Brain metastases: systematic exploration of
prognostic and predictive factors

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Table of contents

1 Declaration 4

2 Abstract 5

3 Zusammenfassung 6

4 Introduction 7
  4.1 Epidemiology of brain metastases 7
  4.2 Pathobiology of the brain metastases 11
  4.3 Clinical prognostic factors in brain metastases 14
  4.4 Treatment of brain metastases 19

5 Aims of this thesis 24

6 Results 25
  6.1 Brain Metastases free survival differs between breast cancer subtypes. 25
  6.2 Brain-only metastatic breast cancer is a distinct clinical entity characterized by favourable median overall survival time and a high rate of long-term survivors. 33
  6.3 Prognostic significance of Ki67 proliferation index, HIF1 alpha index and microvascular density in patients with non-small cell lung cancer brain metastases 39
  6.4 Preoperative diffusion-weighted imaging of single brain metastases correlates with patient survival times. 50
  6.5 Invasion patterns in brain metastases of solid cancers. 59
  6.6 Characterization of the inflammatory response to solid cancer metastases in the human brain. 69

7 Discussion 83
  7.1 General discussion 83
  7.2 Conclusion & future prospects 86

8 Material & Methods 87
  8.1 Materials 87
  8.2 Methods 87

9 List of figures 93
1 Declaration

The present doctoral thesis was carried out at the Department of Medicine I (Medical University of Vienna) and the Institute of Neurology (Medical University of Vienna) in cooperation with the Center of Brain Research (Medical University of Vienna), the Clinical Institute of Pathology (Medical University of Vienna), 3rd Medical Department (Paracelsus Medical University Hospital Salzburg) Department of Neurology, (Paracelsus Medical University Salzburg), Landes-Nervenklinik Wagner-Jauregg and the Department of Neuropathology (University of Tuebingen). The tissue preparation and the immunohistochemical stainings were kindly supported by Carina Dinhof (Department of Medicine 1, Medical University of Vienna), Irene Leisser (Institute of Neurology, Medical University of Vienna), Elisabeth Dirnberger (Institute of Neurology, Medical University of Vienna), Bettina Jesch (Clinical Institute of Pathology, Medical University of Vienna) and Marinanne Leisser (Center of Brain Research, Medical University of Vienna). Preparation of immunohistochemical staining for integrins was performed in cooperation with Jens Schittenhelm (Department of Neuropathology, University of Tuebingen). Analysis of diffusion-weighted imaging was performed within the diploma thesis of Thomas Spanberger (Department of Medicine I, Medical University of Vienna) under supervision of Daniela Prayer (Department of Radiology, Medical University of Vienna). Evaluation of immunohistochemical preparation, assessment of clinical data and interpretation of results as well as preparation of the original papers was performed by Anna Sophie Berghoff (Department of Medicine I, Medical University of Vienna) under the supervision of Matthias Preusser (Department of Medicine I, Medical University of Vienna) and with support of the mentors Johannes A. Hainfellner (Institute of Neurology) and Peter Birner (Clinical Institute of Pathology, Medical University of Vienna)
2 Abstract

Brain metastases are an increasing clinical challenge in modern oncology and their adequate therapy represent an unmet medical need. While new treatment options significantly influenced the survival prognosis of patients with advanced extracranial solid cancers, the prognosis of patients with brain metastases remains poor due to the limited treatment options. Deeper insight in the involved mechanisms of the brain-metastatic cascade and precise patient selection based on survival prognosis are important for successful conduction of clinical brain metastasis trials.

In the frame of this doctoral thesis we investigated clinical, pathological and radiological characteristics of patients with brain metastases. We identified prognostically relevant radiological, like diffusion weighted imaging, and tissue based, like Ki67 proliferation index and microvascular density, factors in patients with brain metastases. Further, we showed that patients with HER2 positive and triple negative metastatic breast cancer develop brain metastases earlier during their course of disease and that brain-only metastatic breast cancer might be a distinct disease pattern associated with long term survival. We gained a further insight on the invasion patterns of brain metastases into the surrounding brain parenchyma and characterized the inflammatory response to brain metastases.

In conclusion, the results of the doctoral thesis provide novel insight into the pathological and radiological characteristics of brain metastases and their clinical relevance. Our data may provide a basis for future studies including prospective clinical brain metastases specific trials.
3 Zusammenfassung


Zusammenfassend könnten die durch die vorliegende Arbeit erworbenen Kenntnisse neue Aufschlüsse über Prognosefaktoren sowie den klinischen Krankheitsverlauf bei PatientInnen mit Hirnmetastasen generieren. Dieses neue Wissen kann in Zukunft eingesetzt werden um klinische, Hirnmetastasen spezifische Studien zu planen.
4 Introduction
4.1 Epidemiology of brain metastases

Brain metastases (BM) are an increasing challenge in modern oncology. Despite recent advances in the management of cancer patients, BM are still a devastating complication with an enormous impact on overall survival and also on the quality of life of patients.

Historically, BM are described as a rare and late complication in patients with metastatic cancer. However, recent epidemiologic investigations show that the incidence of BM is constantly increasing over the last decade [1-3]. Reasons for this phenomenon are probably manifold and only poorly investigated. Patients with metastatic cancer live longer mainly due to the increased control of the extracranial cancer by improved systemic treatment strategies. Thus, patients who might have died earlier to their extracranial, metastatic disease, nowadays live long enough to experience the symptomatic manifestation of BM [4]. Numerous of the new targeted therapies are postulated to be unable to cross the blood-brain barrier. As a consequence, while the extracranial disease is controlled, the brain is a sanctuary site for the cancer cells [5]. The lack of blood-brain barrier penetration is especially evident in monoclonal antibodies like trastuzumab (anti HER2, breast cancer) or pertuzumab (anti HER2, breast cancer) [6]. Therefore, a significantly increased incidence of BM was described for some cancer disease after the introduction of the new targeted treatment [7]. Although screening for BM is not recommended for most solid cancers, patients with mild neurological symptoms receive high resolution imaging at an earlier stage due to the wide availability of magnet resonance imaging, resulting in the early detection of oligosymptomatic BM [4, 8].

The propensity for BM differs between the solid cancers. Lung cancer is the most frequent cause of BM followed by breast cancer, melanoma, kidney and colorectal cancer [2, 9]. Within the primary cancer, further subgroups defined by the presence of certain molecular characteristics like receptor expression or gene mutation, with increased propensity for BM development exist.
In lung cancer, the histological subtype is the first most important risk factor for development of BM. Patients with small cell lung cancer (SCLC) have the highest risk for the development of BM, as up to 70% of patients develop BM [10]. Due to this highly elevated risk, SCLC is the only subpopulation with a clear indication for prophylactic whole brain radiation in order to prevent BM development [11].

Incidence among patients with metastatic non-small cell lung cancer (NSCLC) is about 20% to 40% and differs according to the further histological and molecular characteristics, which influence survival, treatment modalities as well as prognosis and risk for BM development [12]. Patients with squamous cell carcinoma develop BM less frequently than patients with non-squamous carcinoma [13]. A higher propensity for BM and especially for the development of multiple, miliary BM was described for patients with EGFR mutation [14]. Similar frequencies of FGFR amplification, ALK translocation and ROS1 gene rearrangements were investigated for primary lung cancer and matched BM samples, indicating that these molecular aberrations do not increase the risk of BM [15-17].
Depending to the molecular subtype, which is defined according to overexpression of the steroid receptors (oestrogen receptor (ER), progesterone receptor (PR)), amplification and subsequent overexpression of HER2 and the proliferation rate, BM incidence varies from 5% to 30% among patients with metastatic breast cancer [18]. Luminal A subtype is characterized by the overexpression of ER, facultative overexpression of PR and a low proliferation index and associated with infrequent occurrence of BM [19]. The HER2 subtype is defined by overexpression of the HER2 receptor and BM frequently occur during the course of the disease [5]. The triple negative subtype is defined by the absence of any receptor overexpression and associated with the highest propensity of BM [20].

Melanoma is the third most common cause of BM, with a BM incidence of up to 40% [12, 21, 22]. Risk factors for the development of BM in patients with melanoma are thickness, ulceration and presence of mitosis in the primary melanoma as well as location of the primary in the head and neck region and elevation of lactate dehydrogenase (LDH) [23, 24]. Little is known on molecular characteristics increasing the propensity of BM in melanoma patients. The most common and clinically relevant mutation in melanoma, point mutation of v-Raf murine sarcoma viral oncogene homolog B1 (BRAF V600E), was shown to have the same incidence in BM like in extracranial melanoma, suggesting that the BRAF mutation might not be involved in the brain metastatic cascade [25].

Renal cell carcinoma is the fourth most common cause of BM, as about 17% of patients suffering of renal cell carcinoma develop symptomatic BM [26-28]. Characteristically, BMs are a late event in the clinical course. The first diagnosis of BM can be years after the initial diagnosis of renal cell carcinoma [29]. Interestingly enough, in contrast to the other common causes of BM, the incidence of BM in metastatic renal cell carcinoma patients is postulated to be decreasing probably due to the improved systemic treatment strategies including anti-angiogenic tyrosine kinase inhibitors [27, 30]. So far, no molecular factors increasing the incidence of BM in renal cell carcinoma were identified.
BM are a rather infrequent complication of colorectal cancer with an incidence of 2 to 8%. However the incidence was reported to be rising over the last decade [8]. The improved control of the extracranial disease is supposed to be the main cause for the recent increase, as due to the longer survival, patients who would otherwise have died to the extracranial disease, actually experience the occurrence of the late complication of BM [31]. Risk factors for the development of BM are, left sited (rectal) located primary tumour and long-standing pulmonary metastases [32]. No molecular risk factors for the development of BM in patients with advanced colorectal cancer have been identified yet.

Rarely BMs are caused by other primary, extracranial tumours like ovarian cancer, prostate cancer, bladder cancer, uterus cancer or gastro-oesophageal cancer [8]. Frequency of BM in these entities is estimated to be about 1 to 2%. However, also in these rare entities, the incidence of BM was reported to be rising over the last decade. About 10% of BM patients suffer of BM from unknown primary tumours [33].
4.2 Pathobiology of the brain metastases

The propensity of metastatic spread is a hallmark of malignant cancers [34]. A single cancer cell has to overcome several critical obstacles before the successful establishment of a macrometastasis. The "seed and soil" theory postulates that brain metastatic colonization is not only influenced by certain characteristics of the tumour cell (the seed), but also by the microenvironment of the brain parenchyma (the soil) [35]. In terms of the "seed", specific gene expression patterns of brain metastasizing tumour cells were identified that significantly differ from the gene expression patterns of bone metastases in breast cancer model [36]. The "soil", the brain parenchyma, the pre-existing brain vascular structures as well as astrocytes and microglia influence the establishment of BM [37-39]. The understanding of the involved molecular mechanism is the prerequisite for the identification of possible `druggable´ targets, especially in the prevention of BM.

The disconnection of a single tumour cell or a group of tumour cells from extracranial tumour formations (either primary tumour or extracranial metastasis) in a process called epithelial-to-mesenchymal transformation (EMT) is the first mandatory step in the brain metastatic cascade. The process of EMT is characterized by the loss of E-cadherin, an adhesion molecules, as well as the induction of motility [40].

The dissemination of tumour cells to the brain parenchyma does solely occur via the blood stream, as the brain lacks lymphatic vessels. Therefore, tumour cells have to manage survival and adapt to the changed microenvironment within the blood stream. Several preclinical studies indicate, that tumour cells might aggregate with platelets and leucocytes in order to survive [41].

The passage through the blood brain barrier is the next critical step in the brain metastatic cascade. Here, tumour cells were shown to rest at vascular branching points, presumably due to the reduced shear forces of the blood flow, and use similar mechanisms as leukocytes in the adhesion cascade to cross the blood brain barrier [40]. The involved adhesion molecules like selectins, integrins, chemokines, heparanases and matrix metallopropeases, represent several theoretically targetable molecules [42].
After the successful passage of the blood brain barrier BM tumour cells have to manage intraparenchymal growth. A real time mouse model of BM using a multi-photon laser-scanning microscope through a chronic cranial window revealed capacious information on the behaviour and properties of BM forming tumour cells after the passage of the blood brain barrier [43]. Tumour cells were shown to stay in close contact with the microvessels directly after the passage through the blood brain barrier and induce either neoangiogenesis or growth via vascular co-option alongside the pre-existing brain vascular structures. The angiogenic pattern differed depending on the primary tumour subtype. BM from NSCLC were shown to induce early angiogenesis in the outgrowth from micro- to macrometastases, which can be inhibited by anti-angiogenic treatment. BM from melanoma presented with growth via vascular co-option and anti-angiogenic treatment did not influence the outgrowth of macrometastases [43]. The growth via vascular co-option is characterized by collective tumour cell migration along pre-existing vessel and relies on integrin signalling. In vivo experiments of integrin beta 1 inhibition resulted in prevention of adhesion to the vascular basement membrane und BM outgrowth was attenuated [37, 44]. In line, application of the alpha v integrin inhibitor Intetumumab in a breast cancer BM rat model prevented BM formation and decreased the number of BM [45].

Induction of neo-angiogenesis relies on activation of the vascular endothelial growth factor (VEGF)/hypoxia-inducible factor 1 alpha (HIF 1 alpha) pathway. Hypoxia, induced by fast proliferation an insufficient corresponding neoangiogenesis and can be measured using the HIF 1 alpha index, increases VEGF expression and in consequence endothelia cell proliferation and blood vessel formation [46]. The resulting vascular formations show pathologic features in their morphology as well as in their growth pattern [47]. Vascular structures as well as vascular density were shown to differ between the primary sites, as melanoma BM were shown to have a lower number of microvessels when compared to carcinomas of the lung or breast [48]. Besides the formation of new blood vessels, the VEGF/HIF 1 alpha axis further influences blood vessel permeability and resulting peritumoural oedema as well as treatment response [49, 50]. Further, high HIF 1 alpha index is associated with resistance to radiotherapy and chemotherapy [51].

So far the time points of detachment from the extracranial tumour lesion, passage of the blood-brain barrier and outgrowth from micro- to macrometastasis are uncertain. In
general, BM are considered a late complication in metastatic cancer. However, screening studies revealed a high incidence of asymptomatic BM [52]. This finding suggests that the detachment of tumour cells and the passage of the blood-brain barrier actually occur early during the disease course but the tumour cells do not grow from the micrometastasis to the macrometastasis status for a certain time. In line, a real time mouse model of the establishment of BM showed that brain metastatic cells are able to stay dormant in the perivascular niche and persist as asymptomatic micrometastases over prolonged time periods [43]. Therefore molecular characteristic involved in passage of the blood brain barriers and the outgrowth of macrometastases are potentially ‘druggable’ targets in order to prevent the occurrence of symptomatic BM. Understanding to these key regulators and the time course of brain involvement are precondition to establish clinically applicable BM preventive strategies [53].

Growth and invasion depends further on the interaction with the brain microenvironment including astrocytes, microglia and the immune system. BM tumour cells were shown to recruit astrocytes for their own advantage. Through physical contacts astrocytes were postulated to lead to a chemoprotection of BM tumour cells [39]. The central nervous system is considered an immunoprivileged organ, what might inhibit the inflammatory response to BM. Data on experimental metastases in murine brain suggest that activated microglia, which are the main effector cells of the brain specific immune system, have tumour cytotoxic effects, although some publications have also indicated pro-neoplastic microglia effects in glioma [54, 55]. Microglia cells function involves innate as well as adaptive immune responses [56, 57]. Interaction of the adaptive immune response, namely B- and T-cell, and BM formation has not been investigated yet. However, density of T-cell infiltration was postulated as a prognostic factor in various frequent primary tumours of BM [58-61].
4.3 Clinical prognostic factors in brain metastases

Accurate and realistic survival estimation is mandatory for treatment decision in patients with BM. Although survival prognosis of patients with BM is in general poor, some patients do experience long-term survival despite the presence of BM. On the other hand some patients experience fast deterioration and do not profit of intensified treatment strategies. Therefore survival estimation in patients with BM is an important requirement for adequate selection of patients for clinical trials as prognostic homogeneity is crucial for the clinical utility of studies [62, 63].

Several clinical characteristics were identified from databases of the Radiotherapy and Oncology Group (RTOG) BM studies and compiled in a prognostic score. The first score, recursive partitioning analysis (RPA), includes the parameters age (< 65 years; > 65 years), Karnofsky performance status and presence of extracranial metastases [64]. The second score, the graded prognostic assessment (GPA), calculates a prognostic score out of age (> 60; 50-59: < 50), Karnofsky Performance Score (< 70; 70-80; 90-100), presence of extracranial metastases (present vs. absent) and number of BM (> 3; 2-3; 1) [65]. The GPA was validated in a cohort of almost 2000 patients, further analysis however revealed that the histology of the primary tumour highly impacts the survival prognosis. Most included patients suffered of BM from NSCLC and the subgroup analysis showed that depending on the tumour of origin the clinical prognostic parameters differ.

The third score, the diagnosis specific graded prognostic assessment (DS-GPA), is based on the survival data of almost 4000 patients included in clinical trials of the RTOG. The DS-GPA was conducted in order to acknowledge the individual prognostic factors for each primary tumour histology [66-68].
Table 1: Diagnosis specific prognostic assessment (DS-GPA; adapted from [68]). DS-GPA class is calculated by adding the score for each characteristic.

<table>
<thead>
<tr>
<th>Score</th>
<th>Lung cancer</th>
<th>Breast cancer</th>
<th>Melanoma</th>
<th>Renal cell carcinoma</th>
<th>Gastrointestinal cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age, years</td>
<td>Age, years</td>
<td>Karnofsky performance status</td>
<td>Karnofsky performance status</td>
<td>Karnofsky performance status</td>
</tr>
<tr>
<td></td>
<td>&lt; 60</td>
<td>&lt; 60</td>
<td>&lt; 70</td>
<td>&lt; 70</td>
<td>&lt; 70</td>
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<tr>
<td></td>
<td>&gt; 60</td>
<td>&gt; 60</td>
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</tr>
<tr>
<td></td>
<td>&lt; 50</td>
<td>&lt; 50</td>
<td>50-60</td>
<td>70-80</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>&gt; 3</td>
<td>&gt; 3</td>
<td>70-80</td>
<td>70-80</td>
<td>2-3</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>2-3</td>
<td>Absent</td>
<td>Luminal A</td>
<td>Luminal B</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

In lung cancer (non-small cell and small cell lung cancer) the factors age (< 60; 50-60; < 50), Karnofsky Performance Score (< 70; 70-80; 90-100), presence of extracranial metastases (present vs. absent) and number of BM (> 3; 2-3; 1) showed significant impact on survival prognosis. Estimation of median survival ranges from 3.0 month in the least favourable group compared to 14.8 months in patients in the most favourable prognostic group [68]. So far, the DS-GPA does not separate between small cell and non-small cell lung cancer, although data from real life cohorts postulate a prognostic impact of the underlying histology [69]. Actually molecular subtypes of NSCLC like EGFR mutation, alk translocation or ROS1 gene rearrangements are not included in the survival estimation. However, first data from
real life cohorts postulates, that EGFR mutated NSCLC might have an improved survival prognosis [70, 71].

In breast cancer the impact of the breast cancer subtypes (luminal A and B, HER2 and triple negative) on survival prognosis led to their inclusion in the DS-GPA calculation. Therefore, the DS-GPA for breast cancer is based on the parameter age (≥ 60; < 60), Karnofsky Performance Score (≤ 50; 60; 70-80; 90-100) and breast cancer subtype (triple negative; luminal A; HER2; luminal B). Survival prognosis for patients with BM from breast cancer ranges from 3.4 months in patients of the least favourable group to 25.3 months in patients in the most favourable prognostic group [68]. The presence of extracranial metastases was not included in the DS-GPA for breast cancer, as no survival impact was found for this parameter. However, the DS-GPA for breast cancer is based on a highly selected cohort of patients, eligible for inclusion in a clinical trial. Indeed, approximately only half of the included breast cancer patients suffered of extracranial disease indicating an inclusion bias.

The DS-GPA for melanoma BM and for renal cell carcinoma BM does only include Karnofsky Performance Score (< 70; 70-80; 90-100) and number of BM (> 3; 2-3; 1). Median estimated survival in the least favourable group is 4.4 months for melanoma and 3.3 months in renal cell carcinoma compared to 13.2 months for melanoma and 14.8 months for renal cell carcinoma in the most favourable prognostic group [68]. Again, status of extracranial disease was not included in the prognostic assessment. However, for the instance of melanoma evidence exists that BM as first metastatic site might represent a distinct clinical entity with reduced survival prognosis [72].

In gastrointestinal cancer BM only Karnofsky Performance Score (<70; 70; 80; 90; 100) was identified as impacting the survival prognosis. The median survival in the least favourable prognostic group is 3.1 months and 13.5 months in the most favourable group [68]. However, number of BM and presence of extracranial metastases were identified as prognostic marker in real life cohort, indicating the importance of further investigation of prognostic factors [73]. In addition, the definition of gastrointestinal cancers is rather inaccurate, as the clinical course and management of the various cancer types originating in the gastrointestinal tract (e.g. colorectal cancer, gastric cancer, oesophageal cancer, pancreatic cancer etc.) are distinct. So far little is known on specific prognostic parameters in patients with BM of
colorectal, gastric or oesophageal cancer. In these cancer types several molecular and histological subtypes correlate with different survival and need to be investigated also upon their impact on BM prognosis.

Although the DS-GPA is frequently used for the definition of inclusion criteria in clinical trials and is based on a huge database, it has to be taken into account that validation in real life cohorts of adequate size is urgently warranted. The included patients were all highly selected for the inclusion in clinical trials, suggesting that the clinical prognostic factors might differ in real life. For example, approximately only half of the patients suffered of extracranial disease and over two thirds of the patients suffered of less than three BM [68]. Validation and extension of clinical prognostic factors in real life cohorts is currently on-going.

Table 2: Estimated survival according to DS-GPA class (adapted from [68])

<table>
<thead>
<tr>
<th></th>
<th>DS-GPA Class I</th>
<th>DS-GPA Class II</th>
<th>DS-GPA Class III</th>
<th>DS-GPA Class IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lung cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score</td>
<td>0-1.0</td>
<td>1.5-2.0</td>
<td>2.5-3.0</td>
<td>3.5-4.0</td>
</tr>
<tr>
<td>Median OS (months)</td>
<td>3.0</td>
<td>5.5</td>
<td>9.4</td>
<td>14.9</td>
</tr>
<tr>
<td><strong>Breast Cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score</td>
<td>0-1.0</td>
<td>1.5-2.0</td>
<td>2.5-3.0</td>
<td>3.5-4.0</td>
</tr>
<tr>
<td>Median OS (months)</td>
<td>3.4</td>
<td>7.7</td>
<td>15.1</td>
<td>25.3</td>
</tr>
<tr>
<td><strong>Melanoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score</td>
<td>0-1.0</td>
<td>1.5-2.0</td>
<td>2.5-3.0</td>
<td>3.5-4.0</td>
</tr>
<tr>
<td>Median OS (months)</td>
<td>3.4</td>
<td>4.7</td>
<td>8.8</td>
<td>13.2</td>
</tr>
<tr>
<td><strong>Renal cell carcinoma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score</td>
<td>0-1.0</td>
<td>1.5-2.0</td>
<td>2.5-3.0</td>
<td>2.5-4.0</td>
</tr>
<tr>
<td>Median OS (months)</td>
<td>3.3</td>
<td>7.3</td>
<td>11.3</td>
<td>14.8</td>
</tr>
<tr>
<td><strong>Gastrointestinal cancer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score</td>
<td>0-1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Median OS (months)</td>
<td>3.1</td>
<td>4.4</td>
<td>6.9</td>
<td>13.5</td>
</tr>
</tbody>
</table>

At present estimation of survival prognosis is only based on clinical parameters. However, radiological and tissue based findings might actually add valuable information. Radiological findings represent growth and invasion of a brain metastasis and can be easily assessed, suggesting that further investigation might reveal further includable parameters.
The size of the peritumoural oedema was identified as a prognostic factor in a well-defined and homogenous cohort of patients with single brain metastasis and neurosurgical resection as first line treatment [74]. Oedema of less than one centimetre correlated with a median survival of 5 months, compared to 19 months with a large oedema of over one centimetre and corsage of the midline. Size of oedema showed correlation with microvascular density, as large peritumoural oedema was associated with high microvascular density [74]. Therefore, the peritumoural oedema might be a surrogate marker representing the angiogenic and growth pattern.

Consideration of radiological finding might add additional value in terms of treatment directions and survival estimation.
4.4 Treatment of brain metastases

The treatment of BM mainly relies on local strategies including surgery, radiosurgery and whole brain radiation, while systemic therapies depending on the primary tumour have a smaller impact in the clinical management of BM patients so far [75].

First line treatment decisions in patients with newly diagnosed BM mainly rely on the primary tumour, number of BM, size of BM and Karnofsky performance score. Patients with only one singular BM are candidates for either surgery or radiosurgery, depending on the size of the BM. BM with a size over 3 cm have an increased risk of radionecrosis and should therefore be treated with surgery [76]. Patients with unknown primary tumour are candidates for surgery in order to obtain histology for further treatment strategies [76]. Patients with one to three BM are candidates for surgery in combination with radiosurgery or radiosurgery alone depending on size and location of BM. A combined treatment might be suitable for patients with BM over 3 cm or in well accessible areas for neurosurgery [77].

The value of whole brain radiation therapy after the local treatment of one to three BM is a matter of discussion. Only one prospective phase III study addressed the matter and could not identify a benefit in terms of overall survival [78]. However, brain progression free survival was significantly increased in patients receiving whole brain radiation therapy.

The first line treatment approach in patients with over three BM is whole brain radiation therapy [53]. However, the value of whole brain radiation therapy is matter of intensive discussion, as it is associated with impairing side and long-term effects like decline in neurocognitive function, memory loss, leukoencephalopathy and resulting decline in quality of life [79-81]. Especially in patients with favourable survival prognosis even upon the diagnosis of BM the value of first line whole brain radiation therapy is questioned [63]. A first line systemic treatment approach using a systemic compound with effect on BM might be more favourable as patients may actually experience the long-term side effects of whole brain radiotherapy [62]. Hippocampal sparing whole brain radiation is a further option in order to avoid the side effect of memory loss. The method of hippocampal sparing whole brain radiation therapy was proven to be safe and valid, as BM are hardly ever observed in the
hippocampal area. However, the hippocampal sparing method is technically difficult and time consuming, making it only feasible in selected cases [82-84].

