plasmin activator receptor
UV:	Ultraviolet
VEGF(R):	Vascular endothelial growth factor (receptor)
VGP:	Vertical growth phase
WHO:	World Health Organization

List of publications

The present thesis is based on the following papers, which are referred to by their Roman numerals:

I. Cornelia Schuster, Hans P. Eikesdal, Hanne Puntervoll, Juergen Geisler, Stephanie Geisler, Daniel Heinrich, Anders Molven, Per E. Lønning, Lars A. Akslen, Oddbjørn Straume. Clinical efficacy and safety of bevacizumab monotherapy in patients with metastatic melanoma: predictive importance of induced early hypertension. *PLoS One.* 2012;7(6):e38364

II. Cornelia Schuster, Lars A. Akslen, Oddbjørn Straume. Expression of Heat Shock Protein 27 in Melanoma Metastases is Associated with Overall Response to Bevacizumab Monotherapy: Analyses of Predictive Markers in a Clinical Phase II Study. *PLoS One*. 2016; 11(5):e0155242

III. Cornelia Schuster, Tomasz Stokowy, Lars A. Akslen, Oddbjørn Straume. The predictive value of angiogenic proteins in serum samples from patients with metastatic melanoma treated with bevacizumab monotherapy. (*Manuscript submitted)*

Contents

1. INTRODUCTION

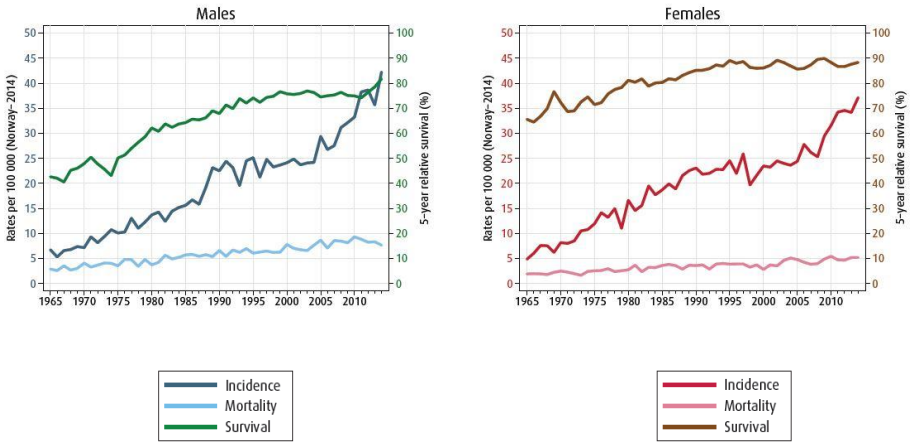
1.1 Epidemiology

The incidence of cutaneous malignant melanoma (CMM) has been increasing worldwide among fair-skinned populations over the last decades[1-4]. GLOBOCAN reported the highest incidence rates in Australia (40.3 per 100.000) and New Zealand (30.5 per 100.000), followed by a rate of over 10 per 100.000 in Northern and Western Europe and Northern America[5]. In contrast, incidence rates are 10 to 20-fold lower in non-white populations[5,6]. In general, the number of localized thin melanomas is increasing in white populations and especially among younger women[7].

The incidence rate in Norway is among the highest worldwide and has been increasing approximately tenfold since the 1950s with a temporary stabilization in the 1990s[8]. Incidence rates in men rise above those for women at the age of fifty years[8]. In total, 2.003 new cases of malignant melanoma were reported in the latest update from the Norwegian Cancer Registry in 2014; the age standardized incidence rate was 37.1 for women and 42.2 for men per 100.000 person-years during the period from 2010 to 2014[9]. CMM is the second most common cancer in young adults (25-49 years old) in Norway[9].

The relative survival rate was higher in women and in younger patients[9]. The latest survival rate was almost 90% for localized melanoma but only about 18% in melanoma patients with distant metastases[9]. The latest age-standardized mortality rate was 7.7 for men and 5.2 for women per 100.000 person-years in Norway[9]. The mortality rate for melanoma is been among the highest in Europa[10]. **Figure 1** illustrates the incidence, mortality and survival of malignant melanoma in Norway since the 1960s.

Figure 1. Incidence, mortality and survival for malignant melanoma in Norway[9]



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1.2 Etiology and risk factors

1.2.1 Phenotypic factors

The predisposed phenotypes for development of malignant melanoma are described by skin type I and II[11]. People with this skin types are characterized by green eyes, fair skin, freckles, blond or red hair and the tendency to get sunburned very easily. A big meta-analysis verified significantly increased risk for malignant melanoma for these phenotypical features[12]. Type and amount of melanin pigment produced by melanoncytes determine the color of skin and hair[13]. The melanocortin-1 receptor (MCR1) is expressed in melanocytes and melanoma cells and is one of the best known regulators of pigmentation[13,14]. Variations in the *MCR1* gene are associated with phenotypic characteristics of skin type I and II[15,16].

Furthermore, increasing numbers of melanocytic nevi and dysplastic nevi are independent risk factors for cutaneous melanoma[7,17,18]. The relative risk for melanoma was more than six times higher in patients with more than five atypical

nevi in a meta-analysis from case-controlled studies[19]. Architectural disorder and cytologic atypia are major criteria in definition of dysplastic nevi[20,21]. Clinical characteristics of dysplastic nevi defined by Tucker et al. in a large case-control study[18] were: size >= 5 mm, presence of a flat component as well as variable pigmentation, irregular outline and fuzzy borders.

B-K mole syndrome[22], familial atypical multiple-mole melanoma (FAMMM) syndrome[23] and dysplastic nevus syndrome[24] describe nevus phenotypes in melanoma-prone families. Clark, Lynch and Elder use different criteria in their reports and still today, there is no consensus about required diagnostic features[25]. The NIH consensus conference requires melanoma in first or second-degree relatives, > 50 clinical atypical nevi and distinct histological features for diagnosis of DNS. Thereby, this consensus is the only one that includes histology in diagnosis of FAMMM syndrome[25].

Another known risk factor are large congenital melanocytic nevi, defined by a diameter greater than 20 cm (Hale, EK BR J Dermatology 2005). Those nevi can develop into early onset melanomas in ca. 2-3%, a mean age of 12.6 years is reported in a recent review at diagnosis[26,27].

1.2.2 UV radiation

UV radiation is a well-known risk factor for cutaneous melanoma. UVA radiation has longer wavelength (320 - 400nm), represents the major part of solar radiation and can penetrate deeper in the tissue than UVB radiation with shorter wavelength (280-320 nm). Both UVA and UVB radiation can induce cyclobutane pyrimidine dimers that are mutagenic. UVA radiation induces in addition oxidative DNA damage in melanocytes[28-30]. The most common UV- induced DNA damages are C>T and CC>TT transitions, also called fingerprint mutations. This kind of mutations accounts for the majority of somatic mutations in melanoma[31]. UV radiation is also the reason for that melanoma has the highest rate of somatic mutations among all cancer types[32]. Furthermore, *CDKN2A* and *TP53* mutated melanomas showed an increased rate of UV fingerprint mutations[33].

Meta-analyses and case-control studies showed that the history of sunburn has significant impact on melanoma risk[34-36] and this association was seen during all periods of lifetime (childhood/adolescence and adulthood)[36-38]. However, several migration studies investigated the association between age of migration to lower latitude or more sunny destinations and risk of melanoma. Migration during childhood was correlated to a higher melanoma risk compared to migration at later ages[39]. These observations were used to postulate that increased sun exposure during childhood is associated to higher risk for melanoma.

Another risk factor is intermittent sun exposure, probably because of DNA damage due to relatively high dose UV radiation on insufficient protected skin[34,36,37]. Possibly, people living in higher latitudes experience more intermittent sunburn while traveling to more sunny regions. However, the pattern of sun exposure must be looked at in more detail, since chronic, often occupational sun exposure was inversely associated to melanoma[34,37] and repeated weekend exposure was protective except for people with red hair[35]. Conversely, others found a positive association between increased occupational sun exposure and melanoma on the head and neck[36,40].

The use of sunbeds is also an accepted risk factor for melanoma[41] and this risk is especially high for younger people as shown in several studies[42-45]. The European Code against cancer recommends avoiding sunbeds[46] also the FDA restricts the usage of indoor tanning devices to persons above the age of 18 years[47]. In Norway, sunbed use is not allowed for people younger than 18 years[9].

There is ongoing controversial discussion about the role of Vitamin D in cutaneous melanoma. Several epidemiologic studies and cohort studies suggest that higher vitamin D levels may be associated with lower incidence of cancer and other diseases and better outcome[48]. Synthesis of vitamin D in keratinocytes is catalyzed by UV radiation and *in vitro* studies showed different anti-tumor effects[48,49]. A recently published meta-analysis did not find any significant association between vitamin D serum levels or dietary intake and the risk for melanoma; however, an inverse

correlation between Breslow thickness and vitamin D serum levels was described in some of the included studies[49].

Acral lentiginous melanoma is the most common subtype among blacks[50]. However, the highest incidence of this subtype is reported in Hispanics[50]. It is suggested, that more pigmented skin protects better against UV radiation but there is ongoing discussion about the mechanisms[51].

1.2.3 Hereditary risk factors

About 5 -10% of melanomas are caused by germline mutations and in approximately 20 - 40% of families with hereditary predisposition the mutation can be identified[52,53]. Hereditary melanoma is often characterized by early onset, occurrence of multiple primary melanomas and the presence of dysplastic nevi[52]. The tumor suppressor gene cyclin-dependent kinase inhibitor 2A (*CDKN2A*) encodes two distinct proteins by alternatively splicing[54]. About 20-40% of melanomas with hereditary predisposition are caused by *CDKN2A* mutations and a geographically variability in penetrance is described[52,53,55]. The *CDKN2A* mutations are found in a high percentage of patients with FAMMM-syndrome; furthermore, these mutations comprise also increased risk for pancreatic cancer[53]. Melanoma risk is lower for *CDKN2A* carriers in the general population without familial melanoma history[55,56].

Other high penetrance genes are Cyclin-dependent-kinase-4 (*CDK4*), BRCA1 associated protein-1 (*BAP*1), Protection of telomeres 1 (*POT1*), adrenocortical dysplasia protein homolog (*ACD*)/telomeric repeat binding factor 2 interacting protein (*TERF2IP*) and Telomerase RT (*TERT*). *CDK4* is probably the best described melanoma predisposition gene along with *CDKN2A*. Compared to mutations in *CDKN2A*, the point mutation in *CDK4* is less frequent but its penetrance is quite high (74%) at the age of 50 years[57]. In addition, a high frequency of *BRAF* mutations is described in melanomas with *CDK4* mutations[58]. Increased risk of developing other cancer types in addition, has been reported in melanoma patients with *BAP1* or *POT1* mutations[55].

Germline mutations with medium penetrance are less likely oncogenic driver genes on their own but cross-signaling between several of these alleles may increase melanoma risk[55]. Melanocortin 1 receptor gene (*MC1R*) mutation is associated with a two-fold increase[52]. The presence of more than one *MC1R* variant doubled the melanoma risk in *CDKN2A* mutation carriers, and carriers of multiple *MC1R* variants had a four time higher melanoma risk[59]. Other medium penetrance genes are the microphthalmnia-associated transcription factor (*MITF*) which is an important regulator of pigment cells, and solute carrier family 45, member 2 (*SLC45A2*) variants that are associated with more pigmented skin[55]. Furthermore, loci of low penetrations genes that are associated with increased melanoma susceptibility, were identified in Genome-wide association studies (GWAS)[60,61], others were reported in a recent review[55]. Finally, various hereditary syndromes which include also raised occurrence of melanoma are known. Xeroderma pigmentosum is an autosomal recessive syndrome in which DNA damage caused by UV radiation cannot be repaired properly. The Li-Fraumeni Syndrome, familial retinoblastoma syndrome, Werner Syndrome and *BRCA2* associated breast/ovarian cancer are also described in this context[52,53].

1.3 Clinical aspects in primary melanomas

Early diagnosis of primary melanomas is essential since thin tumors have excellent prognosis. Friedman et al. defined characteristic clinical features, the so-called ABCD- criteria, already in 1985[62]. "Evolving" was added as fifth feature about 20 years later, and describes any change due to size, shape, color surface features or symptoms over time[63]. The ABCDE criteria are shown in **Table 1**.

Table 1. The ABCDE criteria

A	Asymmetry
B	Irregular Borders
C	Multiple colors
D	Diameter > 6 mm
E	Evolving (with respect to size, shape, shades of color, surface features or symptoms)

Modified by N. R. Abbasi, 2004[63]

These characteristics are useful tools to distinguish melanomas from benign nevi when clinical or dermatoscopic examination of pigmented lesions is performed. In addition, the ABCDE-criteria are used in public education campaigns. A recent review confirms high sensitivity and specificity of the ABCDE-criteria among dermatologists and improved utilization among general practitioners after specific training. However, it is more doubtful how useful the criteria are for laypersons[64].

The "*ugly duckling*" sign describes a nevus that does not share the common features of all other nevi in an individual patient and therefore attracts special attention during examination of the integument[65]. These lesions are highly suspicious for melanoma and should be further investigated. The inter-observer agreement/sensitivity for detection of "ugly duckling" nevi was high[66]. Other alarming features for melanoma

suspicion are bleeding, itching or pain of nevi as well as de-novo appearance of quickly growing pigmented lesions.

1.4 Development, classification and staging

1.4.1 Developmental aspects

Melanocytes are melanin containing dendritic cells that derive from neural crest cells and migrate to the epidermis of the trunk along the dorsolateral pathway during embryogenesis[67,68]. Skin melanocytes which settle predominantly in the dorsal and lateral body walls as well as the limbs derive from Schwann cell precursors that follow the dorso-ventral pathway[67].

1.4.2 Tumor pathogenesis

The Clark model describes how melanocytes develop to benign and later on dysplastic nevi resulting in malignant melanoma. Transmigration of melanoma cells from the epidermis into the papillary and reticular dermis is typical in the vertical growth phase (VGP). Further uncontrolled proliferation of melanocytes in the epidermis and loss of contact to keratinocytes due to a loss of E-cadherin characterizes the radial growth phase[68,69]. This pathogenesis is often seen in melanomas developing after non-cumulative sun-induced damage[70]. However, melanomas often develop from normal skin as a result of UV radiation induced DNA damage that is not adequately repaired[28,70]. Whereas severely damaged keratinocytes undergo apoptosis, substantially damaged melanocytes do survive and divide before mutations are repaired properly[28]. Also, other oncogenic events like loss-of-function alterations of tumor suppressor genes or epigenetic alterations play a role in melananomagenesis[70]. Furthermore, solar stimulated UV radiation in general suppresses immune response, and UVA as well as UVB radiation contribute by various specific mechanisms[71].

1.4.3 Histological classification

Clark et al. described the histology of *superficial spreading melanoma* (SSM), *nodular melanoma* (NM) and the *lentigo maligna melanoma* (LMM) for the first time in 1969[72]. During the Cancer Conference in Sydney in 1972, a classification and nomenclature was standardized by pathologists[73]; the current classification of melanoma includes *acral lentiginous melanoma* (ALM) as the fourth major histologic subtype. SSM is the most common subtype in Caucasians, diagnosed in 65-70% of all primary melanomas and mostly seen on sun exposed sides like the trunk and the lower limbs. NM accounts for about 15-20%, followed by LMM (13%) which is often diagnosed on long-term sun exposure skin in elderly people[74,75]. ALM is the rarest type (2-3%) and predominantly found on the palms, soles and nail beds; the proportion of this subtype is much higher in blacks (36%) and non-Hispanic Whites[75,76].

1.4.4 Molecular classification

During the last two decades, several somatic mutations associated to distinct subtypes of melanoma has been discovered. The mitogen-activated protein kinase (MAPK) pathway and the PTEN/AKT pathway play a key role in cell proliferation in melanocytic lesions.

BRAF mutations are seen in 50- 60% of melanomas, and the V600E mutation in exon 15 is the most commonly observed alteration[77-79]. *BRAF* mutations are frequently found in SSM and NM in younger patients on intermittent sun-exposed skin, and are often associated with the presence of acquired melanocytic nevi and metastasize preferably to regional lymph nodes[70,80]. The $BRAF^{V600E}$ mutation is the most common driver mutation[81]. It is found in a high percentage of melanocytic nevi, suggesting that this mutation occurs early in melanomagenesis[15,70,82].

NRAS mutations account for about 15% of all somatic mutations in melanomas on non-chronic sun damaged skin (non-CSD), CSD, acral and mucosal melanomas[70,78]. Mutations in this gene are most common in codon 61[78,83]. Presently, there are

controversial observations regarding association between *NRAS* mutation and sun exposure, body site of primary melanoma, histopathological subtype or other histologic features[80]. *NRAS* mutations have been associated with significantly greater Breslow depth compared to wild type or *BRAF* mutated melanomas[78]. Compared to *BRAF* mutations, *NRAS* mutations are non-significantly more frequent in elderly patients[79].

In 2015, mutant *NF1* and Triple wild type were suggested as two additional subtypes in the genomic classification of cutaneous melanoma[84]. *NF1* mutations are loss of function mutations that result in alternatively activation of the MAPK pathway. The Triple wild type encloses a heterogeneous subgroup of melanomas that lacks *BRAF, NRAS* or *NF1* mutations[84]. *KIT, GNAQ* or *PTEN* mutations are included in this group among others[84]. *KIT* mutations are seen in approximately 15% of acral, mucosal and CSD-melanomas. They arise often in elderly people (>60 years) and are not correlated to the presence of acquired melanocytic nevi[70,80]. *GNAQ* or *GNA11* mutations are exclusively observed in uveal and blue-nevus like melanomas[70]. *PTEN* mutations are described in non-CSD, acral and mucosal melanomas[70]. *PTEN* and *BRAF* mutations are often present concurrently, resulting in co-activation of the MAPK and the PI3K pathway[15].

1.4.5 pTNM classification of melanoma

The pathologic tumor-node-metastases (pTNM) classification in its 7th edition summarizes prognostic variables that should be reported when primary melanomas are histologically assessed[85,86].

Primary tumors (T) are classified by tumor thickness and the absence or presence of ulceration. In addition, the mitotic rate (mitoses/mm^2) was implemented to distinguish between stage T1a and T1b, see **Table 2**. The level of invasion as defined by Clark[72] is the weakest prognostic factor; it is therefore only relevant to determine stage T1b when the mitotic rate cannot be investigated properly.

Tumor thickness by Breslow is measured from the top of the granular layer of the epidermis to the deepest infiltrating tumor cells[87].

Tumor ulceration is defined as "the interruption of the surface epithelium involved by the tumor"[88].

The *mitotic rate* is the number of mitoses assessed in an area corresponding to 1 mm^2 within a hot-spot area in the dermis[86,89,90].

Table 2. T-classification in cutaneous melanoma

T classification	**Thickness (mm)**	**Ulceration/mitosis**
Tis	not applicable	not applicable
T1	≤ 1.0	a: +/- ulceration and mitosis < 1/mm^2 b: + ulceration or mitosis ≥ 1/mm^2
T2	1.01 - 2.0	a: +/- ulceration b: + ulceration
T3	2.01 - 4.0	a: +/- ulceration b: + ulceration
T4	> 4.0	a: +/- ulceration b: + ulceration

Modified from Balch et al.[86]

The N-stage is defined by the number of affected regional lymph nodes and include in transit metastases and microscopic satellites (**Table 3)**. Metastases that are only documented pathologically are "microscopic" nodal metastases and are in contrast to

macroscopic metastases that are diagnosed by clinical or radiologic examination. There is no threshold for a minimum size of microscopic metastases[86]. The site of distant metastases and serum levels of lactate dehydrogenase (LDH) define M-categories (**Table 4**)[86].

Table 3. N-classification in cutaneous melanoma

N classification	**Number of metastatic nodes**	**Nodal metastatic burden**
N0	0	not applicable
N1	1	a: micrometastases* b: macrometastases^
N2	2 - 3	a: micrometastases* b: macrometastases^
N3	4+ or matted nodes or in transit met/satellites with metastatic nodes	c: in transit metastases or satellites without metastastic nodes

* diagnosed after sentinel node biopsy; ^ defined as clinically detectable nodal metastases pathologically confirmed.

Modified from Balch et al.[86]

Table 4. M-classification in cutaneous melanoma

M classification	Site	Serum LDH
0	no distant met[1]	not applicable
M1a	distant skin, subcutaneous or nodal met	normal
M1b	lung metastases	normal
M1c	all other visceral met any distant met	normal elevated

1 met: metastases. Modified from Balch et al.[86]

1.4.6 Sentinel node biopsy

Sentinel node biopsy (SLNB) in melanoma was described for the first time in 1992[91]. Briefly, a radiotracer is injected intradermally around the primary melanoma the day before surgery; additional blue dye injection in the beginning of the surgical procedure is optional. The node with highest radioactivity is identified with a gamma probe and removed[92].

Common guidelines from The American Society of Clinical Oncology (ASCO) and Society of Surgical Oncology (SSO) published in 2012, recommend SLNB for patients with intermediate-thickness melanomas (Breslow thickness 1-4 mm) regardless of the anatomic site, and the performance of complete lymph node dissection (CLND) in case of positive SLNB[93]; the AJCC staging system emphasizes SLNB also for patients with stage T1b[86]. The Norwegian guidelines are in accordance with the international recommendations[94].

The recently published 10-year follow-up data from a phase 3 trial that randomized patients with intermediate-thickness melanomas for SLNB versus observation did not show significant difference in melanoma specific survival rates when node positive

and node negative patients were analyzed together[95]. Within the group who underwent SLNB however, positive sentinel-node status was the strongest predictor of disease recurrence and melanoma specific death in patients with intermediate-thickness or thick melanomas[95]. About 20% of patients with intermediate-thickness melanomas had positive node biopsies[95]. Thus, the number of unnecessary SLNBs should be reduced. There is ongoing research to find additional clinicopathological variables that can identify the patients who will benefit from SLNB. Breslow thickness, lymphovascular invasion and localization of the primary tumor on the trunk were recently associated with patients at risk for positive sentinel nodes[96]. Meves et al. suggests a model that combines clinicopathologic variables like age, Breslow thickness and ulceration with a gene signature linked to cell-adhesion genes to predict the likelihood of sentinel node metastases[97].

The ongoing EORTC Minitub trial and the MSLT-2 trial are investigating the role of CLND vs observation in patients with positive SLNB[98,99].

In conclusion, SLNB is an established staging modality in patients with intermediate-thickness melanomas but there is an ongoing discussion regarding patients with thin melanomas. Additionally, markers for better prediction of sentinel node positivity are needed. Also the benefit of CLND in node positive patients remains to be investigated further.

1.5 Tumor biology - important hallmarks of cancer in melanoma

The hallmarks of cancer define important features of tumors that enable their growth and ability to metastasize and describe their interaction with the microenvironment[100]. These characteristics include sustained proliferation, resistance to cell death, activation of invasion and metastasis, inducing angiogenesis and evasion from immune response[100]. Several of these traits also play an important role in melanoma development and progression. Furthermore, melanomas have a very high number of

somatic mutations that contribute to enhanced proliferation and tumor growth due to activation of oncogenes and inhibition of tumor suppressor genes[32,70].

