Experimental modeling and novel therapeutic strategies in melanoma brain metastasis

Terje Sundstrøm

Dissertation for the degree of philosophiae doctor (PhD)

University of Bergen, Norway

2015

Dissertation date: June 9th 2015

LIST OF ABBREVIATIONS

3D	3-dimensional
5-ALA	5-aminolevulinic acid
AAAS	American Association for the Advancement of Science
ACT	Adoptive cell transfer
ADC	Apparent diffusion coefficient
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
APC	Antigen-presenting cell
APOE	Apolipoprotein-E
ATP	Adenosine triphosphate
B7-H3	B7 homolog 3
BBB	Blood-brain barrier
BCL2A1	Bcl-2-related protein A1
bFGF	Basic fibroblast growth factor
BLI	Bioluminescence imaging
BRAF	Serine/threonine-protein kinase B-raf
BRMS1	Breast cancer metastasis-suppressor 1
BTB	Blood-tumor barrier
CI	(Mitochondrial) Complex I
CC22	Chemokine (C-C motif) ligand 22
CD44v6	CD44 splicing variant 6
CDK4	Cyclin-dependent kinase 4
CDKN2A	p16INK4A inhibitor of CDK4
cMAP	Connectivity Map
CNS	Central nervous system
COT	Serine/threonine kinase Cot
CRAF	RAF proto-oncogene serine/threonine-protein kinase
CT	Computed tomography
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	C-X-C chemokine receptor type 4

Da	Dalton (unit)
DNA	Deoxyribonucleic acid
DWI	Diffusion weighted imaging
ECM	Extracranial metastases
EDNRB	Endothelin receptor B
EFSA	European Foods Safety Authority
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERBB2	Receptor tyrosine-protein kinase erbB-2
ERK	Extracellular signal-regulated kinase
ET3	Endothelin-3
FDA	Food and Drug Administration
GBM	Glioblastoma multiforme
GEMMs	Genetically engineered mouse models
GI	Gastrointestinal
GPA	Graded prognostic assessment
GRAS	Generally Recognized As Safe
GTPases	Guanosine triphosphate hydrolases
Gy	Gray (unit)
H1	Human melanoma brain metastasis cell line 1
HDL	High-density lipoprotein
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HIF1α	Hypoxia-inducible factor 1α
HPSE	Heparanase
HR	Hazard ratio
HSP90	Heat shock protein 90
IGF-1R	Insulin-like growth factor 1 receptor
IL-1β	Interleukin-1β
IL-2	Interlukin-2
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KPS	Karnofsky performance status
L1CAM	L1 cell adhesion molecule

LDH(A/B)	Lactate dehydrogenase (A/B)
LINAC	Linear accelerator
LXRβ	Liver X receptor β
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MDM4	Protein Mdm4
MEK	Mitogen-activated protein kinase kinase
MITF	Microphthalmia-associated transcription factor
miRNA	MicroRNA
MHC	Major histocompatibility complex
MMP-2	Matrix metalloproteinase-2
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
mTOR	Mammalian target of rapamycin
NAA	*N*-acetylaspartate
NT-3	Neurotrophin-3
NF1	Neurofibromin
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
NPC1L1	Niemann-Pick C1 Like 1
NRAS	Neuroblastoma RAS viral oncogene homolog
NSCLC	Non-small cell lung cancer
OS	Overall survival
OXPHOS	Oxidative phosphorylation
p38α	Mitogen-activated protein kinase 14
p53	Cellular tumor antigen p53
PA	Plasminogen activator
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDGFRβ	Platelet-derived growth factor β
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase, isoenzyme 1
PET	Positron emission tomography

PFS	Progression-free survival
PGC1α	Peroxisome proliferator-activated receptor γ coactivator 1-α
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PWI	Perfusion weighted imaging
rCBV	Relative cerebral blood volume
Rac1	Ras-related C3 botulinum toxin substrate 1
RCC	Renal cell carcinoma
RCT	Randomized controlled trial
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RPA	Recursive partitioning analysis
RR	Response rate
RTOG	Radiation Therapy Oncology Group
SCLC	Small cell lung cancer
SDF-1α	Stromal cell-derived factor 1α
shRNA	Short hairpin RNA
SOCS-1	Suppressor of cytokine signaling 1
SPION	Superparamagnetic iron oxide nanoparticles
SRS	Stereotactic radiosurgery
STAT	Signal transducer and activator of transcription
T-DM1	Trastuzumab emtansine
TCA	The citric acid cycle
TCGA	The Cancer Genome Atlas
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TIL	Tumor-infiltrating lymphocyte
TMZ	Temozolomide
TNFα	Tumor necrosis factor alpha
TrkC	Tropomyosin receptor kinase C
US	Ultrasound
UV	Ultraviolet

VCAM-1	Vascular cell adhesion molecule-1
VEGF(A/R)	Vascular endothelial growth factor (A/receptor)
WBRT	Whole brain radiotherapy
WT	Wild-type
ZO-1	Tight junction protein ZO-1

SCIENTIFIC ENVIRONMENT

The work presented in this PhD thesis was carried out at the K. G. Jebsen Brain Tumour Research Centre, Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen.

Some of the work was done during a research stay at the Ferrara laboratory, Department of Biomedical Engineering, University of California Davis, USA.

The Western Norway Regional Health Authority provided funding through a PhD scholarship and a personal overseas research grant.

I have been affiliated with the Departments of Biomedicine and Clinical Medicine, University of Bergen, and the Department of Neurosurgery, Haukeland University Hospital.

ACKNOWLEDGMENTS

"Mellow is the man who knows what he's been missing"
Over the Hills and Far Away, Led Zeppelin (1973)

Metastasis literally means beyond stillness (*meta*: beyond, *stasis:* stillness), in many ways descriptive of the field of basic cancer research itself. The complexity of unanswered and answered questions in this field is both fascinating and daunting. This PhD work has been a great experience for me, and I have learned many lessons that I will take with me throughout life.

I am truly grateful to all my colleagues and staff at the K. G. Jebsen Brain Tumour Research Centre for all their help and creativity. I would especially like to thank Francisco Azuaje, Heidi Espedal, Patrick N. Harter, Erlend Hodneland, Sindre Horn, Tina Pavlin, Lars Prestegarden, Gro Vatne Røsland, Kai Ove Skaftnesmo, Jobin K. Varughese and Ingvild Wendelbo for their dedication and collaboration. I am particularly obliged to Professor Rolf Bjerkvig for bringing me into his reality distortion field where everything is possible. I would like to extend my sincerest thanks and appreciation to Professor Frits Thorsen for his persistent support and for giving me the independence to pursue my research interests. I am also deeply grateful to Professor Morten Lund-Johansen for his clinical perspectives and reality check meetings.