Systemic cytotoxic therapies are postulated to have only a minor impact in the treatment strategy of BM patients due to the difficulty of penetrating the blood brain barrier [85]. However, the blood brain barrier might be at least leaky in BM or even in parts disrupted, so that systemic therapies might have at least partial effect [86-88]. Irrespective, patients with BM were systematically excluded from clinical trials in the last decade and resulting little is known about the value of some systemic therapies in BM patients. Recently, the urgent need for BM specific trials was pointed out by scientific clinical committees like the EORTC (European Organization for Research and Treatment of Cancer) Brain Metastasis Platform [62].

Although, lung cancer is the most common cause of BM, there is a lack of specific lung cancer BM trials. As in theory, EGFR tyrosine kinase inhibitors are able to cross the blood brain barrier due to their small molecule size. Some small studies investigated the value of EGFR tyrosine kinase inhibitors based therapies [89-92]. Treatment with EGFR tyrosine kinase inhibitors was proven to be safe, also sequenced after application of whole brain radiotherapy [14, 89, 93-96]. In the extracranial disease a greater impact as observed for patients harboring an EGFR mutation, with response rates up to 80%, indicating a therapeutic value of EGFR tyrosine kinase inhibitors also in patients with BM [89]. A phase II study on pemetrexed and cisplatin as first line treatment approach in asymptomatic NSCLC BM patients revealed effectiveness with an cerebral response rate of 41.9% [97]. A first line systemic treatment approach using pemetrexed-cisplatin based therapy might be feasible in selected NSCLC BM patients.

In view of the rising incidence of BM in HER2 positive breast cancer patients, especially since the introduction of trastuzumab, systemic treatment of this subpopulation with very favourable survival prognosis even upon the diagnosis of BM has been in the center of research. Trastuzumab, although in theory unable to cross the blood brain barrier, showed effect and impact on overall survival in patients with BM from HER2 positive breast cancer [98, 99]. This finding underscores, that the blood brain barrier is disrupted in BM and therapies with high molecular size have at least partly effect [87, 100]. In terms of HER2 targeted therapies, some trials were
conducted to investigate the value of the HER2 tyrosine kinase inhibitor lapatinib, which in theory could better cross the blood brain barrier due to its small molecular size. The LANDSCAPE trial investigated the combination of lapatinib and the orally available cytotoxic drug capecitabine in newly diagnosed BM patients suffering of HER2 positive breast cancer [101]. The combination therapy showed response rates of 65% in the selective cohort of low to asymptomatic patients with multiple BM and the whole brain radiation therapy could be postponed for 8 months [101]. This is of clinical significance as patients with BM from HER2 positive breast cancer have a median survival of 7 to 24 months upon the diagnosis of BM and are therefore at very high risk of experiencing the late side effects of an up front whole brain radiation therapy [68, 79].

Several new emerging drugs changed the treatment of metastatic melanoma dramatically through the last decade. The identification of BRAF V600E mutations and the possibly to selectively inhibit the mutated BRAF by the BRAF inhibitors vemurafenib or dabrafenib, facilitate the first targeted therapy in metastatic melanoma [21, 25, 102, 103]. BRAF inhibition was shown to be also feasible in patients with BM from metastatic melanoma. Response rate of up to 40% were observed in BM for single agent dabrafenib, suggesting that systemic therapy is valid treatment option in patients with BM from melanoma, especially under consideration of the radio resistance of melanoma [104]. However, the duration of response is limited and patients experience progress in median after 4 months [104]. Recently, the addition of a MEK inhibitor to the BRAF inhibitor was shown to reduce the BRAF inhibitor induced side effects (especially the occurrence of secondary basal carcinoma) and prolong the progression free survival [105]. However, no data on the combination of BRAF and MEK inhibitor for patients with melanoma BM have been generated so far in clinical trials.

An emerging treatment option in patients with metastatic melanoma is the application of immune checkpoint inhibitors [21, 106, 107]. Immune checkpoint inhibitors can be applied irrespective of the BRAF mutation status [108, 109]. Immune checkpoint inhibitors, like the CTL4 antibody ipilimumab or the PD1 antibody nivolumab, boost the host immune response by inhibiting the inhibitors signals of the T-cell response, induced by the immunosuppressive properties of the tumour. Therefore, the “real” immune response is unmasked by inhibiting the tumour immune inhibiting properties.
[110, 111]. However, no immediate tumour shrinkage is evident after application of immune checkpoint inhibitors, as the immune response and the therapeutic effect need several months to reveal the full potential. Therefore, patients with fast progressing highly symptomatic disease and short survival prognosis are unlikely to benefit of an immune checkpoint based therapy [108]. The response to immune checkpoint inhibitors differs from the response to cytotoxic chemotherapy by the observation that about 20% of patients experience a long-term response up to years after the induction of the immune checkpoint inhibitor [112]. The right selection of patients, furthermore the decision whether therapeutic benefit is expected from the immune checkpoint inhibitor based therapy or a fast acting, tumour shrinking treatment approach is needed, is currently the matter of intense research. In this context, level of LDH, Karnofsky performance score and the presence of BM were postulated as negative predictors for the benefit of an immune checkpoint inhibitor based therapy [108, 113, 114]. Although, patients with melanoma BM have in general a very impaired survival prognosis, some patients have a slow progressing, asymptomatic disease and might profit from an immune checkpoint inhibitor based treatment strategy. A phase II trial showed a response rate for up to 20% for ipilimumab monotherapy in patients with not otherwise treated melanoma BM [115]. It has to be taken into account that the majority of patients was asymptomatic and did not need any steroid therapy or was symptom free and on stable steroid dosage. An Italian population based experience on the extended access program of ipilimumab supported these findings, suggesting that immune checkpoint inhibitors might be a valid treatment option in selected melanoma BM patients [116]. In terms of predictive markers for the response to immune checkpoint inhibitors several studies are ongoing. Currently, the immune score is based on the density of CD3 and CD8 positive tumour infiltrating lymphocytes is investigation. Further, PD-L1 expression on tumour cells is considered as predictive marker for response to immune checkpoint inhibitors [60, 117]. However, none of these have yet been investigated in BM.

No BM specific trials have been conducted for the more infrequent causes of BM like colorectal or kidney cancer.

Besides the tumour specific treatment, most patients need additional supportive, symptom controlling therapies including steroids and antiepileptic drugs. According to the current standard of research, no prophylactic antiepileptic treatment should be
applied, especially regarding possible side effects. Only patients who experienced at least some seizure should be treated for at least six months and than re-evaluated [118]. The main symptoms of BM are caused by space occupation due to the tumour surrounding oedema. Steroids are frequently applied for reduction of the peritumoural oedema. However, steroid cause several severe side effects like iatrogenic Cushing syndrome, myopathy, diabetes and psychological disorders. Due to this unfavourable side effect of steroids, other systemic treatment approaches of oedema reduction should be explored. Favourable results with the application of VEGF antibody bevacizumab in patients lacking further local treatment options and suffering of large peritumoural oedema were reported in case reports [119].

A promising and emphasizing new approach in BM research is the prevention of BM [62]. Targeted therapies might be able to act on circulating tumour cells preventing the crucial steps of the brain metastatic cascade rather than to be active in already established BM, that are in least in part protected by the blood brain barrier [40]. This theory is supported by several preclinical studies. NSCLC BM were shown to highly depend on neoangiogenesis and application of the VEGF antibody bevacizumab before the establishment of BM efficiently inhibited the outgrowth of micrometastases to macrometastases [43]. Interestingly, bevacizumab based treatment was shown to reduce the incidence of BM as first site of recurrence in patients with advanced NSCLC [62, 120]. Application of the integrin inhibitor intetumumab was shown to reduce number and size of BM in a breast cancer BM mouse model [45]. However, efficacy of intetumumab in various frequent primaries of BM has not been investigated yet. Clinical investigations have populated a BM prophylactic value for various substances. Treatment with EGFR tyrosine kinase inhibitors was shown to reduce the incidence of BM in patients with EGFR mutated NSCLC and treatment with the VEGF receptor tyrosine kinase inhibitor sorafenib was found to reduce the incidence of BM in patients with advanced renal cell carcinoma [27, 121]. Only one randomized trial investigated the prophylactic value of target therapies. A BM prophylactic value was postulated for lapatinib as compared to trastuzumab as lapatinib is able to cross the blood brain barrier. However, no significant difference in the occurrence of BM as first site of recurrence was observed [52]. Future studies might focus on the approach to prevent BM.
5 Aims of this thesis

a. Which clinical characteristics at the time of diagnosis of BM influence survival?

b. Do established prognostic indices, which have largely been developed in patients enrolled in clinical trials, have value in a real-life population of patients with BM?

c. Does the expression of HIF 1 alpha, Ki67 proliferation index and microvascular density as well as angiogenic pattern in BM specimens correlate with treatment response and survival?

d. Do radiological findings correlate with tissue based characteristics and survival in patients with BM?

e. Do the invasion patterns of BM in the surrounding brain parenchyma correlate with primary tumour type?

f. Characterization of the adaptive and innate inflammatory response in and around BM. Does the inflammatory pattern differ between primary tumour types?
6 Results
6.1 Brain Metastases free survival differs between breast cancer subtypes.

Prologue
BM are a frequent complication in breast cancer [6, 122]. In order to guide the development of preventive trials, a better knowledge on the clinical course of patients developing BM is needed. The breast cancer subtypes have a known impact on the propensity for BM as well as on the prognosis upon the diagnosis of BM [19]. However, the impact of the breast cancer subtype on time to development of BM has not been investigated. In general, BM are regarded a late complication during the metastatic disease. However some patients present with early development of symptomatic BM. In the paper “Brain Metastases free survival differs between breast cancer subtypes” we investigated the time from diagnosis of the metastatic breast cancer to the development of BM in order to identify patients developing particularly early or late BM during the clinical course. Patients with triple negative breast cancer had a significantly shorter time till development of BM compared to patients with HER2 positive or luminal breast cancer [123].
Brain metastases free survival differs between breast cancer subtypes

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BACKGROUND: Brain metastases (BM) are frequently diagnosed in patients with HER-2-positive metastatic breast cancer; in addition, an increasing incidence was reported for triple-negative tumours. We aimed to compare brain metastases free survival (BMFS) of breast cancer subtypes in patients treated between 1996 until 2010.

METHODS: Brain metastases free survival was measured as the interval from diagnosis of extracranial breast cancer metastases until diagnosis of BM. HER-2 status was analysed by immunohistochemistry and reanalysed by fluorescent in situ hybridisation if a score of 2+ was gained. Oestrogen-receptor (ER) and progesterone-receptor (PgR) status was analysed by immunohistochemistry. Brain metastases free survival curves were estimated with the Kaplan–Meier method and compared with the log-rank test.

RESULTS: Data of 213 patients (46 luminal/124 HER-2/43 triple-negative subtype) with BM from breast cancer were available for the analysis. Brain metastases free survival differed significantly between breast cancer subtypes. Median BMFS in triple-negative tumours was 14 months (95% CI: 11.34–16.66) compared with 18 months (95% CI: 14.46–21.54) in HER-2-positive tumours (P = 0.001) and 34 months (95% CI: 23.71–44.29) in luminal tumours (P = 0.001), respectively. In HER-2-positive patients, co-positivity for ER and HER-2 prolonged BMFS (26 vs 15 months; P = 0.033); in luminal tumours, co-expression of ER and PgR was not significantly associated with BMFS. Brain metastases free survival in patients with lung metastases was significantly shorter (17 vs 21 months; P = 0.014).

CONCLUSION: Brain metastases free survival in triple-negative breast cancer, as well as in HER-2-positive/ER-negative, is significantly shorter compared with HER-2/ER co-positive or luminal tumours, mirroring the aggressiveness of these breast cancer subtypes.


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Keywords: advanced breast cancer; brain metastases; carcinomatous meningitis; human epidermal growth factor receptor 2 (HER-2)-positive breast cancer; triple-negative disease

In the last decade, overall survival of metastatic breast cancer patients has improved due to advances in systemic treatment (Lin and Winer, 2007; Kiely et al., 2011). Despite this success, the rising incidence of brain metastases (BM) as late complication became a major clinical problem (Weil et al., 2005). Increasing incidence was reported for triple-negative tumours. We aimed to compare brain metastases free survival (BMFS) of breast cancer subtypes in patients treated between 1996 until 2010.

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Brain metastases decrease quality of life and increase morbidity and mortality. Currently, survival of patients with BM ranges from 2 to 16 months (Weil et al., 2005).

Prognosis and clinical behaviour of breast cancer differs between subtypes (Perou et al., 2000; Sorlie et al., 2001; Kennecke et al., 2010). Patients with triple-negative tumours, defined by the absence of oestrogen-receptor (ER), progesterone-receptor (PgR) and Her-2-receptor expression, are at higher risk of being diagnosed with BM compared with the luminal or HER-2-positive subtypes (Heitz et al., 2009). HER-2-positive patients, on the other hand, have a higher incidence of BM than patients with HER-2-negative breast cancer (Sanna et al., 2007). Especially since the introduction of trastuzumab, a growing incidence of symptomatic BM was reported. As trastuzumab cannot penetrate through the blood–brain barrier due to its molecular weight, a tumour cell sanctuary is created. Furthermore, trastuzumab improves systemic disease control, which leads to a 'unmasking' of BM in patients who would otherwise have died from progression of systemic disease.

Apart from triple-negative or HER-2-positive disease, established risk factors for the development of BM are young age at first diagnosis, presence of lung metastases and short disease-free interval (Weil et al., 2005).

Treatment of BM remains challenging and consists of surgery, whole-brain irradiation, radiosurgery and systemic therapy (Weil et al., 2005). Surgery or radiosurgery is an option for patients with
one to three metastases. Whole-brain irradiation, while offering activity also in patients with >3 metastases, causes long-term sides effects such as memory loss and cognitive impairment. Effect of systemic therapy is limited by the blood–brain barrier. Thus, limited therapy options for symptomatic BM substantiates the urgent need for better understanding of risk factors and possibilities of prevention.

Importantly, treatment with lapatinib resulted in a decreased incidence of BM in HER-2-positive disease (Geyer et al, 2006). Other preventive measures such as prophylactic cranial radiotherapy, while well established in small-cell-lung cancer, is not routinely used in breast cancer, as no survival benefit was observed so far (Saip et al, 2009). Even screening for BM is not a part of routine follow-up, as no evidence for a benefit from early detection exists (Niwinska et al, 2007). This, however, might be rather due to the lack of appropriate selection criteria for a potential screening cohort. Therefore, a more precise definition of patients and breast cancer subtypes at high risk for early development of BM is needed (Heitz et al, 2009).

The objective of this study therefore was to determine clinical and histopathological risk factors associated with early development of BM. This might identify a high-risk population deriving the largest benefit from screening and prevention.

PATIENTS AND METHODS

Two Austrian centres contributed information relating to demographics, case history and survival. Data were processed at the Medical University of Vienna, Austria. This retrospective analysis was conducted in accordance with the ethical regulations of the Medical University of Vienna and approval by the local ethics committee was obtained.

Patients

Patients treated for symptomatic BM from breast cancer between 1996 and 2010 were identified from a breast cancer database. No routine screening for BM was conducted, and none of the patients available for this analysis participated in trials of BM screening or prevention. Data were analysed as of August 2011.

Hormone-receptor and HER-2 status

Oestrogen-receptor and progesterone-receptor status was assessed by immunohistochemistry (ERz antibody, clone 1D5, Dako A/S, Glostrup, Denmark; and PR antibody, Dako A/S). Receptor expression was estimated as the percentage of positively stained tumour cells. Results were given as 1+, 2+ and 3+ positive or negative staining, with a cutoff value of <10% positive tumour cells (Hammond et al, 2010). HER-2 status was assessed by immunohistochemistry (Herceptest; Dako A/S) or dual colour fluorescent in situ hybridisation (FISH; PathVision HER-2 DNA probe kit, Vysis Inc., Downers Grove, IL, USA). Tumours were classified as HER-2-positive if they had a staining intensity of 3+ on the Herceptest; if a score of 2+ was gained, tumours were reanalysed by FISH (Wolff et al, 2007).

Breast cancer subtypes

Breast cancer subtypes were defined according to the results of the immunohistochemical analysis. Tumours heralding hormone-receptor expression in the absence of HER-2-receptor over-expression were summarised as belonging to the luminal subtype, without further differentiation. The HER-2 subtype was defined by overexpression of the HER-2 receptor and/or amplification of the HER-2/neu gene. Tumours were defined as triple-negative in the absence of ER, PgR as well as HER-2 expression (Anders et al, 2011; Duan et al, 2011).

Treatment plan and patient evaluation

In metastatic patients, routine re-evaluation of patients’ tumour status was performed every 3 months with contrast-enhanced CT scans of the chest and the abdomen, with additional work up if indicated. In patients with early breast cancer, follow-up was done according to local protocol. Brain imaging was performed only when symptoms of CNS metastases or carcinomatous meningitis occurred. Brain metastases were diagnosed by CT and/or MRI and histologically confirmed in case neurosurgery was performed. Carcinomatous meningitis was defined as enhancement of the meninges as detected by MRI and/or detection of tumour cells in the cerebrospinal fluid. Metastatic breast cancer and BM were treated according to the current evidence-based standard of care including surgery, radiotherapy, systemic therapy, targeted therapy and endocrine treatment (Beslja et al, 2007). Follow-up of BM was conducted every 3 months with either contrast-enhanced cranial CT or MRI scans.

Study end points

We defined brain metastases free survival (BMFS) as the interval from diagnosis of metastatic disease until the development of BM. Therefore, patients with BM as first site of metastatic disease were excluded from analysis of BMFS. Furthermore, we analysed the association of breast cancer subtypes with brain as first site of disease progression, number of BM, time to development of BM (<24 months vs ≥48 months), and development of carcinomatous meningitis.

Statistical analysis

Brain metastases free survival was estimated by the Kaplan–Meier product limit method. To test the differences between BMFS curves, the log-rank test was used. For correlation of two parameters, the χ2-test and the likelihood ratio were used. Two-tailed P-values <0.05 were considered to indicate statistical significance. Variables exhibiting significance (P<0.05) or near significance (P<0.09) at univariate analysis were included into a Cox proportional hazards models.

The association of the following variables with BMFS were investigated using univariate analysis: breast cancer subtype (luminal vs triple-negative vs Her-2-positive), presence of pulmonary metastases, presence of any visceral metastases, age at primary diagnosis (>65 years; <35 years), grading (grades 1 and 2 vs 3), stage at primary diagnosis (localised vs metastatic) and time to progression after first diagnosis of early breast cancer (<24 months vs ≥24 months). Correlation analysis was performed for subtype and BM as first site of recurrence, time to progression to the brain (<24 months, >48 months), number of BM (1–3 vs ≥3 BM) and presence of carcinomatous meningitis.

All statistics were calculated using statistical package for the social sciences (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

Overall, 250 patients with BM from breast cancer were identified from two Austrian centres between 1996 and 2010 (absolute incidence of breast cancer in Austria 1996–2010: 68 661 patients). Thirty-seven patients had to be excluded due to incomplete information about breast cancer subtype (e.g., missing data concerning Her-2 status, hormone-receptor status). Therefore, 213 patients were available for this retrospective analysis.

According to the immunohistochemical analysis of the primary tumour, patients were divided into three groups: luminal subtype, HER-2 subtype and triple-negative subtype. Forty-six patients...
Table 1 (Continued)  Patient characteristics (a) without BM and (b) with BM as first site of progression

### Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>(a) Entered patients (n = 169)</th>
<th>(b) Entered patients (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td><strong>%</strong></td>
<td><strong>n</strong></td>
</tr>
<tr>
<td><strong>Median age at first diagnosis (years)</strong></td>
<td>50</td>
<td>54</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>25–82</td>
<td>27–79</td>
</tr>
<tr>
<td><strong>Age &gt;65 years</strong></td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td><strong>Age &lt;35 years</strong></td>
<td>17</td>
<td>4</td>
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<td><strong>Grade 3 tumour</strong></td>
<td>116</td>
<td>31</td>
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<tr>
<td><strong>Invasive ductal carcinoma</strong></td>
<td>135</td>
<td>31</td>
</tr>
<tr>
<td><strong>Stage IV</strong></td>
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<td>12</td>
</tr>
<tr>
<td><strong>Subtype</strong></td>
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<tr>
<td>Luminal subtype</td>
<td>36</td>
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</tr>
<tr>
<td>HER-2 subtype</td>
<td>102</td>
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</tr>
<tr>
<td>Triple-negative subtype</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
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<td>31</td>
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<tr>
<td>Adjuvant endocrine therapy</td>
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<td>13</td>
</tr>
<tr>
<td>Adjuvant trastuzumab</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td><strong>Median time to progression (months)</strong></td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>0–166</td>
<td>1–5</td>
</tr>
<tr>
<td><strong>Visceral metastases</strong></td>
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<td><strong>Brain as first site of metastatic disease</strong></td>
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<td>0</td>
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<tr>
<td><strong>Median metastatic sites</strong></td>
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<tr>
<td><strong>Range</strong></td>
<td>1–5</td>
<td></td>
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<tr>
<td><strong>Palliative chemotherapy before BM</strong></td>
<td>152</td>
<td>39</td>
</tr>
<tr>
<td><strong>Palliative endocrine therapy before BM</strong></td>
<td>63</td>
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<tr>
<td><strong>Palliative trastuzumab before BM</strong></td>
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<td><strong>Palliative lapatinib before BM</strong></td>
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<td><strong>Response to systemic therapy at time of BM diagnosis</strong></td>
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<tr>
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<tr>
<td>PD</td>
<td>27</td>
<td>6.1</td>
</tr>
<tr>
<td><strong>Median BM free survival (months)</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
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<tr>
<td><strong>Median OS from first diagnosis (months)</strong></td>
<td>58.5</td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
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<tr>
<td><strong>Median OS from diagnosis of metastatic disease (months)</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
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<tr>
<td><strong>Median OS from diagnosis of BM (months)</strong></td>
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</tr>
<tr>
<td><strong>Range</strong></td>
<td>0–81</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease; BM = brain metastases; OS = overall survival. Characteristics grading, staging, subtype are from time point of first diagnosis. Characteristics metastatic sites are from time point of diagnosis of brain metastases.

In patients with lung metastases, median BMFS was 17 months (95% Cl: 14.10–19.90) compared with 21 months (95% Cl: 15.45–26.55) in patients with no evidence of lung metastases (P = 0.014, log-rank test) (Figure 2).

### Brain metastases free survival

Median BMFS was 19 months (95% Cl: 15.18–22.82) in the population of 169 patients with metastatic breast cancer who did not have BM as first site of progression. Univariate analysis revealed a significant difference in median BMFS between breast cancer subtypes. In the luminal subtype, median BMFS was 34 months (95% Cl: 23.71–44.29) compared with 18 months (95% Cl: 14.46–21.54) in the HER-2-positive subtype (P = 0.001, log-rank test) and 14 months (95% Cl: 11.34–16.66) in the triple-negative subtype (P = 0.001, log-rank test) (Figure 1).

In patients with lung metastases, median BMFS was 17 months (95% Cl: 14.10–19.90) compared with 21 months (95% Cl: 15.45–26.55) in patients with no evidence of lung metastases (P = 0.014, log-rank test) (Figure 2). In patients with time to extracranial progression after first diagnosis of early breast cancer of <24 months, median BMFS was significantly shorter compared to patients with time to extracranial progression after first diagnosis over 24 months (14 vs 24 months; P < 0.001, log-rank test). None of the other variables included into the univariate model displayed a significant influence on BMFS (Table 2).
In the multivariate analysis of BMFS, presence of lung metastases and breast cancer subtype as well as time to extracranial progression after first diagnosis of early breast cancer retained statistical significance. Hazard ratio (HR) for non-luminal breast cancer subtypes was 1.51 (95% CI: 1.17 – 1.95; P = 0.002, Cox proportional hazards model), 1.39 (95% CI: 1.01 – 1.93; P = 0.047, Cox proportional hazards model) for presence of lung metastases and 1.49 (95% CI: 1.07 – 2.08; P = 0.019, Cox proportional hazards model) for time to progression after first diagnosis of early breast cancer of < 24 months, respectively.

\( \chi^2 \)-test and likelihood ratio

The likelihood ratio of developing BM as first site of metastatic disease did not differ significantly between the breast cancer subtypes (luminal subtype 21.7%; HER-2 subtype 17.7%; triple-negative subtype 20.7%; \( P = 0.372, \chi^2 \)-test).

On the other hand, the likelihood of being diagnosed with BM in <24 months (BMFS < 24 months) correlated significantly with the breast cancer subtype. Within the luminal subtype, 30.6% (11 patients) of patients developed BM in <24 months; in the HER-2 subtype, 59.4% (60 patients) and in the triple-negative subtype, 77.4% (24 patients) of patients had a BMFS of <24 months (\( P < 0.001, \chi^2 \)-test). Furthermore, the likelihood of BMFS > 48 months again correlated significantly with the breast cancer subtype. Only one patient (3.2%) within the triple-negative subtype had a BMFS > 48 months, while 12 patients (17.6%) of the HER-2 group and 12 patients (33.3%) of the luminal group had a BMFS of > 48 months, respectively (\( P = 0.006, \chi^2 \)-test).

In all, 92 (48.7%) patients had over three BM at first diagnosis of BM. Accordingly, 32.3% of patients had a single metastasis, 9.5% had two BM and 9.5% three BM. The number of BM at time of first diagnosis of BM did not differ between the subtypes. In all, 24 patients (58.3%) within the luminal subtype had three or less metastases, corresponding numbers for the HER-2-positive and triple-negative subtypes are 50.9% and 50.0%, respectively (\( P = 0.666, \chi^2 \)-test).

The likelihood ratio for the development of carcinomatous meningitis again significantly correlated with breast cancer subtype. In all, 19.6% (nine patients) of the luminal subtype compared with 3.2% (four patients) of the HER-2 subtype and 9.3% (four patients) of the triple-negative subtype developed carcinomatous meningitis (\( P = 0.002, \chi^2 \)-test).

BMFS in subsets of the HER-2-positive subtype

In HER-2-positive patients, we further analysed whether HER-2/ER co-positivity or trastuzumab-based therapy had any influence on BMFS. In patients who received trastuzumab-based therapy before the development of BM, median BMFS was 17 months (95% CI: 13.41 – 20.53) compared with 21 months (95% CI: 8.53 – 33.47) in HER-2-positive patients who had not received trastuzumab-based treatment (\( P = 0.939, \log \text{-rank test} \)). Therefore, trastuzumab did not prolong BMFS.