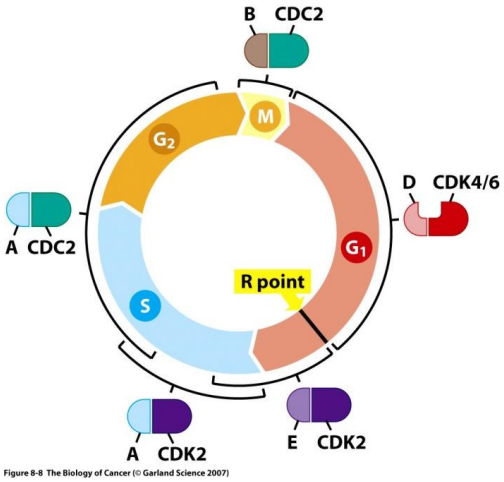
1.5.1 Cell cycle regulation

The mammalian cell cycle consists of four phases, the first gap phase (G1), the synthetic phase (S), the second gap phase (G2) and mitosis (M). In addition, there is a non-proliferating G0 state. The G1-phase starts after the previous cell division is completed and the decision about entering a new growth phase or becoming quiescence is made. This decision depends on extracellular growth-regulating signals and cannot be diminished after passing the restriction point (R-point) in the late G1-phase. At this point it is determined if the cell stays in G1, switches to G0 or moves on to late G1 and S-phase. Growing cells replicate DNA during S-phase, continue into G2-phase and finally start mitosis. The M-phase includes prophase, metaphase, anaphase and telophase. DNA replication and division of chromatids are extremely critical steps in cell division[101]. Checkpoints in G1, at G1/S transition, at G2/M transition and at the metaphase/anaphase transition ensure that every single step in each phase is performed exactly before moving on[101,102]. Cyclin-depending kinases (CDKs) together with their corresponding regulatory subunits, the cyclin protein, are important players in cell cycle regulation. These CDKs can be inhibited by various CDK inhibitors. p16, p15, p18 and p19 specifically inhibit CDK4/6; p21, p27 and p57 inhibit the four other complexes shown in **Figure 2**.

The G1/S checkpoint seems to be crucial in melanomagenesis and progression whereas alterations in the retinoblastoma protein (Rb) pathway are relatively common. Mutations in the p53 pathway are less frequent [102].

Numerous drugs that target single steps in cell cycle control are currently under investigation[103].

Figure 2. Pairing of cyclins with cyclin-dependent kinases during the cell cycle



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pRB pathway

pRb, p16, Cyclin D1 and p27 are important elements in the pRB pathway. pRB is the gatekeeper at the R-point in late G1-phase. In its unphosphorylated state, it is bound to E2F transcription factors and acts as a growth suppressor. When phosphorylated by D-CDK4/6 during G1, it releases the transcription factors and becomes functionally inactive after hyperphosphorylation by E-CDK2 in late G1. As a consequence, it loses its inhibitory function and cells can pass through into S-phase[101,102]. Inherited mutations in Rb predispose to melanoma and other tumors[102].

p16 is coded by the *CDKN2A* gene and its binding to CDK4/6 inhibits the formation of the active D-CDK4/6 complex; consecutively, Rb will not be phosphorylated and the cell remains in G1 phase[102].

p27 regulates activation of D-CDK4 and E-CDK2[102].

Cycline D1 is coded by the *CCND1* gene and its binding to CDK4/6 activates the kinase. *CCND1* amplification impact on melanoma progression is controversially[102].

p53 pathway

p53 is encoded by the *TP53* gene and is entitled "the guardian of the genome". p53 becomes activated in case of DNA damage, and either arrests the cell in G1 phase or induces apoptosis. When DNA damage occurs, p14 transfers the signal to MDM2 which in turn activates release of p53 from the MDM2/p53 complex; finally, p53 is activated by phosphorylation[102]. Various frequencies of *TP53* mutations are reported in melanoma and there is evidence for UV radiation related genesis of gene alterations[102]. In contrast to other cancers, p53 protein expression in melanoma can be increased independently from *TP53* mutation status, indicating that stress related to DNA damage or hypoxia might induce upregulation[102].

p21 is encoded by the *RAS* gene and is the main mediator of p53 induced growth suppression. Upregulation of p21 results in an increased number of p21-D1-CDK4 complexes which inhibits action of D1-CDK4, and thereby also phosphorylation of Rb[102]. Alterations in the *p21* gene are rare in melanoma and other tumors. Increased expression of p21 in melanoma is shown in the majority of studies with decreasing frequency in metastatic lesions[102].

In case of severe DNA damage, p53 triggers apoptosis, mainly through the intrinsic apoptotic program by upregulation of pro-apoptotic factors[104]. *p53* activates expression of apoptotic protease activating factor 1 (APAF-1) and expression of members of the Bcl-2 family. One of these proteins induces cytochrome C release from the mitochondria which in turn activates caspase 9. Cytochrome C, caspase 9 and APAF-1 form the apoptosome. Caspase 9 is activated in this complex and promotes consecutively activation of other down-stream caspases resulting in apoptosis of the cell[105]. Furthermore, p53 can initiate apoptosis via the extrinsic pathway by inducing expression of transmembrane death receptors like FasR or TNFR1[106].

Mitogen-activated protein kinase (MAPK) pathway

Activation of receptor tyrosine kinases (RTK) on the cell surface by growth factors initiates the MAPK pathway. Interaction of RTK with RAS, a membrane bound GTPase, starts the intracellular signaling cascade. RAS activates RAF that phosphorylates MEK which in turn activates ERK. Phosphorylated ERK affects various downstream targets resulting in increased proliferation, survival, invasiveness and angiogenesis[107]. BRAF and NRAS mutations are mutually exclusive.

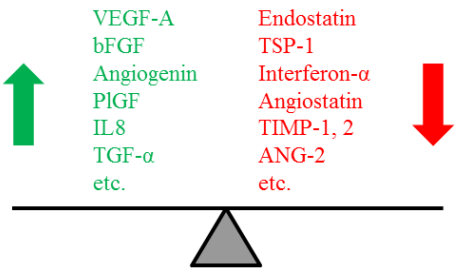
Phosphatidylinositol 3-kinase (PI3K) pathway

c-KIT is another RTK that can activate the MAPK or PI3K pathway. RAS can alternatively activate PI3K which phosphorylates AKT via PIP3. Activated AKT promotes cell survival, proliferation, angiogenesis and other processes[15,108]. The tumor suppressor PTEN antagonizes the activity of PI3K by dephosphorylating PIP3.

1.5.2 Angiogenesis

Angiogenesis is the formation of new vessels from preexisting vessels. Folkman observed that tumor growth beyond a diameter of 1-2 mm^2 depends on neovascularization and entitled the responsible growth factor "tumor-angiogensis factor (TAF)"[109,110]; his group suggested that the crosstalk between TAF and endothelial cells (ECs) promotes angiogenesis[111]. The process of angiogenesis and its role in tumor growth and metastasis is described in detail by Bielenberg and Zetter[112]. Furthermore, it was observed that avascular tumors persisted in a stable "dormant" status for many years but grew exponentially when they became vascularized[113,114]. The balance between pro and anti-angiogenic factors determines if tumor dormancy or neovascularization and growth dominate tumor biology[115]. The change in vascularization status was called the "angiogenic switch". **Figure 3** gives an overview of important factors in this balance.

Figure 3. Balance between pro-and anti-angiogenic factors



Based on "Angiogenesis: regulators and clinical applications"[116]

Algire was the first to observe vascularization in melanoma transplanted to the mouse in a transparent chamber in 1943[117]. High vascularization in primary melanomas is correlated with worse prognosis and is an independent factor in multivariate analysis in line or even superior to tumor thickness[118-120]. Furthermore, it has been shown that several growth factors, integrins and matrix metalloproteinases play an important role in melanoma angiogenesis[121].

Vascular permeability factor (VPF) was purified in 1983[122] and later on renamed as vascular endothelial growth factor A (VEGF-A)[123]. The VEGF-A family consists of five members, VEGF-A to VEGF-D and placenta growth factor (PlGF), and belongs to the platelet-derived growth factor supergene family[124]. VEGF-A plays a key role in physiological and pathological angiogenesis, activates proliferation and migration of endothelial cells, induces vascular leakage and vasodilatation[125,126]. Alternative splicing of the *VEGF-A* gene results in several pro-angiogenic isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$) as well as anti-angiogenic VEGF-A_{xxx}b isoforms that have a different C-terminus[127]. $VEGF_{165}$ is the most common isoform and exists heparin-bound or secreted[125]. Hypoxia, several cytokines and growth factors as well as oncogenes or tumor suppressor genes upregulate VEGF expression[128]. VEGF binds to the tyrosine kinase receptors VEGFR-1 and 2 on ECs and tumor cells; its binding to VEGFR-2 is an important step in angiogenesis and activates further downstream signaling via PIK3 and

phospholipase C_y pathways[129]. Conversely, binding of $VEGF_{xxx}b$ to VEGFR-2 seems to inhibit the key functions of VEGF-A[127]. VEGF-A expression is reported in primary melanomas as well as metastases[119]. $VEGF_{xxx}b$ expression was observed in normal dermis and other normal tissues, however, it is downregulated or absent in tumor tissue[127]; absence of $VEGF_{xxx}b$ characterized primary melanomas that did metastasize compared to non-metastasizing cases[130].

Basic fibroblast growth factor (bFGF) was described for the first time in 1984[123,131]. bFGF is involved in various oncogenic mechanisms, here we focus on its role in angiogenesis. bFGF binds to tyrosine kinase FGFRs on the surface of endothelial cells and activates their proliferation and migration as well as extracellular matrix (ECM) degradation[132]. Inhibition of bFGF and FGFR-1 mediated signaling abolished angiogenesis in human melanoma xenograft tumors[133]. Furthermore, high microvessel density in primary melanomas is associated with expression of bFGF[134]. There is also evidence for a synergistic effect of FGF and VEGF signaling in angiogenesis[135].

Recently, the pro-angiogenic role of Heat Shock protein 27 (HSP27) was indicated in cell-line studies, xenografts and patient samples[136]. HSP27 is a family member of small heat shock proteins that maintain cell survival under stressful conditions[137]. Various physical, biological or chemical stress signals can increase intracellular HSP27 concentration and influence its oligomerization status. HSP27 prevents aggregation of misfolded proteins, inhibits apoptosis and interacts with different parts of the cytoskeleton[137,138]. Downregulation of HSP27 in an angiogenic breast cancer cell line resulted in decreased secretion of VEGF-A and bFGF, and a non-angiogenic phenotype *in vivo*[136]. Furthermore, strong expression of HSP27 is reported as a poor prognostic factor in various solid tumors[136,139,140] and is associated with resistance to chemotherapy[141,142].

The urokinase plasmin activator receptor (uPAR) plays a role in proliferation, extracellular matrix degeneration, cell motility and invasion[143]. It is anchored to GPI in the cell membrane and lacks a transmembrane domain so that it depends on

interaction with other proteins to transfer signals[143]. VEGF-A induced re-localization of uPAR to the leading edge of endothelial cells contributes to angiogenesis[144]. uPAR is not detectable in benign nevi and early stage melanoma, but increased expression is reported in more advanced primary lesions and metastases[145].

Interleukin 1b (IL1b) is a pro-inflammatory cytokine produced by tumor cells and other cells in the microenvironment. It contributes to angiogenesis by interaction with VEGF-A, endothelial cells and myeloid cells[146]. Examples for this interaction are the regulation of expression of VEGF-A and its receptors on ECs by IL1b and synergistic effects of IL1b with VEGF-A by increasing gene-expression of growth factors and cytokines on ECs[146].

Activin A belongs to the transforming growth factor-b superfamily and its role in angiogenesis is more controversial and may depend on tumor type and the microenvironment. Activin A stimulates expression of VEGF-A and bFGF *in vitro*[147,148] and corneal neovascularization was observed *in vivo*[149]. On the other hand, Activin A suppressed endothelial cell growth and decreased angiogenesis in a gastric cancer model[150].

1.5.3 Invasion and metastasis

The ability of cancer cells to invade the surrounding tissue and metastasize to distant sites in the body is another key feature. Adequate expression of cell adhesion molecules maintains a stable state in the basal layer of tissues and controls the interaction with the ECM[100]. One of the best known adhesion molecules in epithelial cells is E-cadherin. Loss of E-cadherin and upregulation of N-cadherin characterizes many carcinomas[100]. Various transcriptional repressors of the E-cadherin gene have been described, *e.g.* Snail, Slug, SIP1and E2a[151] as well as Twist[152]. The acquirement of more mesenchymal like attributes together with the downregulation of epithelial traits during an epithelial mesenchymal transition (EMT) and the reversion of this process, mesenchymal-epithelial transition (MET), are key processes during metastasizing[151]. EMT-like processes have also been described in melanoma[153].

The "*seed and soil"* theory proposed by Stephen Paget in 1889 is widely accepted. Progenitor cells, metastatic cells or cancer steam cells can be "seeds" evolved from the primary tumor that may establish metastasis in proper niches or organ microenvironments ("soils")[154]. Metastasizing includes local invasion into blood vessels or lymphatic vessels, transportation of multi-cell aggregates to distant sites, arrest in the capillary bed and subsequent extravasation into organ parenchyma and finally, establishment in the new microenvironment and further proliferation[154]. This process can be interrupted by the antitumor host response and is therefore completed only in a very limited number[154]. Nowell[155] suggested that acquired genetic variability during tumor progression together with selection pressure results in more aggressive tumor cells. The concept of *clonal origin of metastases* has been verified by others and may explain heterogeneity in response to chemotherapy and course of disease[154].

Melanomas metastasize mainly through the lymphatic system but also via haematogenic dissemination to more distant sites like lungs, liver and brain[156]. Various EMT related transcription factors play a role in phenotypic heterogeneity and plasticity of melanomas[157]. Since melanomas are of intermediate mesenchymal nature, expression of EMT transcription factors alters depending on the phenotype [158] as also shown in various *in vitro* and *in vivo* models[153,159]. Expression of E-cadherin in normal melanocytes is essential for their contact with keratinocytes in the epidermis, however, its loss is characteristic for more invasive late stage melanomas[153].

The role of cancer stem cells (CSC) in the pathogenesis of melanoma metastasis is still under investigation; however, disease progression, ability of metastasizing and poorer prognosis seem to be associated with the presence of CSCs[160]. CSCs in melanoma are also named malignant melanoma initiating cells (MMICs). CSCs are characterized by the following traits: the ability to initiate tumor growth, the capacity to self-renew and the ability to differentiate into tumor cells[160]. Two major models include CSCs in their explanation of tumor growth and progression[161]. In the hierarchical model, the phenotype of CSCs is stable and self-renewable but cells arising from these CSCs lose the ability of self-renew. In contrast, there is the

stochastic model in which signals from the microenvironment determine the fate of the cancer cells and cells can switch between being a non-stem cell and being a CSC[161]. ABCB5 and CD271 are established markers for MMICs in melanoma[160], others are still under investigation. Increased expression of CD271 promotes immune evasion and expression of ABCB5 is characteristic in chemo-resistant cells. Furthermore, MMICs may promote invasion and metastasis due to interaction with EMT and MET and further mechanisms are under investigation[160]. Other melanoma stem cell markers are CD20, CD133 and ABCG2[161].

1.5.4 Immune system

There are three phases of interaction between tumor cells and the immune system from the onset of the first tumor cells until the existence of a tumor in progression[162]. First, the innate and adaptive immune system recognizes and eliminates tumor cells. During the following equilibrium between remaining tumor cells and the immune system, the tumor can stay in dormancy over many years. However, the genetically instable tumor cells and the surrounding host immune system continue interaction with each other. This process is defined “immune editing”. Finally, the tumor can escape from the immune system and proliferates uncontrolled[162]. Escape mechanisms include secretion of cytokines that inhibit or mediate immune response, induction of an immune suppressive environment and alteration of important antigens and molecules on the surface of the tumor cells themselves[162-164]. Interference of dentritic cell maturation, inappropriate presentation of tumor antigens, deficiency of costimulatory molecules involved in T-cell activation or recruitment of immune-suppressive myeloid-derived suppressor cells (MDSCs) are other approaches to evade the host immune system[163,164].

Melanoma is a highly immunogenic tumor and many of the mentioned mechanisms play an important role in its progression as well in successful treatment. Vascular endothelial growth factor (VEGF) can be secreted by cancer cells and cancer associated fibroblasts[165]. VEGF recruits MDSC and macrophages to the tumor, inhibits maturation of dentritic cells and prevents T-lymphocyte invasion[125,166,167].

High pretreatment levels of VEGF were associated with decreased OS in melanoma patients treated with ipilimumab[168]. Other mechanisms of melanoma cells that are associated with immune escape are expression of FasL[169] and inadequate expression of HLA class I molecules[170].

1.6 Prognostic and predictive factors in melanoma

A *prognostic* biomarker defines the risk for the likely outcome of disease at time of diagnosis independently of further treatment[171,172]. Conversely, a *predictive* biomarker identifies the likelihood for response to a particular therapy[171].

1.6.1 Prognostic markers

Histopathological markers

The current AJCC staging system provides important prognostic information with impact on tumor staging. The database contains prospective data on 30,946 patients with stage I, II and III and 7,972 patients with stage IV melanoma from 17 major medical centers[86,173].

Tumor thickness as defined by Breslow[87] is still one of the most important factors to determine T-category and is the strongest predictor for survival in multifactorial analysis[86]. 10-year survival was 96% for patients with 0.01-0.05 mm thick melanomas and 42% for patients with melanomas thicker than 6.00 mm.

Mitotic rate was the second most powerful predictor of survival in a multifactorial analysis and was therefore introduced to the AJCC staging system in 2009[86]. A threshold of at least 1 mitosis per mm^2 showed greatest impact on survival. The presence of mitosis is especially important to distinguish stage T1a and T1b. Another marker for mitosis is phosphohistone H3 (PHH3) that was an independent prognostic factor for OS when investigated in a series of nodular melanomas. Its prognostic value was even superior to the assessment of mitotic rate and this marker should be further validated in greater series[174].

Ulceration of the primary melanoma was another important prognostic factor. The survival rates for patients with an ulcerated melanoma are quite similar to those for patients with a non-ulcerated melanoma in the next higher T-category[86]. Originally, ulceration was defined as the deficiency of intact epithelium. Recent research suggests a particular biologic entity for ulcerated melanomas characterized by epidermal defect, distinct elements in host response and affection of the surrounding epidermis. Furthermore, the presence of ulceration was identified as an independent beneficial predictive factor in several studies investigating adjuvant treatment with interferon[175].

Tumor thickness, mitotic rate and ulceration predict 10-year survival in T1 tumors. The level of invasion which was part of the staging system for many decades is the weakest prognostic factor in multivariate analysis in stage I and II tumors and therefore only relevant to categorize patients to T1b if the mitotic rate cannot be determined[86].

Melanoma growth pattern (vertical versus horizontal tumor growth) is not an independent prognostic factor even though melanomas other than SSM and NM may have a fairly different prognosis due to various growth patterns.

Vascular invasion describes the presence of tumor cells within the lumen of vessels and was described as independent prognostic marker in primary melanomas[176-178] whereas others did not find any independent prognostic impact[179,180].

Microvessel density (MVD) is defined as the number of proliferating vessels or endothelial cells in hot-spot areas of the tumor. MVD is an independent prognostic factor correlated to worse overall survival and disease free survival (DFS)[119,181].

Presence of *tumor necrosis* in primary melanomas was shown to be an independent prognostic marker[182,183].

Tumor-infiltrating lymphocytes (TIL) are assumed to be a favorable prognostic factor in terms of anti-tumor immune response since Clark et al.[184] described a positive

association between the presence of TIL and better OS but their role as a prognostic marker is still discussed controversially[179,185-187].

The TNM staging system is used for all histologic subtypes[85]. In contrast to previous staging categories, all patients with microscopic melanoma metastasis in lymph nodes (lymphatic system) are classified as stage III. The *number of positive nodes* has significant prognostic impact as shown by Balch in the latest AJCC classification[86].

In patients with stage IV melanoma, the *site of distant metastasis* as well as *LDH level* has prognostic impact. Thus, patients with elevated LDH level are classified as M1c independently the localization of distant metastasis. The 2-year overall survival rate is 40% in patients with normal LDH compared to 18% in patients with elevated LDH[86].

Molecular markers

Recently, a population based study including 912 patients did not find prognostic impact of *BRAF* or *NRAS* mutation status in primary melanomas on melanoma specific survival compared to wild-type tumors[188]. These findings confirm results from previously published studies[78,189]. Conversely, the presence of *NRAS* or *BRAF* mutations was reported as poor prognostic factors by others[190-192]. Ki67 and p53 expression as well as loss of p16 expression were reported as independent prognostic markers[193]. In addition, protein as well as DNA and RNA-expression has been assessed in blood and tissue samples of melanoma patients to identify potential prognostic markers[187,194-196]. To date, LDH is the only one included in the AAJC staging system[85].

Clinical factors

Age has been reported as an independent prognostic factor in several studies, even though various cut-off points to distinguish between younger and elderly patients were used[197,198]. Thicker and ulcerated melanomas were more often diagnosed among elderly patients; however, thickness and age were the only independent prognostic markers in a multivariate analysis by Austin et al.[197]. Age was also an independent

prognostic factor for OS within each thickness subgroup in the large dataset used for validation of prognostic factors by the AJCC in 2001[199].

Clark was the first who observed better prognosis among women compared to men in his publication from 1969[72]. *Gender* has been determined as an independent prognostic factor in several studies during the last decades[90,178]. Joosse et al. confirmed a lower risk of progression in female patients with significantly reduced risk of visceral metastases compared to men and showed that the survival advantage persisted after progression of disease[200]. It looks like the survival benefit of women is independent of differences in detection or diagnostic delay as well as age at diagnosis, ulceration and female hormone-status[200].

A correlation between *anatomic site* and better outcome has been reported in several studies; melanomas on the limbs have in general better prognosis than those located on the trunk or head and neck[198,199].

1.6.2 Predictive markers

The *BRAF*^{V600E} mutation is the best established predictive marker with high response rates to treatment with BRAF inhibitors[201]. Furthermore, *KIT* mutated melanomas can be successfully treated with a KIT inhibitor[202]. The predictive value of programmed death-ligand 1 (PD-L1) expression is still under investigation. Sunshine and Taube found a correlation between PD-L1 expression by tumor cells and objective response in average 45% of the patients with solid tumors treated with anti-PD-1 or anti-PD-L1 inhibitors[203]. Since 15% of the patients with PD-L1 negative tumors responded to treatment, further investigation is needed to determine the role of PD-L1 as a predictive marker[203]

1.7 Treatment of melanoma

1.7.1 Primary melanoma

Surgical treatment

Surgery is the gold standard in treatment of primary cutaneous melanoma. Excision of a suspicious lesion is the method of choice to obtain complete histologic diagnosis[86,204]. In case of melanoma, complete excision with adequate margins should be performed whereupon Breslow thickness is the determining factor. Excision margins with up to 5 cm were common from the beginning of the last millennium until the 1970s[205]. Recommendations changed dramatically over time and current guidelines from Europe, America and Australia accepted much narrower margins as shown in **Table 5**[206].