I would especially like to thank Professor Emeritus Knut Wester for his inspiration and mentorship over many years, and for introducing me to the worlds of science and neurosurgery.

I am glad to have good friends and colleagues at the Department of Neurosurgery, and I am thankful to be working with such devoted and stimulating colleagues.

I would like to thank the Western Norway Regional Health Authority for granting me the PhD scholarship and the personal overseas research grant, which I used to visit the laboratory of Professor Katherine W. Ferrara at UC Davis.

I would like to thank my parents Eva and Jan for their relentless support and encouragement during this endeavor and all others.

Finally, I am deeply grateful to my wife Edith for all her efforts on my behalf, and my children Anna, Ola and Einar who put up with my long hours seeing this project to completion.

Bergen, March 2015

Terje Sundstrøm

TABLE OF CONTENTS

1. ABSTRACT

Melanoma patients carry a high risk of developing brain metastases and improvements in survival are still measured in weeks or months. The aim of this thesis was to study the biology of melanoma brain metastasis and find new therapeutic approaches. In **Paper I**, we reviewed the current literature on animal models of brain metastasis. Many models are available and have provided valuable insights, but technical and biologic limitations have hampered clinical translation. In **Paper II**, we reported on the development and validation of a new experimental brain metastasis model. This model featured MRI-based automated quantification of nanoparticle-labeled melanoma cells in the mouse brain after intracardiac injection. We proposed that this model could help to increase the reproducibility and predictivity of mechanistic and therapeutic studies of melanoma brain metastasis. In **Paper III**, we examined the temporal, spatial and functional significance of lactate dehydrogenase A (LDHA) in melanoma brain metastasis. We found that LDHA expression was hypoxia-dependent, but did not affect tumor progression or survival *in vivo* or in a large patient cohort. In **Paper IV**, we applied genomics-based drug repositioning and carried out a comprehensive *in vitro* and *in vivo* screening of potential anti-melanoma brain metastasis compounds. We found the cholesterol analogue β-sitosterol to inhibit the growth of brain metastases and improve survival in established and preventive scenarios across several *in vivo* models. β-sitosterol provided broad-spectrum suppression of the important mitogen-activated protein kinase (MAPK) pathway and reduced mitochondrial respiration through Complex I inhibition. Notably, increased mitochondrial respiration is a key mediator of intrinsic and acquired resistance to established MAPK-targeted therapies. Together, **Papers I** and **II** showed that the study of melanoma biology and brain metastasis requires reproducible and predictive animal models. By applying such models in **Papers III** and **IV**, we revealed novel insights into the biology and therapy of melanoma brain metastasis, and suggested that mitochondrial respiration might play an imperative role in tumor progression and treatment resistance.

2. PUBLICATION LIST

Paper I

Daphu I, Sundstrøm T, Horn S, Huszthy PC, Niclou SP, Sakariassen PØ, Immervoll H, Miletic H, Bjerkvig R & Thorsen F**. In vivo animal models for studying brain metastasis: value and limitations.** Clinical & Experimental Metastasis 2013; 30: 695-710.

Paper II

Sundstrøm T, Daphu I, Wendelbo I, Hodneland E, Immervoll H, Skaftnesmo KO, Lundervold A, Jendelova P, Babic M, Sykova E, Bjerkvig R, Lund-Johansen M & Thorsen F. **Automated tracking of nanoparticle-labeled melanoma cells improves the predictive power of a brain metastasis model.** Cancer Research 2013; 73: 2445-2456.

Paper III

Sundstrøm T, Espedal H, Harter PN, Fasmer KE, Skaftnesmo KO, Horn S, Hodneland E, Mittelbronn M, Weide B, Beschorner R, Bender B, Rygh CB Lund-Johansen M, Bjerkvig R & Thorsen F. **Melanoma brain metastasis is independent of lactate dehydrogenase A expression.** Neuro-Oncology 2015; Mar 19 [Epub ahead of print].

Paper IV

Sundstrøm T, Varughese JK, Prestegarden L, Azuaje F, Røsland GV, Skaftnesmo KO, Ingham E, Even L, Tam S, Tepper C, Petersen K, Ferrara KW, Tronstad KJ, Lund-Johansen M, Bjerkvig R & Thorsen F. **β-sitosterol provides broad-spectrum therapeutic suppression of melanoma brain metastasis.** Manuscript submitted.

3. FIGURES AND TABLES

Figures

Tables

4. INTRODUCTION

4.1. METASTASIS

Metastasis is the most ominous hallmark of cancer being responsible for >90% of cancer mortality[1]. This multistep process whereby tumors spread from their primary site to form secondary tumors at distant sites is also the most enigmatic[2]. This cascade of events requires successful cancer cell invasion, intravasation into blood and lymphatic vessels, survival during transit through these vessels, arrest and extravasation into distant organs, and multiplication from micrometastatic to macrometastatic lesions within the organ parenchyma (**Fig. 1**).

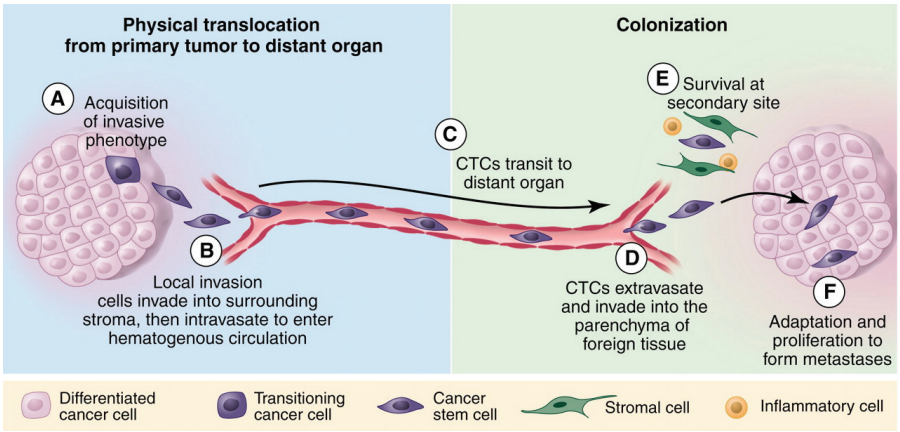

Figure 1 The metastatic process. Each step in this cascade is driven by the acquisition of genetic and/or epigenetic alterations and requires intricate cooperation between cancer cells and stromal cells. Hematogenous dissemination is the primary route to distant organs. Circulating tumor cells (CTCs) denote cancer cells with stem-like properties (e.g. enhanced tumorigenicity, self-renewal potential). From Chaffer et al.[2]. Reprinted with permission from the American Association for the Advancement of Science (AAAS).