In patients with ER/HER-2 co-positive tumours, median BMFS was 26 months (95% CI: 16.40 – 35.60) and therefore significantly
Brain metastases in breast cancer
A Berghoff et al

Figure 3 Kaplan-Meier estimates for BMFS. Median BMFS in HER-2/ER co-positive patients was 26 months (95% CI: 16.40–35.60) compared to (15 months; 95% CI: 10.77–19.23) in patients with HER-2-positive/ER-negative disease (P = 0.033, log-rank test).

In the field of BM prevention in Her-2-positive disease, promising results of lapatinib were reported, a dual tyrosine-kinase inhibitor of EGFR and HER-2 (Cameron et al, 2008). Other preventive strategies such as prophylactic cranial irradiation currently have no role in breast cancer treatment, as supporting data are missing (Saip et al, 2009). Also, screening for BM is not established, since early detection of BM was not found to influence survival henceforth (Niwinska et al, 2010). This, however, might result from the inclusion of patients at relatively low risk for developing BM into the respective clinical trials; therefore, a better definition of risk groups is warranted as first step to establish effective strategies of screening and prevention.

Clinical and translational research redefined breast cancer as a heterogeneous disease, divided into different subtypes defined by divergent gene expression profiles. In daily clinical practice, grading as well as immunohistochemical assessment of hormone-receptor status, Her-2, and Ki-67 are usually used as approximation. Therefore, breast cancer is assigned to the luminal, the HER-2 or the triple-negative phenotype at first diagnosis. This classification influences estimation of prognosis and treatment decisions (Perou et al, 2000; Sorlie et al, 2001, 2003). In the present study, we show that different breast cancer subtypes associate with time to development of BM. Patients with triple-negative disease had a significantly shorter BMFS (14 months) compared with 34 months in patients with luminal tumours (P = 0.001). Previously, the triple-negative subtype was identified to have a higher overall risk of developing BM; furthermore, BM are diagnosed relatively early during the course of disease (Pestalozzi et al, 2004; Heitz et al, 2009). Here, we could demonstrate tremendous differences of BMFS in triple-negative disease in comparison to luminal tumours, as BMFS of luminal subtypes is almost doubled. This finding indicates that triple-negative breast cancer warrants further research of BM-preventive strategies (Pestalozzi, 2009).

A higher incidence of BM was observed in HER-2-positive disease as well. Different authors suggested a connection to trastuzumab, a monoclonal antibody targeting the extracellular domain of HER-2. As trastuzumab cannot penetrate the blood–brain barrier, the CNS becomes a safe haven for tumour cells (Clayton et al, 2004). Also, improved control of systemic disease may eventually lead to the ‘unmasking’ of BM (Lin and Winer, 2007). In our analysis, BMFS within the HER-2 subtype was 18 months and was significantly different from the other two subtypes (P = 0.001). Compared with luminal cancers, shorter BMFS was observed in Her-2-positive disease, while BMFS was longer compared with triple-negative tumours. No influence of trastuzumab-based therapy on BMFS was observed. This finding indicates that biological behaviour rather than systemic treatment defines the risk for early or late development of BM in patients with HER-2-positive breast cancer (Burstein et al, 2005; Pestalozzi et al, 2006; Lin and Winer, 2007).

Several studies postulated the absence of ER expression as an unfavourable factor for the probability of developing BM (Slimane et al, 2004; Weil et al, 2005). Therefore, we performed an analysis of BMFS in the HER-2-positive subtype in dependence of ER expression. Patients with ER-negative/HER-2 co-positive disease have shown to have significantly longer BMFS compared with patients with ER-negative/HER-2-positive disease (26 months vs 15 months; P = 0.033). This once again shows that the Her-2-positive phenotype comprises heterogeneous subtypes.

Brain metastases are usually diagnosed rather late in the course of metastatic disease (Weil et al, 2005). Previous studies indicate a correlation of visceral and pulmonary metastases and the occurrence of BM (Weil et al, 2005; Kennecke et al, 2010). Our findings further support this investigation, as pulmonary metastases remained a significant risk factor associated with shorter BMFS in the Cox regression model (HR 1.49; P = 0.016). Therefore, we suggest that patients with triple-negative tumours and pulmonary metastases might be the most suitable
group for prospective trials investigating strategies of screening and prevention.

The number of BM is an important factor for prognosis as well as treatment, as surgery or radiosurgery is usually only applied in patients with oligometastatic (1–3 metastases) disease (Kamar and Posner, 2010; Niwinska et al., 2011a, b). Recently, an influence of breast cancer subtypes on the number of BM at first diagnosis was postulated. Oestrogen-receptor-positive patients, according to one study, might be more likely to develop oligometastatic brain involvement (Garg et al., 2011). In our homogenous, large collective, however, we cannot support those findings; the likelihood for oligometastatic involvement did not differ between the breast cancer subtypes.

Carcinomatous meningitis, just like BM, occurs late during the course of the disease and treatment options are very limited (de Azevedo et al., 2011). While breast cancer subtype influences overall survival after the diagnosis of carcinomatous meningitis, little is known about risk factors (Lee et al., 2011; Niwinska et al., 2011a, b). In our study, patients with luminal subtype were at higher risk for the development of carcinomatous meningitis compared to patients with HER-2 or triple-negative disease (19.6% vs 3.2% vs 9.3%; P = 0.002). Although the small sample size has to be taken into account, this apparent contradiction to solid BM warrants further investigation.

In conclusion, our study shows that patients with triple-negative as well as patients with ER-negative/HER-2-positive disease are at highest risk for developing BM early during their course of disease. The risk is further raised by the presence of pulmonary metastases. This analysis might help in defining the optimal breast cancer patient population for future prospective trials of BM screening and prevention.

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REFERENCES


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6.2 Brain-only metastatic breast cancer is a distinct clinical entity characterized by favourable median overall survival time and a high rate of long-term survivors.

Interlude

Survival estimation is the basis for treatment decisions in patients with newly diagnosed BM [124]. Further, the precise definition of prognostically homogenous cohorts is needed for the establishment of clinically meaningful clinical trials [63, 125]. The DS-GPA for patients with BM from breast cancer includes breast cancer subtype, age and Karnofsky performance score [68]. Of notice, the DS-GPA is based on the data of patients eligible for inclusion in a clinical trial and might therefore represent an inclusion bias. Therefore, we aimed to investigate clinical prognostic factors in a real life cohort of patients with BM from breast cancer. Interestingly, we identified patients with brain only metastatic breast cancer as a distinct prognostic subgroup with frequent occurrence of long-term survival [126]. Therefore, information on the status of the extracranial disease might add additional value in the prognostic evaluation of patients with BM from breast cancer.
Brain-only metastatic breast cancer is a distinct clinical entity characterised by favourable median overall survival time and a high rate of long-term survivors

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BACKGROUND: The clinical course of breast cancer patients with brain metastases (BM) as only metastatic site (brain-only metastatic breast cancer (BO-MBC)) has been insufficiently explored.

METHODS: All breast cancer patients with BM treated at our institution between 1990 and 2011 were identified. For each patient, full information on follow-up and administered therapies was mandatory for inclusion. Oestrogen receptor, progesterone receptor and Her2 status were determined according to standard protocols. Statistical analyses including computation of survival probabilities was performed.

RESULTS: In total, 222 female patients (26% luminal; 47% Her2; 27% triple negative) with BM of MBC were included in this study. In all, 38/222 (17%) BM patients did not develop extracranial metastases (ECM) during their disease course and were classified as BO-MBC. Brain-only-MBC was not associated with breast cancer subtype or number of BM. The median overall survival of BO-MBC patients was 11 months (range 0–69) and was significantly longer than in patients with BM and ECM (6 months, range 0–104; \( P = 0.007 \)). In all, 7/38 (18%) BO-MBC patients had long-term survival of \( > 3 \) years after diagnosis of BM and long-term survival was significantly more common in BO-MBC patients as compared with BM patients with ECM (\( P < 0.001 \)).

CONCLUSIONS: Brain-only metastatic behaviour occurs in around 17% of breast cancer with BM and is not associated with breast cancer subtype. Exploitation of all multimodal treatment options is warranted in BO-MBC patients, as these patients have favourable prognosis and long-term survival is not uncommon.

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Keywords: metastatic breast cancer; brain metastases; brain-only metastatic behaviour; prognosis; overall survival; breast cancer subtypes

Metastatic breast cancer (MBC) is the leading cause of cancer-related death in women (De Vita et al, 2012). The overall survival (OS) of patients with MBC constantly improved over the past decades mainly due to advances in systemic treatment (Kiely et al, 2011). Despite these advances, the development of brain metastases (BM) remains a severe and devastating complication decreasing quality of life and increasing morbidity and mortality (Well et al, 2005). The incidence seems to be rising as up to 40% of patients will develop BM during their course of disease (Well et al, 2005; Pestalozzi et al, 2006). Treatment options for patients with BM are limited. Local treatment approaches include surgery, radiosurgery and radiotherapy depending on number of BM, status of systemic disease and performance status of the patient (Lin et al, 2004). Although some trials postulate a positive impact of systemic treatment, the true effect of systemic therapy approaches on BM remains unclear (Bartsch et al, 2007, 2012; Lin et al, 2008). Survival of BM patients varies with breast cancer subtypes, however, the median OS remains limited ranging from 3.5 to a maximum of 25.3 months (Sperduto et al, 2012).

Despite the general poor prognosis, we observed patients with BM from MBC with long-term survival of \( > 36 \) months after the first diagnosis of BM at our institution. As some patients presented with isolated brain metastatic disease in the absence of extracranial disease (brain-only (BO) MBC) during their course of disease, we hypothesised that patients with BO metastatic behaviour might be a distinct entity.

We undertook this study to define the clinical characteristics and course of patients with BO-MBC and to compare it to patients with BM and extracranial metastatic disease.

MATERIALS AND METHODS

Patients
We identified all breast cancer patients with BM treated at our institution between 1990 and 2011. Diagnosis of BM was
performed by cranial computed tomography and/or cranial magnetic resonance imaging (MRI). All patients were treated according to the current evidence-based standard of care for systemic disease as well as for BM. Treatment approaches included radiosurgery, (whole brain) radiation therapy, surgical approaches, targeted as well as endocrine therapy and chemotherapeutic agents. For each patient, full information on clinical characteristics, follow-up and administered therapies was mandatory for inclusion.

Breast cancer subtypes
Oestrogen receptor (ER), progesterone receptor (PR) and Her2 status were assessed by immunohistochemistry (ERα antibody, clone ID5, Dako A/S, Glostrup, Denmark; and PR antibody, Dako A/S, Herceptest; Dako A/S) with a fully automated multi-modal slide-staining system (Ventana Benchmark ULTRA, Ventana, A/S, Herceptest; Dako A/S, Glostrup, Denmark; and PR antibody, Dako A/S, Herceptest; Dako A/S) with a fully automated multi-modal slide-staining system (Ventana Benchmark ULTRA, Ventana, Tucson, AZ, USA). Oestrogen receptor, PR and Her2 status were determined according to standard protocols (Wolff et al, 2007; Hammond et al, 2010). Breast cancer subtypes were defined as luminal subtype in the presence of ER and/or PR expression and the absence of Her2 expression, as Her2 subtype in the presence of Her2 expression regardless of ER and PR expression, and as triple-negative subtype in the absence of ER, PR and Her2 expression.

Study endpoints
Brain-only-MBC patients were defined as breast cancer patients with BM and the absence of extracranial metastases (ECM) during the entire course of the disease. We assessed the incidence of BO-MBC in relation to clinical characteristics including patient age, breast cancer subtypes (Her2-positive, triple-negative and luminal subtypes), number of BM and evaluated OS times. Overall survival was defined as time from first diagnosis of BM by computed tomography and/or MRI scan till death of any cause.

Statistical analysis
For correlation of two parameters the \( \chi^2 \) test was used. Two-tailed P-values <0.05 were considered to indicate statistical significance. For univariate survival analysis the Kaplan–Meier product limit method was used. To test differences between curves the log-rank test was applied. For multivariable survival analysis a Cox regression model was used. All statistics were calculated using statistical package for the social sciences (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS
Patients characteristics
In total, 222 female patients with a median age of 49 years at first diagnosis of breast cancer (range 26–79) and a median age of 53 years at first diagnosis of BM (range 26–83) were included in this study. All patients presented with symptomatic BM as none of the patients underwent screening for BM. Median time from first diagnosis of breast cancer to diagnosis of BM was 46.9 months (range 0–200). In all, 98/222 (44%) had neurosurgical resection and 22/222 (10%) radiosurgery as first local treatment approach for BM. In total, 180/222 (81%) were treated with whole brain radiotherapy (WBRT). In all, 73/180 (41%) patients received WBRT as adjuvant therapy after neurosurgical resection, 30/180 (17%) after radiosurgery and 77/180 (43%) as the only local treatment approach for BM. Overall, 85/180 (38%) received systemic therapy after diagnosis of BM. Table 1 lists further patients characteristics.

Metastatic pattern
In total, 60/222 (27%) patients had BM as first site of recurrence. Of 60 patients with BM as first site of recurrence, 22 (37%) developed further systemic metastases during their course of disease. In all, 38/222 (17%) of BM patients did not develop ECM during their disease course (median follow-up time 7 months; range 0–104) and were classified as BO-MBC cases. Table 1 lists further details on the metastatic pattern.

Brain only metastatic pattern
Brain-only metastatic behaviour was neither associated with breast cancer subtype (\( P=0.198; \chi^2 \) test) nor with number of BM (\( P=0.110; \chi^2 \) test). Further, prior trastuzumab-based therapy did not correlate with BO metastatic behaviour (\( P=0.090; \chi^2 \) test). Distribution of diagnosis-specific graded prognostic assessment (GPA) class did not differ between the BO-MBC cohort and patients with extracranial disease (\( P=0.784; \chi^2 \) test). Patients with BO-MBC were more likely to have neurosurgical resection as first-line therapy for BM (\( P=0.002; \chi^2 \) test) and less likely to receive chemotherapy after diagnosis of BM compared with patients with ECM (\( P=0.016; \chi^2 \) test).

Overall survival
The median OS from diagnosis of BM in the entire cohort was 11.8 months (range 0–104). Overall survival in luminal subtype was 9 months, 7 months in Her2 subtype and 6 months in triple-negative subtype (\( P=0.47; \log\text{-}rank \) test). At a median follow-up of 7 months after first diagnosis of BM (range 0–104) 202/222 (91%) patients had died. The median OS of BO-MBC patients was 11 months (95% CI 8.5–13.5) and was therefore significantly longer than in patients with BM and ECM (6 months; 95% CI 3.8–8.2; \( P=0.007, \log\text{-}rank \) test) (Figure 1). In multivariable analysis with diagnosis-specific GPA and number of BM, BO metastatic behaviour remained a significant prognostic factor of OS (hazard ratio 0.6; \( P=0.029; \) Cox regression model). In the BO-MBC cohort, Karnofsky performance status >70 (\( P=0.02; \) log-rank test), single BM (\( P<0.001; \) log-rank test) and ER expression (\( P=0.014; \) log-rank test) were associated with favourable OS in univariate analysis and included in multivariate analysis. In multivariate analysis Karnofsky performance status >70 (hazard ratio 0.07; \( P=0.01; \) Cox regression model) and single BM (hazard ratio 0.13; \( P=0.003; \) Cox regression model) remained significant (Table 2). In all, 7/38 (18%) BO-MBC patients had long-term survival of ≥3 years after diagnosis of BM. Compared with patients with the presence of extracranial disease, long-term survival was significantly more common in BO-MBC patients (\( P<0.001; \chi^2 \) test).

DISCUSSION
Our data show that BO-MBC is a distinct clinical breast cancer entity with favourable median OS time of 11 months compared with 6 months in BM patients with additional ECM. Interestingly, we observed survival of ≥36 months in 7/38 (18%) patients with BO-MBC, indicating that long-time survival is possible and not uncommon in this patient population. Our data stress that intensive therapy with exploitation of all multimodal treatment approaches is warranted in breast cancer patients presenting with metastatic disease confined to the central nervous system. This conclusion is well in line with the situation in non-small cell lung cancer, where stage IV patients with exclusive oligometastatic cerebral disease and limited primary tumour also constitute a good prognosis subgroup that can be treated with curative intent (Pfannschmidt and Dienemann, 2010). High Karnofsky index, the presence of only one BM and positive ER expression were
favourable prognostic factors in our cohort of BO-MBC patients and may help to adapt clinical management strategies. The pathobiological explanation for BO metastatic involvement in breast cancer remains unclear. Previous studies have shown that the Her2-positive and the triple-negative breast cancer subtypes are characterised by relatively high incidences of BM (Kennecke et al, 2010). However, in our study we found no correlation of

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<tr>
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<td>Triple-negative</td>
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<td>Her2-targeted therapy before BM</td>
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Abbreviations: BM = brain metastases; BO = brain-only; DS-GPA = diagnosis-specific graded prognostic assessment; ECM = extracranial metastases; OS = overall survival; WBRT = whole brain radiotherapy.

Figure 1 Overall survival from diagnosis of BM in patients with BO metastatic behaviour (11 months; 95% confidence interval (CI) 8.47–13.59) compared with patients with present ECM (6 months; 95% CI 3.81–8.19).

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<th>Table 2</th>
<th>Multivariate survival analysis in BO-MBC cohort</th>
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<td>Hazard ratio</td>
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<td>Karnofsky performance score at first diagnosis of BM</td>
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<td>Number of BM</td>
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<td>ER expression</td>
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Abbreviations: BM = brain metastases; BO-MBC = brain-only metastatic breast cancer; CI = confidence interval; ER = oestrogen receptor.

Brain-only metastatic breast cancer

AS Berghoff et al


Clinical Studies
breast cancer subtype with BO metastatic behaviour. The distribution of breast cancer subtypes within our BO-MBC cohort was equalised, as approximately one third of the patients belonged to the Her2-positive, one third to the triple-negative and one third the luminal subtype, respectively. It is interesting to note that prior trastuzumab-based therapy did not correlate with BO metastatic behaviour, although trastuzumab is thought to favour the development of BM owing to its inability to cross the blood-brain barrier (Bendell et al, 2003; Musolino et al, 2011). Patients with triple-negative and luminal breast cancer subtypes developed BM significantly earlier during their disease course than patients with Her2-positive disease in our study (Berghoff et al, 2012). Further studies are needed to clarify the molecular mechanisms of the selective brain tropism of metastatic spread in some breast cancer patients. As postulated by the 'seed and soil' hypothesis, the development of this distinct metastatic behaviour may be explained by the interaction between specific tumour cells ('the seed') and the microenvironment of the brain ('the soil') (Fidler, 2011).

So far, only a few studies focusing on BM as first site of recurrence were conducted (Boogerd et al, 1993, 1997; Niwinska et al, 2011; Dawood et al, 2012). Dawood et al (2012) documented a high incidence of BM as first site of recurrence in a population of triple-negative breast cancer patients with stage I to III, but in contrast to our study, no further analysis of the clinical course after diagnosis of BM was performed. Boogerd et al (1993) showed that breast cancer patients with single BM in the absence of ECM have improved OS after intensive local treatment compared with BM patients with ECM at first diagnosis of BM. However, in this study no differentiation of breast cancer subtypes and characterisation of prognostic factors was performed. To our knowledge, our study is the first to investigate the incidence and clinical course of contemporary breast cancer patients with BM as first site of recurrence with a focus on patients with BO-MBC. However, our study has some limitations that have to be considered in the interpretation of the data. First, only retrospectively collected data were available for our analysis and we included patients diagnosed and treated with MBC over a long period (1990–2011). Changes in clinical management such as the introduction of new therapy standards (e.g., trastuzumab, lapatinib for Her2-positive MBC) or diagnostic procedures (e.g., cranial MRT) during this period may have influence our results. However, the date of diagnosis of both groups, BO-MBC and BM with ECM, was distributed evenly over the entire study period making a bias arising from differences in clinical management improbable. In any case, analysis of data from prospective clinical trials might be useful to validate our findings.

In our series, 60/222 (27%) patients had BM as first site of recurrence and more than one third of these patients, i.e., 22/60 (37%) developed ECM after diagnosis of BM. Overall, 38/222 (17%) patients experienced BO metastatic disease in the absence of ECM during their course of disease. Our data show that patients with BO metastatic behaviour represent a distinct clinical entity with a better survival prognosis from diagnosis of BM compared with BM patients with additional ECM. We could not identify any factors predicting for BO metastatic behaviour, but identified high Karnofsky index, the presence of only one BM and positive ER status as favourable prognostic factors in BO-MBC patients. Long-term survival is not uncommon and was achieved in a fifth of BO-MBC patients, exploitation of all multimodal treatment options is warranted in patients with BM as first site of recurrence. Future studies are needed to clarify the role of systemic therapies with novel targeted agents in relation to established local therapy approaches like neurosurgery, radiosurgery and radiotherapy.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCES


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6.3 Prognostic significance of Ki67 proliferation index, HIF1 alpha index and microvascular density in patients with non-small cell lung cancer brain metastases.

Interlude
Survival estimation in patients with NSCLC BM is based on clinical prognostic factors including number of BM, Karnofsky performance score, age and status of extracranial disease [68]. So far no tissue based characteristics are involved in the prognostic evaluation of NSCLC BM patients. Re-evaluation of tissue based characteristic in metastatic sites might add valuable additional information, as little is known so far on the accordance of primary tumour and matched BM [14, 127]. Therefore, we concentrated on the identification of tissue based prognostic factors in patients with NSCLC BM in the paper “Prognostic significance of Ki67 proliferation index, HIF1 alpha index and microvascular density in patients with non-small cell lung cancer brain metastases”. We identified low median Ki67 proliferation index as well as high median microvascular density as favourable tissue based prognostic factors indicating that inclusion of tissue based characteristic might add additional value to already existing clinical prognostic assessments. We also observed, that patients receiving whole brain radiation therapy survived shorter when the BM tumour tissue revealed a high HIF 1 alpha expression [128].
The development of brain metastases (BM) is a devastating complication in cancer patients, for which there are only limited treatment options. Unfortunately, the incidence of BM has increased over the last decade. Non-small cell lung cancer (NSCLC) is the primary tumor most frequently responsible for BM and approximately 40% of patients suffering from metastatic NSCLC eventually develop BM [1].

The prognosis upon diagnosis of BM is poor, with a median overall survival (OS) of only a few months [2]. Several prognostic scores have been established in order to provide survival estimations for patients with newly diagnosed BM. These prognostic scores rely mainly on established clinical prognostic factors such as age, Karnofsky Performance Status, status of extracranial disease and number of BM [2–5]. The diagnosis-specific graded prognostic assessment score (DS-GPA) is an established prognostic score for survival estimation in patients with newly diagnosed NSCLC BM [2]. Herein, the calculated median OS from diagnosis of BM ranges from 3 months in the least favorable group, to 14.8 months in the most favorable group [2]. Tissue-based prognostic factors are not currently included in any of the established prognostic scores for BM patients.

Neoangiogenesis, hypoxia and proliferation are hallmarks of cancer. These factors have been shown to influence patients’ prognosis and response to therapy in many tumor types, including primary and metastatic NSCLC [6–10]. However, the prognostic value of these parameters in NSCLC BM has not been systematically studied.

In the present study we investigated the association of Ki67 tumor cell proliferation index, the expression of hypoxia-inducible factor 1 alpha (HIF-1 alpha) and the expression of CD34 (as an endothelial marker) with outcome parameters in order to explore their prognostic value. The study cohort comprised a large and well-defined series of NSCLC patients treated with first-line neurosurgical resection upon diagnosis of BM.

Methods

Patients

All patients diagnosed with NSCLC BM having undergone first-line neurosurgical resection between January 1990 and February 2011 were identified from the Neuro-Biobank, Medical University of Vienna. Histological confirmation of BM originating from NSCLC was mandatory for inclusion. Clinical data, including clinical prognostic factors, were identified by chart review. DS-GPA was calculated based on clinical factors [2, 3]. Survival data was obtained from the National Cancer Registry of Austria database and the Austrian Brain Tumor Registry [11]. The ethics committee of the Medical University of Vienna approved the study (vote 078/2004).
Tissue-based analysis

Formalin-fixed and paraffin-embedded tissue blocks were assembled according to standard laboratory practice. Tissue blocks were cut into 3-µm slices with a microtome. Immunohistochemistry was performed using an automated horizontal slide processing system (Autostainer Plus Link; Ki67; Dako Denmark, Glostrup, Denmark) and a fully automated multimodal slide staining system (Benchmark ULTRA; HIF-1 alpha, CD34; Ventana Medical Systems, Tucson, AZ, USA) according to standard protocol [12–14]. In brief, slides underwent heat-induced epitope retrieval in pH6.0 citrate buffer in pH8.0 buffer (CD34: 36 min). Afterwards, sections were incubated with antibody: HIF-1 alpha: polyclonal rabbit purified anti-human HIF-1 alpha/610959 BD Transduction Laboratories™ (BD Biosciences, East Rutherford, NJ, USA) 1:10; Ki67: monoclonal mouse Ki67 clone MIB-1/M7240 (Dako), 1:200; CD34: Novocastra™ lyophilized mouse monoclonal antibody endothelial cell marker (CD34) (Novocastra™, Leica Biosystems, Wetzlar, Germany) 1:50.

For the Ki67 proliferation index, 500 cells were counted within the area of strongest staining to give the percentage of positive cells (0–100 %) [12]. HIF-1 alpha score was calculated according to the modified H-score [15–17]. HIF-1 alpha intensity groups were defined as follows: 0 = no appreciable staining in the tumor cell nucleus; 1 = barely detectable staining intensity in the nucleus; 2 = moderate staining intensity distinctly in the tumor cell nucleus; 3 = strong staining intensity of the tumor cell nucleus. For each intensity group the fraction of cells (0–100 %) was recorded. The HIF-1 alpha index was calculated by multiplying the intensity by the fraction of cells producing this intensity, producing a total range of 0–300. The mean microvascular density (MVD) was defined by the number of CD34-positive vessels within the area of the highest density at a 200x magnification (“hot spot”) [18]. Furthermore, the vascular pattern was analyzed in the CD34 staining to differentiate between the “angiogenic type” defined by the predominance of sprouting vessels (characterized by multilayer endothelium) and the “silent type” defined by predominance of vessels with thin monolayer endothelium. Specimens with an equal distribution of angiogenic and silent types were defined as the “balanced type” [17].