Table 5. Recommended excision margins

T stage by Breslow thickness	NCCN, CCO, CMA, ESMO, ANC	Norwegian guidelines[1]
Tis, in situ	0.5 cm	0.5 cm
T1 < 1.0 mm	1.0 cm	1 cm
T2 1.01 – 2.0 mm	1-2 cm	1 cm
T3 2.01 – 4.0 mm	2 cm*	2 cm
T4 > 4.01 mm	2 cm	2-3 cm

NCCN: National Comprehensive Cancer Network; CCO: Cancer Care Ontario; CMA: Canadian Medical Association; ESMO: European Society for Medical Oncology; ANC: Australian Cancer Network.

*except ANC: 1-2 cm. Table modified from Fong[206]; 1: Nasjonalt Handlingsprogramm[94]

For in situ melanoma, there exists no randomized trial, so the recommendation is based on the consensus of an expert panel. Randomized trials that investigated

different margins in intermediate thickness melanomas (1-4 cm) did not find any difference in OS[205,206]; however, Thomas et al. found a higher rate of local recurrence among the group with 1 cm margins compared to the group with 3 cm margins[207].

An ongoing neo-adjuvant trial investigates the clinical and pathological response of the combination of the BRAF inhibitor vemurafenib and the MEK inhibitor cobimetinib in patients with BRAF mutated melanoma with palpable lymph node metastases (clinicaltrials.gov NCT02036086).

1.7.2 Treatment of regional lymph nodes

Sentinel node biopsy is recommended for patients with melanoma thickness 1-4 mm as described above[93].

Any enlarged lymph node discovered by clinical examination or ultrasound should be biopsied for further diagnostics. Complete lymph node dissection should be performed if nodal metastases are confirmed and distant metastases were not detected[204]. Adjuvant radiotherapy after lymphadenectomy showed significant lower recurrence rates but increased morbidity without OS benefit. This treatment should therefore mainly be considered in patients with high risk nodal-disease[206].

1.7.3 Adjuvant treatment

10-year survival rate is < 50% among patients with AJCC stage IIC, IIIB and IIIC[86]. As a consequence, several clinical trials have investigated treatment options for this patient group over the last decades.

The efficacy of *immune therapy* has been evaluated for interferon alpha in several trials, testing low dose, high dose or pegylert interferon to improve the outcome for this patient group. Two recently published meta-analyses by Mocellin et al. showed improved DFS and OS for treatment with interferon alpha[208,209]. However, current guidelines do not agree about recommendation of adjuvant interferon alpha. This is probably caused by conflicting results about OS benefit in many studies[206,210,211]. Ipilimumab, an inhibitor of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4),

was the first drug that showed improved OS in metastatic melanoma. Based on the encouraging results, this drug is also investigated in adjuvant trials. Recently published results from the randomized, placebo controlled EORTC 18071 trial showed significantly improved recurrence-free survival in patients with high risk stage III melanoma[212]. However, 52% discontinued treatment with Ipilimumab, so risk-benefit, cost effectiveness and OS benefit should be evaluated in future. Ipilimumab and the PD-1 receptor inhibitor nivolumab are currently under investigation for adjuvant treatment (clinicaltrials.gov: NCT02388906, NCT02437279).

Targeted therapy achieved encouraging response rates in patients with BRAF mutated metastatic melanoma. Recurrence free survival time of adjuvant treatment with the BRAF inhibitor dabrafenib (clinicaltrials.gov NCT01682213) or vemurafenib (BRIM8, clinical trials.gov NCT01667419) is currently tested.

Since angiogenesis is important in tumor growth and metastases, the benefit of anti-angiogenic treatment with bevacizumab monotherapy was investigated in high risk patients after complete resection. Interim analysis of this randomized phase III trial showed improved PFS in the treated group; data for the primary endpoint, OS benefit after 5 years, are pending[213].

1.7.4 Treatment of local recurrence

In-transit metastases are lesions that occur > 2 cm from the primary melanoma and are seen in 4-14% of melanoma patients[206]. Solitary, localized lesions can be excised[204] or treated locally with interferon alpha injections, topical application of imiquimod or radiotherapy[206]. Hyperthermic isolated limb perfusion (HILP) and isolated limb infusion (ILI) are other options in case of multiple lesions on the extremities[214]. Complete response rates are higher in HILP[215] compared to ILI[214,216]; however, HILP is a more complicated and costly procedure with a higher right of adverse side effects. In general, all mentioned therapies provide local disease control but no benefit in OS[206].

1.7.5 Treatment of metastatic disease

Five-year survival for patients with metastatic disease is poor and depends on localization of distant metastases and LDH level[86]. Targeted therapy and immune checkpoint blockade revolutionized the outcome of patients with stage IV melanoma dramatically after many decades without any drug that could improve OS and low response rates to available treatment options.

Surgery

Surgery can improve OS in all categories of stage IV disease if complete resection (R0) can be achieved, as shown in multiple studies for different metastatic sites. This approach should be considered in patients with solitary or few localized metastases and good performance status. Tumor volume doubling time within 60 days is a good marker to assess aggressiveness of metastases and should be taken in consideration when patients are selected for metastasectomy[217].

Targeted therapy

BRAF is mutated in about 50% of melanomas, resulting in an upregulation of the MAPK pathway. BRAF inhibitors vemurafenib and dabrafenib were approved for treatment of metastatic melanoma in 2011 and 2013, respectively. Each of these drugs shows high response rates with improved PFS and OS compared to chemotherapy with dacarbazine in randomized phase III trials[201,218-220]. However, the curves for PFS in patients treated with vemurafenib and dacarbazine meet each other after about 20 months[218] and converge to each other after about seven months in the dabrafenib study[219]. Acquired resistance is a major problem in treatment with BRAF inhibitors[221-223]. The MAPK pathway can be activated in an alternative manner by mutations in *MEK* or BRAF independent MEK activation. The efficacy of the MEK inhibitors trametinib and cobimetinib was tested as single agents as well as in combination with BRAF inhibitors. The combination therapy with BRAF and MEK inhibitors is superior to single drug treatment due to response rates, PFS and OS[220]. Furthermore, the number of cutaneous squamous cell carcinomas was lower among patients treated with combination therapy[220].

To date, no targeted therapy for *NRAS* mutated melanomas is established. Promising results were observed in in a phase II study with the MEK inhibitor binimetinib, and a randomized phase III trial in *NRAS* mutated melanoma is ongoing[224]. Furthermore, early clinical trials for combination of MEK and CDK4 inhibition or MEK and PI3K/Akt inhibition are ongoing[224].

Immunotherapy

Inhibition of key immune checkpoints was the first treatment that improved overall survival in patients with stage IV melanoma. The best known examples are inhibition of CTLA-4 and the PD-1 receptor on T-cells as well as the PD1 ligand (PD-L1) on the surface of tumor cells. These inhibitory receptors modulate the immune system independently from each other and their inhibition results in enhanced anti-tumor immune response and increased OS. CTLA-4 regulates T-cell proliferation and migration to the tumor; PD-1 and PD-L1 interact with T-cells at the tumor site[225]. Randomized controlled phase III studies demonstrated OS benefit for patients treated with ipilimumab[226,227]; in addition, durable long term survival benefit was seen for 18-26% of the patients with a plateau from the third year[228,229]. Efficacy of PD-1 inhibition was published for the first time in 2010[230] and confirmed in another phase I study in 2012, in which response was seen in 1 out of 4 melanoma patients[231]. A recently published phase III trial comparing nivolumab with chemotherapy after progress on ipilimumab or a BRAF inhibitor showed a response rate of 38% in pretreated melanoma patients[232].

Combination of CTLA-4 and PD-1 inhibition in a phase I study showed promising response rates[233] which were confirmed in a recently published randomized phase III trial[234]. Several studies combining ipilimumab and PD-1 or PD-L1 inhibitors as well as combinations of immunotherapy wit targeted or anti-angiogenic therapy are ongoing. To date, no predictive marker is established for any of the immune-therapeutic options.

Autologous tumor-infiltrating T-lymphocytes (TIL) are used in adoptive cell transfer immunotherapy. This treatment alternative showed objective response rates of about

50% in patients with metastatic melanoma and 20% of the responders had durable remissions[235]. Based on the promising results from clinical phase II studies, a randomized multicenter phase III trial is under development[235].

Chemotherapy

Dacarbazine, an alkylating agent, is the only FDA approved chemotherapeutic agent for treatment of metastatic melanoma and it was widely used for many decades in lack of other treatment options. The overall response rate was about 15% in a meta-analysis of randomized trials but OS benefit was never shown[236]. Today it is still a treatment option in *BRAF* wild type patients who do not respond to immune therapy. Patients with *BRAF* wild type had improved clinical benefit and overall survival in a single arm, single institution study with dacarbazine[237]. Temozolomide, orally administrated dacarbazine, shows overall response rates comparable to dacarbazine but penetrates into the CNS and is preferable in case of brain metastases[236]. Several other chemotherapeutic agents were tested as single drugs or in combination but none showed OS benefit[238].

Anti-angiogenic treatment

Monoclonal antibodies as well as tyrosine kinase inhibitors have been investigated for anti-angiogenic treatment of metastatic melanoma[239-241]. Bevacizumab, a humanized monoclonal antibody against VEGF-A, was investigated in combination trials and as single agent. Monotherapy did not meet the primary objective in a randomized study by Varker et al.[242]. Addition of bevacizumab to chemotherapy showed favorable outcomes, but significant benefit could not yet be confirmed. In the BEAM trial that compared chemotherapy alone versus chemotherapy and bevacizumab, OS was significantly longer in the combination group (12.3 vs 8.6 months), but follow up analyses after four additional months could not confirm the survival benefit. Furthermore, non-significant benefit in PFS was seen among the patients with combination therapy[243].

Interferon alfa (IFN α) has anti-angiogenic activity in low doses; IFN α in non-cytostatic concentrations down-regulated bFGF concentration and angiogenesis in

vitro and in *vivo*[244,245]. Furthermore, successful treatment of pediatric hemangiomas was described[246]. In melanoma, IFN α did not show any additional benefit when given together with bevacizumab[242].

1.8 Clinical studies

1.8.1 Classification of clinical studies

There exist four different types of clinical studies: phase I, phase II, phase III and phase IV-trials. In phase I trials the new drug is given to a small number of patients in increasing dosage to find the maximum tolerable dose, the most favorable application schedule and to describe the toxicity profile[247]. Because of the toxicity of cancer drugs, all testing is done in patients and not in healthy volunteers. Pharmacokinetic and pharmacodynamics parameters are assessed in addition[248].

Safety and potential efficacy of the new drug are investigated in phase II studies. Since sample size is limited, efficacy should be validated in lager randomized phase III studies[247].

Phase III trials are conducted to confirm efficacy of the drug of interest and to record the incidence of adverse events in a greater number of patients. Usually, the trials are randomized and often stratified to ensure balanced groups. “Comperative efficacy trials” assess the question if the new drug improves outcome compared to current standard treatment or placebo[247]. “Equivalency trials” are another type of phase III studies. They investigate if the new drug is equivalent to the established treatment[247].

1.8.2 Response evaluation

Changes in tumor burden are assessed clinically or radiologically to evaluate response to treatment. The response evaluation criteria in solid tumors (RECIST)-guidelines are an important tool in this process and secure that the same criteria are used globally. These response evaluation criteria in solid tumors were published in 2000 for the first time and updated in 2008[249]. Since the clinical effects of new

immune therapeutics cannot necessarily be adequately evaluated by the classical RECIST criteria, new immunological response criteria were suggested[250].

1.8.3 Evaluation of critical adverse events

Critical adverse events (CAE) that occur during investigation of a new drug in clinical trials must be recorded continuously and are reported in the safety profile of the study. These criteria are published by the National Cancer Institute and are regularly updated[251]. In the protocol of every clinical trial, it has to be defined which version of the Common Terminology of CAE (CTCAE) is used. Critical events are graded from 1 (asymptomatic or weak symptoms) to 5 (death).

2. PURPOSE OF THE STUDY

2.1 General aim

When this clinical phase II trial was initiated at Haukeland University Hospital, chemotherapy was the only established treatment for patients with metastatic melanoma. During recent years, new drugs have become available and high response rates to BRAF inhibitors and various treatment options, including immune checkpoint blockade, show promising results[220,228,234,252]. However, there are still limited treatment options for patients who are not suitable for immunotherapy, do not harbour the *BRAF* mutation or progressed to first line treatment. The aim of this thesis was to investigate the efficacy of anti-angiogenic treatment with bevacizumab monotherapy in patients with metastatic melanoma and to identify potential predictive markers.

2.2 Specific aims

1. To investigate efficacy and safety of treatment with bevacizumab monotherapy in patients with metastatic melanoma in a clinical phase II study (**Paper I**).

2. To examine the predictive value of angiogenesis related markers in tissues and blood samples of patients treated with bevacizumab monotherapy, and to study the correlation between angiogenesis markers (MVD, pMVD, GMP) and treatment response (**Paper II**).

3. To analyze serum samples by a multiprotein array to identify potential predictive markers, and to perform further validation by single protein assays in blood samples and by immunohistochemistry in metastases (**Paper III**).

3. MATERIALS AND METHODS

3.1 Study design and ethics

The PhD-project is based on a clinical phase II trial (ClinicalTrials.gov NCT00139360) run at the Oncology Department at Haukeland University Hospital in Bergen, Norway. This study is an open labeled, single arm, single institution clinical trial where patients with metastatic melanoma in progression were treated with bevacizumab monotherapy until disease progression or intolerable toxicity. The dosage of the drug was 10mg/m^2, given every 14 days. Computer tomography (CT) was performed every 8th week for response evaluation. Biopsies and blood samples for research purposes taken before the first treatment were investigated. The study design was based on the two-stage design for clinical phase II trials by Simon[253]. Response of the study drug had to be shown in patients that progressed on standard treatment before bevacizumab could be given first line. The study was approved by the regional ethic committee (processing number: 05/329) and was conducted in accordance with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. All patients signed informed consensus before enrollment.

3.2 Patients

52 patients at Haukeland University Hospital were screened for inclusion in the study between April 2005 and March 2010. Eligibility criteria are listed in **Table 6**, more detailed information can be found in the study protocol published on ClinicalTrials.gov NCT00139360.

Table 6. Eligibility Criteria

Inclusion Criteria	Exclusion Criteria
Histologically confirmed metastatic disease in progression	more than 1 prior chemotherapy regimen for metastatic disease
WHO-performance status 0-2	prior interferon alpha or IL-2 for metastatic disease
Age >18 years	pregnant or lactating patients
Able to undergo outpatient treatment	clinical evidence of coagulopathy
Clinically and/or radiographically measurable disease according to RECIST	psychological, familial, sociological or geographical condition potentially hampering compliance
>4 weeks since adjuvant interferon alpha	brain metastases
Recovered from prior chemotherapy	symptomatic congestive heart failure
absolute granulocytes > 1.0 x 109/L	unstable angina pectoris or cardiac arrhythmia
platelets > 100 x 109/L	history of thrombosis
bilirubin < 1.5 x upper normal limit	non-steroidal anti-inflammatory medications
Normal serum creatinine	uncontrolled hypertension
INR < 1.5	full-dose anticoagulants (INR>1.5) or heparin, or daily treatment with aspirin (>325 mg/day)

3.3 Response assessment and toxicity

Treatment response was evaluated according to RECIST published in 2000[254]. Main outcome measures were complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Measurement is based on the longest diameter of target lesions and response is defined by the relative change in the sum of these

diameters. In case of CR, the target lesion has completely disappeared. PR is achieved when the sum of target lesion diameters decreased at least 30%. On the other hand, PD is reported when the sum increases by at least 20%[254]. Best overall response (BOR) is the best response observed between treatment start and disease progression. Based on these criteria, we defined different types of responses. Objective response (OR) includes CR and PR; this was the primary endpoint in the study. Disease control (DC) describes patients with CR, PR or SD for at least 6 months. BOR is not applicable in patients who progressed clinically before first CT evaluation. Time to progression (TTP) is defined as the time from enrolment to PD or death due to melanoma. Progression free survival (PFS) is the time from inclusion until PD. Overall survival (OS) is the time from inclusion until death. Response status by August 2011 was reported in **Paper I** and **II**; in **Paper III**, the observation time was extended to September 2013.

Critical adverse events were graded according to CTCAE, version 3.0 defined by the National Cancer Institute[251] and were recorded every 2nd week. Early hypertension (EH) was defined as hypertension ≥ grade 1 according to CTCAEv3.0 diagnosed before the first response evaluation at eight weeks.

3.4 Sampling of tissue and blood samples

Paraffin-embedded tissue from 32/35 primary tumors was obtainable for immunohistochemically staining as performed in **Paper II**. The three missing blocks included one patient with unknown primary tumor, one patient with ocular melanoma treated with brachytherapy and one not trackable tissue block. Because of insufficient material, 30/35 primary tumors were available for immunohistochemical investigations in **Paper III**. Furthermore, 35/35 metastases were accessible for analyses. If several biopsies were taken at various time points, the metastasis diagnosed closest to date of inclusion was chosen (median 5 days). Core needle biopsies mainly taken from lung, liver, abdominal or pelvic organs were taken in 17/35 patients. In addition, 18/35 excisional biopsies from skin metastases or lymph nodes were available.

Furthermore, blood samples were taken before the first treatment with bevacizumab (median 2 days). From the 35 patients, 28 serum samples and 29 plasma samples were collected at baseline. Serum samples were taken in 10 ml gel free tubes. After clotting for 30 minutes, the tube was centrifuged at 1600 x g for 10 minutes at 4°C and plasma was frozen at -20°C. Plasma samples were taken in EDTA-tubes, centrifuged at 1600 x g for 10 minutes at 4°C and plasma was frozen at -20°C. Blood samples were aliquoted when used for analysis for the first time. In addition, routine samples including LDH were taken from all patients at time of inclusion and before every new cycle.

3.5 Clinico-pathologic variables

The following variables were recorded: anatomical site of primary tumor and of metastasis, sex, age, WHO performance status, M-stage, number of previous treatment lines for metastatic disease, presence of proteinuria, diagnosed hypertension before enrollment, blood pressure before treatment start and before every further treatment with bevacizumab, anti-hypertensive medication used at time of inclusion and changes during treatment with bevacizumab. Furthermore, the number of treatment cycles with dacarbazine and/or bevacizumab was recorded.

Hematoxylin and eosin-stained sections from all primary melanomas were reclassified by LAA and CS blinded for response data. The following histopathologic features were reported: histologic type[72,73], thickness by Breslow[87], ulceration[88], mitotic rate[86,90], Clark level of invasion[72], growth phase[184], presence or absence of vascular invasion[255], regression[184] and necrosis[183] as well as tumor infiltrating lymphocytes (TIL) categorized as absent, non-brisk or brisk[184,256]. In addition, the presence of TIL in metastases was recorded by OS and CS in hematoxylin and eosin-stained sections from metastases.

3.6 Analysis of mutation status

Direct Sanger sequencing was used for BRAF and NRAS mutation screening. Tumor tissue was manually dissected from three paraffin sections (10µm), followed by DNA-extraction with the E.Z.N.A. Tissue DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA). Amplifying of BRAF exon 11 and 15 and NRAS exon 1 and 2 by PCR was performed with previously described primers. The sequence reactions were done with the Terminator Cycle Sequencing kit, BigDye version 1.1 (Applied Biosystems, Foster City, CA, USA), and were analyzed on an ABI PRISM 3100 Genetic analyzer, applying Sequencing Analysis software, version 3.7 (both from Applied Biosystems).

3.7 Immunohistochemistry

Immunohistochemical staining was performed on 4-5µm thin tissue sections from primary melanomas and metastases. Mono staining was done with primary antibodies for HSP27, VEGF-A, VEGF165b, bFGF, anti-factor VIII (F-VIII), uPAR, Activin-A and IL1b. In addition, double staining with F-VIII and Ki67 was performed for assessment of angiogenesis. First, the sections were deparaffinized in xylene and different alcohol dilutions. After rehydration, antigen retrieval was performed by proteinase K, heating the specimens in the microwave or the pressure cooker. Endogenous enzyme activity was inhibited by blocking with peroxidase inhibitor, dual endogenous enzyme block or protein block before incubation with the primary antibody. A detailed overview including detection antibodies and chromogens is provided in **Table 7**. Tissues from different cancer types were used as positive controls. For negative controls, primary antibodies were omitted or specific blocking peptides, for HSP27, VEGF-A and bFGF, were used.

Table 7. Immunohistochemical staining methods

Antibody	Provider	Epitope retrieval	Dilution	Incubation
HSP27 pAb sc-1048 (goat)	Santa Cruz	MW 6th sense 20 min, pH6	1:100	30 min, RT
VEGF-A pAb sc-152 (rabbit)	Santa Cruz	MW 6th sense 20 min, pH9	1:50	60 min, RT
VEGF165b mAb ab14994 (mouse)	Abcam	MW 6th sense 20 min, pH9	1:100	Overnight, 4°C
FGF-2 pAb sc-1390 (rabbit)	Santa Cruz	Pressure cooker, pH9	1:50	60 min, RT
Von Willebrand Factor pAb A0082 (rabbit)	Dako	Proteinase K, 5 min	1:800	30 min, RT
Von Willebrand Factor pAb A0082 (rabbit) and Ki67 mAb (mouse) M7240	Dako	MW 6th sense 20min, pH6	1:500 and 1:200	60 min, RT
Activin A pAb A-1594 (goat)	Sigma Aldrich	MW 6th sense 20min, pH8	2,5µg/ml	60 min, RT
IL1b pAb sc-7884 (rabbit)	Santa Cruz	MW 6th sense 20min, pH6	1:50	120 min, RT
uPAR mAb ADG-3937 (mouse)	American Diagnostics	MW 6th sense 20min, pH6	1:100	Overnight, 4°C

pAb: polyclonal antibody; mAb: monoclonal antibody; MW: Microwave;

RT: Room temperature

3.8 Evaluation of staining results

For evaluation of immunohistochemical staining, sections were subjectively screened in a light microscope to avoid areas of ulceration, necrosis or scarring within the tumor. Cytoplasmic staining intensity was recorded for VEGF-A, $VEGF_{165}b$, HSP27, bFGF, uPAR, IL1b and Activin A, nuclear staining was additionally recorded for bFGF and $VEGF_{165}b$. Furthermore, the proportion of positive tumor cells, respectively endothelial cells in case of VEGF-A were recorded. Staining intensity

was graded as absent (0), weak (1), moderate (2) or strong (3)[257]. The proportion was rated as "no positive tumor cells" (0), "less than 10% positive tumor cells" (1), "10-50% positive tumor cells" (2) or "more than 50% positive tumor cells" (3). The staining index (SI) is the product of intensity and area, ranging from 0-9; SI was evaluated by two observers (CS, OS) blinded for response data.