Primary tumors can often be cured by surgical resection and adjuvant chemo- and radiotherapy, whereas metastatic disease is often incurable due to its extent and resistance to available therapies[1]. Thus, future improvements in cancer treatment and patient prognosis are largely reliant on continued innovation seeking to prevent or reverse cancer metastasis.

4.2. BRAIN METASTASIS

4.2.1. Epidemiology

The exact prevalence and incidence of brain metastases based on population studies are unavailable[3]. Despite the incompleteness of data and inadequate ascertainment of cases, most studies indicate that the number of patients with brain metastases has been increasing and will continue to increase in coming years[4,5,a].

4.2.1.1. Prevalence

Symptomatic brain metastases develop in 8.5-9.6% of all adults with cancer[6,7]. The true prevalence is probably much higher, as asymptomatic patients are not diagnosed, symptomatic brain metastases are not reported in patients with widespread disease, and patients with brain metastases are misdiagnosed as having cerebrovascular disease or other neurological conditions[3,8]. Historical autopsy series have generally reported higher frequencies of brain metastases than that reported in population-based studies. In an autopsy study of breast cancer patients, only 31% of the cases were diagnosed or suspected before death[9]. Large autopsy series have revealed brain metastases in 15-41% of cancer patients[10,11]. However, the current prevalence is difficult to establish due to low autopsy rates (<5%)[8].

4.2.1.2. Incidence

The estimated incidence of brain metastases in the United States (US) is 7-14 persons per 100,000 per year (22,000-44,000 persons per year)[12]. A population-based study from the period 1935 through 1968 from Rochester in the US reported an incidence rate of 11.1 per 100,000 per year[10]. A national survey study from the US reported an incidence rate of 8.3 per 100,000 between 1973 and 1974[13]. A population-based study from Scotland conducted in 1989-1990 reported an incidence rate of 14.3 per 100,000; only 11% of cases had pathological confirmation and brain metastases accounted for 48% of all intracranial tumors[14]. This study also showed an exponential increase in incidence rates until age 74 and thereafter a decline. The age-adjusted incidence of hospitalization due to brain metastases doubled from 7 to 14 persons per 100,000 per year in Sweden between 1987 and 2006[15]. In a large retrospective cohort

[a] http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/index

study from the US, the annual number of surgical resections for brain metastases increased by 79% from 3,900 in 1988 to 7,000 in 2000[16].

Several factors contribute to the observed increase in incidence of brain metastases[17]. The first is the introduction and rapidly increasing availability of neuroimaging, in particular of magnetic resonance imaging (MRI); 20 years ago, only 2% of cancer patients underwent MRI as compared to 64% of patients today[18]. Many cancer patients undergo surveillance brain imaging in the absence of symptoms, and many clinical trials mandate MRI screening to exclude patients with brain metastases[3]. Second, there has been a steady increase in the incidence of cancers with a predilection for brain metastasis, such as melanoma[a]. Third, cancer patients live longer due to earlier detection and better treatment, and the population at risk of developing brain metastases therefore increases; this is especially important for lung and breast cancer, which display decreasing overall incidences[17,b]. For instance, patients diagnosed with breast cancer in Sweden in the period 2004-2006 were at a 44% increased risk of being admitted with brain metastases as compared to patients diagnosed in the period 1998-2000[19]. Fourth, many targeted therapies have limited bioavailability in the brain; observations suggest an increasing incidence of brain metastases in human epidermal growth factor receptor 2 (HER2)-positive breast cancer patients treated with trastuzumab, a substance that has limited ability to pass the blood-brain barrier (BBB)[20,21] and hence creates a "sanctuary site" for tumors to develop within the central nervous system (CNS)[8].

4.2.1.3. Number and location

Historical autopsy series have revealed a single brain metastasis in 47% of cancer patients[11]. In a surgical series of 309 patients with brain metastases, 72.1% of patients had one metastasis, and most of these patients had a controlled primary tumor and no other metastases[22]. Surgical series are of course biased towards limited disease both intra- and extracranially, as well as a good performance status and a lower age distribution. Clinical series of cancer patients undergoing treatment for brain

[a] http://seer.cancer.gov/statfacts/html/melan.html
[b] http://seer.cancer.gov/statfacts

metastases are less biased, and have shown multiple brain metastases in 47% of cases[23] and more than three metastases in 41% of cases[24].

Multiple brain metastases are more frequently seen in patients with lung cancer and melanoma, whereas breast, renal and colorectal cancers are more frequently associated with a single brain metastasis[23,25].

The distribution of brain metastases is generally in accordance with blood flow and tissue volume: cerebrum 80%, cerebellum 15% and brain stem 5%[25]. However, studies suggest that lung and breast cancer are more prone to cause cerebellar metastases than renal cancer, gynecological cancers and, particularly, melanoma[26-28].

Most patients (60-80%) with brain metastases have concurrent systemic metastases, of which pulmonary metastases are most frequent[25,29,30].

4.2.1.4. Causative primary cancers

Any kind of cancer can disseminate to the brain[30]. Lung cancer, breast cancer and melanoma account for 67-80% of all brain metastases[14,22-24,27,30,31]. The most common reported cause of brain metastases has been lung cancer (39-56%; of which 6-15% is small cell lung cancer (SCLC) and 24-44% is non-small cell lung cancer (NSCLC)), followed by breast cancer (13-44%), melanoma (6-11%), colorectal cancer (3-9%) and renal cancer (2-6%); however patterns are evolving and there are also substantial geographical variations[3,6,18,32]. In a population-based study from the Detroit area in the US of patients diagnosed with cancer in the period 1973 to 2001, it was estimated that 19.9% of lung cancer patients developed brain metastases followed by melanoma (6.9%), renal cancer (6.5%), breast cancer (5.1%) and colorectal cancer (1.8%)[6]. In a study from Norway on patients with brain metastases, comparing the periods 1983-1989 and 2005-2009, Nieder et al. described a reduction in lung cancer (52% versus 40%), increase in melanoma (5% versus 9%), increases in colorectal and kidney cancers (8% versus 24%), and a stable incidence of breast cancer (17%)[18].

Usually, brain metastases develop in patients with a known history of cancer or brain metastases precede a diagnosis of cancer somewhere in the body. However,

sometimes (2-14%) the cancer of origin is not found, even at autopsy[14,23,29-31,33-35]. In a German study looking at 5,074 patients with brain metastases who were diagnosed and treated in 2008, 7.5% of patients had unknown primaries[29].

For patients with a known history of cancer, one should not presume that a single brain lesion is synonymous with a brain metastasis. In a randomized clinical trial assessing the efficacy of surgical resection for a single brain metastasis, 11% of patients were diagnosed with a primary CNS tumor (glioblastoma multiforme (GBM) and low-grade astrocytoma), abscess or inflammatory process[36].