Statistical analysis

The Spearman’s rank correlation coefficient was used to assess monotone associations between two continuous variables. For assessing group differences, \( \chi^2 \)-square, paired and unpaired t-, Mann–Whitney U and Kruskal–Wallis tests were used as appropriate. A significance level of 0.05 was applied. OS of patients was estimated with the Kaplan–Meier product limit method and group differences were assessed with the log-rank test. The median was used as the cutoff value for continuous variables entered in univariate analysis.

Variables with significant results in univariate analysis were entered into a multivariate Cox proportional hazards model. Overall and partial measures of dependence (R squared, R\(^2\) values) were computed according to Kent and O’Quigley [19, 20]. Due to the exploratory and hypothesis-generating design of the present study, no adjustment for multiple testing was applied [21].

For calculation of the tissue GPA (tGPA) prognostic score a multivariate Cox regression model was used. For graphical representation, the patient cohort was divided into three equal sized classes according to the terciles of the tGPA.

All statistical analyses were performed with the Statistical Package for the Social Sciences version 20.0 software (IBM, SPSS, Armonk, NY, USA) and SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics

A total of 230 patients (151 male, 79 female) with a median age of 56 years (range 33–78 years) at first diagnosis of NSCLC BM were included. All patients underwent neurosurgical resection as first line therapy for newly diagnosed BM. Histology was as follows: 165/230 (71.7 %) patients had adenocarcinoma, 29/230 (12.6 %) squamous cell carcinoma, 15/230 (6.5 %) adenosquamous carcinoma, 17/230 (7.4 %) large cell carcinoma and 4/230 (1.7 %) patients had unknown histology. A history of cigarette smoking was reported by 169/230 (73.5 %) patients. Further patient characteristics are listed in Table 1.

Tissue-based findings in brain metastasis specimens

Median Ki67 proliferation index was 39.8 % (range 5–97 %), median HIF-1 alpha index was 60 (range 0–270) and median MVD was 71/0.7 mm\(^2\) (range 7–298/0.7 mm\(^2\)). Of all specimens, 102/230 (44.3 %) showed a predominance of microvascular sprouting (angiogenic type); 62/230 (27.0 %) specimens showed a silent angiogenesis with predominance of mature vessels and without signs of angiogenesis (silent type); 61/230 (26.5 %) specimens showed an equal distribution of angiogenic and silencing vascular patterns (Kruskal–Wallis test, \( p = 0.311 \)). Furthermore, no correlation between Ki67 proliferation index and MVD (Spearman’s correlation coefficient \( r_{s} = 0.049 \), \( p = 0.459 \)) or vascular pattern (Kruskal–Wallis test, \( p = 0.572 \)) was present. No correlation between histology and MVD (Kruskal–Wallis test, \( p = 0.587 \)).

Among BM specimens, no correlation was observed between Ki67 proliferation index and MVD (Spearman’s correlation coefficient \( r_{s} = 0.039 \), \( p = 0.556 \)) or vascular pattern (Kruskal–Wallis test, \( p = 0.587 \)). Furthermore, no correlation between HIF-1 alpha index and MVD (Spearman’s correlation coefficient \( r_{s} = 0.049 \), \( p = 0.459 \)) or vascular pattern (Kruskal–Wallis test, \( p = 0.572 \)) was present. No correlation between histology and MVD (Kruskal–Wallis test, \( p = 0.311 \)), HIF-1 alpha index (Kruskal–Wallis test, \( p = 0.321 \)) or vascular pattern (\( \chi^2 \)-square test, \( p = 0.799 \)) was evident. Weak correlation was observed
Prognostic significance of Ki67 proliferation index, HIF-1 alpha index and microvascular density in patients with non-small cell lung cancer brain metastases

Abstract

**Background.** Survival upon diagnosis of brain metastases (BM) in patients with non-small cell lung cancer (NSCLC) is highly variable and established prognostic scores do not include tissue-based parameters.

**Methods.** Patients who underwent neurosurgical resection as first-line therapy for newly diagnosed NSCLC BM were included. Microvascular density (MVD), Ki67 tumor cell proliferation index and hypoxia-inducible factor 1 alpha (HIF-1 alpha) index were determined by immunohistochemistry.

**Results.** NSCLC BM specimens from 230 patients (151 male, 79 female; median age 56 years; 199 nonsquamous histology) and 53/230 (23.0%) matched primary tumor samples were available. Adjuvant whole-brain radiation therapy (WBRT) was given to 153/230 (66.5%) patients after neurosurgical resection. MVD and HIF-1 alpha indices were significantly higher in BM than in matched primary tumors. In patients treated with adjuvant WBRT, low BM HIF-1 alpha expression was associated with favorable overall survival (OS), while among patients not treated with adjuvant WBRT, BM HIF-1 alpha expression did not correlate with OS. Low diagnosis-specific graded prognostic assessment score (DS-GPA), low Ki67 index, high MVD, low HIF-1 alpha index and administration of adjuvant WBRT were independently associated with favorable OS. Incorporation of tissue-based parameters into the commonly used DS-GPA allowed refined discrimination of prognostic subgroups.

**Conclusion.** Ki67 index, MVD and HIF-1 alpha index have promising prognostic value in BM and should be validated in further studies.

**Keywords**

Survival · Whole-brain radiation therapy · Proliferation · Angiogenesis · Hypoxia

### Prognostische Signifikanz von Proliferationsindex Ki67, HIF-1α-Index und mikrovaskulärer Gefäßdichte bei Patienten mit zerebralen Metastasen eines nicht-kleinzelligen Lungenkarzinoms

**Zusammenfassung**


**Methoden.** Neurochirurgische Resektion zerebraler NSCLC-Metastasen wurden in dieser Studie untersucht. Die Gefäßdichte („microvascular density“, MVD), der Ki67-Proliferationsindex sowie der HIF-1α-Index wurden mittels immunhistochemischer Methoden analysiert.


**Schlussfolgerung.** Die Analyse des Ki67-Proliferationsindex, der Gefäßdichte sowie des HIF-1α-Index sollte in die prognostische Beurteilung von Patienten mit zerebralen NSCLC-Metastasen inkludiert werden.

**Schlüsselwörter**

Überleben · Ganzhirnbestrahlung · Proliferation · Angiogenese · Hypoxie

between Ki67 proliferation index and HIF-1 alpha index (Spearman’s correlation coefficient 0.298, p < 0.001).

**Comparative analyses of brain metastases and corresponding primary tumors**

Tumor tissue from the corresponding primary tumor was available in 53/230 (23.0 %) cases. Median Ki67 proliferation index of the primary tumor was 39% (range 4–79%) and did not significantly differ from the BM Ki67 proliferation index (paired t-test, p = 0.897). Median primary tumor MVD was 65/0.7 mm² (range 26–179/0.7 mm²), which was significantly lower than in BM (71/0.7 mm²; paired t-test, p = 0.032). Median primary tumor HIF-1 alpha index was 30 (0–210), which was significantly lower than in BM (60; paired t-test, p = 0.013).

**Survival analyses**

**Impact of parameters on time to diagnosis of brain metastases**

Time to diagnosis of BM was only evaluated in patients with subsequent diagnosis of BM and no synchronous diagnosis of primary tumor and BM (n = 103). No impact of Ki67 proliferation index, HIF-1 alpha index, MVD or vascular pattern of the primary tumor on time to development of BM (TTBM) was observed. Pa-
Patients with large cell carcinoma histology (median TTBM: 4 months) developed BM earlier than patients with adenocarcinoma (median TTBM: 12 months), squamous cell (median TTBM: 11 months) or adenosquamous cell carcinoma histology (median TTBM: 9 months; log-rank test, \( p = 0.052; \) Fig. 2). Furthermore, patients with large cell carcinoma (11/17, 64.7\%) or adenocarcinoma (99/165, 60.0\%) had more synchronous diagnosis of NSCLC and BM than patients with adenosquamous (7/15, 46.7\%) or squamous cell carcinoma (8/29, 27.6\%; \( \chi^2 \)-square test, \( p = 0.009 \)).

### Impact of clinical characteristics on overall survival from diagnosis of brain metastasis

DS-GPA showed a statistically significant correlation with OS measured from first diagnosis of BM. Patients with DS-GPS class 1 had a median OS of 17 months, compared to 7 months in class 2, 5 months in class 3 and only 1 month in patients with class 4 (log-rank test, \( p < 0.001; \) Fig. 3a). However, no significant difference in OS was observed between DS-GPA class 2 and DS-GPA class 3 (7 vs. 5 months; log-rank test, \( p = 0.205 \)).

Histology significantly influenced survival prognosis. Patients with adenocarcinoma had a median OS of 9 months from diagnosis of BM, compared to 8 months in patients with large cell histology, 6 months in patients with adenosquamous histology and 4 months in patients with squamous histology (log-rank test, \( p = 0.008; \) Fig. 3b).

Patients scheduled for adjuvant WBRT after neurosurgical resection of BM had a significantly longer median OS than patients without adjuvant WBRT after neurosurgical resection of BM (9 vs. 5 months; log rank test, \( p < 0.001 \)). Patients with a single BM received WBRT significantly less frequently after neurosurgical resection (100/164, 61.0\%) than did patients with 2–3 BM (38/47, 80.9\%) or > 3 BM (15/18, 83.3\%; \( \chi^2 \)-square test, \( p = 0.012 \)). Furthermore, patients receiving chemotherapy after diagnosis of BM survived significantly longer than patients not receiving chemotherapy (15 vs. 6 months; log-rank test, \( p = 0.005 \)). Patients receiving combination therapy comprising WBRT

### Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Entire population (( n = 230 ))</th>
<th>No. patients</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at first diagnosis of lung cancer, years (range)</td>
<td>56 (33–78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology of primary tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Adenocarcinoma</td>
<td>165</td>
<td>71.7</td>
<td></td>
</tr>
<tr>
<td>– Squamous cell carcinoma</td>
<td>29</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td>– Adenosquamous carcinoma</td>
<td>15</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>– Large cell carcinoma</td>
<td>17</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>– Unknown</td>
<td>4</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Stage IV primary tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Yes</td>
<td>145</td>
<td>63.0</td>
<td></td>
</tr>
<tr>
<td>– No</td>
<td>85</td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td>Surgery for primary tumor before diagnosis of BM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Yes</td>
<td>76</td>
<td>33.2</td>
<td></td>
</tr>
<tr>
<td>– No</td>
<td>153</td>
<td>66.8</td>
<td></td>
</tr>
<tr>
<td>Number of extracranial metastatic sites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– 0</td>
<td>180</td>
<td>78.3</td>
<td></td>
</tr>
<tr>
<td>– 1</td>
<td>32</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>– ≥ 2</td>
<td>18</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Visceral metastases before first diagnosis of BM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Yes</td>
<td>28</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>– No</td>
<td>202</td>
<td>87.8</td>
<td></td>
</tr>
<tr>
<td>Number of chemotherapy lines before first diagnosis of BM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– 0</td>
<td>181</td>
<td>78.7</td>
<td></td>
</tr>
<tr>
<td>– 1</td>
<td>40</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>– ≥ 2</td>
<td>9</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Time from first diagnosis of primary tumor to first diagnosis of BM, months (range)</td>
<td>11 (1–162)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median age at first diagnosis of BM, years (range)</td>
<td>57 (34–78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPA class at first diagnosis of BM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– I</td>
<td>64</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td>– II</td>
<td>114</td>
<td>49.6</td>
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<tr>
<td>– III</td>
<td>47</td>
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</tr>
<tr>
<td>– IV</td>
<td>5</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Status of primary tumor at first diagnosis of BM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– No evidence of disease</td>
<td>55</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td>– Partial response</td>
<td>6</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>– Stable disease</td>
<td>32</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>– Progressive disease</td>
<td>10</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>– Synchronous first diagnosis of primary tumor and BM</td>
<td>127</td>
<td>55.2</td>
<td></td>
</tr>
<tr>
<td>WBRT after surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Yes</td>
<td>153</td>
<td>66.5</td>
<td></td>
</tr>
<tr>
<td>– No</td>
<td>76</td>
<td>33.0</td>
<td></td>
</tr>
<tr>
<td>– Unknown</td>
<td>1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy after diagnosis of BM</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>– Yes</td>
<td>79</td>
<td>34.3</td>
<td></td>
</tr>
<tr>
<td>– No</td>
<td>146</td>
<td>63.5</td>
<td></td>
</tr>
<tr>
<td>– Unknown</td>
<td>5</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Event</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Yes</td>
<td>203</td>
<td>88.3</td>
<td></td>
</tr>
<tr>
<td>– No</td>
<td>27</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td>Median overall survival from first diagnosis of lung cancer, months (range)</td>
<td>14.5 (0–168)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median overall survival from first diagnosis of BM, months (range)</td>
<td>8.0 (0–141)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BM brain metastasis, GPA graded prognostic assessment score, WBRT whole-brain radiation therapy
and chemotherapy after surgery had improved survival (15 months) compared to patients receiving WBRT (8 months) or chemotherapy alone (14 months; log-rank test, \( p < 0.001 \); Fig. 3c).

A synchronous diagnosis of BM and primary NSCLC had been made for 127/230 (55.2%) patients. In this cohort, patients treated with surgical treatment of both primary tumor and BM had a more favorable OS compared to patients in whom only the BM was resected (15 vs. 7 months; log-rank test, \( p = 0.004 \); Fig. 3d).

**Impact of tissue-based characteristics on overall survival from diagnosis of brain metastasis**

Patients with a low Ki67 proliferation index in the BM tissue had an improved survival compared to patients with high Ki67 proliferation index (10 vs. 6 months; log-rank test, \( p = 0.011 \); Fig. 4a). Patients with a low HIF-1 alpha index in the BM tissue had more favorable OS than patients with a high HIF-1 alpha index (11 vs. 7 months; log-rank test, \( p = 0.013 \); Fig. 4b). Patients with high MVD in the BM tissue survived significantly longer than patients with low MVD (10 vs. 6 months; log-rank test, \( p = 0.049 \); Fig. 4c). The vascular pattern did not show a significant impact on prognosis (log-rank test, \( p = 0.850 \)).

In the cohort of patients treated with WBRT after neurosurgery, patients with a low HIF-1 alpha index had a more favorable median OS than patients with a high HIF-1 alpha index (15 vs. 7 months; log-rank test, \( p = 0.004 \); Fig. 5a). However, HIF-1 alpha expression had no impact on OS in the cohort of patients without WBRT (log-rank test, \( p = 0.904 \); Fig. 5b). A trend was observed in multivariate interaction analysis of WBRT and HIF-1 alpha index (Cox regression model, \( p = 0.074 \)).

**Multivariable analysis of overall survival**

According to the results of univariate analysis, we entered the following parameters into multivariate survival analyses using the Cox regression model: DS-GPA, primary tumor histology, chemo-
therapy (yes/no), adjuvant WBRT (yes/no), HIF-1 alpha index, Ki67 proliferation index and MVD. DS-GPA, WBRT, HIF-1 alpha index, Ki67 proliferation index and MVD remained independent prognostic parameters for survival from diagnosis of BM in multivariable analysis (Table 2). Furthermore, the tissue-based characteristics were shown to add statistically significant information to the model (Cox regression model, three degrees of freedom, \(p < 0.001\)). Overall, the R\(^2\) measure showed that the seven prognostic factors explained 34.04% of the variability in OS time. After accounting for the effects of the clinical prognostic factors and the other respective tissue-based characteristics, the partial R\(^2\) values for MVD, HIF-1 alpha index and ki67 proliferation index were 2.72, 2.18 and 4.38%, respectively (Table 2).

**Calculation of a prognostic score including tissue-based characteristics**

To illustrate the potential of improved prognostication by incorporation of tissue-based prognostic parameters into the DS-GPA, we calculated a tGPA. Using the terciles as cutoffs, the entire cohort was divided into three tGPA classes (class 1: 76 patients; class 2: 77 patients; class 3: 77 patients). A statistically significant association was observed between tGPA and median OS (class 1: 15 months, class 2: 9 months, class 3: 4 months; log-rank test, \(p < 0.001\); Fig. 5c). As no significant discrimination was observed between DS-GPA class 2 and DS-GPA class 3, we applied the tGPA to this group of patients (n = 161). Herein, tGPA showed a statistically significant discrimination. Patients with tGPA class 1 had median OS of 11 months; for patients with class 2 this was 8 months and patients with tGPA class 3 had a median OS of 5 months (log-rank test, \(p < 0.001\); Fig. 5d).

**Discussion**

In this project we investigated for the first time the prognostic value of Ki67 pro-
1.0 Ki67 proliferation index
HIF 1 alpha index Microvascular density

- p=0.049
- <71/0.7 mm² (n=117)
- >71/0.7 mm² (n=113)
- <60 (n=116)
- >61 (n=114)

p=0.011 p=0.013
- <39.8% (n=113)
- >39.8% (n=117)

**Survival Functions**

- Overall survival from diagnosis of BM (months)

**Fig. 4**

- Overall survival (OS) from diagnosis of brain metastases (BM) according to Ki67 proliferation index.
- OS from diagnosis of BM according to HIF-1 alpha index.
- OS from diagnosis of BM according to microvascular density (MVD).

**Fig. 5**

- Overall survival (OS) from diagnosis of brain metastases (BM) in patients receiving adjuvant whole-brain radiation therapy (WBRT) after surgery of BM related to HIF-1 alpha index.
- OS from diagnosis of BM in patients not receiving adjuvant WBRT after surgery of BM related to HIF-1 alpha index.
- OS from diagnosis of BM related to tissue graded prognostic assessment (tGPA) class.
- OS from diagnosis of BM in patients with GPA class 2 related to tGPA.
Table 2 Results of multivariate survival analyses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exp(B)</th>
<th>95 % CI</th>
<th>P-value</th>
<th>Partial R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS-GPA</td>
<td>1.693</td>
<td>1.374–2.087</td>
<td>&lt; 0.001</td>
<td>12.33 %</td>
</tr>
<tr>
<td>Histology (categorical)</td>
<td></td>
<td></td>
<td></td>
<td>1.40 %</td>
</tr>
<tr>
<td>- Adenocarcinoma</td>
<td>0.657</td>
<td>0.739–1.139</td>
<td>0.134</td>
<td></td>
</tr>
<tr>
<td>- Squamous cell carcinoma</td>
<td>0.865</td>
<td>0.307–1.442</td>
<td>0.402</td>
<td></td>
</tr>
<tr>
<td>- Adenosquamous cell carcinoma</td>
<td>1.378</td>
<td>0.799–2.377</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Large cell carcinoma</td>
<td>0.753</td>
<td>0.545–1.041</td>
<td>0.086</td>
<td>4.91 %</td>
</tr>
<tr>
<td>Chemotherapy (yes/no)</td>
<td>0.591</td>
<td>0.428–0.816</td>
<td>0.001</td>
<td>3.26 %</td>
</tr>
<tr>
<td>HIF-1 alpha index (continuous)</td>
<td>1.003</td>
<td>1.000–1.005</td>
<td>0.050</td>
<td>2.18 %</td>
</tr>
<tr>
<td>Ki67 proliferation index (continuous)</td>
<td>1.013</td>
<td>1.005–1.021</td>
<td>0.002</td>
<td>4.38 %</td>
</tr>
<tr>
<td>MVD (continuous)</td>
<td>0.996</td>
<td>0.993–1.000</td>
<td>0.031</td>
<td>2.72 %</td>
</tr>
<tr>
<td>DS-GPA diagnosis-specific graded prognostic assessment, WBRT whole-brain radiation therapy, MVD microvascular density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Our findings underscore the heterogeneity of tumor behavior among NSCLC subtypes and the relevance of these differences for the biology and clinical course of BM. Firstly, we found a higher proliferation rate in squamous, compared to non-squamous BM tumors—a finding that mirrors the situation in primary NSCLC [26]. In line with these results, we found that patients with squamous cell BM had a poor outcome, with median OS reaching only 4 months and thereby significantly shorter than that observed for other tumor types (6–9 months).

Interestingly, we found a profound effect of tumor histology on the time to BM development from first diagnosis of NSCLC. Patients with large cell carcinomas developed BM within a median 4 months from diagnosis of the primary tumor, while in other tumor types BM came evident only after median follow-up times of more than 11 months. To the best of our knowledge, this result has not been described previously and warrants further investigation. It is of note that a relatively high incidence of BM has been documented in adenocarcinoma and large cell carcinoma previously [31]. Additionally, a higher incidence of synchronous diagnosis of primary tumor and BM was evident for large cell and adenocarcinoma in our series, thus further highlighting the higher propensity of BM in these histological entities.

Concerning cases with synchronous diagnosis of BM and primary NSCLC tumor, we observed a strong effect of surgical strategy on patient outcome. Patients treated with resection of both the CNS and the primary tumors fared significantly better than patients treated with neurosurgery only. Our study is limited by its retrospective nature; in particular, the prescription bias with respect to the administered therapies may be a potential issue of concern resulting from poorly standardized therapy approaches in patients with BM. Furthermore, statistical power may be an additional issue and prospective, randomized trials are urgently warranted to reappraise our findings. However, compared to previous studies, we were able to include and investigate an appreciable sample size. Some small phase II prospective trials already exist and underscore our findings: these have shown that a subgroup of patients with synchronous diagnosis of NSCLC and oligometastatic disease benefits from multimodal treatment including BM and lung resection [32–34].

We observed significant longer survival in patients receiving a multimodal therapy approach including surgery, chemotherapy and WBRT as compared to patients receiving only adjuvant WBRT after surgery. Although a selection bias cannot be ruled out, our findings once more underscore the shaky and ambiguous value of adjuvant WBRT after neurosurgical resection of NSCLC BM [35, 36]. A prospective phase III trial failed to demonstrate an impact of adjuvant WBRT on OS in patients with one to three BM treated with neurosurgical resection or radiosurgery [37]. However, time to intracra-
nial disease progression was significantly prolonged in patients receiving WBRT. Considering the relative radiosensitivity of NSCLC and the long-term side effects of WBRT (e.g., neurocognitive decline), a predictive marker for patients profiting from adjuvant WBRT would be of clinical relevance [38–40]. Our data suggest that the HIF-1 alpha index, which is frequently used as a surrogate marker for hypoxia, might serve as a predictive marker for WBRT. This is in good agreement with the results of previous studies indicating the value of HIF-1 alpha expression as a predictor for the response to radiotherapy in various primary tumors [41].

Our results clearly suggest the need for prospective studies to further investigate whether stratification of BM patients for adjuvant WBRT based on HIF-1 alpha expression is a feasible strategy.

Conclusion

In order to make appropriate personalized and prognostic-based treatment decisions, prognostic scoring systems have to be as precise as possible and include all relevant prognostic aspects. To date, established prognostic scores only include clinical prognostic factors and do not include radiological or pathological findings [2–4, 42]. In the present study, we could demonstrate the impact of proliferation, MVD and hypoxia on survival in our cohort of patients with NSCLC BM. We illustrate by example that the addition of tissue-based parameters to traditional prognostic scores based on clinical parameters alone may improve discrimination between prognostic subgroups. Therefore, the inclusion of tissue-based prognostic factors should be considered, but needs to be validated in independent patient cohorts and prospective studies.

Acknowledgements. We thank Carina Dinhof, Gerda Reckeen, Irene Leisser, Ursula Rajky and Bettina Jesch for excellent technical assistance. The costs for this project were covered by the research budget of the Medical University of Vienna. This study was performed within the PhD thesis project of Anna Sophie Berghoff in the PhD program "Clinical Neuroscience (CLINS)" at the Medical University Vienna.

Compliance with ethical guidelines


References


6.4 Preoperative diffusion-weighted imaging of single brain metastases correlates with patient survival times.

Interlude
As previously mentioned survival estimation in patients with newly diagnosed BM is based on clinical characteristics [68]. Radiological parameters have not yet been included in the prognostic assessment, but might add valuable information as they correlate with histological characteristics, providing an indirect insight in a tumour’s microarchitecture. Diffusion weighted imaging visualizes the mobility of water molecules in the extracellular space. A hyperintense diffusion weighted imaging represents a low diffusion capacity i.e. a restricted mobility of water molecules in the extracellular space [129]. On the cellular basis, hyperintense diffusion weighted imaging was shown to correlate with high cellularity, poor differentiation and dense stromal matrix [129-131]. In the study “Preoperative diffusion-weighted imaging of single brain metastases correlates with patient survival times” we aimed to investigate the prognostic impact and tissue based correlation of diffusion weighted imaging in a cohort of patients with singular BM and neurosurgical resection as first line treatment approach. We observed that hyperintense preoperative diffusion weighted imaging correlated with high density of reticulin fibers in the tissue based analysis and an impaired survival prognosis [132].
Preoperative Diffusion-Weighted Imaging of Single Brain Metastases Correlates with Patient Survival Times

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Abstract

Background: MRI-based diffusion-weighted imaging (DWI) visualizes the local differences in water diffusion in vivo. The prognostic value of DWI signal intensities on the source images and apparent diffusion coefficient (ADC) maps respectively has not yet been studied in brain metastases (BM).

Methods: We included into this retrospective analysis all patients operated for single BM at our institution between 2002 and 2010, in whom presurgical DWI and BM tissue samples were available. We recorded relevant clinical data, assessed DWI signal intensity and apparent diffusion coefficient (ADC) values and performed histopathological analysis of BM tissues. Statistical analyses including uni- and multivariate survival analyses were performed.

Results: 65 patients (34 female, 31 male) with a median overall survival time (OS) of 15 months (range 0–99 months) were available for this study. 19 (29.2%) patients presented with hyper-, 3 (4.6%) with iso-, and 43 (66.2%) with hypointense DWI. ADCmean values could be determined in 32 (49.2%) patients, ranged from 456.4 to 1691.8*10^-6 mm^2/s (median 969.5) and showed a highly significant correlation with DWI signal intensity. DWI hyperintensity correlated significantly with high ADCmean values (30 months), respectively. In multivariate survival analysis, patients with hyperintense DWI (3 months) and low ADCmean values (7 months) had significantly worse OS than patients with iso/hypointense DWI (16 months) and high ADCmean values (30 months), respectively. In multivariate survival analysis, high ADCmean values retained independent statistical significance.

Conclusions: Preoperative DWI findings strongly and independently correlate with OS in patients operated for single BM and are related to interstitial fibrosis. Inclusion of DWI parameters into established risk stratification scores for BM patients should be considered.