3.9 Assessment of angiogenesis markers

Microvessel density (MVD) was assessed as described by Weidner[258] on sections double stained for F-VIII and Ki67. Thereby, we focused on areas with high intensity of F-VIII, defined as hot-spot areas[258]. Ulcerated and necrotic parts within the tumor were avoided. In addition, the number of *proliferating microvessels* (pMVD) was co-registered in the same high power fields (HPFs). Proliferating vessels co-express Ki67 in the nucleus and F-VIII in the cytoplasm of endothelial cells (ECs)[259]. Finally, the *vascular proliferation index* (VPI) was calculated as the ratio between pMVD and MVD (%)[259]. MVD and pMVD were reported as the number of microvessels per mm^2. Sections were screened for hot-spot areas at magnifications x40 and x100; counting of MVD and pMVD was performed at magnification x400 in three HPFs. *Glomeruloid microvascular proliferations* (GMPs) are focal glomerulus-like aggregates of at least 15 related, multilayered F-VIII positive ECs. Presence or absence of GMPs was recorded in sections stained with F-VIII and hematoxylin at magnification x100 in four consecutive HPFs[260,261]. Assessment was done simultaneously by OS and CS blinded for response data.

3.10 Analysis of blood samples

3.10.1 Enzyme linked immunosorbent assay (ELISA)

ELISA-kits based on the sandwich technique with pre-coated antibodies were used to measure the concentration of VEGF-A_{165} (DVE00 by R&D), HSP27 (ADI-EKS-500 by Enzo Life science), bFGF (DFB50 by R&D), uPAR (DUP00, R&D) and Activin A (DAC00B, R&D) in serum, respectively plasma samples. The assays were

performed according to the manufacturer's manual and the absorbance was measured at the wavelength of 450nm in a microplate reader (ELx808, Absorbance Microplate reader, BioTek Instruments, Inc.) immediately after the stop solution was added. All samples were run in duplicates. If the measured absorbance was above the highest standard, samples were diluted until they fit into the standard absorbance. Final sample concentrations that were below the minimal detection dose (MDD) as defined in the manufacturer's manual were handled as zero measurements.

3.10.2 Multiplex array

This quantibody array (Quantibody Human Angiogenesis Array 1000, RayBiotech, Inc. Norcross/GA, USA) contains 60 angiogenesis-related proteins that are spotted on glass slides as quadruplicates. Briefly, all wells were blocked with sample diluent before incubation with serum samples for two hours. Washing was followed by incubation with the detection antibody and finally, Cy3 equivalent was added. When the slides were completely dry, imaging was done with a microarray scanner (GenePix 4000B, Axon Instruments) at different photomultiplier tube gains. Interslide normalization for the most suitable scan was performed by RayBiotech.

3.11 Statistics

As mentioned above (3.1), the trial design was based on the two-stage clinical trial design by Simon[253] to determine sample size. The calculation was based on a 10% type I error rate and 90% power, and the assumption that treatment with bevacizumab would have a CB rate of 30%. These conditions required a minimum sample size of 35 patients.

Statistical analyses were performed with SPSS, version 22 (SPSS Inc., Chicago). Pearson's chi-square test was used for comparison of two categorical variables. Non-parametric tests were used for all analyses since not all data followed normal distribution. Associations between ordinal or continuous variables and categorical variables were assessed by Mann-Whitney U Test (MWT) or the Kruskal-Wallis Test (KWT). Paired Mann-Whitney Test (pMWT) was used to calculate the association

between proteins in the primary tumor and metastases by the assumption that these samples are matched pairs. Strength and direction of correlations between two interval scaled continuous or ordinal variables or one of each type were investigated by Spearman's rho correlation. Kaplan-Meier curves were constructed for time-to-event endpoints such as PFS and OS, and significance in differences was calculated by log-rank test. The significance threshold was 0.05 for all tests. When continuous variables were categorized, the median was used as cut point if not described elsewise.

Log2 and quantile normalization was performed in serum concentrations from the multiplex array[262]. Outliers were detected by Dixon test. Then, unsupervised Ward hierarchical clustering was performed. Finally, supervised analysis of particular proteins was conducted.

4. MAIN RESULTS

Paper I

The efficacy and safety of bevacizumab monotherapy in patients with metastatic melanoma in progression was investigated in a clinical phase II study. 52 patients were screened and 35 were finally enrolled. 14/35 patients were treated with dacarbazine before inclusion in the study. One of those patients was previously also included in a study that assessed the efficacy of dacarbazine and ipilimumab. In 21/35 patients, bevacizumab was given in first line. *BRAF* mutations were detected in 46% and *NRAS* mutations in 26% of the samples.

6/35 patients (17%) showed objective response (OR) to treatment with bevacizumab; 1/6 had complete response (CR) and 5/6 had partial response (PR). In addition, five more patients had stable disease (SD) > 6 months. In total, 31% of the patients had disease control (DC) and 69% (24/35) had progressive disease (PD). The treatment was well tolerated, no treatment related deaths occurred. However, bevacizumab had to be stopped in three patients according to grade 3 and grade 4 toxicities as defined in CTCAEv3.0. In addition, treatment had to be interrupted in one patient with symptomatic grade 3 toxicity. Hypertension according to CTCAEv3.0 was diagnosed in 14/35 patients after treatment with bevacizumab started. Early hypertension (EH) was significantly associated with DC, PFS and OS. Furthermore, all patients with stage M1a or M1b disease (7/7) had DC to treatment with bevacizumab compared to 4/28 patients with stage M1c disease. Normal levels of LDH were correlated to DC. BRAF or NRAS mutation status, age, sex or performance status were not correlated with response to treatment.

Paper II

In **Paper II**, we investigated the potential predictive value of angiogenic factors in blood and tissue samples for response to bevacizumab monotherapy. Tissue expression of vascular endothelial growth factor (VEGF-A), the anti-angiogenic splicing variant $VEGF_{165}b$, basic fibroblast growth factor (bFGF) and heat shock

protein 27 (HSP27) was investigated by immunohistochemistry. In addition, microvessel density (MVD), proliferating microvessel density (pMVD), vascular proliferation index (VPI) and the presence of glomeruloid microvascular proliferations (GMPs) were recorded. Strong cytoplasmic expression of HSP27 in tumor cells of metastases was correlated with objective response (OR) to bevacizumab monotherapy (p=0.044, MWT). Furthermore, disease control (DC) was seen among patients with strong HSP27 expression in primary melanomas. MVD was significantly higher in primary melanomas of patients with DC compared to non-responders (p=0.042, MWT). Serum concentration of VEGF-A, bFGF and HSP27 as well as intratumoral expression of the other angiogenic factors was not associated with response to bevacizumab.

Paper III

The purpose of this paper was to detect angiogenic factors in serum samples that could predict treatment response to bevacizumab monotherapy. A multiplex protein array was used to identify candidate proteins in baseline samples. Fold-change differences between responders and non-responders were calculated based on their serum concentrations. To validate our findings, candidate markers were further assessed in single ELISA assays, and their tissue expression in metastases was investigated by immunohistochemistry. Relevant proteins investigated in **Paper II** were included in the analyses. Serum concentration of Activin A, IL1b, uPAR and VEGF-A was associated with response to treatment when assessed by multiplex array, but these findings could not be validated by ELISA. Strong intratumoral tissue expression of Activin A, IL1b and uPAR in metastases was associated with response. Furthermore, we found a correlation between the number of proliferating vessels and tissue expression of Acitvin A, IL1b and uPAR. These findings indicate that those proteins are involved in VEGF-A related angiogenesis and are potential predictors for treatment with bevacizumab monotherapy in patients with metastatic melanoma.

5. DISCUSSION

5.1 Discussion of materials and methods

5.1.1 Patients, study design and samples

This was a single arm, single institution trial to show the effectiveness of bevacizumab monotherapy in an era where dacarbazine was the only established treatment for patients with metastatic melanoma. Fifty-two patients were screened and finally, 35 patients were enrolled in this clinical phase II study. The relatively high number of screening failures was due to brain metastases, co-morbidity or withdrawal of informed consent. Brain metastases were defined as exclusion criteria for treatment with bevacizumab because of increased risk for bleedings. Nevertheless, the number of patients needed to show benefit of bevacizumab monotherapy compared to dacarbazine was reached. Since this was a single center clinical trial and the number of screening failures was relatively high, the inclusion time ranged from 2005 to 2010. A clear advantage of this study is the availability of matched primary tumors and metastases, blood samples, clinical data and follow-up data.

To be able to investigate predictive markers that are specific to VEGF targeted treatment, the administration of bevacizumab as single drug was chosen. In many other studies in melanoma and other cancer types[243,263-266], bevacizumab was given in combination with chemotherapy. This complicates the identification of predictive markers for anti-angiogenesis treatment. However, candidate markers that were identified will have to be validated in randomized trials before they can be introduced to clinical practice.

For ethical reasons, the two step design by Simon was used. 14/35 patients were treated with dacarbazine first line before inclusion in the study. One of the 14 patients was originally included in a clinical study with dacarbazine +/- ipilimumab and continued with dacarbazine monotherapy before bevacizumab was given. When efficacy of treatment with bevacizumab monotherapy was shown in these patients, treatment with bevacizumab was given first line in the remaining 21/35 patients. The

study investigated efficacy of bevacizumab monotherapy in patients with metastatic melanoma in progression. Response evaluation was therefore performed in the total number of patients regardless of the line of treatment. Since treatment with dacarbazine may have influenced tumor biology [267], differences in protein expression between treatment lines were investigated intratumoral and in serum samples and descriptive data were reported.

5.1.2 Immunohistochemistry

All antibodies were first tested on whole tissues sections of melanomas, tissue recommended as positive controls and tissue microarrays containing cores of various normal tissues as well as different tumor tissues. The time of tissue fixation in formalin is an unknown variable when working with archival paraffin embedded tissue blocks. Different fixation times can result in inhomogeneous immunohistochemical staining. To optimize demasking of the epitopes, different retrieval methods like proteinase K, cooking in different buffers with pH 6, pH 8 or pH 9 and heating in the microwave or the pressure cooker, were tested. To determine the best staining for each antibody, different dilutions of the primary antibody as well as varied incubation times were used. Endogenous phosphatase or peroxidase was inhibited prior to incubation with the primary antibody. Detection systems based on alkaline phosphatase (AP) or horseradish peroxidase (HRP) in combination with different chromogens were tested. 3-Amino-9-ethylcarbazole (AEC) was preferable used for visualization in HRP-based staining protocols. Final counterstaining with hematoxylin was applied when mono-staining was performed. Biopsies that easily fell off the glass slides during the staining process were incubated at 58°C degrees overnight.

5.1.3 Evaluation of staining methods and assessment of angiogenesis markers

Immunohistochemical staining was evaluated in a semiquantitative manner as described in chapter 3.8. For HSP27, VEGF-A and bFGF assessment was done simultaneously at a double light microscope by CS and OS. The other factors were

rated by CS and indistinct cases were discussed with OS. The amount of representative tumor tissue was sparse in some of the core needle biopsies from metastatic lesions, though biopsies were taken in accordance with contemporary clinical practice. The intratumoral staining for HSP27, VEGF-A and bFGF could be evaluated in all metastases. Due to the limited amount of tumor tissue in some samples, staining for $VEGF_{165}b$ and uPAR could be evaluated in 34/35 metastases, staining for IL1b in 33/35 and staining for Activin A in 32/35 metastases.

Recording the intratumoral protein expression by the SI as described, takes both the intensity and the area of protein expression into account. This method may capture protein expression more completely than recording the staining intensity only. This index is established as a robust method in our group. However, the lack of standardized staining protocols and the multitude of various manual and digital evaluation methods are important issues when results from different studies are compared.

Assessment of MVD and pMVD was performed as described in chapter 3.9. Before recording MVD in patient samples, counting of MVD and pMVD was practiced in a series of colon cancer sections stained for CD31, F-VIII and F-VIII/Ki67. The MVD and pMVD count in this test material was recorded by experienced colleagues previously, so that the inter-observer agreement by kappa and Spearman`s rho could be calculated. When satisfactory kappa and Spearman`s rho correlation was achieved for intra- and inter-observer correlations in the colon series, assessment in the patient samples was performed by CS. Due to the limited amount of tissue in small primary tumors and several needle biopsies of metastases, three HPFs were chosen to determine MVD and pMVD in hot-spot areas. Difficult cases in the patient samples from the melanoma series were evaluated together with OS. Two of the 35 metastases contained insufficient amount of material to evaluate three representative high power fields, so MVD and pMVD were reported in 33/35 cases.

5.1.4 Analyses of blood samples

The choice of sample type is an important issue already in the planning phase of a clinical study. The sampling protocol should be as standardized and simple as possible to make sure that there is little variation in handling of the samples at this stage. Advantages and disadvantages of additives in various tubes should be taken into consideration because they can be an issue in later analyses. The use of serum samples eludes this problem but proteins can be bound or released by different cells during clotting. It is for example shown that VEGF is released by platelets and that this process results in significantly higher serum concentrations of VEGF compared to plasma concentrations[268,269]. Our results were in line with this, when VEGF was measured in EDTA plasma and serum samples. However, low VEGF-A concentrations in plasma samples can be below the detection limit of the assay. In our material, all serum samples were within the detectable range but 6/29 plasma samples had to be handled as zero measurements. As discussed by George et al.[270], serum may therefore be the preferable sample type anyway. However, biomarker analyses have been performed in various types of blood samples[271,272]; this may be one reason why still no predictive markers for treatment with bevacizumab have been established.

Furthermore, ELISA kits from different manufacturers are pre-coated with different antibodies resulting in various detection sensitivity and different final protein concentrations. Commercial ELISA kits are often not established in research with human samples and sensitivity for binding the protein of interest in such samples may not be high enough, especially if the expected concentration is low. In single protein ELISA, reactivity of the kit with the antigen is visually traceable and cross reactivity is less relevant. However, up to 240µl sample or more can be needed to analyze one protein in duplicate and insufficient binding of the antigen to the antibody epitope can be problematic. In the Quantibody array, less than a third part of sample volume was sufficient to perform the assay in quadruplicates. Another advantage was the possibility to perform multiple scans to figure out the best adjustments of the laser scanner for most of the proteins. Though, finally one scan had to be chosen for interslide normalization and further analyses. Since different detection methods and

different antibodies were used in ELISA and the multiplex array, the final concentrations of proteins assessed by both methods were not necessarily correlated. This demonstrates once more the difficulties in detecting reliable validated predictive markers.

5.1.5 Statistics

As mentioned in chapter 3.11, the sample size was calculated to show efficacy of bevacizumab monotherapy in patients with metastatic melanoma. The analyses of predictive markers have to be interpreted with caution due to low sample size and the findings need to be validated in a larger randomized study. At the same time, some analyses may not have been able to detect existing differences between responders and non-responders.

The choice of cut-off points in continuous variables like the staining index is also an issue of discussion. We used the Mann-Whitney U test to distinguish between responders and non-responders. However, when predictive markers should be used in clinical practice, the definition of a cut-off point will be necessary.

We did not correct for multiple testing. In **Paper II**, the analyses were performed based on pre-specified hypotheses. The candidate proteins we investigated were involved in VEGF-A related angiogenesis and treatment with a VEGF-A inhibitor was given. **Paper III** was based on a boarder screening approach to identify potential predictive markers. However, the sixty proteins investigated by the multiplex array were angiogenesis related. Potential candidate proteins were further validated by an additional test method like single ELISA assays or immunohistochemistry.

5.2 Discussion of results

5.2.1 Angiogenesis and anti-angiogenic treatment in melanoma

Tumor growth beyond a few square millimeters and metastatic progression depend on angiogenesis[100,110,273]. Activation of the angiogenic switch seems to play a role in the transition from horizontal to radial growth phase in primary melanomas[274].

Furthermore, increased vascularization was shown to be an adverse prognostic factor for OS[120,176,275]. In addition to these studies in archival material and preclinical models[276,277], Srivastava et al. measured the Doppler flow signal in primary melanomas[278]. A recently published study with 15 years follow up time reported 39% melanoma related cases of death in patients with Doppler flow positive primary lesions compared to no melanoma related deaths and no recurrences in the Doppler flow negative group[278]. Conversely, others did not show any correlation between angiogenesis and melanoma outcome[279,280].

However, multiple clinical trials investigated various anti-angiogenic treatment options as referred to in chapter 1.7. In our material, 6/35 patients with metastatic melanoma in progression had objective response (OR) to bevacizumab monotherapy; in addition, 5 more patients had stable disease (SD) for at least 6 months (**Paper I**). One patient had complete response (CR) and is still disease free without receiving further treatment. These findings are in contrast to the results from Varker et al.[242] who observed no partial or complete response in patients treated with bevacizumab monotherapy. This may be explained by the low sample size (n=16). An overall response rate of 17%, as reported in our material, is in line with the findings of other studies combining bevacizumab with fotemustine (OR 15%)[281], temodalozide (OR 16%)[282] or dacarbazine (OR 19%)[283]. A similar overall response rate (19%) was also reported after monotherapy with the selective VEGF-R inhibitor axitinib[284]. When bevacizumab is given in combination with carboplatin and paclitaxel, higher overall response rates were achieved (33%)[263], 25.5%[243]).

Development of early hypertension (EH), i.e. before the first evaluation, predicted response to treatment (**Paper I**) and was observed in 63.6% of all responders (7/11) compared to 12.5% of the patients with disease progression. Among the responders, 4/6 patients with overall response developed EH. The median progression free survival time and overall survival were significantly longer in patients with EH (**Paper I**). Also among patients with metastatic melanoma who were treated with the VEGF-R inhibitor axitinib, induced hypertension predicted better PFS and OS[284]. Bevacizumab-induced hypertension was also predictive for outcome in several other

studies in that bevacizumab was given in combination with other drugs; even though, some findings were contradictory[285]. The controversial findings could be explained by various time points and techniques of blood pressure measuring, various definitions of hypertensive events, differences in anti-angiogenic treatment and different tumor types. Lambrechts et al. investigated VEGF-A pathway and hypertension associated SNPs in a pooled analysis from patients receiving bevacizumab-containing treatment. Several candidate markers were identified; however, statistical significance was not maintained after correction for multiple testing[286]. In general, it is not unproblematic to use an adverse event like hypertension as predictive biomarker as discussed by Jubb et al.[285]. Adverse events may become severe, need additional treatment or result in interruption or termination of treatment. Therefore, upfront predictive markers are preferable to avoid starting ineffective therapy.

Normal LDH and M1a or M1b-stage were other predictive factors in our material. The correlation between a normal level of LDH at baseline and better response was also reported by Fruehauf et al.[284]. Furthermore, the level of LDH and M-stage are well established prognostic factors[86] and characterize less aggressive melanomas. We did not find any correlation between the *BRAF* or *NRAS* mutation status and response to treatment. So, anti-angiogenic treatment could be an option for *BRAF* wild type patients who are not eligible for immunotherapy or patients who progressed on first line treatment with BRAF inhibitors or immune modulating drugs. A recently published phase II trial showed significantly better OS in *BRAF* wild type patients compared to *BRAF* mutated patients treated with axitinib and subsequently administration of paclitaxel/carboplatin[287].

5.2.2 Candidate markers of angiogenesis

VEGF-A plays an important role in physiologic and pathological angiogenesis by activation of endothelial cell proliferation and migration, and promotion of vessel dilatation and permeability[126]. VEGF-A and its receptors are not expressed in normal melanocytes but are overexpressed in melanoma cells[279,280,288]. We observed

intratumoral VEGF-A expression in nearly all primary melanomas and metastases (**Paper II**). The quantification of protein expression did not correlate with response to treatment with bevacizumab monotherapy. Since VEGF-A is a ligand, it can be intracellular, extracellular or receptor bound. Because of the ubiquitous existence of ligands, it is more difficult to quantify their presence and biological activity compared to proteins that are exclusively seen in a particular cellular compartment. However, in primary melanomas, strong VEGF-A expression was correlated to a higher number of proliferating vessels (pMVD) and in metastases, VEGF-A expression was associated to a higher number of microvessels (MVD) (**Paper II**). These findings confirm a role of VEGF-A dependent angiogenesis in a subgroup of primary melanomas and metastases. VEGF-A contributes also to angiogenesis by re-localization of uPAR to the leading edge of migrating endothelial cells[144,289]. In our material, strong intratumoral uPAR expression in metastases was associated to better OR, a higher MVD, presence of GMPs and stronger expression of HSP27 in metastases (**Paper III**).

In addition to VEGF-A, we investigated expression of the anti-angiogenic splicing variant $VEGF_{165}b$. This isoform binds to bevacizumab with the same affinity as VEGF-A[290] and is strongly expressed in normal tissue but seems to be down-regulated in tumors[127,280,291]. In primary melanomas that metastasized, no expression of $VEGF_{165}b$ was recorded, even though it was expressed in peritumoral normal epidermis[130]. In our material, there was no correlation between $VGEF_{165}b$ expression in primary melanomas or metastases and response to treatment with bevacizumab. Furthermore, there was neither a positive nor a negative correlation to VEGF-A expression in primary tumors or metastases (**Paper II**).

VEGF-A expression in tumors is regulated by numerous growth factors, cytokines and HIF1-α[126]. HSP27 is another regulator of VEGF-A expression[136] as described in chapter 1.7. Stressful conditions can result in HSP27 triggered degradation of a cytoplasmic protein complex that inhibits the transcription factor NF-κB[292]. As a consequence, NF-κB can be translocated to the nucleus and activates transcription of VEGF. Downregulation of HSP27 in an angiogenic breast cancer cell line resulted in

reduction of VEGF-A secretion and a non-angiogenic phenotype *in vivo*[136]. Based on these results, we wanted to investigate the role of HSP27 in our material. We found that strong expression of HSP27 in metastases was correlated to better objective response to treatment with bevacizumab monotherapy (**Paper II**). Still, HSP27 and VEGF-A expression in melanoma cells were not significantly correlated. It could be that this correlation would be positive with a larger samples size. Additionally, various factors are involved in angiogenesis and may be expressed at different levels at a certain time point[293].

Another important factor in angiogenesis is bFGF[132]. Like VEGF-A it is expressed in most melanoma cells but not by normal melanocytes[279]. Intratumoral bFGF expression in primary melanomas or metastases did not show any correlation to treatment response in our study (**Paper II**). Furthermore, there was no association of cytoplasmic bFGF expression and VEGF-A expression in our material.

The proangiogenic role of IL1b and its interaction with VEGF-A and bFGF *in vivo* and *in vitro* is reviewed by Voronov et al.[146]. In our material, strong IL1b expression in metastases was correlated to strong expression of VEGF-A, bFGF and Activin A as well as a higher number of proliferating vessels (**Paper III**). Furthermore, patients with strong intratumoral expression of IL1b in metastases responded better to bevacizumab and had better PFS (**Paper III**).

Patients with OR to bevacizumab showed also stronger Activin A expression in metastases than non-responders and we observed an association to the expression of other proangiogenic proteins and a higher number of proliferating vessels in metastases (**Paper III**). This is in line with findings from others who described a role of Activin A in expression of VEGF-A and bFGF as well as increased tubulogenesis[148,149,294].