4.2.2. Diagnosis

Early detection of brain metastases is important to maximize the efficacy of available therapies and to minimize the morbidity of these treatments[17]. Brain metastases are established indicators of poor prognosis and there are no effective preventive measures; vigilant clinical monitoring is thus required for early diagnosis and minimization of neurological injury[17]. MRI is the most important modality and brain metastases are typically detected using contrast-enhanced T1-weighted (T1w) sequences. The definite diagnosis is made by standard histopathological and molecular analyses of surgical tissue specimens (resection or biopsy). Several imaging techniques, which at present are being developed preclinically, aim at early detection of brain metastases (see section 4.4.2.2.).

The appearance of a single brain metastasis can be very similar to e.g. a GBM with peripheral contrast enhancement and central necrosis. Two advances in MRI technology can be helpful to differentiate between primary and metastatic tumors: magnetic resonance spectroscopy (MRS) and perfusion-weighted imaging (PWI). The choline to *N*-acetylaspartate (NAA) ratio from MRS spectra and the PWI-derived relative cerebral blood volume (rCBV) are similar within high-grade gliomas and brain metastases, but different in the peritumoral zones. Both the choline to NAA ratio and rCBV measurements are higher around high-grade gliomas due to their infiltrative growth, whereas brain metastases have close to normal choline to NAA ratios and rCBV measurements due to their circumscribed, non-infiltrative growth[37,38].

Diffusion-weighted imaging (DWI) can indicate if a lesion is a brain metastasis or a brain abscess. Abscesses typically have low apparent diffusion coefficient (ADC) ratios and display high signal intensity (restricted diffusion) on DWI, whereas cystic brain metastases have high ADC ratios and low signal intensity on DWI[39]. MRS is less specific and more time-consuming, but can also show different spectra between abscesses and brain metastases with abscesses displaying elevated levels of acetate, succinate, lactate and amino acids such as valine, leucine and isoleucine[40]. These amino acids are not seen in the spectra of brain metastases.

4.2.3. Treatment

Treatment of brain metastases is multidisciplinary and based on a selective use of radiation and surgery[17]. Surgery or stereotactic radiosurgery (SRS) are the preferred options for patients with a newly diagnosed solitary brain metastasis and a good prognosis. A surgical approach is favored by mass effect (particularly relevant for metastases in the posterior fossa), superficial and/or accessible location, maximal diameter >3-4 cm and diagnostic uncertainty. SRS is favored for patients with poor performance status and prognosis, deep and/or inaccessible location, maximal diameter <2-3 cm and close proximity to eloquent brain structures. Patients with 2-4 brain metastases are typically treated with SRS and/or whole brain radiotherapy (WBRT). Patients who progress after local therapy should be considered for systemic therapy and/or WBRT. Molecularly targeted therapies and immunotherapies offer great promise for defined subsets of patients.

Figure 2 shows a suggested evidence-based treatment algorithm as put forward by Meier in 2014[41]. A number of other factors influence decision-making, including physician and patient preferences (quality of life versus overall survival (OS)). Standardized diagnostic and treatment guidelines for brain metastases (1-3, >3 and leptomeningeal) are available through the National Comprehensive Cancer Network (NCCN)[a].

[a] http://www.nccn.org/professionals/physician_gls/pdf/cns.pdf

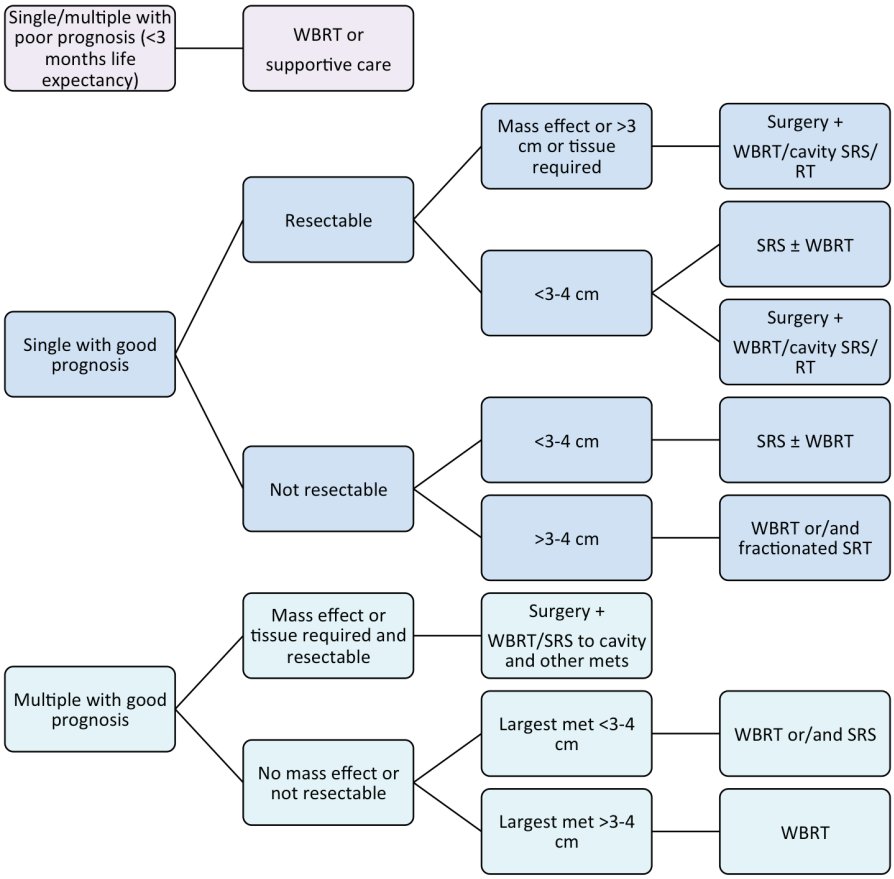

Figure 2 Treatment algorithm of single and multiple brain metastases. At all stages, consider clinical trial participation and systemic therapy. Surgery should be followed by radiotherapy, whereas adding WBRT to SRS is optional (including for patients with 2-4 brain metastases). WBRT, whole brain radiotherapy; SRS, stereotactic radiosurgery; RT, localized fractionated radiotherapy; met, metastasis. Adapted with permission from Meier R. 2014[41].

Brain metastases management is hampered by the lack of effective chemotherapy beyond the BBB and inevitable concerns of radiation and surgery on surrounding brain structures[5,42,43]. Furthermore, patients with brain metastases are often excluded from clinical trials, leaving us uncertain about the effects of new therapeutic modalities[44-46]. New and innovative research approaches and treatment strategies are needed to improve the outcome of brain metastasis patients[5,17,47].