Introduction

Metastases to the brain are a frequent complication of cancer and are associated with high morbidity and mortality. Primary tumor types vary in their propensity to form brain metastases (BM) with lung cancer, breast cancer and melanoma showing the highest incidences of central nervous system (CNS) involvement. [1,2] Treatment so far relies mainly on surgery and radiotherapy, although some targeted drugs have shown clinically meaningful activity in distinct molecular tumor subtypes and are beginning to enter clinical practice. [3,4,5].

The prognosis of BM patients is poor with median overall survival times of only few months. Several risk stratification scores have been developed such as the recursive portioning analysis (RPA), the graded prognostic assessment (GPA) and the diagnosis specific graded prognostic assessment (DS-GPA). [6,7,8] These scores are based on parameters with established prognostic impact including the Karnofsky performance status (KPS), patient age, status of the primary tumor, presence of extracranial metastases and the number of BM. [6,7,8] Median overall survival (OS) from diagnosis of BM varies extensively from 3 months in the least favourable group, up to 25.3 months in the most favourable groups which includes also long term survivors. [8,9] Neuroradiological variables, with the exception of the number of BM, are not considered for prognostic risk stratification so far.
Magnetic Resonance Imaging (MRI) using pre- and post-contrast T1-weighted imaging, T2-weighted imaging and fluid attenuated inversion recovery (FLAIR) is the modality of choice for radiological evaluation of brain tumors. [10] Increasingly, additional advanced radiological techniques like magnetic resonance spectroscopy (MRS), perfusion MRI, or diffusion-weighted imaging (DWI) are used to characterize brain lesions in order to provide further clinically relevant information. [11,12] DWI is an MRI method based on the visualisation of the mobility of water molecules in the extracellular space. A low diffusion capacity due to a restricted mobility of the water molecules in the extracellular space results in a hypointense signal in DWI and low apparent diffusion coefficient (ADC) values. In contrast, a high diffusion capacity due to an increased mobility of water molecules results in a hypo- or isointense DWI signals and high ADC values. [12] DWI parameters have been shown to correlate with various histopathological characteristics such as tumor type, tumor grade, Ki67 tumor cell proliferation index, cellularity, or amount of interstitial fibrosis and survival prognosis in several intra- and extracranial tumor types. [13,14,15,16,17,18,19,20,21]. However, the prognostic value of DWI and its correlation with histomorphological findings in patients with BM has not been systematically studied so far.

In the present study, we investigated the prognostic impact of DWI signal intensity and performed a correlative analysis with tissue-based parameters in a homogenous cohort of patients with single BM and surgery as first line treatment for BM.

Patients and Methods

Ethics Statement

The study was approved by the local ethics committee of the Medical University of Vienna, Austria. No written consent was given by the patients for their information to be stored in the database and used for research, because this study was performed in a retrospective manner in line with local regulations. The institutional ethics committee waived the need for written informed consent from the participants for this project (Ethics committee protocol number 641/2011).

Patients

We identified all patients with radiologically proven single BM who underwent surgery as a first-line-therapy for a single BM between April 2002 and December 2010 and whose presurgical MR work-up included DWI. Availability of at least one tissue block for research purposes with viable BM tissue and full information on the clinical course including date of diagnosis, administered therapies, Karnofsky performance score, GPA and date of death or date of last follow-up investigation were mandatory for inclusion. All clinical parameters were retrieved by chart review and from the database of National Cancer Registry of Austria and the Austrian Brain Tumor Registry. [22].

Imaging Analysis

All imaging analyses were performed by one investigator (TS) blinded to all clinical and histological data. In conventional MRI (contrast-enhanced and native T1-weighted images, FLAIR and T2-weighted images, as available) the maximum diameter and localization of the single BM was determined. In DWI, the BM was semiquantitatively judged to be either hypointense, isointense, or hyperintense in comparison to normal non-pathological brain tissue. In BM which showed heterogeneous signal behaviour, diffusion intensity was graduat-ed upon the predominant (>70% of the metastasis) signal behaviour. In the cases with available ADC maps, ADC values were derived as described previously in up to 5 non-overlapping areas of interest (each at least 50 mm²) in solid, non-necrotic, non-macrohemorrhagic areas of the BM. In each case, the mean ADC value (ADCmean) was calculated from the ADC values of all areas of interest. [13].

Tissue Based Analysis

Tissue based analysis was performed blinded to clinical and radiological data. Histological confirmation of BM was evaluated on routinely performed hematoxylin and eosin (H&E) stained tissue sections by a specialist in neuropathology. Cellularity was evaluated semiquantitatively on an H&E section as low, moderate and high. Gomori silver impregnation stain for reticulin was performed according to laboratory standard. The amount of extracellular reticulin fibers was semiquantitatively grouped as follows: prominent interstitial fibrosis (more than 25% of the tumor tissue displaying a dense interstitial meshwork of reticulin fibres); little interstitial fibrosis (less than 25% of the tumor tissue displaying a dense interstitial meshwork of reticulin fibres). Ki67 (antibody MIB1, Dako, Glostrup, Denmark) immunostaining and analysis was performed as previously published. [23] Ki67 proliferation index was obtained by counting 500 cells and giving the percentage of positive cells (0–100%). [23] Differentiation of the tumor tissue was divided into well, moderately and poorly differentiated based on the tumor organization, the cell polymorphism and the mitotic activity.

Statistical Analysis

For correlation of parameters the Spearman's correlation coefficient, Chi square test or the Mann-Whitney U test were used as appropriate. Overall survival (OS) was defined as time from first diagnosis of BM until death or last day of follow up. For all tests, a two-sided p-value of <0.05 was considered as statistically significant. For univariate survival analysis Kaplan-Meier curves and the log-rank test were used. Variables that showed statistically significant prognostic value at univariate survival analysis were entered in multivariate survival analysis using the Cox regression model.

The statistical software package SPSS version 19 (SPSS Inc, Chicago, IL, USA) was used for all calculations.

Results

Patients' Characteristics

65 patients (31 female, 34 male) with a median age of 59 years (range 33–80) at first diagnosis of BM were available for this study. All patients had a single BM and surgery as first line treatment for BM. 45/65 (69.2%) of patients had adjuvant whole brain radiotherapy (WBRT) and 28/65 (43.1%) adjuvant chemotherapy after surgery of BM. Table 1 lists further patients' characteristics.

Imaging Analysis

19/65 (29.2%) of patients presented with hyperintense, 3/65 (4.6%) with isointense and 43/65 (66.2%) with hypointense DWI signals. Clinical characteristics including primary tumor types, size of BM, patient age, status of primary tumor, presence of extracranial metastases and GPA did not differ between DWI signal intensity groups (p>0.05; Chi square test; table 2).

ADC maps were available for 32 patients. The median ADCmean value was 969.47*10⁻⁹ mm²/s. ADC values strongly
correlated with signal intensity in isotropic DWI (p<0.001, Mann-Whitney U test; table 2). Clinical characteristics including primary tumor types, size of BM, patient age, status of primary tumor, presence of extracranial metastases, GPA and KPS did not correlate with ADCmean values (p>0.05; Mann Whitney U test).
Tissue Based Findings

14/65 (21.5%) specimens were classified with low, 30/65 (46.2%) with moderate and 21/65 (32.3%) with high cellularity based on H&E histomorphology.

No statistically significant correlation of DWI signal intensity or ADCmean and cellularity was observed (p < 0.05; Chi square test and Mann-Whitney U test, respectively). 3/65 (4.6%) specimens were classified as well differentiated, 12/65 (18.5%) as moderately differentiated and 46/65 (70.8%) as poorly differentiated. No statistically significant correlation of DWI signal intensity or ADCmean and differentiation was observed (p < 0.05; Chi square test and Mann-Whitney U test, respectively). Mean ki67 proliferation index was 44.4% (range 5.4% –89.6%) and did not correlate with DWI signal intensity (p < 0.05; Mann-Whitney U test) or ADCmean values (Spearmans correlation coefficient r = - 0.3, p = 0.09). 24/65 (36.9%) specimens presented with prominent interstitial fibrosis while 41/65 (63.1%) showed little interstitial fibrosis. Semiquantitative DWI signal intensity showed a significant correlation with density of the reticulin network: tumors with restricted diffusion showed higher amounts of interstitial fibrosis and tumors with unrestricted diffusion showed less interstitial fibrosis (p = 0.02; Chi square test; table 2, figure 1).

Survival Analyses

Median OS from first diagnosis of BM to death was 15 months (range 0–99 months) in the entire population.

In univariate analysis, patients with hypo/isointense DWI signal intensity showed a significantly longer survival with a median OS of 16 months (95% CI: 10.79–21.25) than patients with hyperintense DWI signal intensity with a median OS of 5 months (95% CI: 0–12.47; p = 0.029; log rank test; figure 2). Patients with high ADCmean values showed a significantly longer survival with a median OS of 30 months (95% CI: 13.97–46.03) than patients with low ADCmean values with a median OS of 7 months (95% CI: 1.51–12.49; p = 0.02; log rank test; figure 2). Furthermore, primary tumor type, Karnofsky performance score <70, lack of adjuvant WBRT after neurosurgery and high GPA were significantly associated with unfavourable OS in univariate analysis (p<0.05). Adjuvant chemotherapy after surgery for BM had no statistically significant impact on OS (p>0.05).

All factors with statistically significant impact on OS in the univariate analysis were included in multivariate analysis (ADCmean, primary tumor type, KPS and adjuvant WBRT (yes/no)). Primary tumor type as well as KPS and adjuvant WBRT did not remain statistically significant in multivariate analysis. Only high ADCmean values remained as statistically significant independent

### Table 2. Diffusion weighted imaging analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DWI signal intensity</th>
<th>Chi square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypo/isointense</td>
<td>Hyperintense</td>
</tr>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
</tr>
<tr>
<td>Age at first diagnosis of BM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>26 74.3 9 25.7</td>
<td>0.50</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>20 66.7 10 33.3</td>
<td></td>
</tr>
<tr>
<td>Primary tumor type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung cancer</td>
<td>19 76 6 24</td>
<td>0.22</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>6 75 2 35</td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>3 60 2 40</td>
<td></td>
</tr>
<tr>
<td>Kidney cancer</td>
<td>5 100 0 0</td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>3 100 0 0</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>10 53.8 9 46.2</td>
<td></td>
</tr>
<tr>
<td>Status of primary tumor at first diagnosis of BM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synchronous diagnosis</td>
<td>20 69 9 31</td>
<td>0.14</td>
</tr>
<tr>
<td>No evidence of disease</td>
<td>14 73.7 5 26.3</td>
<td></td>
</tr>
<tr>
<td>Stable disease</td>
<td>7 63.6 4 36.4</td>
<td></td>
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<tr>
<td>Progressive disease</td>
<td>5 83.3 1 16.7</td>
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<td>Presence of extracranial metastases</td>
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<td></td>
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<td>yes</td>
<td>13 59.1 9 40.9</td>
<td>0.16</td>
</tr>
<tr>
<td>no</td>
<td>33 76.7 10 23.3</td>
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<td>Karnofsky performance score</td>
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<tr>
<td>&lt;70</td>
<td>1 25 3 75</td>
<td>0.04</td>
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<tr>
<td>&gt;70</td>
<td>45 73.8 16 26.2</td>
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<td>GPA class</td>
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<tr>
<td>I</td>
<td>19 82.6 4 17.4</td>
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</tr>
<tr>
<td>II</td>
<td>10 66.7 5 33.3</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>16 64 9 36</td>
<td></td>
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<td>IV</td>
<td>1 50 1 50</td>
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<td>BM localisation</td>
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<td>Infratentorial</td>
<td>14 73.7 5 26.3</td>
<td>0.74</td>
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<tr>
<td>Supratentorial</td>
<td>32 69.6 14 30.4</td>
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</tr>
<tr>
<td>Size of BM</td>
<td></td>
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<tr>
<td>&lt;3 cm</td>
<td>16 72.7 6 27.3</td>
<td>0.80</td>
</tr>
<tr>
<td>&gt;3 cm</td>
<td>30 69.8 13 30.2</td>
<td></td>
</tr>
<tr>
<td>ADCmean</td>
<td></td>
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<tr>
<td>&lt;969.47*10^-6 mm²/s</td>
<td>8 44.4 10 55.6</td>
<td>0.001</td>
</tr>
<tr>
<td>&gt;969.47*10^-6 mm²/s</td>
<td>15 100 0 0</td>
<td></td>
</tr>
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<td>Cellularity</td>
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<tr>
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<td>9 64.3 5 35.7</td>
<td>0.86</td>
</tr>
<tr>
<td>Moderate</td>
<td>22 73.3 8 26.7</td>
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</tr>
<tr>
<td>High</td>
<td>15 71.4 6 28.6</td>
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<tr>
<td>Differentiation</td>
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<tr>
<td>Low</td>
<td>34 73.9 12 26.1</td>
<td>0.50</td>
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<tr>
<td>Moderate</td>
<td>8 66.7 4 33.3</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3 100 0 0</td>
<td></td>
</tr>
<tr>
<td>Ki67 proliferation index</td>
<td></td>
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<tr>
<td>0–25%</td>
<td>12 75 4 25</td>
<td>0.81</td>
</tr>
<tr>
<td>25.1–50%</td>
<td>15 71.4 6 28.6</td>
<td></td>
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</tbody>
</table>

Table 2. Diffusion weighted imaging analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DWI signal intensity</th>
<th>Chi square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypo/isointense</td>
<td>Hyperintense</td>
</tr>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
</tr>
<tr>
<td>50.1–75%</td>
<td>17 70.8 7 29.2</td>
<td></td>
</tr>
<tr>
<td>75.1–100%</td>
<td>2 50 2 50</td>
<td></td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Little</td>
<td>33 80.5 8 19.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Prominent</td>
<td>13 54.2 11 45.8</td>
<td></td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0055464.t002
Figure 1. T1-weighted and diffusion weighted imaging of a patient with hyperintense DWI signal intensity (A, B) and of a patient with hypointense DWI signal intensity (D, E) and the Gomori silver impregnation stain for reticulin in these patients showing dense reticulin network (C) and scattered reticulin network (F).

doi:10.1371/journal.pone.0055464.g001

Figure 2. Kaplan Meier plots showing the statistically significant association of DWI signal intensity (A), ADCmean values (B).

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In this study, we found a highly significant association of pre-surgical DWI parameters with OS times of patients operated for single BM. Both, DWI signal intensity as assessed semiquantitatively by visual impression and ADCmean values stratified patients into prognostic groups. The median OS of patients with tumors showing hyperintense DWI was 5 months compared to 16 months in patients with iso- or hypointense DWI signals (p = 0.029; log rank test). ADCmean values showed an even stronger separation of risk groups with patients with high ADCmean values showing more than 4-times longer median OS (30 months) than patients with low ADCmean values (7 months; p = 0.008; log rank test). The prognostic impact of ADC values was independent from known prognostic factors including GPA class, the primary tumor type and the KPS and also from postoperative therapy including adjuvant WBRT and chemotherapy in multivariate analysis (Hazard ratio 0.32, 95% CI 0.12–0.91; p = 0.03; Cox regression model; table 3).

Discussion

In this study, we found a highly significant association of pre-surgical DWI parameters with OS times of patients operated for single BM. Both, DWI signal intensity as assessed semiquantitatively by visual impression and ADCmean values stratified patients into prognostic groups. The median OS of patients with tumors showing hyperintense DWI was 5 months compared to 16 months in patients with iso- or hypointense DWI signals (p = 0.029; log rank test). ADCmean values showed an even stronger separation of risk groups with patients with high ADCmean values showing more than 4-times longer median OS (30 months) than patients with low ADCmean values (7 months; p = 0.008; log rank test). The prognostic impact of ADC values was independent from known prognostic factors including GPA class, the primary tumor type and the KPS and also from postoperative therapy including adjuvant WBRT and chemotherapy in multivariate analysis (Hazard ratio 0.32, 95% CI 0.12–0.91; p = 0.03; Cox regression model).

While, to the best of our knowledge, the correlation of DWI parameters with patient outcomes has not been investigated in BM, some studies have postulated a prognostic value of DWI signal intensity in primary tumors. In high grade gliomas a hyperintense DWI signal with low ADCmean values resembles areas of high cellularity with high cytoplasm to nucleus ratio. [14,15] Similar, a correlation of hyperintense DWI signal intensity and poor tumor differentiation was shown for extracranial tumors like lung cancer, breast cancer or rectal cancer. [19,21,24] For the instance of BM, a low ADCmean value was shown to correlate with high tumor cellularity and poor tumor differentiation. [26,27] In our study, we could demonstrate a significant correlation of a prominent interstitial fibrosis with signs of restricted diffusion in DWI, which resembles the impaired mobility of water molecules in the intercellular space. The interstitial reticulin fiber network is part of the fibrotic collagen-rich tumor stroma and our data further emphasize the importance of the microenvironment in the pathobiology of BM. [28] In line with our results, several other tumor types with a restricted diffusion due to dense stromal matrix were shown to have an impaired survival prognosis. [29,30,31].

Our study has some limitations that need to be acknowledged. We performed a retrospective study in a single center and were

| Table 3. Survival analysis from first diagnosis of brain metastasis to death. |
|----------------------------------|-----------------|----------------|-----------------|-----------------|
| Parameter                        | Median OS, months| 95% Confidence interval | Log-rank test | Cox regression model |
| ADCmean                          |                 |                      |                |
| <969.47                          | 7               | 1.51–12.49           | 0.008          | 0.03            |
| >969.47                          | 30              | 13.97–46.03          |                |
| Primary tumor type               |                 |                      |                |
| Lung cancer                      | 21              | 11.81–30.19          | 0.015          | 0.64           |
| Breast cancer                    | 12              | 0–31.40              |                |
| Melanoma                         | 4               | 0.78–7.22            |                |
| Kidney cancer                    | not reached     | not reached          |                |
| Colorectal cancer                | 11              | 0–22.20              |                |
| Others                           | 12              | 5.76–18.24           |                |
| Karnofsky performance            |                 |                      |                |
| <70                              | 1               | 0–3.94               | 0.001          | 0.06           |
| >70                              | 15              | 11.73–24.27          |                |
| GPA class                        |                 |                      |                |
| I                                | 21              | 16.57–25.43          | 0.001          | 0.48           |
| II                               | 21              | 1.77–40.23           |                |
| III                              | 12              | 0.28–23.73           |                |
| IV                               | 0               | -                    |
| WBRT after surgery               |                 |                      |                |
| Yes                              | 18              | 12.66–23.34          | 0.034          | 0.41           |
| No                               | 5               | 0–11.57              |                |

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prognostic factors (Harzard ratio 0.32, 95% CI 0.12–0.91; p = 0.03; Cox regression model; table 3).
able therefore to include at least a limited number of cases, thus restricting the statistical power of our correlative analyses. On the other hand, our approach enabled us to analyse a well-defined patient cohort characterized by single brain metastasis homogeneously treated by neurosurgical BM resection as the initial therapy. Another limitation related to the retrospective nature of our study is the fact that ADC maps could not be retrieved in all cases. In the absence of ADC maps, diffusion restriction as cause of DWI hyperintensity cannot be unequivocally differentiated from other phenomena such as T2-shine through. [32] Further, the accuracy of ADC values is potentially limited due to the usage of different MRI machines. [33] However, we found a strong correlation of ADC values and semiquantitative DWI signal intensity in the cohort of 32 patients of whom both parameters were available. Furthermore, in this cohort the prognostic impact of ADC values was even more pronounced than the semiquantitatively evaluated DWI signal intensity. Still, our findings need to be reproduced in independent data sets, preferably in prospective studies.

In conclusion, we could demonstrate the independent prognostic value of DWI findings in our large homogenous cohort of patients with a single BM and its correlation with tissue based characteristic, indicating the value of DWI signal intensity as an imaging biomarker. Future studies should prospectively evaluate the prognostic value and the inclusion in prognostic scores of DWI parameters.

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Author Contributions

Conceived and designed the experiments: ASB TS AIM MM M. Hutterer AW M. Hackl GW KD CM PB DP MP. Performed the experiments: ASB TS AIM MM M. Hutterer AW M. Hackl GW KD CM PB DP MP. Analyzed the data: ASB TS AIM MM M. Hutterer AW M. Hackl GW KD CM PB DP MP. Contributed reagents/materials/analysis tools: ASB TS AIM MM M. Hutterer AW M. Hackl GW KD CM PB DP MP. Wrote the paper: ASB TS AIM MM M. Hutterer AW M. Hackl GW KD CM PB DP MP.

References


6.5 Invasion patterns in brain metastases of solid cancers.

Interlude
BM are described as well delineated lesions towards the surrounding brain parenchyma [133]. However, preclinical studies suggest that invasion patterns might differ as growth alongside pre-existing vessels was observed in a melanoma BM mouse model [43]. Further, a diffusely infiltrating growth, as observed in glioblastoma, was observed in some cases of small cell lung cancer [134]. Although different invasion patterns, namely expansive growth, multicellular migration and individual cell migration were postulated for extracranial tumours, no study systematically investigated the invasion patterns of BM and the involved molecular factors [135]. In order to investigate the interaction of BM with the surrounding brain parenchyma, we conducted a cohort of BM autopsy specimen containing viable brain metastasis tumour tissue but as well the surrounding brain parenchyma. In the paper “Invasion patterns in brain metastases of solid cancers” we investigated the infiltrative pattern of BM [136]. We observed a well-demarcated growth, growing rather through outwardly extending than through infiltration only in half of the investigated specimens. Expression of adhesion molecule integrin alpha v beta 6, which is known to modulate invasion and inhibit apoptosis, was associated with well-demarcated growth. Growth either via vascular co-option or a glioma like diffuse single cell infiltration was observed in the remaining investigated specimens. The high fraction of BM showing an infiltrative growth pattern has clinical implication as the inclusion of a safety margin in local therapy approaches such as neurosurgery and radiosurgery, has to be discussed in cases with infiltrating growth in order to prevent local recurrences. Concerning the primary tumour, growth via vascular co-option was more frequently observed in melanoma BM, while small cell lung cancer BM showed a high propensity for diffuse single cell infiltration.
Invasion patterns in brain metastases of solid cancers

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Background. Brain metastases are generally considered to be well demarcated from the surrounding brain parenchyma, although infiltrative growth patterns have been observed. We systemically investigated infiltration patterns and expression of adhesion molecules in a large and well-defined series of autopsy cases of brain metastases.

Methods. Ninety-seven autopsy specimens from 57 brain metastasis patients (primary tumor: 27 lung cancer, 6 breast cancer, 8 melanoma, 2 colorectal cancer, 1 kidney cancer, and 13 other) were evaluated for patterns of invasion into surrounding brain parenchyma. Expression of integrins αv; cytoplasmic β3, αvβ3, αβ3, αvβ6, and αvβ8; and of E and N cadherin were evaluated using immunohistochemistry.

Results. Three main invasion patterns were seen: well-demarcated growth (29/57, 51%), vascular co-option (10/57, 18%), and diffuse infiltration (18/57, 32%). There was no statistically significant association of invasion pattern with primary tumor type, although vascular co-option was most common in melanoma brain metastases (4/10). Invasion patterns of different brain metastases of the same patient were highly concordant (P < .001, chi-square test). Distance of infiltration from the main tumor mass ranged from 12.5 μm to 450 μm (median 56.2 μm) and was not significantly different between the vascular co-option and the diffuse infiltration groups. Levels of αvβ6 were significantly higher in the well-demarcated group than in the vascular co-option and the diffuse infiltration groups (P = .033, Kruskal-Wallis test). Expression of αvβ5 in tumor cells was higher in brain metastasis lesions previously treated with stereotactic radiosurgery (P = .034, chi-square test).

Conclusions. Distinct invasion patterns of brain metastases into the brain parenchyma are not specific for primary tumor types, seem to be influenced by expression of αv integrin complexes, and may help to guide clinical decision-making.

Keywords: adhesion molecules, brain metastases, cadherin, integrin, invasion.

Brain metastases are a frequent complication in oncology and affect up to 40% of patients with metastatic cancer.1 While the incidence of brain metastases has shown a constant increase over the last decades, treatment options remain limited and rely mainly on local approaches like surgery, radiosurgery, or whole brain radiotherapy (WBRT).2-4 Better understanding of the pathobiology of brain metastases may lead to novel treatments.

Brain metastases are usually regarded as growing in a well-delineated fashion within the brain parenchyma.5 This notion is based mainly on their neuroradiological presentation with relatively sharp demarcation of contrast-enhancing areas and a generally better delineation than that of malignant gliomas. However, the histological patterns of invasion in brain metastases have so far not been addressed in comprehensive studies, although infiltrative behavior has occasionally been noted.6,7 Clinically,
infiltrative behavior with unclear resection margins is regularly noted by neurosurgeons, and high local recurrence rates after surgery and radiosurgery have been reported for such cases.8,9

In general, cancer cells grow and invade solid tissues in different ways such as expansive growth, multicellular migration, and individual cell migration.10 Migration and invasion require complex regulation of specific molecules, including adhesion molecules (eg, integrins, cadherins), cytoskeletal components (eg, actomyosin), proteolytic enzymes (eg, matrix metalloproteases [MMPs]), and others.10–13 However, the types of invasive behavior of tumor cells have been described mostly in models of non-CNS tissues (eg, skin), and it is unknown whether similar mechanisms are active in the brain, with its distinct microenvironment. The CNS microenvironment differs from that of other solid organs. The brain parenchyma is composed of highly specialized cells (neurons, astrocytes, oligodendrocytes, microglia), and its extracellular matrix (ECM) has a distinct composition. It lacks constituents usually found in solid organs, such as fibronectin and collagen, but it is rich in proteoglycans, tenasin, laminin, heparin/chondroitin/dermatan sulfates, and hyaluronic acid.14

In this study we systematically characterized the invasion patterns of brain metastases and their correlation with the expression of several adhesion molecules in a series of autopsy specimens. Surgery specimens are not suitable for such studies, since in most cases they include no or only little well-preserved brain tissue around the resection margin and are thus not sufficient for investigation of the invasion front and the interaction of cancer cells with the brain parenchyma.

Materials and Methods

Patients

All patients with histologically proven brain metastases who underwent brain autopsy between 1987 and 2011 were identified from the Neuro-Biobank of the Medical University of Vienna. From each patient, at least one representative formalin-fixed and paraffin-embedded tissue block containing tumor tissue and surrounding brain parenchyma was selected. Clinical and demographic data were retrieved by chart review. This study was approved by the ethics committee of the Medical University of Vienna (ethics committee protocol number 078/2004).