5.2.3 Microvessel density

Furthermore, we investigated the role of microvessel density (MVD) as a predictive marker for anti-angiogenic treatment. In addition, we also quantified the density of

proliferating vessels. Whole tissue sections were available for screening for F-VIII hot-spot areas[258] in primary melanomas. These sections gave a more complete overview of hot-spot areas than smaller biopsies from metastases. Further, it is unknown from which part of the metastases the biopsies were taken. Intratumor heterogeneity results in varied metabolic and oxygenic conditions, and small needle biopsies can only capture a part of it. In addition, the material in some of the needle biopsies from metastases was sparse, so the number of proliferating vessels may be underrepresented in some of the samples. We observed significantly higher MVD in primary tumors of patients who responded to treatment with bevacizumab. However, MVD in metastases was not correlated to response (**Paper II**). Activated angiogenesis may characterize more aggressive primary tumors that metastasize, and clonal evolution may result in more angiogenic metastases. This is in line with the finding that high MVD in primary melanomas is an adverse prognostic factor[176,275]. The relevance of MVD as a predictive marker for anti-angiogenic treatment is still under discussion, since MVD is not necessarily a marker for angiogenic dependency of a tumor, and the grade of vascularization does not determine treatment response[293]. In our material, MVD or pMVD in metastases could not predict objective response (OR) to bevacizumab monotherapy. There are different advantages and weaknesses in determining MVD by the Weidner method[295,296]. Selection of the hot-spot area is done subjectively and markers defining the functional status of the endothelial cells are not included. Furthermore, there is no standardized method of MVD assessment, different endothelial cell markers have been used to determine MVD in various cancer types[279,293,297] and there is no consensus whether MVD should be assessed in the periphery or the center of the tumor[293]. We co-registered MVD and pMVD to additionally investigate the role of proliferating vessels as predictive marker.

Alternative angiogenesis independent mechanisms of vascularization may play a role in addition. Those vessels will not be detected by the markers we recorded and will possibly not be abolished by bevacizumab treatment. One mechanism is co-option of existing vessels as observed in gliomas and in well perfused organs like the brain, lung and the liver[296,298 299]. Another mechanism is vascular mimicry, i.e. tumor cells themselves form channels - without the participation of endothelial cells - to sustain

blood supply[300]. This controversial phenomenon is also described in highly invasive and aggressive melanomas[301]. McDonald et al. commented the findings by Maniotis critically[302]. They highlighted among others the importance of choosing established immunohistochemical markers to determine the presence and localization of endothelial cells and perivascular connective tissue correctly. Furthermore, they discussed the need to distinguish properly between intra- and extravascular erythrocytes and consecutively define the presence of a vessel lumen. Insufficient investigations may otherwise result in misinterpretation of findings. Nevertheless, the observation that tumor cells can contribute to vessel like structures has been reported by others previously[302].

5.2.4 Blood concentrations of various angiogenic factors

The concentration of VEGF-A, HSP27, bFGF, uPAR and Activin A in blood samples was analyzed by ELISA, and in addition 60 factors involved in angiogenesis were assessed in a multiplex protein array. Serum samples were used for all analyses; VEGF-A concentration by ELISA was also measured in plasma samples.

None of these angiogenic factors analyzed by ELISA could predict response to treatment with bevacizumab (**Paper II, Paper III**). These findings are in line with the results from others investigating the predictive value of VEGF-A and bFGF in melanoma patients[242,263,303]. Gringol et al. found no association between concentration of VEGF-A at baseline and response to treatment; contrary, low concentration of bFGF was associated with partial response[304]. Serum levels of HSP27 were increased in patients with hepatocellular carcinoma[305], breast cancer[306] and lung cancer[307] compared to healthy controls. However, HSP27 is not established as a predictive marker.

Analyses by the multiplex protein array identified high concentration of Activin A as well as low concentrations of IL1b, uPAR and VEGF-A as potential predictive markers for treatment with bevacizumab (**Paper III**). Although, the concentrations of uPAR and VEGF-A measured by ELISA correlated to those measured by the multiplex array, the association to treatment response was only seen in the multiplex

array. These contradictory findings illustrate the challenges to identify robust predictive markers and may also be explained by technical issues (see 5.1.4).

Furthermore, the serum concentrations of uPAR, IL1b, Activin A and VEGF-A were not correlated to the intratumoral expression of these proteins in metastases. Others also reported discrepancy between local and systemic concentrations of uPAR[308], IL1b[309] and VEGF-A[310]. IL1b is usually not detectable in serum samples of healthy subjects[146] and has to be cleaved intracellularly before it is secreted in its active form. Differences in protein concentrations may also be explained by interactions between the tumor and its systemic environment.

6. CONCLUSIONS

1) Treatment with bevacizumab monotherapy shows efficacy in patients with metastatic melanoma. We observed objective response in 6/35 (17%) patients and disease control in 11/31 (31%) of the patients (**Paper I**).

2) Development of early hypertension is associated with response to treatment (**Paper I**).

3) Response to treatment was not associated with *BRAF* or *NRAS* mutation status (**Paper I**).

4) Strong expression of HSP27 protein in metastases is associated with response to treatment (**Paper II**).

5) Microvessel density, the number of proliferating vessels or the presence of GMPs in metastases could not predict response to treatment (**Paper II**).

6) Strong tissue expression of Activin A, IL1b and uPAR proteins in metastases was associated with response to treatment (**Paper III**).

7) Candidate proteins could not be validated as predictive markers in serum samples (**Paper III**).

7. FUTURE PERSPECTIVES

Ongoing clinical trials focus mainly on immune check point blockade and targeted therapy for *BRAF* mutated melanomas as well as various combinations of these treatment options. Even tough, improvements in response rates, progression free survival and overall survival rates have been observed, many biological and mechanistic questions remain unanswered. Important issues are primary and acquired resistance, the role of the tumor microenvironment in progression and metastasis as well as the lack of reliable predictive biomarkers. Furthermore, only about 50% of the melanomas harbor *BRAF* mutations and not all patients are eligible for immune-therapy. So, there is still a need to investigate further treatment options for these patients. We have shown that anti-angiogenic treatment may play a role in a subpopulation of melanoma patients and aim to further investigate the role of anti-angiogenic treatment in a larger randomized clinical trial. In addition, the potential predictive markers we identified in our series have to be validated in a larger patient population. Thereby, validated predictive markers in blood samples would be preferable in clinical practice.

Furthermore, we observed long lasting responses in a couple of patients beyond treatment with bevacizumab. Recent publications discuss the role of VEGF-A in the immune system. Treatment with a VEGF-A antibody may therefore contribute to a more anti-tumorigenic environment, and by this improve patient`s outcome. Clinical trials that combine anti-angiogenic and immune-modulating therapy in melanoma treatment are ongoing. We would like to further investigate VEGF-A as a predictor of response in immune-modulating treatment in a clinical phase IV trial.

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Clinical Efficacy and Safety of Bevacizumab Monotherapy in Patients with Metastatic Melanoma: Predictive Importance of Induced Early Hypertension

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Abstract

Background: VEGF driven angiogenesis plays a key role in cancer progression. We determined the clinical efficacy of bevacizumab monotherapy in patients with metastatic melanoma.

Methods and Findings: Thirty-five patients with metastatic melanoma in progression were enrolled in this phase II, single arm clinical trial. Each patient received bevacizumab monotherapy 10 mg/kg q14 d until intolerable toxicity or disease progression occurred. Clinical efficacy was evaluated as objective response, disease control (DC), and survival. We observed one complete (3%) and 5 partial (14%) responses. In addition, 5 patients experienced stable disease >6 months (14%) while 24 patients had progressive disease (PD, 69%), corresponding to a total DC at 6 months in 11 out of 35 patients (31%). Median progression free survival (PFS) was 2.14 months and median overall survival (OS) was 9 months (1.12–49). Seven of the 11 patients experiencing DC developed early hypertension (<2 months) compared to 3/24 of patients with PD ($P = 0.001$), and hypertension was associated with PFS ($P = 0.005$) and OS ($P = 0.013$).

Conclusion: Bevacizumab monotherapy demonstrated promising clinical efficacy in patients with metastatic melanoma with disease control in 31% of the patients. Induced early hypertension was a marker for clinical efficacy of bevacizumab.

Trial Registration: ClinicalTrials.gov NCT00139360.

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Introduction

Metastatic melanoma is a non-curable condition with limited therapeutic options. Until recently, high dose interleukin-2 and dacarbazine were the only regimens in routine use, with response rates observed in about 10% of unselected patients[1–3]. While the human monoclonal anti CTLA-4 antibody ipilimumab was recently shown to cause a survival benefit in stage IV melanoma [4], the drug was found active in a fraction of patients only. Improved survival was also reported for treatment of metastatic melanoma patients carrying a specific *BRAF* mutation (~40% of all melanoma patients) using the highly selective V600E kinase inhibitor vemurafenib [5]. Thus, while selected patients may benefit from novel treatment options, effective treatment is still not available for a high proportion of melanoma patients. In addition, patients benefitting from conventional (interleukin-2 or dacarbazine) as well as novel (ipilimumab and vemurafenib) therapeutic strategies develop acquired therapy resistance over time, underlining the need for alternative treatment options.

Melanoma progression and metastasis is dependent on angiogenesis [6] and the vascular endothelial growth factor (VEGF) system seems to be particularly important [7,8]. The humanized monoclonal antibody bevacizumab is a highly specific inhibitor of VEGF-A. Bevacizumab significantly prolonged overall survival when given in combination with chemotherapy in colorectal cancer [9] and in non-small cell lung cancer [10]. In addition, responses have been reported in clinical trials evaluating bevacizumab in combination with interferon alpha 2B [11], interferon alpha 2A [12] or chemotherapy[13–15] in patients with metastatic melanoma. Administered as monotherapy, bevacizumab prolonged time to progression given in patients suffering from metastatic kidney cancer [16].

To the best of our knowledge, no clinical trials have been published specifically testing the clinical efficacy of bevacizumab monotherapy in metastatic melanoma. Here, we report the results

from a phase II trial evaluating clinical efficacy of bevacizumab monotherapy in patients with metastatic melanomas.

Methods

Ethics

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. The protocol was approved by the Regional Ethics Committee and the Norwegian Medicines Agency. All participating patients provided signed informed consent before enrolment.

Patients

Between April 2005 and August 2009, 52 patients were screened. Eligibility criteria included histologically confirmed unresectable metastatic melanoma in progression; age >18 years; WHO performance status 0–2; clinically and/or radiographically measurable disease according to RECIST; >4 weeks since adjuvant interferon; no prior interferon or interleukin for metastatic disease; recovered from prior chemotherapy; no major surgery within 28 days; no known brain metastases; absolute neutrophils $>1.0\times10^9$/L; platelets $>100\times10^9$/L; bilirubin, creatinine, INR <1.5×upper normal limit; no symptomatic congestive heart failure, angina pectoris, cardiac arrhythmia, history of thrombosis, uncontrolled hypertension, full dose coumarin-derived anticoagulants or NSAIDS.

Study Design

This was a phase II, open-label, single-arm, single institution clinical trial (ClinicalTrials.gov Identifier: NCT00139360), performed at the Haukeland University Hospital, Bergen Norway. The full protocol is available online as supporting information (Protocol S1). The primary objective was to determine clinical efficacy, as measured by objective response (OR) and disease control (DC) defined as stable disease (with or without an objective tumor shrinkage) after 6 months on therapy. Secondary objectives were to estimate time to progression (TTP), progression free survival (PFS) and overall survival (OS). Finally, we aimed at exploring potential relations between side effects, including acquired hypertension as well as *BRAF*/*NRAS* mutation status as potential predictive factors to clinical response.

Initially, patients were included after confirmed progression on standard first line treatment with dacarbazine (level A, n = 15). Only after objective response was observed on bevacizumab monotherapy, all new patients were subsequently enrolled for first line treatment with bevacizumab (Level B, 20 patients) (Flow diagram S1).

Each treatment cycle consisted of bevacizumab 10 mg/kg IV on day 1 in a 2-weekly schedule. Thus, the chosen dose was higher than the doses used in bevacizumab therapies for normalization of tumor vasculature (5 mg/kg q14d) [17,18] and in line with the dosing of bevacizumab monotherapy used in advanced renal cancer where a survival benefit was indicated (10 mg/kg q14d) [16]. Drug toxicity was assessed after each cycle, while the response rate was evaluated after every 4 cycles. Patients with disease progression or unmanageable toxicity were discontinued and offered further melanoma treatment at the clinician's discretion. Standard clinical parameters (routine biochemistry, urine analysis, blood pressure, WHO performance status) as well as the mutational status for *BRAF* and *NRAS* were assessed for subsequent correlation with clinical outcome.

Response Assessment and Toxicity

The primary endpoint was objective response (OR) defined as complete response (CR) or partial response (PR) according to RECIST [19] as well as disease control (DC) defined as CR + PR and including stable disease (SD) for more than 6 months. Disease stabilization is considered beneficial to patients experiencing melanoma progression at the time of inclusion and DC is frequently included as an additional statistical endpoint in trials investigating new antiangiogenic drugs in which therapeutic activity and clinical benefit are present, even in the absence of radiological tumor shrinkage[20–22]. Importantly, all patients were in clinical and/or radiological progression at the time of inclusion. OR and DC were calculated on the basis of investigator assessment. While confirmed response after 4 weeks was not a protocol requirement, all patients achieving an objective response had a subsequent confirmation at the next routine visit every 8 weeks. Patients with clinical disease progression or death due to melanoma before first radiological progression were recorded as progressive disease (PD), and best overall response (BOR) was not available in these patients. TTP was defined as the time from enrolment to disease progression or death due to melanoma.

Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse events, version 3.0 [23], and were recorded by each 2-week cycle.

Tissue Sampling and DNA Analysis

To evaluate a possible relationship between the most frequent genetic alterations in melanoma and treatment outcome, a targeted mutational analysis was performed for *BRAF* and *NRAS*. Tumor tissue was manually dissected from 3 paraffin sections (10 μm) before extracting DNA with the E.Z.N.A Tissue DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA). *BRAF* exon 11 and 15, as well as *NRAS* exon 1 and 2 were amplified by PCR, and screened for mutations by direct Sanger sequencing. Primers are described elsewhere[24–26]. The sequence reactions were performed using the Terminator Cycle Sequencing kit, BigDye version 1.1 (Applied Biosystems, Foster City, CA, USA), and were analyzed on an ABI PRISM® 3100 Genetic Analyzer, applying Sequencing Analysis software, version 3.7 (both from Applied Biosystems).

Statistical Methods

The optimal two-stage design for phase II clinical trials proposed by Simon [27] was used. The co-primary endpoint DC was used to determine sample size. It was assumed that the new regimen would have a DC rate of 30%. A DC rate of 10% or lower was considered not superior to standard first-line therapy (dacarbazine). With 10% type I error rate and 90% power a total number of 35 patients were entered in the trial.

Two sample t-test and Mann-Whitney U test were used to compare the distribution of continuous variables between two groups such as responders and non-responders. Kaplan-Meier estimates were constructed for time-to-event endpoints such as PFS and OS, and log rank-test was applied for testing differences. Due to the small sample size and the nature of the phase II study, the above analyses were considered exploratory and the results need to be confirmed in future large-scale studies.

Results

Patients

Between April 2005 and August 2009, 52 patients with metastatic or unresectable melanoma in progression were screened and 35 patients were enrolled in this trial. The seventeen screening

failures were most frequently due to brain metastases, co morbidity, or withdrawal of informed consent (Fig 1). During recruitment at level A, 15 patients received bevacizumab as second/third line treatment (after DTIC failure) while additional 20 more patients were included during recruitment level B (first line therapy bevacizumab). Patient characteristics are listed in Table 1.

Responses, PFS and Survival

In the study population of 35 patients, we observed 1 CR (3%), 5 PR (14%), and 5 SD >6 months (14%). Thus, 24 patients (69%) progressed on therapy, including three patients who progressed clinically before radiological tumor evaluation. Best overall response (BOR), measured as the change in the sum of largest diameter of the target lesions is illustrated in Figure 2A. Duration of the responses in relation to patient characteristics is illustrated in Figure 2B. Tumor responses were observed at metastatic sites such as skin, lymph nodes, lung, liver and ovaries (Fig 3).

At 6 months of follow up, 11/35 (31)% of the patients had no sign of melanoma progression. This proportion was 8/20 for the first line patients and 3/15 for the second/third line patients, respectively. By August 2011, median PFS was 2.14 months whereas mean PFS was 7.7 months (range 0.8–30 months), with a median overall survival of 9 months (mean: 13, range: 1.1–49) (Fig 4 A and B). The median number of cycles was 4 (mean: 14, range: 1.0–64). No patients died of causes other than melanoma progression. Six of the patients are still alive, and 5 of them are still on bevacizumab treatment without signs of progression 15–30 months after starting bevacizumab.

Seven of 11 patients with DC developed early hypertension (EH) as defined by CTCAEv3.0. In contrast, only three of 24 (12.5%) patients with progressive disease (PD) developed EH (Chi-square test $p<0.001$). Median time to progression for patients who developed EH following bevacizumab treatment was 11.4 months

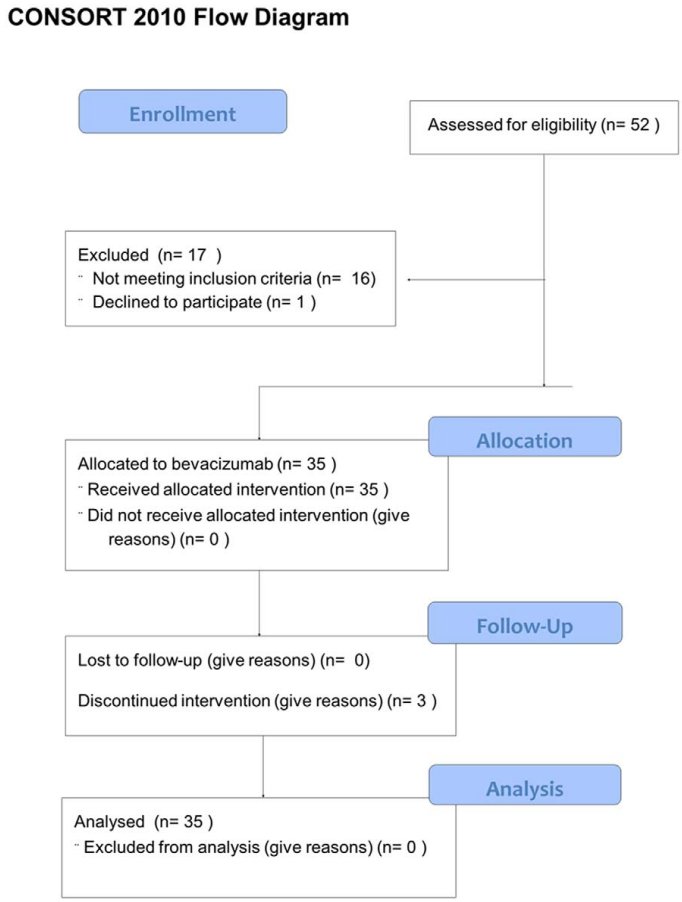

Figure 1. Study flow diagram. Between April 2005 and August 2009, 52 patients with metastatic melanoma were screened. Thirty-five of those patients were eligible according to inclusion criteria and received the study drug.
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Table 1. Baseline Demographic and Clinical Characteristics of Patients.

Characteristics	Study cohort ($n=35$)
Age, years	
Median	63
Range	26–77
Sex - No. (%)	
Male	19 (54)
Female	16 (46)
Stage - No. (%)	
M1a	1 (3)
M1b	6 (17)
M1c	28 (80)
LDH>ULN - No. (%)	
No	14 (40)
Yes	21 (60)
WHO performance status - No. (%)	
0	28 (80)
1	7 (20)
Previous systemic treatments - No. (%)	
0	20 (57)
1	14 (40)
2	1 (3)
Hypertension before treatment - No. (%)	
No	27 (77)
Yes	8 (23)
BRAF exon 15 mutation – No. (%)	
Wild type	20 (57)
V600E	13 (37)
V600K	1 (3)
V600D/V600E Double mutation	1 (3)
NRAS exon 2 mutation - No. (%)	
Wild type	24 (69)
Q61R	4 (11)
Q61L	2 (6)
Q61K	3 (9)
E62E	1 (3)
Not amplifiable	1 (3)

Abbreviations: LDH, lactate dehydrogenase; ULN, upper limit of normal; WHO, World Health Organization.
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compared to 2.0 months in normotensive patients. EH was significantly associated with prolonged PFS (log rank $p = 0.001$, Fig 4C) as well as improved overall survival (log rank $p = 0.005$, Fig 4D). To explore the possible association between the use of different antihypertensive drugs and disease progression we observed that 6 of 7 patients on concomitant beta blockers experienced DC following treatment with bevacizumab monotherapy. In comparison, 3/6 patients who used antihypertensive drugs other than beta blockers, or 2/22 who used no antihypertensive drugs experienced DC (Chi square test $p<0.001$).

Stage M1a and b disease was significantly associated with DC (7/7) as compared with M1c disease (4/28; Chi-square test $p<0.001$). Similarly, 9 of 14 patients with normal levels of lactate dehydrogenase (LDH) at baseline had DC as compared with 2 out of 21 with increased LDH (Chi-square test $p = 0.001$). No significant correlations were found between DC, OR, PFS or OS and *BRAF* or *NRAS* mutation status, performance status, sex or age.

Safety

Bevacizumab monotherapy, given as 10 mg/kg q14d IV was in general well tolerated by the patients. No treatment related deaths were recorded. Treatment was stopped in two patients with CTCAE grade 4 toxicity (1 anaphylactic shock at cycle 2 and 1 lung embolus at cycle 5) and in one patient with grade 3 gastrointestinal toxicity (partial obstruction due to disease progression at cycle 1). Treatment was interrupted in one patient with grade 3 toxicity due to symptomatic left ventricular systolic dysfunction after 16 cycles. All adverse events are listed in Table S1 (online only). No dose reduction, interruption or postponement due to fatigue or hypertension was necessary and no hemorrhage was observed. Bevacizumab was delayed until proteinuria was <2 g/24 h in three patients (<4 weeks), but no treatment was stopped permanently due to proteinuria.

Hypertension according to CTCAEv3.0 was observed in 14 (40%) patients after initiation of bevacizumab. Grade 1:4 (11%), grade 2:3 (9%) and grade 3:7 (20%), respectively. Median time to induced hypertension was 43 days (mean: 59, range: 27–239). We defined early hypertension (EH) as hypertension $\geq$ grade I occurring before 1st tumor response evaluation at 8 weeks. EH was recorded in 10 patients whereas 4 patients developed hypertension at a later time point. As listed in Table 1, 8 (23%) of the patients were treated for hypertension at the time of inclusion. Type of antihypertensive drugs used is listed in Table S2.

Discussion

While some phase II studies have evaluated the use of bevacizumab in concert with interferon alpha 2B [11,28], interferon alpha 2A [12] or chemotherapy [13,14], to the best of our knowledge this is the first study evaluating bevacizumab monotherapy in metastatic malignant melanoma. Our results provide a proof-of-principle that bevacizumab monotherapy is active in metastatic melanoma with a disease control rate of 31% and a 6 months PFS rate of 31%. In consistency with our finding, a recently published study of the multi kinase inhibitor axitinib (including VEGF receptor 1, 2 and 3) given as monotherapy showed a OR rate of 18.8% and a DC rate of 37.5% in a similar patient population [29]. These results are strikingly in line with ours and are in support of a significant subgroup of melanoma patients being susceptible to anti-VEGF strategies. Although not meeting the primary objective of increased median progression free survival, a recently published placebo controlled randomized phase II study showed encouraging overall survival data in metastatic melanoma patients treated with carboplatin and paclitaxel ± bevacizumab [15]. In contrast to our present findings, the patients who benefitted most from that combination were those with increased LDH and M1C disease, possibly indicating different sensitivity between patients groups to combination therapy and monotherapy.