4.2.3.1. Surgery

For many years, surgery was performed on patients who were thought to have a single brain metastasis and an otherwise good prognosis[48,49]. However, the role of surgery was uncertain until Patchell et al. in 1990 showed in a prospective, randomized controlled trial (RCT) that surgery + postoperative radiotherapy was superior to radiotherapy alone for patients with a single brain metastasis; patients receiving the combined treatment lived longer (median 40 weeks versus 15 weeks), had fewer local recurrences (20% versus 50%) and remained functionally independent longer (38 weeks versus 8 weeks)[36]. Previous uncontrolled and retrospective studies had reported conflicting results; some had found a clear benefit from surgery[48,50-56] whereas others had not found a benefit[57-60]. In a 1993 RCT, Vecht et al. verified these findings showing a significant survival benefit (+ four months) of adding surgery to radiotherapy in the treatment of a single brain metastasis[61]. Noordijk et al. reported similar results in 1994 on 63 patients with a single brain metastasis; median survival increased from six to 10 months with the addition of surgical resection[62]. Furthermore, Patchell et al. published a randomized trial in 1998 showing that surgical resection and postoperative radiotherapy was superior to surgical resection alone with a reduced local recurrence rate (10% versus 49%), fewer distant relapses (14% versus 37%) and patients were less likely to die from neurologic causes (14% versus 44%)[63].

Building on these pioneering studies and others, the first evidence-based clinical practice guideline for the treatment of patients with brain metastases was published in 2010[64]. This guideline provides Level I evidence that supports the use of surgical resection + postoperative WBRT as compared to WBRT alone in functionally independent patients who spend less than 50% of time in bed and who have limited extracranial disease. There was insufficient evidence to conclude on management of patients with poor performance status, advanced systemic disease or multiple brain metastases.

There is no established surgical recommendation based on Level I evidence for patients with multiple or recurrent brain metastases. However, studies suggest that in selected patients, surgical resection of all lesions increases survival and confers a

similar prognosis to that of patients operated for a single metastasis[65], and repeat surgical resection of recurrent tumors improves survival and quality of life[66,67].

Evidence-based treatment recommendations are important in surgical decision-making. However, the surgeon must balance the benefits and harms of surgery in each individual patient (*primum non nocere*). This has been clearly underscored in studies of GBM surgery showing three to four months survival reduction from surgically acquired deficits (language or motor)[68], and patients with perioperative complications and new neurological deficits are frequently denied adjuvant chemo- and radiotherapy[69]. Important considerations in brain metastasis surgery are accessibility, size, number, proximity to eloquent brain structures, degree of mass effect, concurrent hydrocephalus and the need for a definitive diagnosis. Likewise important are age, comorbidity, degree of extracranial disease and performance status of the patient. There is no definite threshold to initiate or withhold surgery, but the patient must have a possibility of a reasonably functional outcome. Patients with advanced disease and exhausted treatment options should generally not be subjected to surgical treatment.

Our ability to provide maximally safe and effective surgery for brain metastases has been furthered by significant advances in neuroimaging and surgical technology[70-72]. Intraoperative neuronavigation with three-dimensional (3D) volumetric rendering of tumors and functional structures enables the neurosurgeon to visualize the anatomy and track the location of surgical instruments during surgery thereby providing better precision of craniotomies and tumor resection (**Fig. 3**). Systems for image guidance, like Brainlab® (Brainlab AG) or StealthStation® (Medtronic Inc.), most frequently rely on preoperative MRI and computed tomography (CT) imaging. However, intraoperative imaging updates are also possible through integrated MRI solutions within the operating room as well as real-time ultrasound (US) imaging; these complementary resources can provide valuable feedback on the extent of resection and brain shift during surgery. The standard neurosurgical approach to a brain metastasis is typically microsurgical stripping of the tumor from the surrounding brain parenchyma using conventional white-light microscopy, assisted by preoperative MRI-based neuronavigation and US for deep-seated lesions[73]. Other techniques that can help to optimize the safety and efficacy of surgery include, but are not limited to, awake craniotomy with cortical mapping[74], neurophysiological monitoring, and

photodynamic detection of systemically administered fluorophores like 5-aminolevulinic acid (5-ALA)[75] or fluorescein[76] in tumor tissue. New advanced contrast agents that enable multi-modal imaging of the same probe before and during surgery hold great promise with higher resolution, sensitivity and specificity than conventional technologies, and can also be exploited for drug delivery or phototermal therapy of brain tumors[77].

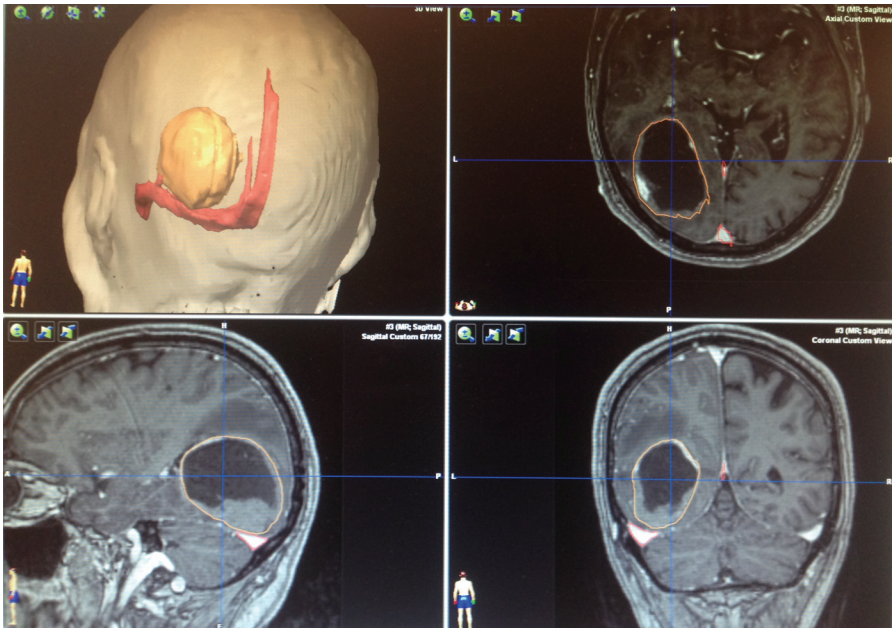

Figure 3 Preoperative outlines of a tumor and functional structures. 3D volumetric rendering of an occipital brain metastasis from lung adenocarcinoma and the adjacent venous sinuses using BrainLab® (Brainlab AG). Illustration by T. Sundstrøm.