Evaluation of Invasion Patterns

Evaluation of invasion patterns was performed on one routinely stained hematoxylin and eosin (H&E) section per tumor block. For enhanced visibility and better evaluation of single tumor cells, immunohistochemistry for cytokeratin (carcinomas) or HMGB45 (melanomas) and for evaluation of vascular structure immunohistochemistry for CD34 was performed on an automated horizontal slide-processing system (AutostainerPlusLink, Dako) using standard protocols in selected cases (Table 1).15–17 Maximal invasion distance of tumor cells from the main tumor mass was microscopically measured on H&E slides with a grid of 25 μm length at 400× magnification.

Immunohistochemistry and Evaluation of Integrins and Cadherins

Immunohistochemistry for the αv subunit, cytoplasmic β3, and αvβ3, αvβ5, αvβ6, and αvβ8 complexes was performed with a fully automated multimodal slide-staining system (BenchMark, Ventana Medical Systems) as described previously.18 In brief, an indirect biotin-avidin system with standard cell conditioner 1 and EDTA pretreatment protocol were used. Signal amplification was achieved with a copper enhancer (iView DAB Detection Kit, Ventana Medical Systems).19 Immunohistochemistry for E cadherin and N cadherin was performed using an automated horizontal slide-processing system (AutostainerPlusLink, Dako). In brief, antigen retrieval was performed with pH9 buffer (Flex TRS high, Dako). Slides were incubated with primary antibody for 1 h for N cadherin (anti-N cadherin antibody, ab18203, solution 1:500, abcam) and overnight for E

Table 1. Antibodies

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>Clone</th>
<th>Dilution</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>αv subunit</td>
<td>EM01309</td>
<td>1:1000*</td>
<td>HT29 colon cancer cell line, DU-145 prostate cancer cell line</td>
</tr>
<tr>
<td>β3 subunit</td>
<td>EM00212</td>
<td>1:500*</td>
<td>Normal kidney, malignant melanoma</td>
</tr>
<tr>
<td>αvβ3</td>
<td>EM22703</td>
<td>1:500</td>
<td>Normal kidney, malignant melanoma</td>
</tr>
<tr>
<td>αvβ5</td>
<td>EM09902</td>
<td>1:800*</td>
<td>Normal colon tissue, HT29 colon cancer cell line</td>
</tr>
<tr>
<td>αvβ6</td>
<td>EM05201</td>
<td>1:1000*</td>
<td>Normal kidney, HT29 colon cancer cell line</td>
</tr>
<tr>
<td>αvβ8</td>
<td>EM13309</td>
<td>1:1000*</td>
<td>Normal peripheral nerve, Ovcar-3 ovarian cancer cell line</td>
</tr>
<tr>
<td>E cadherin</td>
<td>Ab15148</td>
<td>1:30</td>
<td>Skin</td>
</tr>
<tr>
<td>N cadherin</td>
<td>Ab18203</td>
<td>1:500</td>
<td>Liver carcinoma</td>
</tr>
<tr>
<td>CD34</td>
<td>NCL-END</td>
<td>1:500</td>
<td>Glioblastoma</td>
</tr>
<tr>
<td>Pan-cytokeratin</td>
<td>Lu5</td>
<td>1:100</td>
<td>Lung carcinoma</td>
</tr>
<tr>
<td>HMGB45</td>
<td>HMB45</td>
<td>1:50</td>
<td>Melanoma</td>
</tr>
</tbody>
</table>

*Protein concentration 1 mg/mL.
Patients received WBRT during the course of disease, 7/57 (12.3%) received SRS of the brain metastasis investigated in this study, and 17/57 (29.8%) patients received chemotherapy during the course of disease. Upon diagnosis of brain metastases, 34/57 (59.6%) patients were treated with best supportive care. According to autopsy protocols, brain metastases were the cause of death in 19/57 (33.3%) patients. Table 2 summarizes further the patients’ characteristics, and detailed compilation is given in Supplemental Table S1.

**Invasion Patterns of Brain Metastases**

**Histomorphology.**—We delineated 3 distinct invasion patterns: 29/57 (50.9%) brain metastases showed a distinct, well-demarcated border to the surrounding brain parenchyma (well-demarcated group); 10/57 (17.5%); Fig. 1A) brain metastases showed distinct perivascular protrusions of multicellular tumor cell formations from the main tumor mass into the brain parenchyma (vascular co-option group; Fig. 1B); and 18/57 (31.6%) brain metastases showed a diffuse infiltration of single tumor cells into the surrounding brain parenchyma (diffuse infiltration group; Fig. 1C). Generally, the invasion pattern was consistent throughout major parts (>90% of the border) of the tumor/brain border in individual metastases.

In 30/57 (52.6%) cases, multiple distinct brain metastases of the same patient were available for investigation, and the invasion type was generally highly congruent among the lesions (P < .001, chi-square test; Table 3). Median maximal measurable invasion distance of tumor cells from the border of the main tumor mass was 68.7 μm (range 12.5–125 μm) in the vascular co-option group and 56.2 μm (range 12.5–450 μm) in the diffuse infiltration group. The maximal measurable invasion distance was not different between the vascular co-option group and the diffuse infiltration group (P = .486, t-test).

**Correlation with primary tumor type.**—Brain metastases of melanoma tended to grow via vascular co-option more often than other primary tumors (4/8). The most frequent primary tumor in the well-demarcated as well as in the diffuse infiltration group was lung cancer. Brain metastases of small cell lung cancer (SCLC) showed frequently diffuse infiltrative growth (2/3), whereas non-SCLC (NSCLC) grew rather well demarcated (13/24, 54.2%). Squamous NSCLC was more common in the well-demarcated group (4/6), whereas adenocarcinoma NSCLC was equally represented in the well-demarcated (5/12, 41.7%) and the diffuse infiltration group (5/12, 41.7%). See Table 2 for correlation of invasion patterns with primary tumor type.

**Correlation with treatment.**—Complete information on applied therapies after diagnosis of brain metastases was available for 55/57 (96.5%) patients. No statistically significant association was observed between first-line or other brain metastasis treatment and invasion patterns (Table 2). In the diffuse infiltration group, a higher proportion of patients had received WBRT at any time...
point (4/18; 22%) compared with the well-demarcated group (2/28, 7.1%) or the vascular co-option group (1/10). Further, a higher proportion of patients had received chemotherapy during their course of disease in the diffuse infiltration group (8/17, 47.1%) than in the well-demarcated (8/28, 28.6%) or the vascular co-option group (1/10).

**Correlation with clinical characteristics.**—Survival times from first diagnosis of brain metastases were available in 37 patients. Median survival from diagnosis of brain metastases was 2.0 months in the well-demarcated group (n = 19), 1.8 months in the vascular co-option group (n = 5), and 1.8 months in the diffuse infiltration group (n = 13). There was no statistically significant correlation of invasion pattern with survival time from diagnosis of brain metastases (P = .945, log-rank test). Patients in the well-demarcated group had more often a singular brain metastasis at first diagnosis of brain metastases (52%) than the vascular co-option (33.3%) or diffuse infiltration group (35.7%; P = .483, chi-square test). Extracranial metastases were present in 19/57 (33.3%) patients. No difference in the presence of extracranial metastases was observed between the 3 invasion patterns (P = .781, chi-square test). In the cohort of all 56 patients, there was no significant association of invasion pattern with survival time (P = .825, log-rank test).

**Correlation with macroscopic pathology findings and neuroradiology.**—Illustrative correlations of macroscopic pathology and neuroradiological findings with histological invasion patterns are shown in Fig. 1. The low number of available macroscopic photographs (n = 4) and premortal neuroradiological images (n = 5) precluded systematic correlation with histological findings.

**Integrin Expression**

**General description.**—Alpha-v integrins showed strong membranous expression on tumor, vascular, and stromal cells in variable fractions of cases. In general, the majority of specimens showed homogeneous αv integrin expression patterns throughout the tumor tissue, except for αvβ8 expression, which was absent in the majority of specimens (Supplemental Table S1). However, regional accentuation of integrin expression was observed in some specimens. Accentuated expression in perivascular tumor cells was observed in 11/57 (19.3%), 9/57 (15.8%), and 4/57 (7.0%) cases for the αv subunit, αvβ6, and αvβ5, respectively. Perinecrotic overexpression of αvβ6, αv subunit, and αvβ5 was found in 4/57 (7.0%), 2/57 (3.5%), and 2/57 (3.5%) cases, respectively (Fig. 2).

Analyzing expression on vascular structures, we observed αvβ5 integrin expression on all (57/57, 100%)
vessels, including tumoral and peritumoral vessels as well as the vascular structures of the surrounding brain parenchyma. Alpha-vβ3 expression was not observed on the vascular structures of the surrounding brain parenchyma, except randomly on some larger vessels of the meninges. Prominent expression of αvβ3 was observed on angiogenic, sprouting vessels with multilayered endothelium within the tumor and in the peritumoral area. In 30/57

### Table 3. Concordance between first, second, and third brain metastases

<table>
<thead>
<tr>
<th>First Brain Metastasis (n = 57)</th>
<th>Well-demarcated (n = 29)</th>
<th>Vascular Co-option (n = 10)</th>
<th>Diffuse Infiltration (n = 18)</th>
<th>Chi-square Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second brain metastasis (n = 30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-demarcated</td>
<td>14 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>Vascular co-option</td>
<td>1 (17)</td>
<td>5 (80)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Diffuse infiltration</td>
<td>0 (0)</td>
<td>1 (10)</td>
<td>9 (90)</td>
<td></td>
</tr>
<tr>
<td>Third brain metastasis (n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-demarcated</td>
<td>3 (60)</td>
<td>1 (20)</td>
<td>1 (20)</td>
<td>P = .023</td>
</tr>
<tr>
<td>Vascular co-option</td>
<td>0 (0)</td>
<td>3 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Diffuse infiltration</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (100)</td>
<td></td>
</tr>
</tbody>
</table>
(52.6%) specimens, immunoreactivity for αvβ3 of the angiogenic tumor vessels was observed, and in 29/57 (50.9%) specimens, angiogenic vessels in the peritumoral area showed specific immunoreactivity for αvβ3. Specific immunoreactivity for the β3 subunit was observed in angiogenic tumor vessels of 46/57 (80.7%) specimens and in angiogenic vessels in the peritumoral area of 45/57 (78.9%) specimens (Fig. 3). The expression detected for the αvβ3 complex was generally lower than for the β3 cytoplasmic domain. The β3 chain is on 2 integrin complexes, αvβ3 and glycoprotein IIbIIIa (the platelet fibrinogen receptor). Tissue localization suggested that the staining of β3 was not due to platelet deposits or aggregates. The αvβ3 antibody used (EM22703) preferentially binds particular ligated conformation of αvβ3, while the cytoplasmic-β3 antibody (EM00212) does not discriminate. This may explain the difference in staining results between these antibodies.

Fibrous tumoral stroma was observed in 32/57 (56.1%) specimens and showed expression of the αv subunit (32/32, 100%), αvβ3 (2/32, 6.3%), αvβ5 (28/32, 87.5%), and αvβ6 (6/32, 18.8%).

Relative overexpression of αv integrins at the invasion front was not consistently found, but only in some specimens (αv subunit: 8/57, 14.0%; αvβ5: 8/57, 14.0%; αvβ6: 8/57, 14.0%).

**Correlation with invasion patterns.**—Median H-score of αvβ6 was significantly higher in the well-demarcated group (median 90, range 0–300) than in the vascular co-option group (median 0, range 0–120) and the diffuse infiltration group (median 30, range 0–120; P = .033, Kruskal–Wallis test). No correlation of invasion pattern and median H-score of αv subunit, αvβ3, αvβ5, αvβ8, or β3 subunit was observed.

**Correlation with therapy.**—Brain metastases previously treated with SRS presented more frequently with αvβ3 expression in the tumor cells (6/7) than did specimens without prior SRS (21/49, 42.9%; P = .34, chi-square test). Furthermore, the median αvβ5 H-score was significantly higher in brain metastases with prior SRS (60 ± 0; P = .05, Mann–Whitney U-test). No correlation between SRS treatments and αv subunit, αvβ3, αvβ6, αvβ8, or β3 subunit expression was observed. Prior WBRT or chemotheraphy did not show a significant correlation with expression of any of the integrin subunits investigated in this study.

**Cadherin Expression**

Of the tumor specimens studied, 42/57 (73.7%) showed expression of E cadherin, 10/57 (17.5%) showed expression of N cadherin, and 6/57 (10.5%) showed expression of both cadherins. No significant correlation of median H-score of E cadherin or N cadherin and invasion pattern or primary tumor types was observed. Nine of 57 (15.8%) specimens showed increased E cadherin expression at the invasion front. Three of 57 (5.3%)
specimens showed overlapping increased expression at the invasion front of E cadherin and αv, 2/57 (3.5%) showed overlapping expression with αvβ5, and 1/57 (1.8%) specimen showed overlapping expression with αvβ6 at the invasion front. No cadherin expression was observed in the tumor stroma or vascular structures.

Discussion

Brain metastases are an increasing challenge in oncological practice, as survival in many types of solid cancers is increased by novel treatment strategies. The dominant treatment strategies for brain metastases are local and include surgery and radiosurgery. The benefit from local treatments is likely to be heavily affected by the degree to which macroscopically focal disease is truly focal on a microscopic level. Accordingly, the brain metastasis/brain interface may assume major prognostic significance.

Here, we delineate 3 distinct invasion patterns of brain metastases: well-demarcated growth, vascular co-option, and diffuse infiltration. We found a high fraction of cases showing invasive growth via vascular co-option (18%) or single-cell infiltration (32%). These surprising findings challenge the general notion that brain metastases predominantly grow in an expansive and well-delineated fashion, but they are in good agreement with previous results from experimental studies, smaller and less comprehensive investigations on human tissue samples, and clinical observations.

In half of our cases, we observed expansive growth of an outwardly extending tumor mass within the brain parenchyma. Expression levels of αvβ6 were significantly higher in this group of well-demarcated tumors. Integrin αvβ6 is not expressed in healthy adult epithelia but is upregulated in cancer and has been shown to modulate invasion and inhibit apoptosis. However, the exact role of αvβ6 in cancer pathobiology and in particular in brain metastases remains to be determined.

The vascular basement membrane may act as a guiding track for perivasculargrowth of cell collectives. Our results indicate that this invasion behavior is not only present in mesenchymal and epithelial tissues but also occurs in the distinct microenvironment of the CNS. In line with previous studies, we observed vascular co-option most commonly in melanoma brain metastases; however, we found it also in other tumor types, such as NSCLC adenocarcinoma.

Single-cell infiltration into the brain parenchyma of brain-metastatic tumor cells has previously been reported to be characteristic for SCLC. Our data show that this invasion pattern is also not uncommon in brain metastases of other tumor types, including NSCLC adenocarcinoma/squamous cell carcinoma, breast cancer, and melanoma. The high fraction of brain metastasis cases showing infiltrative growth has implications for local therapy options and highlights the need for including a safety margin beyond the neuroradiologically visible tumor borders. In our study, depth of invasion into the CNS parenchyma from the main tumor mass reached up to 450 μm. Of note, SRS uses no margins for treatment, but at 0.4 mm from the prescription isodose line, nearly a full dose is delivered, and therefore the magnitude of invasion has no clear consequence on SRS practice. The selection of patients in need of an extended local treatment approach is challenging, as the current neuroradiological techniques cannot precisely visualize the invasion distance of a given tumor. However, we previously demonstrated that the extent of peritumoral brain edema might function as a surrogate marker for infiltrative tumor growth, as little brain edema was significantly more common in infiltrative brain metastases and correlated with impaired patient survival times. Further prospective studies need to address the prognostic implications of brain metastasis invasion patterns in more detail.

We did not observe a statistically significant correlation of treatment modality with invasion pattern in our cohort. However, a higher proportion of patients in the diffuse infiltration group had received prior radiation or chemotherapy. As our sample size is not adequate for firm statistical conclusions, we cannot exclude that radio- or chemotherapy may select for or produce tumor cells with a higher infiltrative potential, similarly to some observations in primary tumors and gliomas.

Interestingly, brain metastasis lesions previously treated with SRS showed higher expression of αvβ5, a finding that is well in line with previous reports showing that this molecule is essential for tumor growth in preirradiated stroma. Further, we observed a high consistency of invasion behavior between the growth patterns of different brain metastases in individual patients. This again may indicate that intrinsic molecular features of metastases originating from a given primary tumor correlate with certain invasion patterns.

Our results have to be interpreted with caution because the small sample sizes often do not allow firm statistical conclusions. Herein, we concentrated on the descriptive presentation of our results. However, it has to be taken into account that autopsy samples of brain metastases are very rare, and our series displays a rather large cohort compared with previously published studies on autopsy specimens.

We noted prominent expression of αv integrins in many brain metastasis cases. This underscores the important function of this class of molecules in metastatic cancer. Currently, several integrin inhibitors are under clinical development, and promising activity was shown in some of the most frequent primary tumors of brain metastases such as NSCLC and melanoma. Interestingly, the anti-αv-integrin antibody intemumab reduced brain metastasis outgrowth in mice after intracarotid infusion of brain-seeking human epidermal growth factor receptor 2–positive breast cancer cells. Thus, clinical trials specifically investigating the potential of integrin inhibitors for prophylaxis and treatment of brain metastases seem warranted.

Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).
Acknowledgments

We thank Irene Leisser and Gerda Ricken for excellent technical assistance with preparation of tissue specimens. Further we thank Prof Dr Harald Heinzl (Center for Medical Statistics, Informatics, and Intelligent Systems, Medical University of Vienna) for the supervision and advice with statistical analyses. This study was performed within the PhD thesis project of Anna Sophie Berghoff in the PhD program “Clinical Neuroscience (CLINS)” at the Medical University Vienna.

Funding

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Conflict of interest statement. S.L.G. is an employee at Merck-Serono and has a patent application referring to the anti-integrin antibodies used here. M.W. has received research support and honoraria for lectures and service on its advisory board from Merck-Serono. All other authors declare no conflict of interest.

References

6.6 Characterization of the inflammatory response to solid cancer metastases in the human brain.

Interlude

The interaction of the immune system and cancer came into focus of oncology research as immune checkpoint inhibitors, intensifying anti-tumour T-cell response, showed promising clinical responses [115]. Although, the importance of the interaction with the immune system was shown for several pathologies like multiple sclerosis, infection or neurodegeneration as well as for primary brain tumours, only limited insight on the interaction with the immune systems exists for BM [137, 138]. Therefore, we aimed to investigate the interaction of the innate immune system, namely the brain resident microglia, and BM in the established autopsy cohort. In the paper “Characterization of the inflammatory response to solid cancer metastases in the human brain” we observed a dense infiltration with microglia/macrophages at the boarder between BM and surrounding brain parenchyma, while within the tumour tissue only sparse infiltration could be observed [139]. Melanoma BM presented with significantly less peritumoural microglia accumulation compared to NSCLC BM. As microglia function involves phagocytic activity, cytotoxic activity via nitric oxide radical release and activation of adaptive immune response, we further investigated the status of microglia activation. Here we observed high expression of markers associated with phagocytic function, while markers of nitric oxide radical release showed only marginal expression. Expression of major histocompatibility antigen class I, which is needed for antigen presentation in order to activate the adaptive immune response, was frequently presented. However, only sparse infiltration with cells of the adaptive immune response, namely B- and T cells, was observed. Our findings indicate that the microenvironment around BM seems to be ready to alter an immune defence but the BM tumour cells might induce immunosuppressive factors preventing activation of an adaptive immune response.
Characterization of the inflammatory response to solid cancer metastases in the human brain

Anna Sophie Berghoff · Hans Lassmann · Matthias Preusser · Romana Höftberger

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Abstract New immunomodulatory agents showed promising activity in brain metastases (BM). However, little is known about the inflammatory response in BM. New insights are needed to further guide the development of treatment strategies. We investigated 17 human autopic tissue specimens of BM from breast cancer (n = 3), non-small cell lung cancer (NSCLC; n = 5), small cell lung cancer (n = 3) and melanoma (n = 6). Immunohistochemical staining for a comprehensive panel of 21 inflammation-associated markers was performed. Results were quantified by manual counting of the various cell populations in three areas of 0.5 mm² (intratumoral, peritumoral, control region). Profound microglia activation with marked peritumoral accumulation and some intratumoral infiltration of HLA-DR-positive microglia/macrophages was found. A high proportion of these cells showed strong immunoreactivity for phagocytosis associated markers and MHC class 1, while a smaller subgroup of cells expressed molecules involved in radical production. Only few B- and T-lymphocytes were observed in and around BM. The number of CD8-positive T-cells was not correlated to MHC class 1 expression on tumor cells and only a fraction of T-cells showed Granzym B expression. Melanoma BM had significantly less accumulation of peritumoral microglia than NSCLC BM. The inflammatory pattern was independent from treatment of patients with glucocorticoids or radiation. The inflammatory reaction to BM is mainly characterized by activation of microglia/macrophages and shows pronounced upregulation of markers involved in phagocytosis, but seem to be insufficient in activating adaptive immunity. Treatment strategies aimed at activating specific immunity may potentiate immune attack on tumor cells.

Keywords Brain metastases · Microglia · Immune system · Macrophages · Lymphocytes · Adaptive immune system · Innate immune system

Abbreviations

BM Brain metastases
BRAF v-RAF murine sarcoma viral oncogene homolog B1
CTL4 Anti cytotoxic T lymphocyte associated antigen 4
NSCLC Non-small cell lung cancer
EGFR Epithelial growth factor receptor
HER2 In human epidermal growth factor receptor 2
CNS Central nervous system
SCLC Small cell lung cancer
MHC II Major histocompatibility antigen class II
MHC I Major histocompatibility antigen class I
WBRT Whole-brain radiation therapy
NO Nitric oxide
APM Antigen-processing machingery
TGF-beta Transforming growth factor beta
GFAP  Glial fibrillary acidic protein  
IBA-1  Ionized calcium binding adaptor molecule 1  
AIF-1  Allograft inflammatory factor 1  
SIGLEC-11  Sialic acid-binding Ig-like lectin 11  
HMGB1  High-mobility group box 1  
iNOS  Inducible nitric oxide synthase  
NCF-1  Neutrophil cytosolic factor 1

Introduction

Brain metastases (BM) are a severe and devastating complication of solid tumors, occurring in up to 25–40% of patients with metastatic cancer [1–3]. The prognosis of patients with BM is poor with median survival of approximately 2–7 months [4]. However, some patients with favorable prognostic factors, like primary histology of breast cancer with expression of human epidermal growth factor receptor 2 (HER2) and estrogen receptor, age under 60 years and a Karnofsky performance status over 90 at first diagnosis of BM, have median overall survival times of up to 25.3 months [5]. Currently, therapy relies mainly on surgery, radiosurgery and radiotherapy. Most systemic therapies like cytotoxic and novel targeted agents have shown only little or no efficacy against BM [6]. Reasons for this may include inadequate drug penetration through the blood–brain barrier and resistance mechanisms [3, 7]. However, recently novel drugs have shown promising activity in specific subsets of BM, such as inhibitors of v-RAF murine sarcoma viral oncogene homolog B1 (BRAF) in BRAF V600E mutated melanoma, the immunomodulatory anti cytotoxic T lymphocyte associated antigen 4 (CTLA4) antibody ipilimumab in melanoma, epithelial growth factor receptor inhibitors in non-small cell lung cancer (NSCLC) and lapatinib in HER2 positive breast cancer [8, 9]. A better understanding of the pathobiology of BM is likely to support the development of additional active agents for patients with BM.

The immune system plays an important role in the pathophysiology of many central nervous system (CNS) disorders including multiple sclerosis, infection, neurodegeneration, and also neoplastic disease [10–13]. However, while many research groups have studied the inflammatory response to primary brain tumors, only very few data on the role of the immune system in BM are available [14–19]. In this study we aimed at characterizing the inflammatory response to BM in the human brain and performed an immunohistochemical study utilizing autopic tissue specimens of 17 BM patients. Brain tissue is usually not collected at autopsy of BM patients, but we were able to compile this series from a large and well annotated biobank at our institution.

Subjects and methods

Patients and materials

All patients with histologically proven BM of breast cancer, SCLC, NSCLC or melanoma who underwent brain autopsy between 1993 and 2011 were identified from the biobank of the Medical University of Vienna. Clinical and demographic data were retrieved by chart review. Of each patient we selected one representative tissue specimen which included viable BM and surrounding brain tissue. Of each patient one BM tissue block was available, because for routine diagnostic purposes tissue material of one representative BM lesion was sampled. All tissue samples included in this study have been formalin fixed and paraffin-embedded during routine diagnostic work-up. This study was approved by the ethics committee of the Medical University Vienna.

Immunohistochemistry

Tissue blocks were cut into 3–5 μm slices with a microtome. A comprehensive immunohistochemical staining panel was used to characterize microglia and lymphocyte response to BM. Table 1 summarizes markers and immunostaining protocols.

Quantitative evaluation

Regions of interest were defined as follows: BM lesion: within the vital tumor tissue, peritumoral region: brain tissue adjacent to the BM; control region: brain tissue at least 0.5 mm from the BM border. The region of highest density of microglia in the peritumoral region was identified in the staining for HLA-DR in each case. Within this region the density of immunoreactive cells was counted in eight high power fields at a magnification of ×400 (0.5 mm²). The same area was counted in each staining of a case. Within the control region and within the BM the density of immunoreactive cells was counted as well in eight high power fields at a magnification of ×400 (0.5 mm²). Expression of a marker in the tumor cells was evaluated by semi quantitative methods and grouped as sparse (0–10 % of the tumor cells), moderate (11–50 % of the tumor cells) and strong (over 50 % of the tumor cells) staining.