In metastatic melanoma new treatment options have recently emerged targeting *BRAF* [5] or CTLA-4 [4] showing improved overall survival, but these treatments are associated with significant toxicities and costs. In addition, for *BRAF* negative patients or patients with non-immunogenic disease only limited effective treatment options are available. Significantly, in our study there

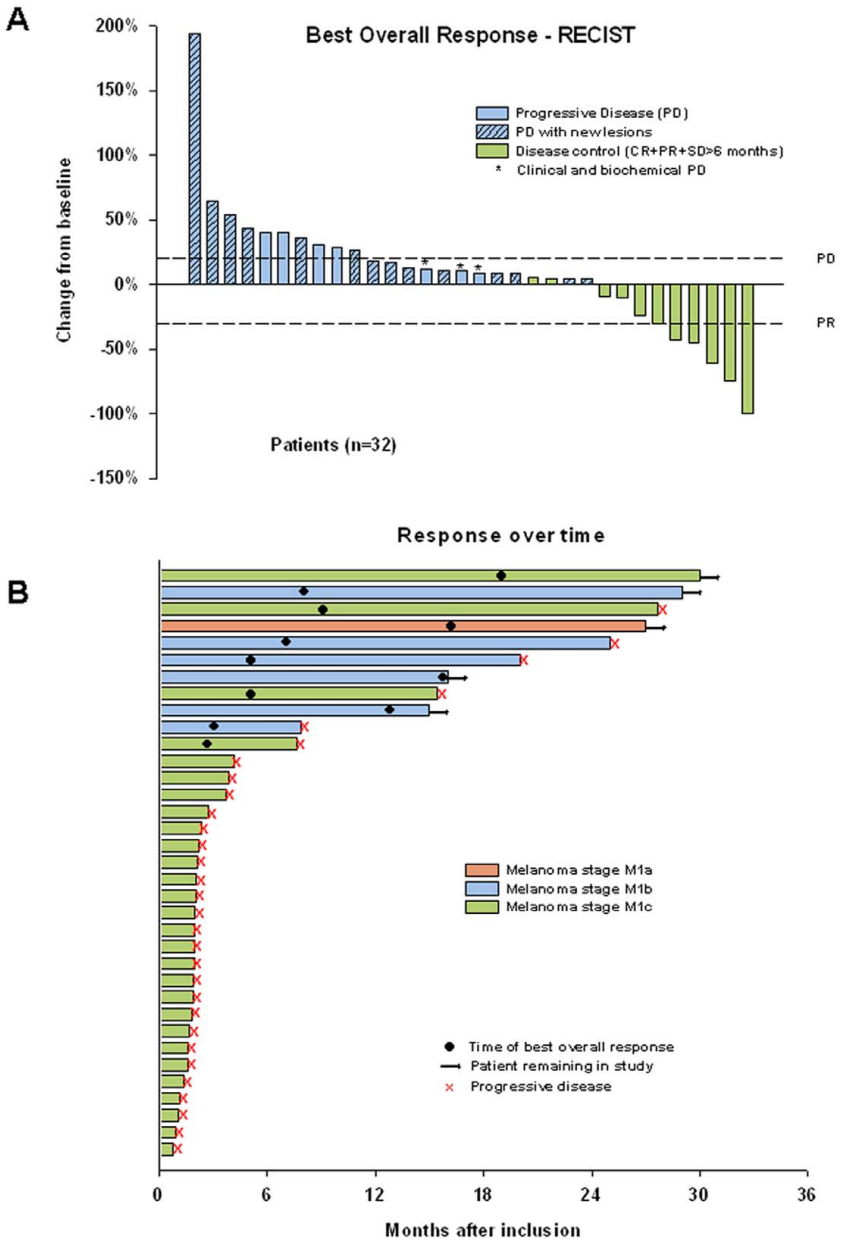

Figure 2. Patterns of response to treatment with bevacizumab monotherapy in metastatic malignant melanoma patients. Panel A shows the best overall response for 32 patients who had undergone at least one tumor assessment measured as the change from baseline in the sum of the largest diameters of each target lesion. Three patients progressed clinically and/or biochemically before first tumor assessment, and are not shown. Negative values indicate tumor shrinkage, and the dashed lines indicate the threshold for a partial response (PR) and progressive disease (PD), respectively. Panel B shows the duration and characteristics of the responses in each patient.
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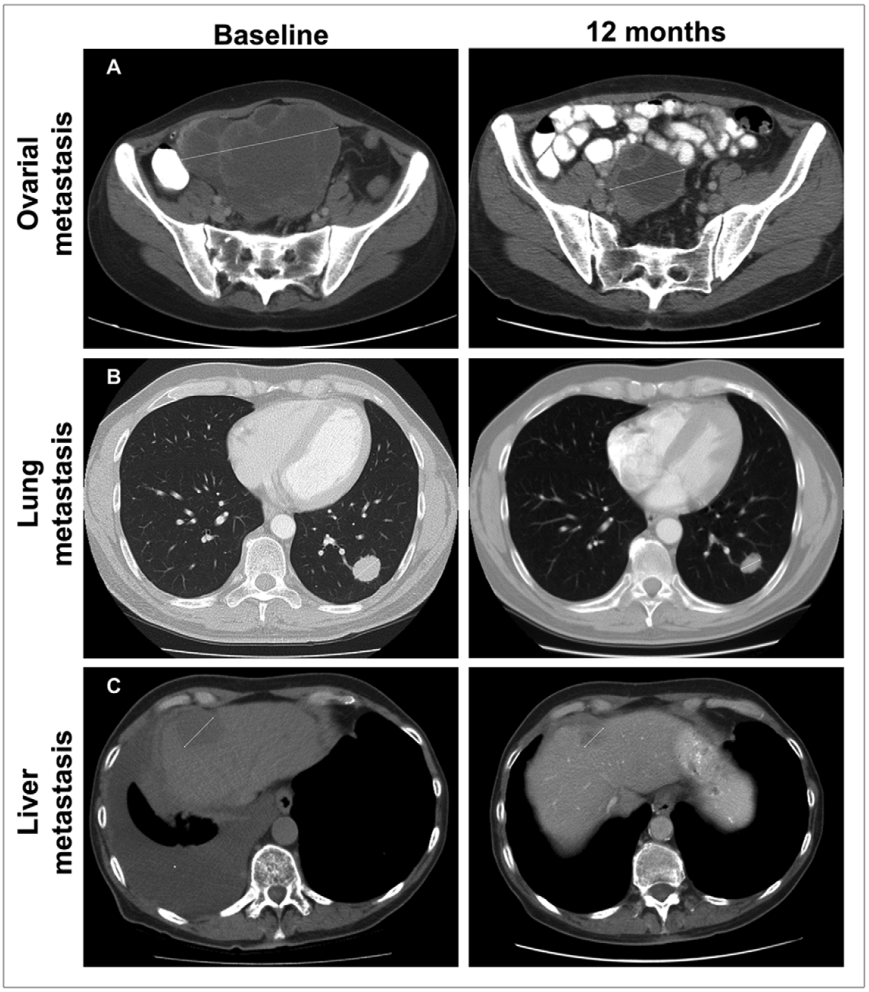

Figure 3. Computed tomography showing partial responses in three different patients at baseline and at 12 months. Panel A shows ovarian metastases in a 43 years old woman. Panel B shows lung metastases in a 50 years old man. Panel C shows liver metastases and pleural effusion (*) in a 70 years old man. Arrows show the largest diameter of the lesions.
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was a subset of patients (14%) showing long-term survival on treatment (>2 years), independent of *BRAF* or *NRAS* mutational status.

Predictive markers for response to antiangiogenic treatment are urgently needed to guide clinical decision making and to target therapy towards well selected subgroups of patients. The present lack of useful predictive biomarkers decrease the likelihood of benefits, cost-effectiveness and therapeutic outcomes [14,30]. We provide evidence that the clinical benefit of bevacizumab monotherapy in metastatic melanoma is almost exclusively limited to those patients who develop early hypertension during treatment. This can in part be explained by the fact that some non-responders did not have sufficient time on bevacizumab to develop hypertension. Still, most of the hypertensive patients (10/14) were recorded with hypertension before the 1st tumor evaluation in week 8 (early hypertension). This phenomenon has been reported for several antiangiogenic drugs [30], and early onset hypertension is one of few markers at the present have been found to predict response to antiangiogenic drugs [30,31].

The causal mechanism behind induced hypertension by antiangiogenic drugs is still elusive. VEGF upregulates nitric oxide [32] and prostacyclin [33], leading to vasodilatation, which is counteracted by bevacizumab. Also, the secondary hypotension following vascular permeability and leakiness caused by VEGF is counteracted by VEGF inhibition [34].

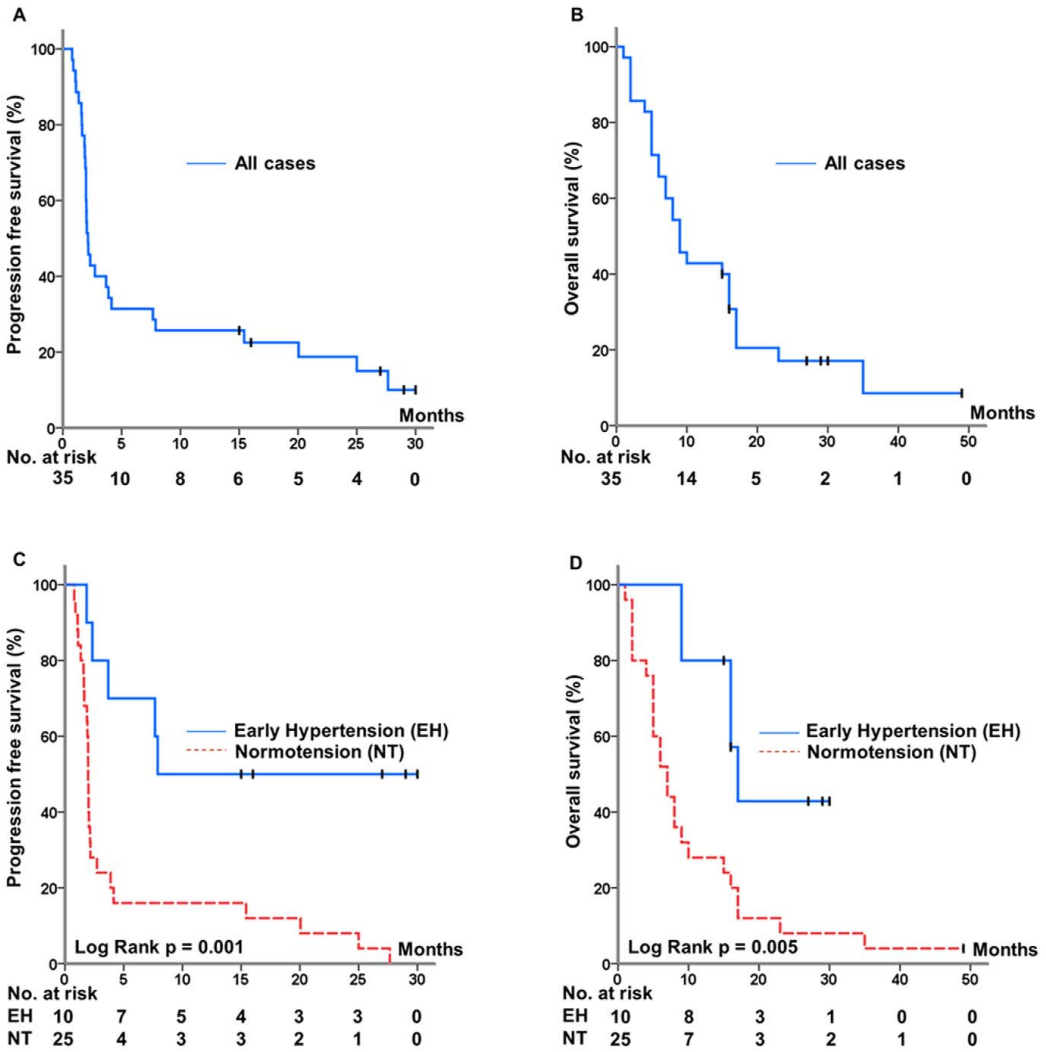

Figure 4. Kaplan Meyer plots of progression free survival (PFS) (A) and overall survival (OS) (B) in 35 metastatic melanoma patients treated with bevacizumab monotherapy. Early hypertension (EH) was significantly associated with PFS (C) and OS (D).
doi:10.1371/journal.pone.0038364.g004

The angiogenic effect of players in the sympathetic nervous system associated with hypertension like norepinephrine (NE), has been reported [35]. Induction of VEGF and HIF-1α expression by NE was completely abolished by the beta blocker propranolol [36], suggesting a possible dual inhibition of VEGF when beta blockers are given together with bevacizumab. Clinical impact of beta blockers in cancer patients has been the focus of several large clinical and epidemiological studies, and these drugs can significantly reduce cancer progression and mortality [37–40], and might represent a promising drug combination with bevacizumab. Interestingly therefore, we found beta blocker use together with bevacizumab to be significantly associated with disease control. Still, this trial was not designed to analyze beta blocker use independently from hypertension, and the data must be interpreted with caution. [41]

In conclusion, bevacizumab monotherapy yielded promising data regarding disease control, progression free survival and overall survival in patients with metastatic melanoma, and the responders were typically characterized by induced hypertension early during therapy.

Supporting Information

Table S1 Drug related toxicities of bevacizumab 10 mg/kg q2w for metastatic melanoma (n = 35). NCI CTCAE v3.0*.
(DOC)

Table S2 Antihypertensive drugs used during treatment in 35 patients.
(DOC)

Flow Diagram S1 CONSORT 2010 Flow Diagram.
(DOC)

Protocol S1 Trial Protocol.
(DOC)

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Author Contributions

Conceived and designed the experiments: OS LA PL. Performed the experiments: CS HE HP JG SG DH OS. Analyzed the data: CS HE HP AM PL LA OS. Contributed reagents/materials/analysis tools: HP AM. Wrote the paper: CS HE HP JG SG DH AM PL LA OS.

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Supplemental Table S1

Table S1. Drug related toxicities of bevacizumab 10mg/kg q2w for metastatic melanoma (n=35). NCI CTCAE v3.0*.

	All grades		Grade 1		Grade 2		Grade 3		Grade 4	
Adverse effect	No.	%	No.	%	No.	%	No.	%	No.	%
Fatigue	5	14	3	9	2	6				
Proteinuria	12	34	9	26	3	9				
Pain	3	9	3	9						
Ileus	1	3					1	3		
Left ventricular systolic dysfunction	1	3					1	3		
Allergy	1	3							1	3
Nausea	2	6	2	6						
Lung embolus	1	3							1	3
Hypertension	14	40	4	11	3	9	7	20		

* National Cancer Institute Common Terminology Criteria for Adverse Events v.3.0.

Supplemental Table S2

Table S2. Antihypertensive drugs used during treatment in 35 patients		
Drug type	No.	%
β1-selective beta blockers	5	14
Non-selective beta blockers	2	6
ACE* inhibitors	2	6
AT II** antagonists	8	23
Calcium antagonists	7	20
Diuretics	7	20
No antihypertensive drugs	22	62
Combinations with beta blockers	5	14
Combinations without beta blockers	6	17
Beta blockers *** without combination	2	6

* Angiotensin converting enzyme. ** Angiotensin II.
*** β1-selective beta blockers

II

PLOS ONE

RESEARCH ARTICLE

Expression of Heat Shock Protein 27 in Melanoma Metastases Is Associated with Overall Response to Bevacizumab Monotherapy: Analyses of Predictive Markers in a Clinical Phase II Study

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Abstract

The aim of this study was to identify potential predictive biomarkers in 35 patients with metastatic melanoma treated with anti-angiogenic bevacizumab monotherapy in a clinical phase II study. The immunohistochemical expression of various angiogenic factors in tissues from primary melanomas and metastases as well as their concentration in blood samples were examined. Strong expression of Heat Shock Protein 27 (HSP27) in metastases correlated significantly with complete or partial response to bevacizumab ($p = 0.044$). Furthermore, clinical benefit, i.e., complete or partial response or stable disease for at least 6 months, was more frequent in patients with strong expression of HSP27 in primary tumors ($p = 0.046$). Tissue expression of vascular endothelial growth factor (VEGF-A), its splicing variant VEGF165b or basic fibroblast growth factor (bFGF) did not correlate with response, and the concentration of HSP27, VEGF-A or bFGF measured in blood samples before treatment did not show predictive value. Further, microvessel density, proliferating microvessel density and presence of glomeruloid microvascular proliferations were assessed in sections of primary tumors and metastases. Microvessel density in primary melanomas was significantly higher in patients with clinical benefit than in non-responders ($p = 0.042$). In conclusion, our findings suggest that strong HSP27 expression in melanoma metastases predicts response to bevacizumab treatment.

Trial Registration

ClinicalTrials.gov NCT00139360

#802630 to LAA, [http://www.forskningsradet.no/en/Home_page/1177315753906]; Norwegian Cancer Society, grant #803149 to LAA, [https://kreftforeningen.no/en/]; and the Helse Vest Research Fund, grant #911873 to LAA, [http://www.helse-vest.no/en/FagOgSamarbeid/Sider/default.aspx]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Cutaneous malignant melanoma (CMM) has shown an increasing incidence worldwide among fair skinned populations during the last decades [1]. Five-year survival in metastatic disease is still poor [2], and it remains to be seen whether new options like targeted treatment [3] or immune checkpoint blockade [4] will improve long term survival rates. Acquired resistance [5] and initial low response rates [6] are still major reasons for poor outcome, and predictive biomarkers in addition to *BRAF* mutation status are needed [3].

Angiogenesis is an important cancer hallmark and treatment target [7, 8]. Preclinical models and clinical investigations have characterized primary melanomas and metastases as highly vascularized [9–11]. Since vascular endothelial growth factor A (VEGF-A) plays a key role in angiogenesis [12, 13] and is expressed in a high proportion of melanomas [9], we conducted a clinical trial with bevacizumab monotherapy, a humanized monoclonal antibody that binds specifically to VEGF-A, in patients with metastatic CMM [14]. As published previously, we observed a clinical benefit rate of 31% [14], indicating that VEGF-A driven angiogenesis is important in a subgroup of these patients. In addition, efficacy of different combinations between bevacizumab and chemotherapy in patients with metastatic melanoma has been reported [15–18]. Bevacizumab is also implemented in the treatment of various other solid tumors but still no predictive biomarkers have been validated [19, 20].

In the present study, we aimed to explore potential predictive biomarkers known to be involved in angiogenesis, and we focused on VEGF-A [12, 13], its splicing variant VEGF165b that binds competitively to VEGFR-2 without phosphorylating pro-angiogenic pathways [21], basic fibroblast growth factor (bFGF) [8] and Heat Shock Protein 27 (HSP27).

HSP27, a small heat shock protein, maintains cell survival under stressful conditions by management of misfolded proteins and prevention of apoptosis [22, 23]. Furthermore, it appears to play an important role in angiogenesis and in tumor cell migration as well as in organization of the cytoskeleton [23, 24]. HSP27 expression is associated with impaired prognosis in melanoma and other tumors as well as resistance to chemotherapy [23, 24]. Previous studies from our group have identified HSP27 as important for tumor dormancy, angiogenesis regulation and tumor progress in cutaneous melanoma and breast cancer [24]. Downregulation of HSP27 in an angiogenic breast cancer cell line resulted in reduced secretion of VEGF-A and bFGF, supporting a HSP27 dependent co-regulation of these factors. Furthermore, the expression of HSP27-related transcription factors phospho-STAT3 and NFkB, involved in regulation of angiogenesis, were significantly reduced in xenograft tumors from HSP27 knockdown cells [24]. Others showed increased secretion of VEGF after endothelial cells were exposed to extracellular HSP27 [25].

Importantly, tissue based angiogenesis markers like microvessel density (MVD), proliferating microvessel density (pMVD), vascular proliferation index (VPI) and presence of glomeruloid microvascular proliferation (GMP) [11, 26–28] were studied. To our knowledge, the present study is the first to indicate that strong tissue expression of HSP27 in melanoma metastases predicts overall response to treatment with bevacizumab monotherapy. However, several other angiogenic markers were not predictive in our study.

Material and Methods

Patients and study design

Thirty-five patients with metastatic melanoma were enrolled in an open-label, single arm phase II study at Haukeland University Hospital, Norway, and were treated with bevacizumab 10 mg/kg q14d until disease progression or intolerable toxicity (ClinicalTrials.gov: NCT00139360).

Study design, eligibility criteria and clinical response data were reported earlier [14]. Fourteen of the thirty-five patients were treated with dacarbazine before they were included in the study. One of these patients received bevacizumab as third line treatment. Twenty-one patients were treated with bevacizumab first line. This approach was based on the two-stage design for phase II clinical trials by Simon [29]. As published previously [14], six of the thirty-five patients had an overall response (OR), *i.e.* complete (CR) or partial response (PR), following treatment with bevacizumab monotherapy. In addition, five more patients had stable disease (SD) for at least six months. Thus, altogether 31% had a clinical benefit (CB), *i.e.* OR and SD. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice, and approved by the Regional Ethics Committee (processing number: 05/329) and the Norwegian Medicines Agency. Informed consent was signed by all patients before enrolment.

Tissue and blood samples

Paraffin embedded tissue of primary tumors was obtainable in 32 of 35 patients. The three cases missing primary tumor tissue blocks represent one primary ocular melanoma treated by radiation therapy, one unknown primary lesion, as well as one undiscoverable tissue block. All primary melanomas were reclassified (LAA, CS) and characterized by the following histopathologic features in hematoxylin and eosin-stained sections: histologic type by Clark, tumor thickness by Breslow, mitotic rate, ulceration, Clark's level of invasion, growth phase, vascular invasion, tumor infiltrating lymphocytes and necrosis [30].

Tissue samples from metastases were available in all patients (35/35). The metastasis diagnosed closest to the date of inclusion (median 5 days) was chosen for further analysis if several metastatic lesions were available. The material includes core needle biopsies (n = 17), mostly taken from lung, liver and abdominal or pelvic organs, as well as excisional biopsies (n = 18) from skin metastases or lymph nodes.

In addition, plasma and serum samples were taken before the first treatment with bevacizumab (median: 2 days); altogether 29 plasma and 28 serum samples were available. EDTA blood was immediately centrifuged at +4°C for 10 minutes at 1600xg, serum samples were processed after clotting for 30 minutes at room temperature. All samples were stored at -20°C and aliquoted when used for analysis.