Numerous studies have been performed on image-guided surgery for the resection of brain tumors, but a recent Cochrane review only identified four RCTs of sufficient quality[78]: one study for intraoperative MRI[79], one study for fluorescence-guided surgery[80] and two studies for neuronavigation[81,82]. No studies on US-guided surgery were deemed eligible for inclusion; 3D US-guided surgery (Sonowand®)[83] has not yet been the subject of a RCT. The Cochrane review concluded that although each of these technologies have their merits like increased extent of resection, the quality of evidence is poor, effects on survival and quality of life are uncertain and studies suffer

from significant reporting biases[78]. Thus, further research is needed to determine the efficacy of these techniques and their individual applicability. Moreover, most of these studies were conducted in patients with high- or low-grade gliomas, hence, the value of these imaging resources are even less clear in brain metastasis surgery. For example, fluorescence-guided resection using 5-ALA does not seem to be reliable in identifying infiltrating parts of metastases[75].

Although surgery plays an indispensable role in the treatment of brain metastases, it is not enough[73]. Local recurrence rates after gross-total resection without subsequent WBRT are about 50-60% with current surgical standards[84,85]. This can of course be ascribed to the surgery itself (e.g. inadequately performed, tumor cell dissemination), but is more likely related to the nature of the disease. For example, cumulative evidence suggests that brain metastases are not as circumscribed and sharply demarcated as we have thought. In an autopsy study, 63% of patients displayed invasive growth patterns, and this was most common with SCLC and melanoma[86]. In a recent surgical series, more than 60% of patients showed tumor extensions and islets in the adjacent brain parenchyma[75]. Taken together, brain metastases should preferentially be resected *en bloc*, there may be a role for supramarginal resection in selected patients and surgery should be combined with SRS or WBRT[73].

4.2.3.2. Whole brain radiotherapy

WBRT has historically been the major alternative to surgical treatment of brain metastases[87]. A landmark paper published in 1954 showed that radiotherapy alleviated symptoms in 63% of patients and provided similar responses in tumors assumed to be radiosensitive as well as radioresistant (e.g. melanoma)[88]. By the 1970s, WBRT had become a mainstay therapy for brain metastases[89]. Moreover, radiotherapy was at the time found to be associated with minimal morbidity and toxicity[90]. WBRT is still a standard of care in combination with other treatments, and it remains the treatment of choice for patients with multiple brain metastases, addressing both macroscopic and microscopic disease[91].

However, there are growing concerns about the adverse effects of WBRT, especially the long-term effects of neurocognitive decline and reduced quality of life[92-94]. WBRT

alone is inadequate over time; in an analysis of 1,200 patients treated with WBRT alone between 1979 and 1993, even the best prognostic group was found to have a median survival of just 7.1 months[95]. Moreover, systemic treatments have progressively improved since the mid-1970s, and the mortality rates of most cancers have decreased, even among patients with metastatic disease[96]. Hence, patients live longer, and the long-term adverse effects of WBRT have gradually become more apparent.

Different dose-fractionation schedules have been utilized in numerous studies, but the most common treatment schedule for WBRT is 30 Gy delivered in 10 fractions over two weeks[91]. This protocol is generally accepted to provide the best trade-off between efficacy and toxicity. WBRT-toxicities are typically classified as acute (within a few days), early-delayed (first weeks to months) or late (after 90 days)[91]. In the acute phase, patients frequently experience fatigue, nausea/vomiting, alopecia, dermatitis and steroid-responsive cerebral edema. Early-delayed symptoms include fatigue and neurocognitive deficits such as memory decline. The late-stage toxicities are usually not self-limited and mild as in the acute and early-delayed stages. The classical biphasic pattern of neurocognitive deterioration begins with a decline around four months after treatment, thereafter a transient improvement before the patients irreversibly deteriorate months to years later with moderate to severe dementia[17,97].

Although various dose-fractionation schedules have failed to demonstrate improved tumor control and patient survival in patients with brain metastases, randomized trials with WBRT in combination with surgery[36,61,62] or SRS[98,99] have. The studies by Patchell et al., Vecht et al. and Noordijk et al. are discussed above[36,61,62]. The Radiation Therapy Oncology Group (RTOG) 9508 phase III randomized trial compared the use of WBRT with or without SRS for patients with one to three brain metastases[98]. This study showed a significant benefit in OS of adding SRS (6.5 months versus 4.9 months) and a stable/better Karnofsky Performance Status (KPS) at six months (43% versus 27%); however, patients with multiple brain metastases did not have better survival, but better KPS scores and less steroid use. For patients with two to four brain metastases, Kondziolka et al. reported a one-year local failure rate of 100% with WBRT alone, but only 8% with the addition of SRS; median time to local failure was six months versus 36 months, respectively[99]. This study also showed a

non-significant survival benefit of adding SRS to WBRT (7.5 months versus 11 months).

In a randomized trial of prophylactic cranial irradiation or not in 286 patients with extensive SCLC, Slotman et al. found that irradiation resulted in an improvement in median survival from 5.4 months to 6.7 months and a reduced risk of symptomatic brain metastases within one year from 40.4% to 14.6%[100]. Irradiation had side effects, but there were no significant differences in global health status between the two groups. In contrast, in a randomized trial by Gore et al., including 356 patients with advanced NSCLC, prophylactic cranial irradiation was not associated with improved one-year OS, even though there was a 2.5 times higher risk of developing brain metastases without irradiation[101]. In this study, the patients showed a considerable neurocognitive decline, although they received a lower dose of WBRT (30 Gy in 15 fractions) than standard.

Several strategies have been investigated to reduce the neurocognitive impact of WBRT. The results of a phase II trial of WBRT with hippocampal sparing were recently reported by Gondi et al. who found significantly less impairment of memory function and quality of life compared with historical series[102]. This technique has also been developed to selectively expose metastatic lesions to higher radiation doses (integrated brain metastases boost)[103], and there are currently several ongoing clinical trials that aim to evaluate this composite technology. Memantine, a N-methyl-D-aspartate (NMDA) receptor antagonist, which is used to treat patients with Alzheimer disease, was recently evaluated in a randomized trial of 508 patients with brain metastases receiving WBRT[104]. Compared to placebo, memantine significantly delayed and reduced neurocognitive deterioration, but did not affect survival (see **Paper IV**).

The combination of WBRT and conventional chemotherapies that can penetrate the BBB has generally produced discouraging results[17]. One of the best studied chemotherapeutic agents that can cross the BBB is the lipid soluble and alkylating agent temozolomide (TMZ). Taken together, the combination of WBRT and TMZ has shown limited or no benefit compared to WBRT alone in four phase II clinical trials[105-108].