Statistical evaluation

Statistical evaluation was performed using SPSS 17.0 statistical software system (SPSS, Chicago, IL). To compare differences between two groups Mann–Whitney U test was performed. P values <0.05 were regarded as significant.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. GFAP</td>
<td>Intermediate filament protein, increase of expression in response to a variety of neurological disorders (reactive gliosis) [44]</td>
<td>Rabbit polyclonal anti-glial fibrillary acidic protein (Z 0334 DakoCytomation)</td>
<td>1:3,000</td>
<td>Proteinase K ready to use DakoCytomation</td>
</tr>
<tr>
<td>2. HLA ABC/MHC I</td>
<td>Antigen presentation for T-cells; expression is essential for the propagation of CD8+ T-cell mediated inflammation [45]</td>
<td>Stam et al. [46]</td>
<td>1:2,000</td>
<td>Tris–EDTA buffer (pH 8.5)</td>
</tr>
<tr>
<td>3. HLA-DR/MHC II</td>
<td>Major histocompatibility antigen class II expressed on microglia cells, necessary for antigen recognition by CD4+ T-cells [47]</td>
<td>Mouse monoclonal anti-human HLA-DR, DQ, DP antigen (M 0775 DakoCytomation)</td>
<td>1:400</td>
<td>Flex TRS low (pH 6)</td>
</tr>
<tr>
<td>4. CD68</td>
<td>Scavenger receptor involved in phagocytosis [48]</td>
<td>Monoclonal mouse anti-human CD68/clone KP1/M0814 (DakoCytomation)</td>
<td>1:5,000</td>
<td>Flex TRS low (pH 6)</td>
</tr>
<tr>
<td>5. CD163</td>
<td>Scavenger receptor involved in phagocytosis [48]</td>
<td>CD163 mouse monoclonal antibody; NCL-CD163 (Novocastra)</td>
<td>1:1,000</td>
<td>Citrate buffer (pH 6)</td>
</tr>
<tr>
<td>6. IBA-1—ionized calcium binding adaptor molecule 1</td>
<td>Calcium binding protein, specifically expressed in microglia cells [49]</td>
<td>Anti IBA 1 (Novocastra)</td>
<td>1:3,000</td>
<td>Tris-EDTA buffer (pH 8.5)</td>
</tr>
<tr>
<td>7. AIF-1—allograft inflammatory factor</td>
<td>Expression under inflammatory conditions involving macrophage and microglia activation [50]</td>
<td>Mouse anti-human/rat AIF-1 (BMA Biomedicals AG)</td>
<td>1:500</td>
<td>Tris-EDTA buffer (pH 8.5)</td>
</tr>
<tr>
<td>8. SIGLEC-11—sialic acid-binding Ig-like lectin</td>
<td>Inhibitory role in inflammatory conditions [51]</td>
<td>Affinity purified rabbit anti-HMG1 polyclonal antibody BD Biosciences Phar mingen TM</td>
<td>1:1,000</td>
<td>Tris-EDTA buffer (pH 8.5)</td>
</tr>
<tr>
<td>9. HMGB1—high-mobility group box 1</td>
<td>Segregation from macrophages which are activated by innate immunity; promotes activation and recruitment of inflammatory cells; stimulates adaptive immune response [33]</td>
<td>Rabbit anti-GLUT 5 polyclonal antibody (AB 1041) Chemicon</td>
<td>1:1000</td>
<td>Citrate buffer (pH 6)</td>
</tr>
<tr>
<td>10. GLUT-5</td>
<td>Increase of expression on microglia in response to any stress [53]</td>
<td>Rabbit anti-inducible nitric oxide synthase (iNOS) polyclonal antibody Chemicon International</td>
<td>1:3000</td>
<td>Tris-EDTA buffer (pH 8.5)</td>
</tr>
<tr>
<td>11. iNOS—inducible nitric oxide synthase</td>
<td>Nitric oxide radical production in inflammatory conditions [54]</td>
<td>p22-phox (FL-195): sc-20781 Santa Cruz Biotechnology, INC</td>
<td>1:100</td>
<td>Citrate buffer (pH 6)</td>
</tr>
<tr>
<td>12. p22phox</td>
<td>Catalytic partner of NOX proteins, membrane regulatory compound of NADPH oxidase [29]</td>
<td>NCF1/p47phox goat anti-human polyclonal antibody (C-terminus) antibody-LSB 2365-LSBio Lifespan Biosciences</td>
<td>1:100</td>
<td>Citrate buffer (pH 6)</td>
</tr>
<tr>
<td>13. NCF-1—neutrophil cytosolic factor 1 (p47-phox, NOXO2)</td>
<td>Cytosolic regulatory compound of NADPH oxidase 2 (NOX2) [55]</td>
<td>Anti-NOX1 rabbit Sigma-Aldrich</td>
<td>1:200</td>
<td>None</td>
</tr>
<tr>
<td>Markers</td>
<td>Function</td>
<td>Antibody</td>
<td>Dilution</td>
<td>Antigen retrieval</td>
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<tr>
<td>---------</td>
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<td>----------------------------</td>
</tr>
<tr>
<td>15. NOXO-1</td>
<td>Cytosolic compound of NADPH oxidase, regulator of NOX 1 [29]</td>
<td>Anti-NOXO rabbit Sigma-Aldrich</td>
<td>1:200</td>
<td>Citrate buffer (pH 6)</td>
</tr>
<tr>
<td>16. CD3</td>
<td>Component of T-cell receptor [56] (all T-cells)</td>
<td>CD3 (Clone SP7) rabbit monoclonal antibody Thermo Scientific</td>
<td>1:2,000</td>
<td>Tris-EDTA buffer (pH 9)</td>
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<tr>
<td>17. CD4</td>
<td>Co-receptor for MHC II [56]</td>
<td>Mouse monoclonal anti-human CD4/clone 4B12/M7310 DakoCytomation</td>
<td>1:100</td>
<td>Flex TRS high (pH 9)</td>
</tr>
<tr>
<td>18. CD8</td>
<td>Co-receptor for MHC I [56]</td>
<td>Monoclonal mouse Anti-Human CD8/clone C8/144b/M7103 DakoCytomation</td>
<td>1:100</td>
<td>Flex TRS low (pH 6)</td>
</tr>
<tr>
<td>19. Granzym B</td>
<td>Component of cytotoxic granules of T-cells, marker for cytotoxic T-cells activation [57]</td>
<td>GZB01; Thermo Scientific</td>
<td>1:1000</td>
<td>Tris-EDTA buffer (pH 9)</td>
</tr>
<tr>
<td>20. CD20</td>
<td>B cell antigen [56]</td>
<td>Mouse monoclonal anti-human CD20cy/clone L26/M0755 DakoCytomation</td>
<td>1:400</td>
<td>Flex TRS low (pH 6)</td>
</tr>
<tr>
<td>21. CD79A</td>
<td>B cell antigen [56]</td>
<td>Mouse monoclonal anti-human CD79 alpha(Clone JCB117/M7050 DakoCytomation</td>
<td>1:100</td>
<td>Flex TRS low (pH 6)</td>
</tr>
</tbody>
</table>
Spearman correlation was used to identify interdependence of variables.

**Results**

**Patients’ characteristics**

Autopsic brain tissue specimens with embedded BM of 17 cancer patients were available for this analysis (histological diagnoses of the primary tumors: 6 melanoma, 3 SCLC, 5 NSCLC, 3 breast cancer). The median age at death was 56 years (range 35–83 years) and the study cohort included nine male and eight female patients. 7 patients had single and 10 patients multiple brain (median 2, range 1–5) metastases. Five patients were treated with whole-brain radiation therapy (WBRT), chemotherapy or a combination of WBRT and chemotherapy for BM. Time from the last treatment (WBRT and/or chemotherapy) till death was at least 1 month (range 1–7 months). Four patients received glucocorticoid therapy within 1 week before death. All other patients had received no specific treatment for BM as their performance status was too poor at the time of diagnosis of BM or first diagnosis of BM was at autopsy. Table 2 lists further demographic and clinical data.

**Tissue analysis**

In all cases we histologically found solid tumor masses embedded in CNS tissue. In 16/17 cases the tumor formations were well-delineated from the surrounding CNS tissue with a clear-cut border between tumor and CNS tissue. Only sparse infiltrating single tumor cells or small tumor cell nests in the brain parenchyma were observed in these cases. One case of SCLC showed rather diffuse and infiltrating growth pattern with a less demarcated border, corresponding to the previously described “pseudogliomatous” growth pattern [20].

**Lymphocyte and plasma cell infiltration**

We observed only very few B and T-cells in and around BM (Fig. 1). Within the BM lesion only few scattered cells were observed with a slightly higher density in perivascular areas. The majority of small round inflammatory cells were identified as T-cells by immunohistochemical methods. More CD8 and CD3 positive cells were observed compared with CD20, CD79a and CD4. Density of CD8 positive T-cell infiltration did not differ between primary tumor types ($P = 0.844$; Kruskal–Wallis test) (Fig. 1). Among the T-cells only a small proportion showed expression of Granzym B. A mean of 4.7 (range 0–27) Granzym B positive cells per 0.5 mm$^2$ were observed within BM compared to 31.3 (range 1–140) CD8 positive cells per 0.5 mm$^2$ (Spearman’s correlation coefficient 0.4; $P = 0.083$). We did not observe unequivocal morphological signs of Granzym B release specifically directed at tumor cells.

T-cell recognize their specific antigen only, when presented in association with major histocompatibility (MHC) molecules. Significantly more microglial cells/macrophages in the peritumoral region stained positive for MHC class I (required for antigen recognition by CD8$^+$ T-cells) compared to the control region ($P = 0.004$; paired $t$ test) (Table 2). Furthermore, 14/17 (87.6 %) BM lesions did demonstrate positive staining for MHC I of the tumor cells [sparse staining: 3/17 (18.8 %) cases; moderate staining: 2/17 (12.5 %) cases; strong staining: 9/17 (56.3 %) cases]. In contrast, only 3/17 (20.0 %) BM lesions showed positive staining for HLA-DR/MHC class II (antigen presentation to CD4$^+$ T-cells) within the tumor cells [moderate: 2/17 (13.3 %) cases; strong: 1/17 (6.7 %) cases]. The number of CD8-positive T-cells did not correlate with MHC I expression on tumor cells ($P = 0.873$; Kruskal–Wallis test).

**Table 2 Patients’ characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients ($n = 17$)</th>
<th>$n$</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>9</td>
<td>52.9</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>47.1</td>
<td></td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>56 (35–83)</td>
<td></td>
<td></td>
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<td>Primary histology</td>
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<tr>
<td>Melanoma</td>
<td>6</td>
<td>35.3</td>
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<tr>
<td>SCLC</td>
<td>3</td>
<td>17.6</td>
<td></td>
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<tr>
<td>NSCLC</td>
<td>5</td>
<td>29.4</td>
<td></td>
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<tr>
<td>Breast cancer</td>
<td>3</td>
<td>17.6</td>
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<tr>
<td>Treatment for BM</td>
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<td>WBRT</td>
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<tr>
<td>Chemotherapy</td>
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<td>5.9</td>
<td></td>
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<td>Glucocorticoid therapy</td>
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<td>No</td>
<td>13</td>
<td>76.5</td>
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<td>Extracranial metastases</td>
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<tr>
<td>Yes</td>
<td>13</td>
<td>76.5</td>
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<td>No</td>
<td>4</td>
<td>23.5</td>
<td></td>
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<td>Location of BM</td>
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<td>Supratentorial</td>
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<td>Infratentorial</td>
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<td>First diagnosis of BM at autopsy</td>
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<td>5</td>
<td>28.4</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>12</td>
<td>71.6</td>
<td></td>
</tr>
<tr>
<td>Median survival from diagnosis of BM, months (range)</td>
<td>1 (0–14)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1 Lymphocyte infiltration of BM. a CD3 immunostaining (magnification ×10), b CD8 immunostaining (magnification ×20), c CD79a immunostaining (magnification ×20), d CD20 immunostaining (magnification ×20), e density of different lymphocytes per 0.5 mm² within BM, f T-cells per 0.5 mm² within BM in different primary tumor histologies.
Expression of markers related to microglia/macrophage activation

Using immunohistochemistry for HLA-DR and IBA-1 we observed a pronounced accumulation of densely packed microglial cells/macrophages in the peritumoral region and around areas of necrosis in all cases (Fig. 2; Table 3). Viable tumor tissue areas and the surrounding normal appearing CNS tissue contained only scattered microglial cells (Fig. 2).

To characterize marker of microglia/macrophage activation, immunohistochemical stainings for Siglec, HMGB1, AIF-1, and GLUT 5 were performed. Compared to the other microglia/macrophage antigens, significantly more cells in the peritumoral region expressed HMGB1 ($P < 0.05$; paired $t$ test). HMGB1 staining was further observed in neurons and in tumor cells. 15/17 (88.2 %) BM lesions showed a strong, homogenous positive staining for HMGB1. HMGB1 was observed in the extracellular space or around necrotic cells in 4/17 (23.5 %) BM lesions.

In further characterization of the microglia/macrophage infiltrates, the majority of the cells in the peritumoral ‘microglial wall’ were identified as macrophages with broad, foamy cytoplasm and strong immunoreactivity for CD68 and CD163 (Fig. 2). A significantly higher proportion of CD163-positive macrophages were identified within the peritumoral microglia wall than in the control region ($P < 0.001$; paired $t$ test) (Table 2). The density of macrophages in the peritumoral region was not correlated to the presence of necrosis ($P = 0.840$; Mann–Whitney $U$ test).

To analyse oxidative burst activation in microglia/macrophages, immunohistochemistry for iNOS, p22phox, NCF-1, NOX-1 and NOXO-1 was performed. Among these, p22phox showed moderate expression, while all other factors were only marginally expressed. No correlation of iNOS expression and the presence of necrosis was observed ($P = 0.521$; Mann–Whitney $U$ test). Overall, in comparison to the cells with positive staining for the macrophages markers CD163 and CD68, relatively few cells showed positive staining for the enzymes involved in oxygen and nitric oxide radical production (Table 2).

iNOS expression showed only marginal correlation with NADPH oxidase marker expression. The strongest correlation was observed for iNOS and NOX 1 (Spearman’s correlation coefficient 0.7; $P = 0.006$) followed by iNOS and p22phox (Spearman’s correlation coefficient 0.6; $P = 0.021$) and iNOS and NCF1 (Spearman’s correlation coefficient −0.6; $P = 0.007$), respectively. No correlation was observed for iNOS and NOXO-1 (Spearman’s correlation coefficient 0.1; $P = 0.749$).

The density of the peritumoral microglia wall differed between primary tumor types. Patients with melanoma had a significantly less dense peritumoral microglia wall with a median of 81.00 (range 44–135) HLA-DR positive cells per 0.5 mm$^2$ compared to 147 (range 131–297) HLA-DR positive cells per 0.5 mm$^2$ in patients with NSCLC ($P = 0.010$, Mann–Whitney $U$ test) (Fig. 3). No significant difference in the density of the microglia wall was found between the other tumor types.

Interestingly, microglia density and expression of microglia activation markers was not significantly influenced by treatment modality, anatomic brain region or presence of necrosis in our small series (Fig. 3).

Astrocytes and reactive gliosis

Reactive gliosis, consisting of GFAP-positive astrocytes with distended cytoplasm and radially arranged processes, were observed in the peritumoral region. Compared to the control region more reactive astrocytes were identified in the peritumoral region (Table 2).

Discussion

The immune systems attracted rising interest in neurooncology as several new approaches in systemic therapy, like dendritic cell vaccination therapy or the anti-CTLA4 antibody ipilimumab, are under investigation and have shown activity in clinical trials for primary and secondary brain tumors [9, 21–23]. However, so far only little is known about the immunologic response within BM. In the present study, we systematically characterized the inflammatory infiltrates in and around BM in human autopsy tissue samples. Our data show that the inflammatory reaction to BM is mainly characterized by activation of microglia/macrophages and shows pronounced upregulation of markers involved in phagocytosis, while the infiltration by T- and B-lymphocytes is very low.

We observed that microglia/macrophages produce dense infiltration in the peritumoral area of BM, whereas intratumoral areas contained comparatively low numbers of inflammatory cells. Interestingly, the inflammatory pattern was independent from tumor localization and treatment with glucocorticoids or radiation in our small patient series. However, we found evidence for differential inflammatory response between tumor types. In our series, melanoma BM had significantly less peritumoral microglia accumulation than NSCLC BM. This finding may be related to the common embryological origin of glial cells and melanocytes.
Fig. 2 Microglia/macrophage activation. a HLA-DR immunostaining in peritumoral region (magnification ×2.5), b HLA-DR immunostaining in peritumoral region (magnification ×20), c iNOS immunostaining in peritumoral region (magnification ×20), d CD163 immunostaining in peritumoral region (magnification ×20), e CD163 immunostaining within BM (magnification ×10), f CD68 immunostaining within BM (magnification ×20)
Microglia cells are the main effector cells of the CNS immune system and their function involves innate as well as adaptive immune responses [11, 12]. Microglia cells may rapidly enlarge and differentiate to macrophages, although macrophages in the CNS may also derive from blood monocytes [12]. Microglia cells produce a high number of signaling molecules including cytokines, proteases and prostanoids upon activation. In addition, activated microglia can induce cytotoxic cell death throughout production of nitric oxide (NO) and superoxide, which are products of the enzymes inducible nitric oxide synthase and NADPH oxidase [24]. Data on experimental metastases in murine brain suggest that activated microglia have tumor cytotoxic effects, although some publications have also indicated pro-neoplastic microglia effects in glioma [25, 26]. So far research on microglia cells and macrophage infiltration in BM from human tissue are sparse and a systematic investigation of microglia activation pattern in different types of solid cancer metastases are lacking [14, 15]. For the better understanding of inflammatory mechanisms and their effects on metastatic tumor cells we analyzed microglia/macrophage activation with a broad panel of established markers in human BM tissue by immunohistochemical methods.

Microglia cells and macrophages are known to release oxygen and nitric oxide radicals, which have been shown to play a major role in neurodegenerative diseases, CNS injury and especially in multiple sclerosis [27, 28]. A previous in vitro study postulated that microglial cells exert tumoricidal activity against brain-metastatic cells through NO release [26]. In contrast, another study found evidence for deficiency in the NO release of microglia cells around surgery specimen of human BM [14]. In our present study we investigated a broad panel of markers involved in the induction of oxidative stress, including iNOS, NOX1, NOXO1, p22phox and NCF-1 [27]. Among these, only p22phox showed moderate expression, while all other factors were only marginally expressed. Since oxygen radical production requires fully assembled NADPH oxidase complexes, the low expression of some of the components (NCF-1, NOXO1) suggests that very little active enzyme is available [29]. However, we observed a correlation of iNOS with markers of the NADPH oxidase. NO reacts with oxygen radicals forming the highly cytotoxic peroxynitrite, which may thus result in at least some NO-related tumoricidity. Interestingly, one study showed that conditioned medium of brain-metastatic colon-carcinoma cells may inhibit NO production of endothelial cells, thus indicating that tumor cells may have protective mechanisms against NO-induced lysis [14, 30]. Taken together, however, our data indicate that cytotoxic microglia activation is minor in BM.

In contrast to the markers of radical production, markers associated with phagocytic activity, namely CD163 and CD68, were consistently and prominently expressed in our study. Our findings are in keeping with data from a previous study, which also showed dense accumulation of microglia cells and especially of CD163 and CD68 positive macrophages at the border between BM and normal CNS tissue in brain-metastatic lung adenocarcinoma [14, 31].

### Table 3 Microglia activation (median number of immunoreactive cells/0.5 mm² (range) in the peritumoral region, within BM lesion and in the control region)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Peritumoral region</th>
<th>BM lesion</th>
<th>Control region</th>
<th>Spearman correlation peritumoral and control region</th>
<th>Spearman correlation peritumoral region and BM lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA DR</td>
<td>131.00 (29–297)</td>
<td>37.00 (11–112)</td>
<td>47.00 (0–154)</td>
<td>0.6*</td>
<td>−0.03</td>
</tr>
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<td>IBA-1</td>
<td>76.50 (3–172)</td>
<td>32.00 (1–240)</td>
<td>43.00 (7–106)</td>
<td>0.3</td>
<td>0.7*</td>
</tr>
<tr>
<td>CD163</td>
<td>74.50 (17–213)</td>
<td>32.00 (1–185)</td>
<td>9.00 (0–122)</td>
<td>0.5</td>
<td>0.7*</td>
</tr>
<tr>
<td>CD68</td>
<td>64.50 (4–205)</td>
<td>37.00 (8–109)</td>
<td>30.00 (9–90)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>iNOS</td>
<td>25.00 (2–142)</td>
<td>9.00 (0–57)</td>
<td>12.00 (0–50)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>p22phox</td>
<td>105.00 (22–268)</td>
<td>46.00 (0–216)</td>
<td>39.00 (17–98)</td>
<td>0.04</td>
<td>0.5</td>
</tr>
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<td>NCF-1</td>
<td>11.00 (0–55)</td>
<td>1.50 (0–30)</td>
<td>9.00 (0–59)</td>
<td>0.6</td>
<td>0.1</td>
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<tr>
<td>NOX-1</td>
<td>34.00 (5–145)</td>
<td>0</td>
<td>9.00 (0–108)</td>
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<tr>
<td>NOXO-1</td>
<td>14.00 (2–102)</td>
<td>0</td>
<td>11.00 (0–50)</td>
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<tr>
<td>Siglec</td>
<td>23.50 (7–94)</td>
<td>2.00 (0–34)</td>
<td>14.00 (0–43)</td>
<td>0.5*</td>
<td>−0.1</td>
</tr>
<tr>
<td>HMGB1</td>
<td>82.50 (25–209)</td>
<td>0</td>
<td>84.00 (27–150)</td>
<td>0.6*</td>
<td>–</td>
</tr>
<tr>
<td>AIF-1</td>
<td>40.00 (0–117)</td>
<td>13.00 (0–80)</td>
<td>13.00 (0–31)</td>
<td>0.7*</td>
<td>0.7*</td>
</tr>
<tr>
<td>GLUT 5</td>
<td>38.50 (4–115)</td>
<td>4 (0–22)</td>
<td>19.00 (2–38)</td>
<td>0.7*</td>
<td>−0.1</td>
</tr>
<tr>
<td>HC-10</td>
<td>62.50 (6–141)</td>
<td>42.00 (17–107)</td>
<td>29.00 (1–113)</td>
<td>0.3</td>
<td>−0.5</td>
</tr>
<tr>
<td>GFAP</td>
<td>43.00 (15–117)</td>
<td>0</td>
<td>27.00 (6–48)</td>
<td>0.6</td>
<td>–</td>
</tr>
</tbody>
</table>

* P < 0.05
Fig. 3  

**a** Density of HLA-DR positive cells per 0.5 mm² in the peritumoral region of different primary tumor histologies.  

**b** Density of HLA-DR positive cells per 0.5 mm² in the peritumoral region in supratentorial and infratentorial location of BM.  

**c** Density of HLA-DR positive cells per 0.5 mm² in the peritumoral region in patients treated with and without WBRT.  

**d** Density of HLA-DR positive cells per 0.5 mm² in the peritumoral region in patients treated with and without chemotherapy.  

**e** Density of HLA-DR positive cells per 0.5 mm² in the peritumoral region in patients treated with and without glucocorticoid therapy.  

**f** Density of HLA-DR positive cells per 0.5 mm² in the peritumoral region in BM with and without necrosis.
Previously, more CD68 positive macrophages were observed in adenocarcinoma BM than in primitive neuro-ectodermal tumors or meningiomas [32]. However, only little is known so far about the factors driving macrophage infiltration in BM. We observed a high expression of the potent macrophage-activating factor HMGB1 in microglia/macrophages but also in the majority of vital tumor cells. In addition HMGB1 was found in apoptotic tumor cells, necrotic tissue areas and in the extracellular spaces. Thus, the release of HMGB1 from disintegrating tumor cells may play an important role in initiating or sustaining microglia/macrophage recruitment to BM [33].

The adaptive immune system came into the focus of cancer research as the density of tumor-infiltrating T-cells were postulated to be associated with better survival in various tumors [34–36]. Furthermore, new approaches in systemic therapy of cancer focus on the recruitment of T-cells like the anti-CTLA4 antibody ipilimumab in melanoma [37]. The healthy human brain contains almost no lymphocytes, but there is evidence for immune surveillance of the normal human CNS by CD3(+/+)CD8(+) lymphocytes, particularly in areas of relatively permissive blood–brain barrier composition [38, 39]. Some brain tumors such as gangliogliomas are known to attract prominent lymphocytic infiltration. For the instance of glioblastomas, the amount of T-cell infiltration was shown to be associated with survival of patients, who received vaccination with dendritic cell immunotherapy [40]. Interestingly, we found only very few B- and T-lymphocytes in and around BM in our patient series. Further, only a small proportion of T-cells did demonstrate sings of cytotoxic activation. CD8-positive cytotoxic T-cells recognize their antigen via the MHC class I molecule on the cell surface and subsequently induce their cytotoxic action. Although we found variable amounts of MHC class I expression on tumor cells, this did not correlate with the intratumoral number of CD8-positive T-cells. Our findings may indicate that the sparse lymphocytic infiltrates correspond mainly to secondarily recruited T- and B-cells that are not antigen specific. BM cells may produce immunosuppressive factors that inhibit antineoplastic activity of the specific immune system, similar to the situation in primary brain tumors [16, 18, 19, 41]. For example, Mehlin et al. [42] presented data suggesting that coordinated downregulation or impaired upregulation of certain components of the HLA class I antigen-processing machin ery may allow astrocytoma cells to evade the hosts’ immune response, even if HLA class I antigen surface expression is not altered. The expression of MHC molecules on microglia and tumor cells could in principle make a direct tumor killing by an antigen-specific immune response possible. Thus, treatment strategies aimed at boosting the response of the specific immune system against BM may increase therapeutic efficacy. Recent examples of such approaches in brain tumors include vaccination strategies and transforming growth factor beta antisense therapy for glioblastoma and the anti-CTLA4 antibody ipilimumab in (brain-) metastatic melanoma [21, 22, 43]. Particularly, immunological intervention using the immunomodulatory anti-CTLA4 antibody ipilimumab has recently shown clinically meaningful activity in melanoma patients with BM [9]. Our findings in fact very nicely complement these data, as we show that an unspecified inflammatory response is in principle activated in the brain by cancer metastases but adaptive immunity is only inadequately elicited. Taken together, the data clearly suggest that activation of specific immunity by immunological interventions in fact provide effective therapeutic potential.

In conclusion the immunological environment in BM seems to be ready to alter an immune defense but the specific stimulus for activation might be missing or might not overcome the anti-inflammatory factors produced by the tumor cells. Further studies are needed to clarify whether approaches aimed at activating specific immunity are sufficient to induce significant antineoplastic immune response in brain tumors including BM.

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Conflict of interest The authors declare that they have no conflict of interest.