Immunohistochemistry

Tissue sections of 4–5 μm were stained with primary antibodies for HSP27, VEGF-A, VEGF165b and bFGF. Furthermore, double staining with anti-factor VIII (F-VIII) and Ki67 was performed for angiogenesis assessment. After deparaffinizing in Xylene and different alcohol dilutions and rehydration, heat mediated or enzymatic antigen retrieval was performed. Endogenous peroxidase and alkaline phosphatase were blocked before incubation with the primary antibody followed by incubation with appropriate HRP-EnVision (DAKO, K4011 or K4007). For staining with HSP27 a secondary rabbit anti-goat antibody (Southern Biotech, Cat. no. 6164–01) was used; for double staining, a secondary goat anti-mouse antibody (Southern Biotech, Cat. no. 1031–04) was used. Details are provided in Table 1. For negative controls, primary antibodies were omitted or specific blocking peptides for HSP27, VEGF-A and bFGF were added. Tissues from different cancer types were used as positive controls.

Evaluation of tissue staining results

Evaluation of HSP27, VEGF-A, VEGF165b and bFGF expression. All sections were subjectively screened in a light microscope (Olympus CX31) at magnifications x40 and x100 to

Table 1. Immunohistochemical staining methods.

Antibody	Provider	Epitope retrieval	Dilution	Incubation
HSP27 pAb sc-1048 (goat)	Santa Cruz	MW 6th sense 20 min, pH6	1:100	30 min, RT
VEGF-A pAb sc-152 (rabbit)	Santa Cruz	MW 6th sense 20 min, pH9	1:50	60 min, RT
VEGF165b mAb ab14994 (mouse)	Abcam	MW 6th sense 20 min, pH9	1:100	Overnight, 4°C
FGF-2 pAb sc-1390 (rabbit)	Santa Cruz	Pressure cooker, pH9	1:50	60 min, RT
Von Willebrand Factor pAb A0082 (rabbit)	Dako	Proteinase K, 5 min	1:800	30 min, RT
Von Willebrand Factor pAb A0082 (rabbit) and Ki67 mAb (mouse) M7240	Dako	MW 6th sense 20min, pH6	1:500 and 1:200	60 min, RT

pAb, polyclonal antibody; MW, microwave; RT, room temperature; mAb, monoclonal antibody.

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determine areas containing at least 50% tumor tissue; areas of ulceration or necrosis within the tumor were avoided. Subsequently, staining intensity and the proportion of positive tumor cells and endothelial cells (ECs) within the area of each high power field (HPF, x400) were recorded using a semi-quantitative grading. Staining intensity was defined as absent (0), weak (1), moderate (2) or strong (3). The proportion was rated as "no positive tumor cells" (0), "less than 10% positive tumor cells" (1), "10–50% positive tumor cells" (2) or "more than 50% positive tumor cells" (3). The staining index (SI) is the product of intensity and area (range 0–9) [9]; SI was used to determine cytoplasmic staining of VEGF-A and HSP27 and to record cytoplasmic and nuclear staining of VEGF165b and bFGF. The SI was evaluated by two observers (CS, OS) blinded for response data.

Evaluation of microvessel density and glomeruloid microvascular proliferations

Assessment of MVD and pMVD was done after dual staining (F-VIII/Ki67). F-VIII positive ECs and microvessels were counted in three HPFs to assess MVD in primary tumors and metastases. Sections were first screened at lower magnification (x40 and x100) for selection of MVD hot-spot areas defined by high intensity of F-VIII [11, 26]. Areas of ulceration or necrosis within the tumor were avoided. Microvessels with co-expression of Ki67 in the nucleus and F-VIII in the cytoplasm of ECs were defined as proliferating vessels. The pMVD was recorded in the same three HPFs chosen for assessment of MVD. Ki67 positive nuclei within the lumen or outside ECs were excluded. Both MVD and pMVD were reported as microvessel per mm^2. In addition, VPI was calculated as the ratio between pMVD and MVD (% of 100) [27].

Assessment of GMP was done after staining with F-VIII and hematoxylin counterstain. GMPs were defined as the presence of focal glomerulus-like aggregates of related multilayered F-VIII positive ECs with a minimum number of 15 cells. After screening the tumor at lower magnification, GMPs were registered by x100 magnification in a maximum of four consecutive HPFs within the area of highest density. Presence or absence of GMPs was finally reported [28].

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed according to the manufacturer`s instructions for HSP27 (Enzo Life Science, ADI-EKS-500) and for bFGF (R&D systems, DFB50) in serum samples as well as for

VEGF-A_{165} (R&D systems, DVE00) in plasma and serum samples. Some samples were diluted to fit within the absorbance of the HSP27-standard curve. Samples were run in duplicates. Referring to the user`s manual, the MDD was less than 0.39 ng/ml for HSP27, 3.0 pg/ml for bFGF and 9.0 pg/ml for VEGF. Results below these limits were considered to be zero.

Statistics

Statistical analyses were performed with SPSS, version 22 (SPSS Inc., Chicago, IL). For comparison of two categorical variables, Pearson`s chi-square test was used. Since not all data followed normal distribution, non-parametric tests were used for all analyses. Continuous and ordinal variables were assessed by the Mann-Whitney U Test (MWT) or the Kruskal-Wallis Test (KWT). Intratumoral protein expression, MVD or pMVD in primary tumors and metastases as well as blood concentrations were independent variables when MWT was performed to calculate the association with response or treatment line. Kruskal-Wallis test was used to calculate the association between Breslow thickness and protein expression in primary melanomas (independent variable). The association of various proteins and angiogenic factors between primary tumors and metastases were calculated by the paired Wilcoxon Test (pWT) by the assumption that samples from the primary melanoma and the metastasis are matched pairs. Strength and direction of correlations between two interval scaled continuous or ordinal variables or one of each type were calculated by Spearman`s rho correlation. The level of significance was defined as $p < 0.05$. When continuous variables were categorized, the median was used as cut-point if no other cut-point is defined. The statistical analyses of this study were performed with pre-specified hypotheses for a limited number of intratumoral proteins related to VEGF-A associated angiogenesis. Thus, we did not correct for multiple testing.

Results

Tissue expression of angiogenic factors

HSP27. HSP27 was expressed in the cytoplasm of melanoma cells in all primary tumors and metastases. The median staining index (SI) was 6 for both primary tumors and metastases ($p = 0.74$; paired Wilcoxon Test, (pWT)). Strong HSP27 expression in metastases was significantly associated with overall response (OR) to treatment with bevacizumab monotherapy. Median HSP27 SI for patients with OR was 7.5 compared to a median SI of 6 in patients with stable disease (SD) or progressive disease (PD) ($p = 0.044$; Mann-Whitney U Test (MWT); Fig 1A and 1B; Table 2). Although some patients with HSP27 overexpression did not respond to treatment, no one with low or absent HSP27 expression belonged to the OR-group. There was also a trend for an association between HSP27 expression and OR in primary tumors ($p = 0.097$; MWT), and HSP27 staining in primary melanomas was significantly associated with CB from bevacizumab treatment ($p = 0.046$; MWT, S1 Table). Histologic features of the primary tumors were not associated with HSP27 expression, nor with response. Median HSP27 staining in metastases was significantly stronger within the group treated in first line with bevacizumab compared to second line treatment (median SI 6 vs 3, $p = 0.008$; MWT, S2 Table).

VEGF-A. Cytoplasmic expression of VEGF-A was assessed separately in melanoma cells and ECs in primary tumors and metastases. All primary melanomas, except one, expressed VEGF-A in tumor cells (median SI = 5), and most metastases as well (33 of 35; median SI = 4). However, the difference in VEGF-A SI between primary tumors and metastases was not significant ($p = 0.71$; pWT). VEGF-A expression in metastases was neither significantly correlated to OR (Fig 2A and 2B) nor to CB (Table 3). Treatment outcome did not depend on VEGF-A expression in primary tumors (S3 Table). VEGF-A expression in metastases did not correlate

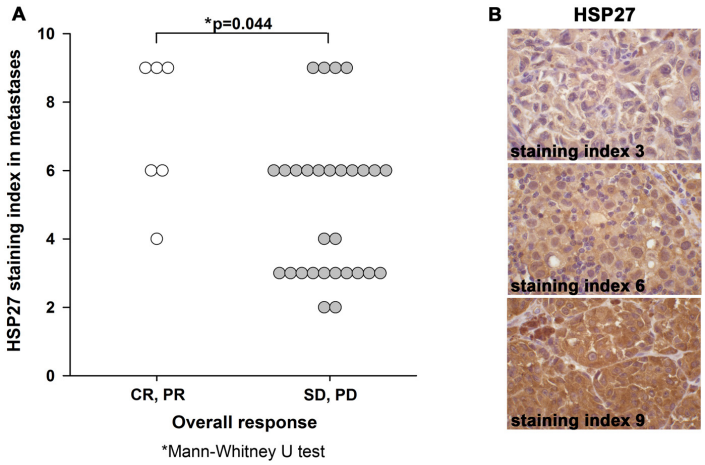

Fig 1. HSP27 expression in metastases predicts overall response to treatment with bevacizumab. (A) HSP27 expression in metastases grouped by treatment response. CR: complete clinical response, PR: partial response, SD: stable disease, PD: progressive disease. (B) Examples for low, moderate and high staining index for HSP27. Original magnification x630.

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significantly with HSP27 expression in metastases. VEGF-A expression in primary tumors was not associated with any of the histologic features. Median VEGF-A expression was significantly stronger in metastases from patients treated with bevacizumab in first line compared to patients treated in second line (median SI 6 vs 3 p = 0.016; MWT, S4 Table).

VEGF-A expression in tumor associated ECs was present in 21/32 (66%) of primary tumors and 15/35 (43%) of metastases. There was no association between EC-VEGF-A expression (primary melanoma or metastases) and response to treatment.

VEGF165b. Both cytoplasmic and nuclear expression of VEGF165b was observed in tumor cells of all primary melanomas and metastases. The median cytoplasmic SI was similar in primary tumors and metastases (p = 0.22; pWT) but the nuclear SI was significantly lower in metastases (p = 0.045; pWT, S1 Fig). The expression level of VEGF165b in primary tumors or metastases was not associated with response to treatment. No associations with histologic features or expression of VEGF-A were observed.

Table 2. Descriptive data for HSP27 expression in metastases.

HSP27 expression in metastases	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean SI[a] +/-SEM[b]	7.2 +/- 0.9	5.0 +/- 0.4	6.3 +/- 0.8	4.9 +/- 0.4
Median SI*	7.5	6	6	5
Minimum SI	4	2	2	2
Maximum SI	9	9	9	9
Number of patients	6	29	11	24

* p = 0.044 (OR), p = 0.15 (CB);
Mann-Whitney U Test.
[a:] Staining index (SI);
[b:] Standard error of mean (SEM).

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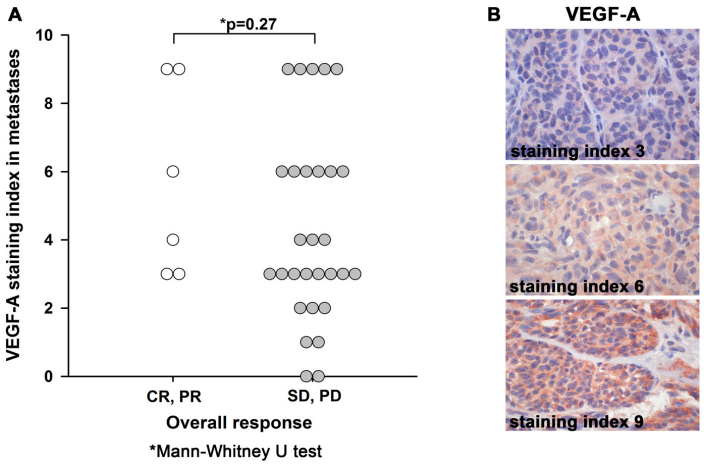

Fig 2. VEGF-A expression in metastases is not associated with overall response to bevacizumab. (A) VEGF-A expression in metastases grouped by treatment response. CR: complete clinical response, PR: partial response, SD: stable disease, PD: progressive disease. (B) Examples for low, moderate and high staining index for VEGF-A. Original magnification x630.

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bFGF. Cytoplasmic bFGF expression was observed in 31/32 primary melanomas and all metastases; nuclear bFGF expression was seen in 30/32 primary melanomas and 28/35 metastases. The median nuclear SI was similar in primary tumors and metastases (p = 0.174; pWT) but the cytoplasmic SI was significantly lower in metastases (p = 0.030; pWT, S2 Fig). There was no association between expression of bFGF in primary melanomas or metastases and response to treatment. Regarding histologic features, primary tumors with a mitotic rate $< 1/mm^2$ showed stronger cytoplasmic and nuclear expression of bFGF (p = 0.03 and p = 0.02; MWT), and strong nuclear expression was associated with the absence of ulceration and lower Breslow thickness (p = 0.014; MWT; p = 0.021; Kruskal-Wallis test (KWT)).

MVD, pMVD, VPI and GMP. Quantification of angiogenesis markers MVD, pMVD and GMP was based on immunohistochemical staining of tumor associated ECs and proliferating ECs. Median MVD was $89/mm^2$ (mean $90/mm^2$) in primary melanomas and $108/mm^2$ (mean $107/mm^2$) in metastases; thus, MVD was significantly higher in metastases than in primary

Table 3. Descriptive data for VEGF-A expression in metastases.

VEGF-A expression in metastases	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean SI[a] +/- SEM[b]	5.7 +/- 1.1	4.3 +/- 0.5	5.4 +/- 0.8	4.2 +/- 0.6
Median SI*	5	3	6	3
Minimum SI	3	0	2	0
Maximum SI	9	9	9	9
Number of patients	6	29	11	24

[a:] Staining index (SI);
[b:] Standard error of mean (SEM)
* p = 0.27 (OR), p = 0.30 (CB);
Mann-Whitney U Test.

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melanomas (p = 0.031; pWT). High MVD in primary tumors predicted CB to treatment with a median MVD of 103/mm^2 in responders vs. 83/mm^2 in non-responders respectively (p = 0.042; MWT), (Fig 3A and 3B; S3 Fig). No significant association was present between MVD in metastases and treatment response. However, high MVD in metastases was correlated to strong VEGF-A expression (p = 0.025; r = 0.39; Spearman).

Median pMVD was significant lower in primary melanomas (3.6/mm^2; mean 5.8/mm^2) than in metastases (8.9/mm^2; mean 9.9/mm^2) (p = 0.01; pWT). There was no association between pMVD and treatment response. Also, median VPI was significantly lower in primary melanomas (5.1; mean 6.1) than in metastases (7.8; mean 10.4) (p = 0.035; pWT). VPI was not associated with treatment response. In primary tumors, strong VEGF-A expression was correlated to higher levels of pMVD and VPI (p = 0.028 and p = 0.027; r = 0.39 for both; Spearman).

GMPs were present in 8/32 (25%) of primary melanomas and 4/34 (12%) of metastases. Presence of GMPs (primary melanomas or metastases) did not predict response to treatment.

Concentrations of angiogenic factors in blood samples

HSP27 concentration in serum samples. All measurements of HSP27 concentration in serum (sHSP27) were above minimal detectable dose (MDD) (median 7.07 ng/ml). sHSP27 concentration was not associated with response to treatment (S11 Table). Notably, there was no association between sHSP27 concentration and tissue expression of HSP27 in metastases, nor with VEGF-A tissue expression. High sHSP27 was correlated with high VEGF-A in plasma and serum as well as with high bFGF in serum (p = 0.004; r = 0.52; p = 0.038; r = 0.39 and p<0.001; r = 0.76; Spearman, respectively). Notably, median sHSP27 was nine fold higher in patients treated with bevacizumab in second line compared with first line (p<0.001; MWT, S12 Table). Further, high sHSP27 was related to high LDH at the time of inclusion (p = 0.039; r = 0.39; Spearman). sHSP27 was not correlated to MVD, pMVD, VPI or GMP in metastases.

VEGF-A concentration in serum and plasma samples. The VEGF-A concentration was above MDD in all serum samples (sVEGF, median 345 pg/ml) and in 23/29 plasma samples (pVEGF, median 52 pg/ml). High VEGF-A concentration in serum was significantly correlated to high VEGF-A concentration in plasma (p<0.001; r = 0.75; Spearman). VEGF concentrations in serum or plasma were not associated with response to treatment (S11 Table). Furthermore, there was no correlation to VEGF-A expression in metastases. Median pVEGF was almost six fold higher in patients treated with bevacizumab in second line compared with first line (p = 0.017; MWT, S12 Table). High sVEGF and pVEGF were associated with high bFGF in serum (p = 0.029; r = 0.41 and p = 0.004; r = 0.52; Spearman) and high baseline LDH (p = 0.018 and p = 0.032; r = 0.4; for both, Spearman). There were no significant correlations between VEGF concentrations in blood samples and tissue based angiogenesis markers (MVD, pMVD, VPI, GMP) in metastases.

bFGF in serum. The bFGF concentration in serum (s-bFGF) was above MDD in 21/28 samples (median 7.8 pg/ml). The concentration of bFGF was not associated with response to treatment (S11 Table). High s-bFGF was correlated to high sHSP27 and high plasma and serum VEGF (see above). bFGF concentration in serum was not associated with expression of any tissue markers in metastases including MVD, pMVD, VPI or GMP status. Notably, median bFGF was significantly higher in serum samples from patients treated in second line (p = 0.002; MWT, S12 Table).

Discussion

Several attempts to identify biomarkers of response to bevacizumab or other anti-angiogenic drugs were recently reviewed by Lambrechts et al. [19]. Validation of potential predictive

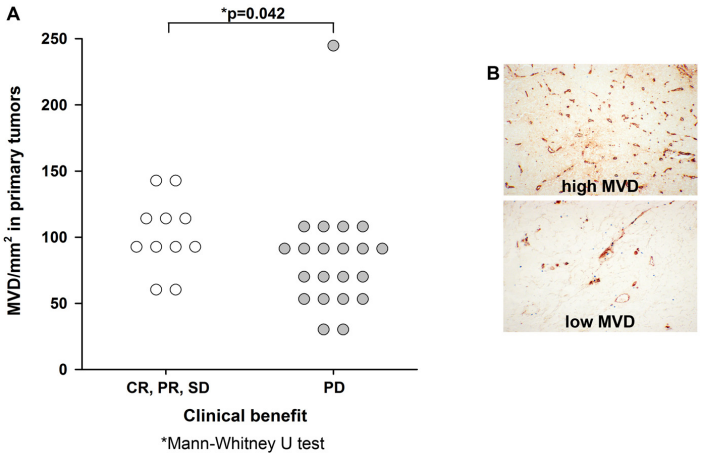

Fig 3. High microvessel density (MVD) in primary tumors predicts clinical benefit to treatment with bevacizumab. (A) MVD in primary melanomas grouped by treatment response. Median MVD in primary melanomas is significantly higher in patients with clinical benefit. CR: complete clinical response, PR: partial response, SD: stable disease, PD: progressive disease (B) Immunohistochemical staining showing high and low MVD. Original magnification x100.

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biomarkers for anti-angiogenic treatment is important to avoid severe side-effects among patients without benefit, and to define treatment indications for optimized and personalized medicine. Since angiogenesis is a complex and dynamic process involving many different growth factors, cytokines and interactions with the stroma [31], there is a challenge to identify individual predictive markers.

Multiple angiogenic factors have been investigated in randomized trials for various cancers [19]. Still, none are currently established as predictive markers. The role of VEGF-A and different VEGF-A SNPs as response predictors has been investigated in clinical trials of bevacizumab. The results are inconsistent, and although associations to improved progression free [32] or overall survival [33] were reported in some trials, none of these markers have been firmly validated [19, 34].

So far, treatment induced hypertension appears to predict clinical benefit in patients treated with anti-angiogenic drugs as reported in metastatic renal cell cancer [35] and metastatic melanoma [14]. In contrast, early hypertension did not predict treatment response in metastatic colorectal cancer when bevacizumab was given in combination with chemotherapy [36].

Here, as a novel finding, strong tissue expression of HSP27 in melanoma metastases was associated with overall response (OR) to bevacizumab monotherapy. Strong expression of HSP27 in metastases characterized all patients with OR, and no patients with weak or absent HSP27 expression were among the responders. Other studies have identified strong HSP27 expression as a prognostic marker for poor outcome in various solid tumors [37–39], and increased intratumoral expression is associated with more advanced tumor stages in ovarian cancer [40]. Furthermore, strong HSP27 expression in vitro and in vivo is associated with resistance to chemotherapy and its downregulation increased sensitivity to treatment [40–43]. HSP27, which is phosphorylated and activated in cells under stressful conditions like hypoxia, is involved in angiogenesis by regulating expression and secretion of VEGF as previously reported [24, 25]. Straume et al. investigated the mechanistic role of HSP27 in angiogenesis in

various models. Knockdown of HSP27 in an angiogenic breast cancer cell line resulted in phenotypic non-angiogenic and microscopic xenograft tumors. Gene expression analysis showed reduced expression of HSP27 as well as of VEGF-A and bFGF [24]. In addition, strong expression of HSP27 was associated with decreased survival in melanoma and breast cancer [24]. Our present finding that strong expression of HSP27 in melanoma metastases is associated with response to anti-VEGF therapy, is in support of HSP27 being a negative prognostic factor and a predictive indicator for treatment response. The reason for this might be that increased HSP27 expression identifies an aggressive melanoma phenotype with an active VEGF dependent angiogenesis that is more sensitive to anti-VEGF treatment.

Although we found a strong correlation between sHSP27 and pVEGF, sVEGF as well as s-bFGF in blood samples taken before treatment with bevacizumab, there was no association between tissue expression of HSP27 in metastases and sHSP27, similar to a previous study on breast cancer [44], and no relationship between blood levels of these factors and response to treatment.

Apart from the significant correlation between high MVD in primary tumors and clinical benefit, we observed no significant associations between VEGF, bFGF (in tissues or blood samples), VEGF165b or tissue based angiogenesis markers and response to treatment. This is in line with the disappointing conclusions from multiple studies of anti-angiogenesis treatment and biomarkers in various cancers [19, 34, 45].

The associations of all assessed intratumoral proteins and angiogenic factors between the primary tumors and metastases were investigated. The median protein expression was similar in primary tumors and metastases except for cytoplasmic bFGF and nuclear VEGF165b. Contrary, the median of MVD, pMVD and VPI was significantly higher in metastases. Based on these findings, a biopsy of the metastatic lesion should be used for investigations of predictive factors.

There was a lack of consistent associations between tissue expression of angiogenesis markers and their levels observed in plasma or serum samples. These findings might be explained by changing tumor-stroma interactions and heterogeneity in primary tumors and metastases, as well as the possibility of clonal evolution and tumor progression in metastatic tumors. Under physiologic angiogenesis, *i.e.* during wound healing, an inverse relation between local and systemic levels of angiogenic factors has been repeatedly observed [46, 47]. These changes might in part explain the challenges in robust quantification of dynamic growth factors, especially ligands like VEGF and bFGF, during cancer progression and treatment.