The use of targeted drugs rather than traditional chemotherapeutic agents is regarded as a more promising approach with reduced systemic toxicity and higher potential for individual stratification of patients to effective therapies[17]. Welsh et al. recently published a phase II trial on 40 patients with NSCLC with brain metastases that were treated with WBRT + the epidermal growth factor receptor (EGFR) inhibitor erlotinib[109]. The authors reported an 86% response rate, few adverse effects and a median survival of 11.8 months; subgroup analyses revealed a median survival of 19.1 months for patients with *EGFR* mutations and 9.3 months for patients with wild-type *EGFR*. In contrast, Sperduto et al. found a median survival of 13.4 months for WBRT + SRS, 6.3 months for WBRT + SRS + TMZ, and 6.1 months for WBRT + SRS + erlotinib in 126 NSCLC patients with one to three brain metastases[110]. These survival differences were not statistically significant, and, importantly, subgroup allocation was not biomarker-based and the control group (WBRT + SRS) displayed much better outcomes than anticipated from previous reports: 6.5 months[98] and 7.5 months[111]. In summary, combinatorial regimens of WBRT, SRS, chemotherapeutic drugs and molecularly targeted drugs for patients with brain metastases are a subject of intense research, and there is a need to define relevant subgroups of patients that adequately benefit from the various combinations.

4.2.3.3. Stereotactic radiosurgery

Noninvasive ablation of cancer cells using focused, high-dose radiation is an option to surgical resection. SRS can be delivered with a Gamma Knife (gamma rays) or a linear accelerator (LINAC; X-rays), and is a non-invasive technique that treats the tumor with minimal radiation exposure to the surrounding healthy tissue. Treatment of brain tumors, including metastases, is typically completed in a single session of 30-60 minutes. In contrast, conventional radiotherapy typically involves multiple sessions and does not spare the surrounding tissue. **Table 1** shows some key SRS studies from the last decade.

Table 1 Selected studies of SRS treatment of brain metastases.

Study	*Pts*	*Mets*	*Dose (Gy)*	*Treatment*	*Local control (%)*	*OS (months)*
Sneed[112]	559	1 to ≥4	NR	SRS+WBRT vs SRS	9/8	NR
Andrews[98]	333	1-3	15-24	WBRT vs WBRT+SRS	71/82	4.9/6.5
Aoyama[111]	132	1-4	18-25	SRS+WBRT vs SRS	89/73	8/7.5
Muacevic[113]	64	1	14-27	S+WBRT vs SRS	82/97	9.5/10.3
Soltys[114]	72	1-4	15-30	S+SRS	79	NR
Brennan[115]	49	1-2	15-22	S+SRS	78	NR
Serizawa[116]	778	1-10	13.5-30	SRS	78-98	NR
Kocher[117]	359	1-3	≥20	S/SRS+WBRT vs S/SRS	NR	11/11
Minniti[118]	101	1	9 x 3 fractions	S+SRS	93	17

Abbreviations: S, surgery; SRS, stereotactic radiosurgery; WBRT, whole brain radiotherapy; OS, median overall survival; NR, not reported; Mets, number of metastases; Pts, number of patients.

SRS has been recommended as the preferred treatment for patients with a limited number of brain metastases and an overall good prognosis[112,119]. In a RCT of 132 patients with one to four brain metastases less than three centimeters in diameter, Aoyama et al. found a similar median survival for SRS alone (8 months) compared to WBRT + SRS (7.5 months). However, there were significantly more tumor recurrences for SRS alone (76.4%) versus WBRT + SRS (46.8%), and salvage therapy was frequently needed in patients that were not treated with up-front WBRT[111]. Chang et al. specifically addressed the benefits and neurocognitive risks from adding WBRT to SRS in 58 patients with one to three brain metastases[120]. The trial was stopped early by the data monitoring committee due to a significantly greater risk of decline in memory and learning function for patients randomized to WBRT + SRS. The authors found a median survival of 15.2 months for SRS alone and 5.7 months for WBRT + SRS, and a local tumor control rate of 67% in the SRS group and 100% in the WBRT + SRS group. In a meta-analysis of RCTs evaluating SRS, WBRT or both for patients with a limited number of brain metastases, Tsao et al. could not find a difference in OS, SRS alone was associated with a better neurocognitive outcome and performance status, and WBRT + SRS was superior in providing both local tumor control and distant brain control[121]. Conclusively, although the addition of WBRT to SRS provides better disease control, patients are probably better off with SRS alone and vigilant control when it comes to

neurocognitive function, performance status and quality of life[84,121,122]. Patients initially treated with SRS alone who experience local or distant relapse should preferably undergo salvage therapy with SRS or WBRT, as OS is similar to that of patients initially treated with WBRT + SRS[121].

It is generally accepted that SRS alone can be considered in patients with more than three brain metastases, and WBRT should still be considered for patients with less than four brain metastases. Interestingly, a recent paper from Japan investigated the efficacy of SRS without WBRT for patients with multiple brain metastases; median survival was 13.9 months for 455 patients with one brain metastasis, 10.8 months for 531 patients with two to four brain metastases, and 10.8 months for 208 patients with five to 10 brain metastases[123]. Survival differences were not significant between patients with two to four and five to 10 tumors, and the authors concluded that SRS might be a valid option instead of WBRT in patients with up to 10 metastases. In a multi-institutional series of 1,921 gamma knife-treated patients between 1975 and 2007, Karlsson et al. found patient age and primary tumor control to be more important predictors of survival than the number of brain metastases; 25 patients survived for more than 10 years[124] (**Fig. 4**).

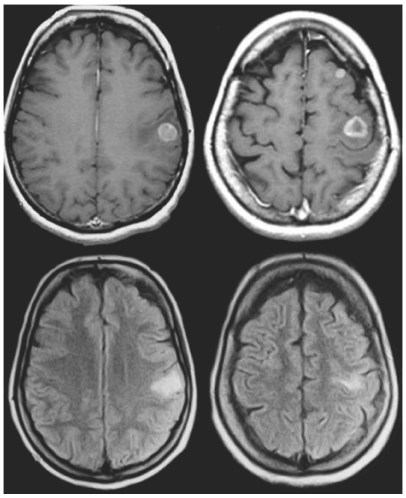

Figure 4 Long-term survivor after gamma knife treatment of multiple brain metastases. This patient underwent gamma knife surgery for nine metastatic lesions in 1994 (**top**; MRI T1-weighted with contrast enhancement), and was alive and tumor-free at the latest follow-up in 2005 (**bottom**; MRI FLAIR images showing only a local high signal reminiscent of previous treatment). Reproduced with permission from Karlsson et al.[124].

Currently, there are no available studies with Level I evidence that compare surgery to SRS, or surgery + SRS to surgery + WBRT (the NCT01372774 trial is currently

recruiting patients to answer the latter issue)[17]. A recent Cochrane review of surgery or SRS + WBRT versus surgery or SRS alone identified five RCTs[63,84,111,120,125], and found that up-front WBRT reduced the risk of brain relapse at one year by 53%, but there was no clear difference in OS or progression-free survival (PFS)[126]. The effects on OS were similar between surgery and SRS, among different WBRT protocols and independent of the number of brain metastases. Study biases and methodological inconsistencies made it difficult to determine whether up-front WBRT had a negative impact on neurocognitive function and quality of life. Moreover, there was only low quality evidence favoring up-front WBRT to surgery and SRS in reducing brain relapse. Nevertheless, there is ample and robust documentation to guide us in clinical decision-making for surgery, SRS and/or WBRT in patients with brain metastases.