References


7 Discussion
7.1 General discussion

In this thesis, clinical and pathological characteristic were examined in a unique and large cohort of patients with BM. We could identify clinical characteristics in the disease course of patients with breast cancer BM, evaluate tissue based characteristics as well as radiological characteristics influencing survival prognosis, investigate the interaction of the innate immune system and BM and describe different invasion patterns of BM. Our findings add valuable information on the involved mechanisms of the brain metastatic cascade and might be used to further guide the development of BM specific trials.

Patients with HER2 positive breast cancer and triple negative breast cancer were shown to develop BM significantly earlier during their clinical course compared to patients with ER positive breast cancer [123]. While the impact of the breast cancer subtypes on overall survival, also upon the diagnosis of BM, is well reviewed, we investigated to our best knowledge for the first time the brain metastasis free survival according to the breast cancer subtype [19, 140]. We emphasized that preventive strategies should be investigated in patients with HER2 positive and triple negative breast cancer. Prophylactic cranial irradiation was discussed especially for patients with triple negative or HER2 positive breast cancer [141, 142]. The CEREBEL studies investigated the potential of lapatinib versus trastuzumab in order to prevent BM as first side of progression. However, no significant difference between the two HER2 directed systemic treatment approaches was observed, although the study did not reach the pre-specified accrual goal [52]. Future studies should focus on the value of new substances in the prevention of BM.

Brain only metastatic breast cancer was identified as distinct metastatic pattern and clinically relevant subtype, as patients might experience long-term survival over 36 months [126]. So far the status of the extracranial disease is not included in the diagnosis specific graded prognostic assessment of patients with BM from breast cancer as it is based on the breast cancer subtype, age and Karnofsky performance score [68]. However, the diagnosis specific graded prognostic assessment is based on the clinical data of patients included in clinical trials of the Radiotherapy and Oncology Group (RTOG). Therefore, the prognostic score might not truly resemble
the relevant prognostic factors in a real life cohort. Our observation emphasizes the need to validate the established clinical prognostic scores in real life cohorts.

We identified high Ki67 proliferation index and high microvascular density as tissue based prognostic factors in NSCLC BM [128]. While Ki67 proliferation index is a known prognostic factor in primary NSCLC, the favourable prognostic impact of high microvascular density in non small cell lung cancer BM is in contrast to the prognostic impact in primary NSCLC [127, 143]. However, a previous study on NSCLC lymph node metastases revealed that the prognostic implication of MVD might change upon metastatic disease, as patients with high MVD in the lymph node metastases presented with an improved survival prognosis compared to patients with low MVD [144]. Therefore, neoangiogenesis as well as its prognostic impact might change due to the changed microenvironment (“seed and soil”) [145]. We further postulated a predictive value for the HIF 1 alpha index in patients receiving whole brain radiation therapy after resection of NSCLC BM [128]. The value of whole brain radiation therapy in NSCLC is currently discussed [63]. In a phase III study, no impact on overall survival for addition of whole brain radiotherapy after neurosurgical resection or radiosurgery of one to three BM was observed [78]. Regarding the side effects of whole brain radiotherapy including neurocognitive impairment and the impact on the quality of life, careful selection of patients profiting of the therapy is needed [147]. The value of HIF 1 alpha index as a predictive marker for whole brain radiotherapy should be investigated in prospective clinical trials.

Preoperative diffusion weighted imaging, which resembles mobility of water molecules in the intercellular space, showed a significant correlation with survival prognosis in patients with singular BM treated with neurosurgical resection as first line treatment approach for newly diagnosed brain metastasis [148]. So far, only clinical factors like number of BM, age, status of extracranial disease and Karnofsky performance score are used for the estimation of the survival prognosis in patients with BM [149]. However, radiological findings might add valuable additional information as they can function as surrogate parameter of tissue characteristics. Indeed, hyperintense diffusion weighted imaging showed correlation with high extracellular fibrosis, measured by the density of reticulin fibers [132]. Importantly, the reticulin fiber network is part of the collagen-rich tumour stroma, underscoring the biological and prognostic importance of the tumour stroma and the involved
microenvironment [35, 150]. Accordingly, presence of a dense stromal matrix was shown to be associated with impaired survival prognosis in frequent primary tumour of BM like triple negative breast cancer [151, 152]. The prognostic value of radiological finding should be investigated within prospective clinical trials.

We were able to characterize invasion patterns of BM in a unique large cohort of autopsy specimens. Here, a well-demarcated border towards the surrounding brain parenchyma was observed in approximately half of the investigated cases. Further, we found a significant fraction of specimens presenting with infiltrative growth via vascular co-option (18%) or gliomas like single-cell infiltration (32%) [136]. So far, the general textbook knowledge classified BM as predominantly growing expansively [153]. However, preclinical BM mouse models postulated the growth via vascular co-option especially for melanoma BM, indication that the growth kinetic might differ according to the primary tumour [43, 145]. In line, we observed a high fraction of melanoma BM growing via vascular co-option [136]. The presence of depth invasion into the surrounding brain parenchyma is of therapeutically importance as local recurrence is a major complication in locally treated BM. Selection of patients needing expanded safety margin treatment e.g. in a local radiosurgical treatment, is challenging as so far no reliable radiological surrogate markers for invasion behaviour could be identified. We observed in a previous study that patients with description of infiltrative growth in the neurosurgical report presented with small peritumoural brain oedema in the preoperative imaging [74]. Further, presence of a small brain oedema correlated with an impaired survival prognosis, underscoring the importance of further clinical investigation on the correlation of radiological finding and BM invasion patterns.

Although the brain is considered as an immune privileged organ, we observed a dense inflammatory reaction to BM, which is characterized by activation of microglia/macrophages [139, 154]. The interaction of cancer and the immune system gained attention in oncology research as recently the introduction of new immune checkpoint inhibitors showed high and durable response rates [155-157]. In patients with BM, response rates of up to 27% were observed, indicating that immune checkpoint inhibitors might be a treatment option in selected patients with BM [107, 115, 116]. Currently, we are investigating the specific immune response including effector, cytotoxic, memory and regulatory T-cell and the prognostic impact in BM.
Although, we were able to investigate a large real life patient cohort our studies have some limitations. The clinical characteristics were collected retrospectively with all the resulting shortcomings. Treatment strategies especially in the systemic therapy improved during the time of investigation as we included patients over a time period of more than 20 years. However, the RTOG studies, which were used for establishment of the clinically used prognostic assessment, were conducted over a comparable time period from 1985-2007 facing similar bias problems. Concerning our histological studies, it would be highly interesting to study the matched primary tumours. However, as patients were treated in different hospitals the tissue was frequently not available. Despite this obstacle, we were still able to investigate a quite large cohort of matched NSCLC samples in comparison to previous studies.

7.2 Conclusion & future prospects

In conclusion, this thesis could provide additional information on the clinical and pathological prognostic factors in patients with BM of solid cancer. Inclusion of the identified clinical, tissue based and radiological factors might have additional value for the prognostic estimation of patients with BM. We could gain insight in the growth characteristics of BM and the interaction of the immune system and BM. Further prospective clinical studies might use the acquired knowledge for the appropriate selection of patients.
8 Material & Methods

8.1 Materials

For conduction of the brain metastasis cohort, all patients with histologically confirmed BM from a solid tumour are identified from the database of Institute of Neurology (Neuropathology), Medical University of Vienna. In each case, histological diagnosis was made during routine diagnostic work-up at the Institute of Neurology by a board-certified neuropathologist. Only patients with distinct intraparenchymal BM are included in further analysis. As a consequence patients with osseous metastases of the scalp are excluded. Further, only patients with availability of at least one formalin-fixed paraffin embed tissue block containing viable brain metastasis tumour tissue were included.

For conduction of the breast cancer BM cohort all patients receiving neurosurgical resection of a BM from breast cancer or receiving whole brain radiation therapy for BM from breast cancer were identified from a clinical database.

First diagnosis of BM of the included patients was between 1990 and 2012. All patients were treated according to the current evidence-based standard of care for primary tumour as well as BM. Treatment for BM was applied either at the Medical University of Vienna, the Christian Doppler clinic (Salzburg) or the Wagner-Jauregg Provincial Neuropsychiatric Clinic (Linz).

8.2 Methods

Tissue based analysis

Tissue stainings

For each included tissue block a hematoxylin & eosin staining according to standard procedure was performed. Further a Gomorri silver impregnation stain was performed according to laboratory standards to investigate reticulin.

Immunohistochemistry

Table 3 gives an overview on performed immunohistochemical protocols and used antibodies.
<table>
<thead>
<tr>
<th>MARKER</th>
<th>ANTIBODY</th>
<th>ANTIBODY DILUTION</th>
<th>ANTIGEN RETRIEVAL</th>
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<td>HIF 1 alpha - hypoxia-inducible</td>
<td>Anti-Human HIF 1 alpha/610959 BD Transduction Laboratories™</td>
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<td>Standard cell conditioner 2 (pH 6) Ventana Benchmark ULTRA</td>
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<td>factor</td>
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<td>Standard cell conditioner 1 (pH8) Ventana Benchmark ULTRA</td>
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<td>1:1000*</td>
<td>Standard cell</td>
</tr>
<tr>
<td>Antigen</td>
<td>Code</td>
<td>Dilution</td>
<td>Secondary Antibody</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>----------</td>
<td>--------------------</td>
</tr>
<tr>
<td>β3 subunit</td>
<td>EM002-12, [160]</td>
<td>1:500*</td>
<td>Standard cell conditioner 1 (pH8) Ventana Benchmark ULTRA</td>
</tr>
<tr>
<td>αvβ3</td>
<td>EM227-03, [160]</td>
<td>1:500</td>
<td>Standard cell conditioner 1 (pH8) Ventana Benchmark ULTRA</td>
</tr>
<tr>
<td>αvβ5</td>
<td>EM099-02, [160]</td>
<td>1:800*</td>
<td>Standard cell conditioner 1 (pH8) Ventana Benchmark ULTRA</td>
</tr>
<tr>
<td>αvβ6</td>
<td>EM052-01, [160]</td>
<td>1:1000*</td>
<td>Standard cell conditioner 1 (pH8) Ventana Benchmark ULTRA</td>
</tr>
<tr>
<td>αvβ8</td>
<td>EM133-09, [160]</td>
<td>1:1000*</td>
<td>Standard cell conditioner 1 (pH8) Ventana Benchmark ULTRA</td>
</tr>
<tr>
<td>E cadherin</td>
<td>Ab15148; Abcam</td>
<td>1:30</td>
<td>EnVision Flex Target Retrieval Solution (pH9) AutostainerPlusLink/ Dako</td>
</tr>
<tr>
<td>N cadherin</td>
<td>Ab18203; Abcam</td>
<td>1:500</td>
<td>EnVision Flex Target Retrieval Solution (pH9) AutostainerPlusLink/ Dako</td>
</tr>
<tr>
<td>Pan-Cytokeratin</td>
<td>Lu5, BMA Biomedicals</td>
<td>1:100</td>
<td>EnVision Flex Target Retrieval Solution (pH6) AutostainerPlusLink/ Dako</td>
</tr>
<tr>
<td>HMBG45</td>
<td>HMB45, Dako</td>
<td>1:50</td>
<td>EnVision Flex Target Retrieval Solution (pH6) AutostainerPlusLink/ Dako</td>
</tr>
</tbody>
</table>

* protein concentration 1mg/ml

**Analysis of immunohistochemical stainings**

All specimens were analyzed with a standard light microscope.
HIF 1 alpha was scored according to the intensity of nuclear staining (none, weak, moderate, strong) and the percentage of cells showing a specific immunohistochemical signal (0-100%) [161]. For conduction of the HIF 1 alpha score the multiplication of the percentage and the corresponding intensity was added, resulting in an HIF 1 alpha index ranging from 0 to 300. Ki67 proliferation index was evaluated by counting 500 cells within the area of the highest density of cells with a specific, nuclear immunohistochemical signal and by giving the percentage of cells showing a specific immunohistochemical signal (0-100%) [162]. The microvascular density was assed by counting the amount of CD34 positive vessels within the area of the highest density at a 20x magnification (“hot spot”) [163]. The vascular pattern was analysed using the immunohistochemistry for CD34 as an endothelial marker. The “active, angiogenic type” was defined by the predominance of sprouting vessels, presenting with a multi-layered endothelium. The “silent type” was defined by the predominance of vessels with thin, mono-layered endothelium. Specimens with no clear predominance of either angiogenic patter were classified as “balanced type”.

For evaluation of inflammatory markers the regions of interest were defined as follows: within the vital brain metastasis tumour tissue, peritumoural region, adjacent brain parenchyma a least 0,5mm from the brain metastasis boarder. Within each region of interest number of immunoreactive cells was counted in eight high power fields at a magnification of x 400 (0.5 mm²).

Analyses of integrin αv subunit, cytoplasmic β3, αvβ3, αvβ5, αvβ6, αvβ8 complexes, and of E cadherin and N cadherin were performed according to previously published standards [164, 165]. In brief, intensity of membranous staining was multiplied by the percentage of cells showing a specific, complete, membranous immunoreactivity, resulting in an H-score ranging from 0 to 300. Further, integrin and cadherin expression of the vascular structures of the surrounding brain parenchyma, tumour vessels, peritumoural vessel and stroma was evaluated semi-quantitatively and recorded as either positive or negative.

**Evaluation of diffusion weighted imaging**
Diffusion weighted imaging was semiquantitatively juggled to be hypointense, isointense or hyperintense compared the non pathological brain parenchyma. In BM presenting with a heterogenous signal behaviour, diffusion intensity was determined
according to the predominant signal behaviour. Further, as available, apparent diffusion coefficient (ADC) values were derived in 5 non-overlapping areas of interest in solid, non-necrotic areas of the investigated brain metastasis. For further analysis, the mean ADC value was calculated for each case [130].

Clinical data
For the secure handling of patient data, a database was programmed using FileMaker Pro (version 11.0v3, FileMaker Inc.). The database is password secured to maintain data privacy protection. A Pub Med search was performed to evaluate clinical factors with potential prognostic impact in patients with BM. Clinical data was obtained by chart review. Survival data was retrieved from the database of the National Cancer Registry of Austria and the Austrian Brain Tumour Registry [166, 167].

Statistical analysis
All clinical data as well as results of immunohistochemical staining were collected in the programmed database. Results were entered into the statistical package for the social sciences (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). Survival time was defined as time from diagnosis of BM to death or last follow up. For estimation survival analysis the Kaplan-Meier product limit method was used. To test differences between groups respective to survival, the log-rank test was used. P-values ≤ 0.05 were considered to indicate statistical significance. Variables that had significant influence on survival in the univariate analysis were entered in a Cox proportional hazard model. For correlation of two parameters the $X^2$-Test, the Student’s T-Test and the Mann-Whitney-U-Test were used as appropriate. Due to the exploratory and hypothesis generating approach of the conducted projects no adjustment for multiple testing was applied [168].
9 List of figures

Figure 1: Frequency of primary tumours causing BM

10 List of tables

Table 1: Diagnosis specific prognostic assessment
Table 2: Estimated survival according to DS-GPA class
Table 3: Overview on used antibodies and protocols
11 References


54. Daginakatte, G.C. and D.H. Gutmann, *Neurofibromatosis-1 (Nf1) heterozygous brain microglia elaborate paracrine factors that promote Nf1-


12 Curriculum vitae

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Date of birth: 13.11.1987 (Bonn/Germany)
Citizenship: Austrian/German

School education
1993-1996 Elementary school, Bonn/Germany
1997-2003 High School, Bonn/Germany
2003-2004 High School, Justin, Tx/USA
2004-2005 High School, Vienna/Austria

Academic education
2005-2011 Medical University of Vienna (n202)
05.07.2011 Medical Doctor (MD) degree
Diploma thesis: Outcome measurements in metastatic breast cancer
(Supervisor: Priv.-Doz. Dr. Rupert Bartsch)
since 01.10.2011 Doctoral program „Clinical Neurosciences“
PhD thesis: Brain Metastases: Brain metastases: systematic exploration of prognostic and predictive factors
(Supervisor: Priv.-Doz. Dr. Matthias Preusser)
Clinical education
10.2011-12.2012 Fellow of Neuropathology, Institute of Neurology, Medical University of Vienna
since 01.01.2013 Fellow of Internal Medicine, Department of Medicine I, Medical University of Vienna

Memberships
Society of Austrian Neurooncology (SANO)
Austrian Society of Haematology and Oncology (ÖGHO)
American Society of Clinical Oncology (ASCO)
European Society of Medical Oncology (ESMO)
European Association of Neurooncology (EANO)
European Organisation of Research and Treatment of Cancer (EORTC)

Publications
45 articles in scientific journals with peer-review system (cumulative impact factor 175.7, H index 8)
16 articles as first author in scientific journal with peer-review system (cumulative impact factor 41.5)
5 oral and 13 poster presentations at international and national scientific meetings

other
2011-2013 Chairperson of the office for postgraduate studies, Austrian Student Union
2009-2011 Chairperson of the office for human medicine studies, Austrian Student Union
2007-2010 Chairperson of the office for social affairs, Austrian Student Union
2010-2011 Member of the academic senate, Medical University of Vienna
2008-2011 Member of the academic committee for human medicine studies, Medical University of Vienna
Publications

All original articles: 45
Cumulative Impact Factor: 175.7
Average Impact Factor: 3.9

First authorship: 16
Cumulative Impact Factor: 41.5
Average Impact Factor: 2.6

Congress Activities
Poster presentations: 13
Oral presentations: 5

Grants and Prices
Poster prices: 4
Travel grants: 5
Research grants: 4
A. First authorship

   Prognostic significance of Ki67 proliferation index, HIF1 alpha index and microvascular density in patients with non-small cell lung cancer brain metastases.
   Strahlenther Onkol. 2014 (in press)
   ISI 2011: IF 4.2

   Invasion patterns in brain metastases of solid cancers.
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   ALK gene aberrations and the JUN/JUNB/PDGFR axis in metastatic NSCLC
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   Clinical Neuropathology practice guide 3-2013: levels of evidence and clinical utility of prognostic and predictive candidate brain tumour biomarkers
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   Preoperative diffusion-weighted imaging of single brain metastases correlates with patient survival times.
   PLOS one 2013;8(2):e55464
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   Characterization of the inflammatory response to solid cancer metastases in the human brain.
   ISI 2011: IF 3.5

Brain-only metastatic breast cancer is a distinct clinical entity characterized by favourable median overall survival time and a high rate of long-term survivors
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11. Berghoff AS, Capper D, Preusser M.

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PD1 (CD279) and PD-L1 (CD274, B7H1) expression in primary central
nervous system lymphomas (PCNSL).
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B. Co-authorship

1. Tschandl P, Berghoff AS, Preusser M, Burgstaller-Mühlbacher S, Okamoto I,
Pehamberger H, Kittler H.
NRAS$^{Q61}$ and BRAF$^{V600E}$ mutations in melanoma-associated nevi and
uninvolved nevi
PLOS one (in press)
ISI 2011: IF 4.1

1. Zagouri F, Sergentanis TN, Bartsch R, Berghoff AS, Chrysikos D, de Azambuja E, Dimopoulos MA, Preusser M.
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carcinomatosis in HER2-positive metastatic breast cancer: A systematic
review and pooled analysis.
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ALK gene translocations and amplifications in brain metastases of non-small cell lung cancer


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BRAFV600E mutant protein is expressed in cells of variable maturation in Langerhans cell histiocytosis.

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J Neurooncol 2013 May;112(3):347-54

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   Immunohistochemical testing of BRAF V600E status in 1,120 tumor tissue samples of patients with brain metastases.
   Acta Neuropathol 2012; 123 (2): 223-33
   ISI 2011: IF 9.3

   Impact of anti-HER2 therapy on overall survival in HER2-overexpressing breast cancer patients with brain metastases.
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   Extent of peritumoral brain edema correlates with prognosis, tumoral growth pattern, HIF1a expression and angiogenic activity in patients with single brain metastases.
   Clin Exp Metastasis. 2013 Apr;30(4):357-68
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   ISI 2011: IF 9.3

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ISI 2011: IF 2.3

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ISI 2011: IF 5.5

International influence of the American ODAC statement upon prescription practice of bevacizumab in metastatic breast cancer.
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ISI 2011: IF 9.7

High plasma-GFAP levels in metastatic myxopapillary ependymoma.
ISI 2011: IF 3.2
Breast cancer brain metastases responding to primary systemic therapy with T-DM1
J Neurooncol 2013 (in press)
ISI 2011: IF 3.2

Ann Oncol 2013 (in press)
ISI 2011: IF 7.4

High rate of FGFR1 amplifications in brain metastases of squamous and non-squamous lung cancer
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ISI 2011: IF 3.4

microRNA Expression Pattern Modulates Temozolomide Response in GBM Tumors with Cancer Stem Cells
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29. Preusser M, Berghoff AS, Hottinger AF
High-grade meningiomas: new avenues for drug treatment?
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C. Manuscripts currently under review
Co-overexpression of HER3 is a predictor of impaired survival in breast cancer patients
Submitted

2. Bartsch R, Berghoff AS, Preusser M, Steger GG, C. Zielinski CC
Anti-angiogenic treatment approaches in breast cancer
Submitted

3. Ilhan-Mutlu A, Berghoff AS, Wrba F, Preusser M
Characterization of Candidate Tissue Biomarkers in a Rare Series of Myxopillary Ependymoma Specimens
Submitted

ETV1 protein expression is not associated with ETV1 gene amplification but with BRAF$^{V600E}$ in brain metastases of malignant melanoma
Submitted

Taxanes plus trastuzumab compared to oral vinorelbine plus trastuzumab in her2-overexpressing metastatic breast cancer patients

Submitted
13 Congress Activities

   Breast cancer brain metastases responding to primary systemic therapy with T-DM1
   Oral Presentation; 3rd Annual Brain Metastases Research and Emerging Therapy Conference
   September 2013, Marseille, France

   Characterization of T-ell infiltrates in brain metastases of solid tumors
   Poster Presentation; 3rd Annual Brain Metastases Research and Emerging Therapy Conference
   September 2013, Marseille, France

   Proliferation and microvascular density predict survival in patients with brain metastases of non-small cell lung cancer.
   Poster Presentation, European Cancer Congress
   October 2013, Amsterdam, Netherland

   Proliferation and microvascular density predict survival in patients with brain metastases of non-small cell lung cancer.
   Poster Presentation, Jahrestagung der Deutschen, Schweizer und Österreichischen Gesellschaft für Hämatologie und Onkologie
   October 2013, Vienna, Austria

4. Berghoff AS
   Clinical practice: Choosing a PCV schedule and toxicity management
   Oral Presentation, Serono Foundation Central Nervous System (CNS) Malignancies Meeting,
   October 2013, Vienna, Austria

Invasion patterns in brain metastases of solid cancers
Poster Presentation; Annual Meeting of the American Society of Clinical Oncology
June 2013, Chicago, USA

Clinical prognostic factors in brain metastases from colorectal cancer
Poster Presentation; Frühjahrstagung der Österreichischen Gesellschaft für Hämatologie und Onkologie
April 2013, Linz, Austria

Co-expression of HER3 is a predictor of impaired survival in HER2-positive breast cancer patients
Poster Presentation; Frühjahrstagung der Österreichischen Gesellschaft für Hämatologie und Onkologie
April 2013, Linz, Austria

Evaluation of invasion patterns and their correlation with integrin expression in alphavbeta brain metastases of solid cancers
Poster presentation; EOCTC-EANO-ESMO trends in Central Nervous System Malignancies
March 2013, Prag, Czech Republic

Characterization of the inflammatory response to solid cancer metastases in the human brain.
Oral presentation; 10th Meeting of the European Society of Neurooncology
September 2012, Marseille, France

Preoperative diffusion-weighted imaging of single brain metastases correlates with patient survival times.
Oral presentation; 17th annual Meeting of the American Society for Neuro-Oncology
November 2012, Washington, USA

Characterization of the inflammatory response to solid cancer metastases in the human brain.
Poster Presentation; Frühjahrstagung der Österreichischen Gesellschaft für Hämatologie und Onkologie
April 2012, Graz, Austria

Characterization of the inflammatory response to solid cancer metastases in the human brain.
Poster Presentation; YSA PhD Symposium
June 2012, Vienna, Austria

Brain-only metastatic breast cancer is a distinct clinical entity characterized by favorable median overall survival time and a high rate of long-term survivors.
Poster Discussion; Congress of the European Society for Medical Oncology
October 2012, Vienna, Austria

Extent of peritumoral brain edema correlates with prognosis, tumoral growth pattern, HIF1a expression and angiogenic activity in patients with single brain metastases.
Poster Presentation; 2nd Annual Brain Metastases Research and Emerging Therapy Conference
September 2012, Marseille, France

Impact of Her2 targeted therapy on overall survival in patients with Her2 positive metastatic breast cancer

Poster Presentation; ASCO Breast Cancer Symposium
September 2011, San Francisco, USA

Oral presentation; Frühjahrstagung der Österreichischen Gesellschaft für Hämatologie und Onkologie
May 2011, Pörtschach, Austria
Grants and Prices

1. Initiative Krebsforschung Grant, Project “Tumorimmunologie von Hirnmetastasen”, 40 000 €, December 2014

2. Hochschuljubiläumsstiftung Grant, Project „Das Immunsystem im Kampf gegen den Krebs“, 7500 €, October 2013

3. Forschungsförderungsstipendium Medical University of Vienna, Project „Inflammatory response in brain metastases of solid tumor“ , 850 €, March 2013

3. Forschungsförderung der Stadt Wien, Project „Preoperative diffusion-weighted imaging of single brain metastases correlates with patient survival times“, 1000 €, January 2013

4. Poster Price 2nd Annual Brain Metastases Research and Emerging Therapy Conference, September 2012, Marseille, France

5. Poster Price Vienna Summer School on Oncology, August 2011, Vienna, Austria

6. Poster Price Jahrestagung der Deutschen, Schweizer und Österreichischen Gesellschaft für Hämatologie und Onkologie, Oktober 2013, Vienna, Austria

7. Poster Price Frühjahrstagung der Österreichischen Gesellschaft für Hämatologie und Onkologie, April 2013, Linz, Austria

4. Travel Grant European Society for Medical Oncology, November 2013, Geneva, Switzerland

8. Methods in Clinical Cancer Research - FLIMS Workshop Travel Grant, June 2013, Flims, Switzerland

9. ASCO Travel Grant Comprehensive Cancer Center Vienna, June 2012, Chicago, USA

10. Forschungsförderungsprogramm "Internationale Kommunikation" der Österreichischen Forschungsgemeinschaft Travel Grant, September 2012, Vienna, Austria

EANO-SNO Travel Grant, November 2012, Washington, USA