Since VEGF-A is a key player in angiogenesis and a specific treatment target on this trial, we also looked at associations between VEGF-A and other markers. VEGF-A tissue expression was significantly associated with microvessel proliferation (pMVD and VPI) in primary melanomas, and with overall microvessel density (MVD) in metastases. Taken together, our findings support an important role of VEGF-A in melanoma angiogenesis and progression as previously indicated [28]. Nevertheless, none of these angiogenesis markers were associated with blood levels of VEGF. Our results are in line with findings by Byrne et al. who reported that VEGF expression in primary breast cancer patients was significantly associated with MVD but not with VEGF concentration in platelet-depleted plasma [48].

The intratumoral expression of some proteins as well as their blood concentrations differed significantly between the patients treated with bevacizumab in first line and second line. Median HSP27 expression in tumor cells in metastases was significantly reduced in patients treated with DTIC in first line followed by bevacizumab when compared to the group of patients treated with bevacizumab in first line. Most responders were found in the latter group [14]. The reason why HSP27 expression was significantly reduced in patients treated with DTIC in first line is not clear. Experimental studies have shown that exposure to both single

and repeated doses of DTIC could select for a more aggressive melanoma phenotype through induction of VEGF and interleukin 8 by mechanisms other than HSP27 [49]. Thus, pretreatment with DTIC in first line might have selected a more complex angiogenic phenotype, more resistant to the specific inhibition of VEGF-A with bevacizumab.

Contrary, serum HSP27 was nine fold higher in patients treated with DTIC in first line followed by bevacizumab. Although, the mechanism of HSP27 secretion is not yet completely understood, release from necrotic cells after exposure to chemotherapy could contribute to increased extracellular levels of HSP27 [50]. Similarly, to sHSP27, the concentrations of pVEGF and s-bFGF were significantly higher in patients treated with DTIC in first line. This is again pointing to the inverse relation between local and systemic levels of angiogenic factors, and suggests that the increased systemic levels of these factors might have other sources than the tumor cells [13].

There are limitations to the present study. One is the low number of patients included, with a relative lack of statistical power. Another problem is the limited amount of tissue available for analysis especially from some metastatic lesions. It is well known that tumor angiogenesis is not evenly distributed and tends to occur in hot-spot areas. The sampling bias might therefore reduce sensitivity and might have impact on the angiogenesis quantification by markers like MVD and pMVD, as well as on the representativeness of intratumoral protein expression, especially regarding small samples from metastatic lesions. However, metastatic melanoma tissue was sampled in accordance with contemporary practice. It remains to be investigated if such tissue samples are sufficiently robust for predictive purposes. On the other hand, since no chemotherapy was given concomitantly, the findings are based on the effects of a single drug. Another advantage is the availability of matched primary tumors and metastases as well as serum and plasma samples. In addition, histologic features of the primary tumors as well as clinical features and follow-up data, including response information, were available. Nevertheless, because of the low number of patients, limited amount of tissue, in some cases of core needle biopsies, as well as the lack of a randomized control group, the results must be interpreted carefully.

In conclusion, our data indicate that strong expression of HSP27 protein in melanoma metastases predicts overall response to bevacizumab monotherapy in patients with metastatic melanoma. In contrast, multiple other angiogenesis markers, examined in tissues and blood samples, showed no relationship with treatment response. Further randomized studies are necessary to validate our findings.

Supporting Information

S1 Fig. Nuclear VEGF165b expression in primary melanomas and metastases.
(TIF)

S2 Fig. Nuclear bFGF expression in primary melanomas and metastases.
(TIF)

S3 Fig. Microvessel density in primary melanomas according to treatment response.
(TIF)

S4 Fig. Proliferating microvessels and glomeruloid microvascular proliferation (GMP).
Proliferating vessels* show positive cytoplasmic staining for Factor VIII (red) and positive nuclear staining for Ki67 (blue). Original magnification x400. GMP: Focal glomerulus-like aggregates of closely associated and multilayered Factor VIII positive endothelial cells. Original magnification x630.
(TIF)

S1 Table. Descriptive data for HSP27 expression in primary tumors.
(DOCX)

S2 Table. Descriptive data for HSP27 expression in metastases according to line of treatment.
(DOCX)

S3 Table. Descriptive data for VEGF-A expression in primary tumors.
(DOCX)

S4 Table. Descriptive data for VEGF-A expression in metastases according to line of treatment.
(DOCX)

S5 Table. Descriptive data for microvessel density (MVD) in primary tumors.
(DOCX)

S6 Table. Descriptive data for microvessel density (MVD) in metastases.
(DOCX)

S7 Table. Descriptive data for proliferating microvessel density (pMVD) in primary tumors.
(DOCX)

S8 Table. Descriptive data for proliferating microvessel density (pMVD) in metastases.
(DOCX)

S9 Table. Descriptive data for vascular proliferation index (VPI) in primary tumors.
(DOCX)

S10 Table. Descriptive data for vascular proliferation index (VPI) in metastases.
(DOCX)

S11 Table. Concentrations of HSP27, VEGF-A and bFGF in blood samples according to overall response.
(DOCX)

S12 Table. Concentrations of HSP27, VEGF-A and bFGF in blood samples according to line of treatment.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CS LAA OS. Performed the experiments: CS LAA OS. Analyzed the data: CS LAA OS. Contributed reagents/materials/analysis tools: CS LAA OS. Wrote the paper: CS LAA OS.

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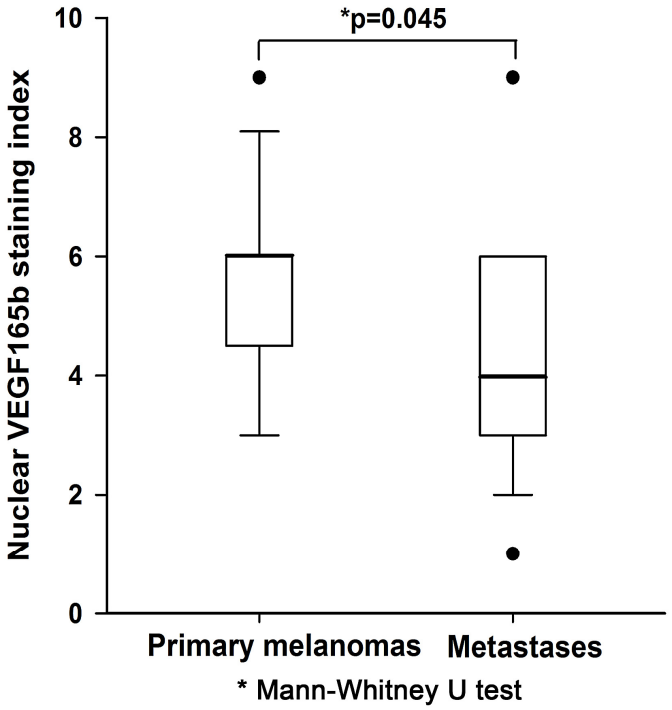
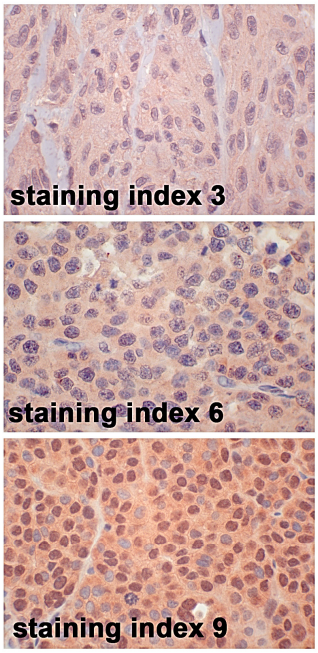
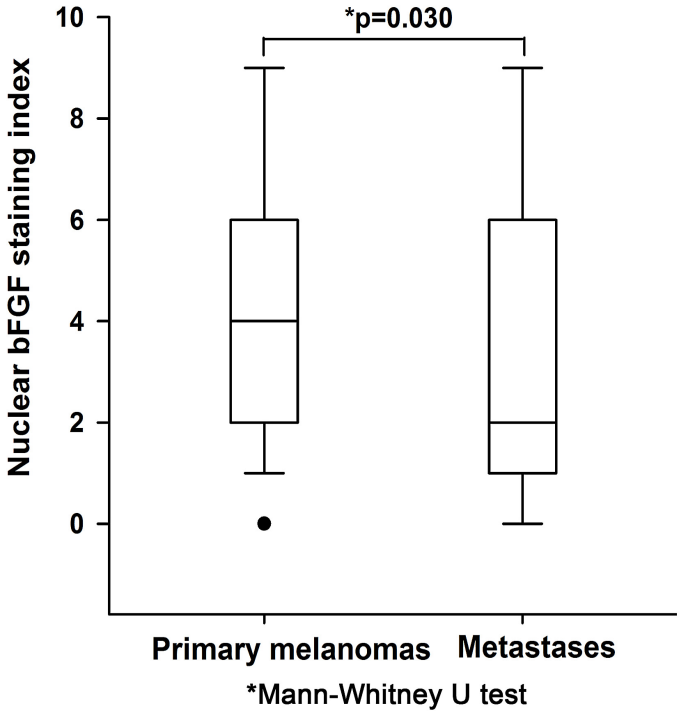
S1A



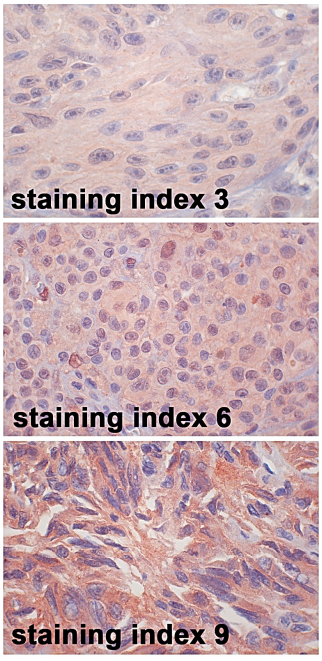
S1B VEGF165b

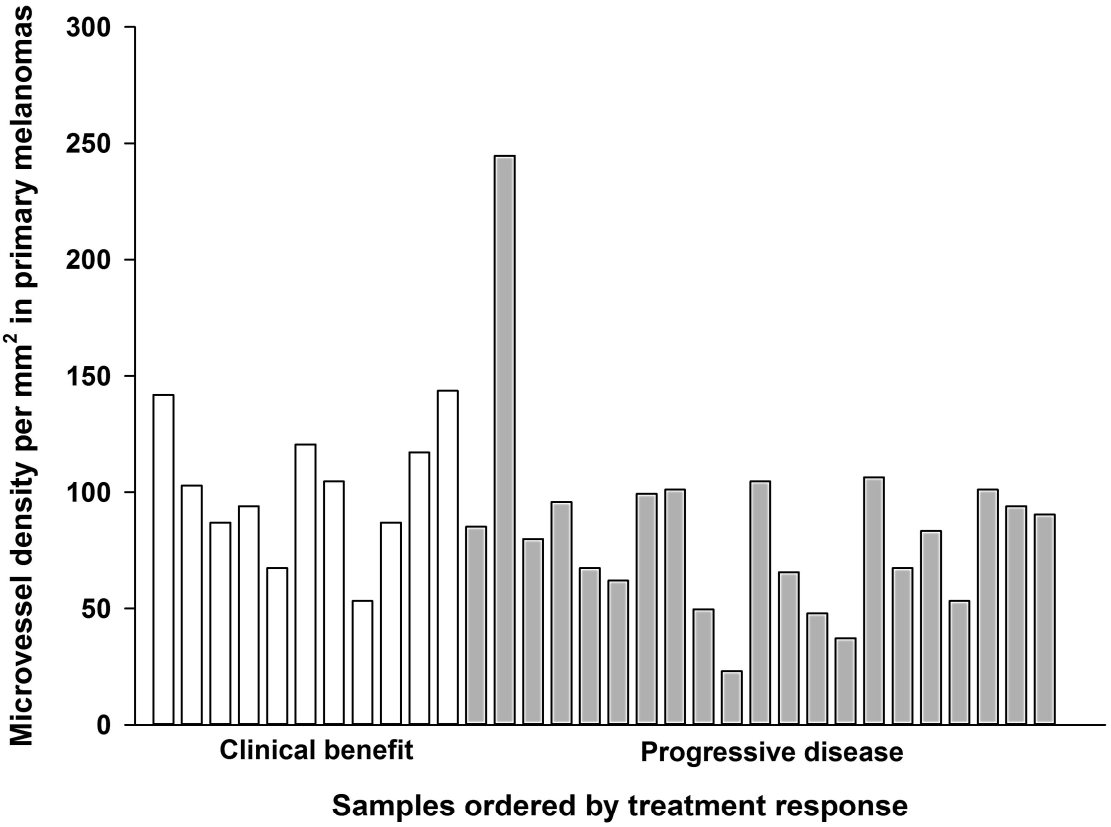


S2A

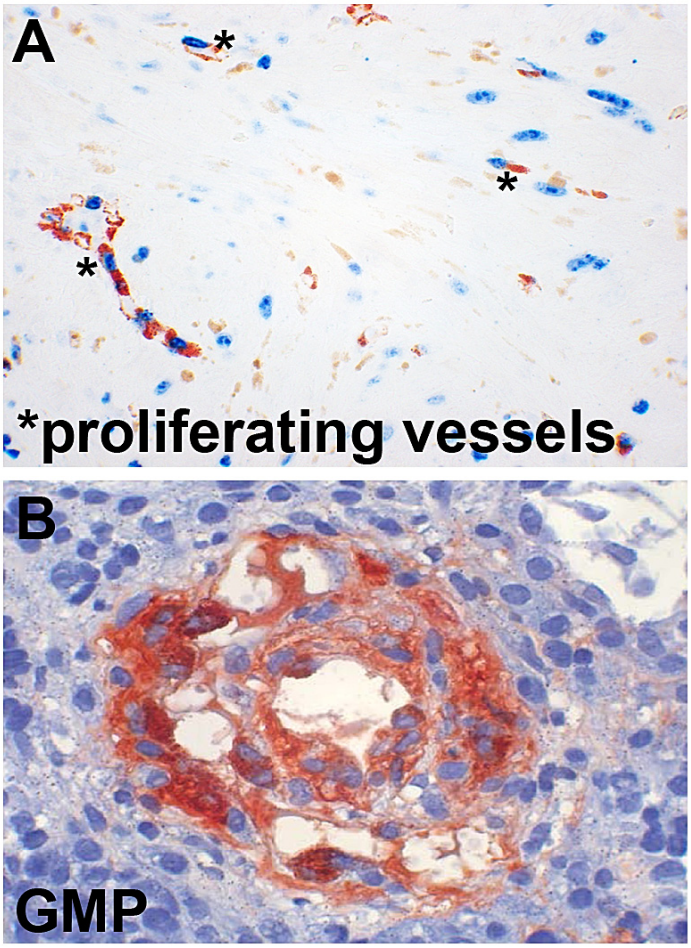

*p=0.030
Nuclear bFGF staining index
10
8
6
4
2
0
Primary melanomas
Metastases
*Mann-Whitney U test

S2B bFGF


staining index 3
staining index 6
staining index 9


Microvessel density per mm2 in primary melanomas
300
250
200
150
100
50
0
Clinical benefit
Progressive disease
Samples ordered by treatment response

S4



S1 Table. Descriptive data for HSP27 expression in primary tumors

HSP27 expression in primary tumors	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean SI[a] +/- SEM[b]	6.5 +/- 0.5	5 +/- 0.3	6.3 +/- 0.5	4.8 +/- 0.3
Median SI*	6	6	6	6
Minimum SI	6	2	3	2
Maximum SI	9	9	9	6
Number of patients	6	26	11	21

a: Staining index (SI); b: Standard error of mean (SEM)

* p=0.097 (OR), p=0.046 (CB); Mann-Whitney U Test.

S2 Table. Descriptive data for HSP27 expression in metastases according to line of treatment

HSP27 expression in metastases	1st line bevacizumab	2nd line bevacizumab
Mean SI[a] +/- SEM[b]	6.2 +/- 0.5	4.1 +/- 0.5
Median SI*	6	3
Minimum SI	3	2
Maximum SI	9	9
Number of patients	21	14

a: Staining index (SI); b: Standard error of mean (SEM)

* p=0.008; Mann-Whitney U Test

S3 Table. Descriptive data for VEGF-A expression in primary tumors

VEGF-A expression in primary tumor	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean SI[a] +/- SEM[b]	5.2 +/- 1.0	5.1 +/- 0.5	5.6 +/- 0.6	4.8 +/- 0.6
Median SI*	5	5	6	4
Minimum SI	3	0	3	0
Maximum SI	9	9	9	9
Number of patients	6	26	11	21

a: Staining index (SI); b: Standard error of mean (SEM)

* p=0.98 (OR), p=0.37 (CB); Mann-Whitney U Test.

S4 Table. Descriptive data for VEGF-A expression in metastases according to line of treatment

VEGF-A expression in metastases	1st line bevacizumab	2nd line bevacizumab
Mean SI[a] +/- SEM[b]	5.4 +/- 0.5	3.2 +/- 0.7
Median SI[*]	6	3
Minimum SI	2	0
Maximum SI	9	9
Number of patients	21	14

a: Staining index (SI); b: Standard error of mean (SEM)

* p=0.016; Mann-Whitney U Test.

S5 Table. Descriptive data for microvessel density (MVD) in primary tumors

MVD in primary tumor	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean MVD +/- SEM[a]	89.2 +/- 10.3	90.1 +/- 8.4	101.7 +/- 8.5	83.8 +/- 9.6
Median MVD[*]	94.9	88.7	102.8	83.3
Minimum MVD	53.2	23.1	53.2	23.1
Maximum MVD	120.6	244.7	143.6	244.7
Number of patients	6	26	11	21

a: Standard error of mean (SEM); * p=0.62 (OR), p=0.042 (CB); Mann-Whitney U Test.

S6 Table. Descriptive data for microvessel density (MVD) in metastases

MVD in metastases	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean MVD +/- SEM[a]	104.6 +/- 18.2	107.1 +/- 10.4	93.4 +/-14.7	112.4 +/- 11.2
Median MVD[*]	97.5	108.2	86.0	115.3
Minimum MVD	60.3	24.8	28.4	24.8
Maximum MVD	154.3	212.8	154.3	212.8
Number of patients	6	27	10	23

a: Standard error of mean (SEM); * p=0.98 (OR), p=0.38 (CB); Mann-Whitney U Test.

S7 Table. Descriptive data for proliferating microvessel density (pMVD) in primary tumors

pMVD in primary tumor	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean pMVD +/- SEM[a]	5.0 +/- 2.0	6.0 +/- 1.4	5.2 +/-1.4	6.2 +/- 1.6
Median pMVD[*]	4.4	3.5	3.6	3.6
Minimum pMVD	0	0	0	0
Maximum pMVD	14.2	30.1	14.2	30.1
Number of patients	6	26	11	21

a: Standard error of mean (SEM); * p=0.98 (OR), p=0.85 (CB); Mann-Whitney U Test.

S8 Table. Descriptive data for proliferating microvessel density (pMVD) in metastases

pMVD in metastases	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean pMVD +/- SEM[a]	15.7 +/- 5.0	8.6 +/- 1.5	9.8 +/- 3.8	9.9 +/- 1.6
Median pMVD[*]	11.53	7.1	5.3	8.9
Minimum pMVD	5.3	0	0	0
Maximum pMVD	35.5	31.9	35.5	31.9
Number of patients	6	27	10	23

a: Standard error of mean (SEM); * p=0.16 (OR), p=0.48 (CB); Mann-Whitney U Test.

S9 Table. Descriptive data for vascular proliferation index (VPI) in primary tumors

VPI in primary tumor	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean VPI +/- SEM[a]	5.7 +/- 1.9	6.2 +/- 1.2	5.3 +/- 1.4	6.5 +/- 1.4
Median VPI[*]	4.6	5.1	4.1	5.3
Minimum VPI	0	0	0	0
Maximum VPI	13.6	21.6	13.6	21.6
Number of patients	6	26	11	21

a: Standard error of mean (SEM); * p=0.98 (OR), p=0.82 (CB); Mann-Whitney U Test.

S10 Table. Descriptive data for vascular proliferation index (VPI) in metastases

VPI in metastases	Overall response (OR)	No OR	Clinical benefit (CB)	No CB
Mean VPI +/- SEM[a]	15.5 +/- 3.8	9.2 +/- 1.8	10.1 +/- 3.2	10.5 +/- 2.0
Median VPI[*]	15.4	7.6	7.8	7.8
Minimum VPI	3.5	0	0	0
Maximum VPI	29.0	46.2	29.0	46.2
Number of patients	6	27	10	23

a: Standard error of mean (SEM); * p=0.11 (OR), p=0.83 (CB); Mann-Whitney U Test.

S11 Table. Concentrations of HSP27, VEGF-A and bFGF in blood samples according to overall response

	HSP27 (serum, ng/ml)		VEGF-A (plasma, pg/ml)		VEGF-A (serum, pg/ml)		bFGF (serum, pg/ml)	
Overall response	**yes**	**no**	**yes**	**no**	**yes**	**no**	**yes**	**no**
Mean	**30.6**	**14.1**	**72.2**	**114.1**	**342.0**	**391.3**	**7.1**	**7.1**
SEM[a]	**23.9**	**3.0**	**56.7**	**29.5**	**121.9**	**66.4**	**2.6**	**0.9**
Median*	**4.8**	**7.4**	**14.0**	**74.0**	**255.5**	**381.0**	**7.2**	**8.2**
Minimum	**0.9**	**1.1**	**0**	**0**	**66.0**	**18.0**	**0**	**0**
Maximum	**149.0**	**49.2**	**353.0**	**615.0**	**901.0**	**1110.0**	**15.4**	**11.8**
Number of samples	**6**	**22**	**6**	**23**	**6**	**22**	**6**	**22**

a: Standard error of mean (SEM); p=0.49 (HSP27), p=0.28 (VEGFA, plasma), p=0.72 (VEGF-A, serum), p=0.89 (bFGF); *Mann-Whitney U test.

S12 Table. Concentrations of HSP27, VEGF-A and bFGF in blood samples according to line of treatment

	HSP27 (serum, ng/ml)		VEGF-A (plasma, pg/ml)		VEGF-A (serum, pg/ml)		bFGF (serum, pg/ml)	
Bevacizumab treatment	**1st line**	**2nd line**	**1st line**	**2nd line**	**1st line**	**2nd line**	**1st line**	**2nd line**
Mean	**7.1**	**29.8**	**53.3**	**169.6**	**267.1**	**511.9**	**4.6**	**10.0**
SEM[a]	**3.1**	**10.5**	**18.3**	**48.5**	**49.2**	**100.3**	**1.2**	**0.7**
Median*	**2.5**	**22.4**	**20**	**118.0**	**189.0**	**415.0**	**6.7**	**9.5**
Minimum	**0.9**	**6.0**	**0**	**0**	**18.0**	**113.0**	**0**	**7.3**
Maximum	**49.2**	**149.0**	**214**	**615.0**	**547.0**	**1110.0**	**10.7**	**15.4**
Number of samples	**15**	**13**	**16**	**13**	**15**	**13**	**15**	**13**

a: Standard error of mean (SEM); p<0.0005 (HSP27), p=0.017 (VEGFA, plasma), p=0.072 (VEGF-A, serum), p=0.002 (bFGF);
*Mann-Whitney U test.