4.2.3.4. Systemic therapy

Future advances in brain metastasis therapy will most likely come from improvements in systemic therapy. However, there is currently no Level I evidence comparing systemic therapy to surgery or radiation in the management of brain metastases[127]. Patients with brain metastases are often excluded from clinical trials[44-46]. Brain metastasis patients have frequently been subjected to a range of previous treatments at the time of diagnosis and the tumors might already be resistant to targeted therapies when they need them the most[127]. Randomized studies that are focused on brain metastases are scarce and often small with variable endpoints[127]. Furthermore, preclinical data clearly indicate that chemotherapeutic and molecularly targeted agents are better at preventing brain metastases than shrinking macroscopic lesions[5]. Preservation of neurological structures and function is unquestionably the best strategy, but preventive treatment also raises a number of controversies around patient eligibility, resistance development, toxicity issues and clinical trial design that remain to be resolved.

At present, there is no standard cytotoxic chemotherapy for the treatment of brain metastases[17]. Brain metastases that cannot be controlled with surgery or radiotherapy are therefore treated with the same cytotoxic chemotherapies used to treat extracranial disease. Some agents known to penetrate the BBB, such as TMZ, procarbazine, irinotecan, topotecan and carboplatin, are also employed on an empirical basis for the

treatment of brain metastases, even if these agents are not considered standard therapies for the primary cancer *per se*. A recent review of 21 clinical trials investigating the use of TMZ in patients with brain metastases revealed variable but better response rates when TMZ was combined with WBRT (8.8-95.9%) and/or other anticancer drugs (0-42.8%), as compared to single agent TMZ therapy (4.2-10%)[128].

Molecularly targeted therapies have already become established treatments for subgroups of patients with specific molecular drivers of cancer progression. Approximately 50% of melanoma patients have activating mutations in the *BRAF* gene, and the serine/threonine-protein kinase B-raf (BRAF) inhibitors vemurafenib and dabrafenib have been shown to produce tumor regression and improved survival in *BRAF*-mutated patients with metastatic melanoma[129,130] (**Fig. 5**).

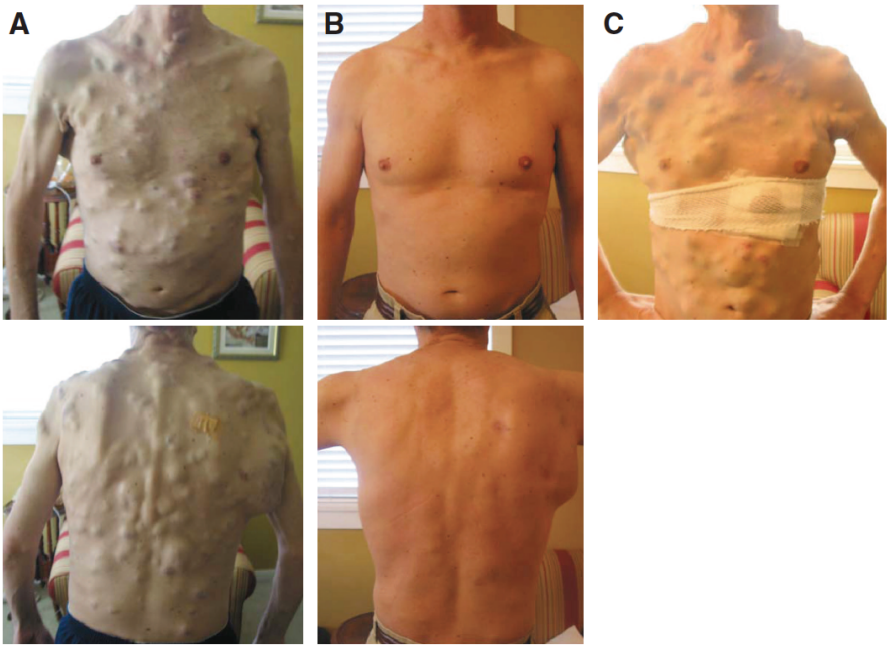

Figure 5 A 38-year-old patient with *BRAF*-mutant melanoma and subcutaneous metastases. Photographs were taken (**A**) before initiation of vemurafenib, (**B**) after 15 weeks of therapy with vemurafenib, and (**C**) after relapse, after 23 weeks of therapy. Reproduced with permission from Wagle et al.[138].

The *ERBB2* gene, which encodes the growth factor receptor HER2, is amplified and HER2 is overexpressed in about 30% of patients with breast cancer; trastuzumab, a HER2 monoclonal antibody, has been found to prevent tumor progression and prolong survival in such patients with metastatic disease[131]. Activating mutations in the *EGFR* gene are present in approximately 10-60% of patients with NSCLC, and the EGFR inhibitors erlotinib and gefitinib have been shown to restrain tumor progression and improve survival in patients with *EGFR*-mutated metastatic NSCLC[132,133]. Furthermore, about 5% of patients with NSCLC have activating rearrangements in the *ALK* gene, and the anaplastic lymphoma kinase (ALK) inhibitors ceritinib and crizotinib have been shown to produce tumor regression and increase PFS in patients with metastatic NSCLC and *ALK*-rearrangements[134,135].

At present, there are about 40 different monoclonal antibodies or protein kinase inhibitors in the Norwegian Pharmaceutical Product Compendium (Felleskatalogen AS), and this list is steadily increasing. Continued advancements in molecular characterization and development of targeted therapies for various cancers will undoubtedly have important ramifications for brain metastasis. Some of the molecular drivers identified are even associated with an increased propensity of brain metastasis, and the development of specific inhibitors is therefore especially warranted. Patients with advanced *HER2*-positive breast cancer have for example a 30-50% risk of developing brain metastases, but trastuzumab with a molecular weight of about 148 kDa is unable to penetrate the BBB and is ineffective in treating established brain metastases[136,137]. Survival improvements associated with the profound extracranial responses of molecularly targeted drugs increases the patients' time at risk of developing brain metastases, and the specific activity of these drugs against brain metastases is an increasingly relevant issue of future research.

Systemic drug therapy of brain metastases has a number of challenges. A key challenge is the poor bioavailability of drugs due to the presence of the BBB at the level of the brain vascular endothelium[139]. Moreover, cancer cells that have extravasated to the brain parenchyma, but not yet developed into a macroscopic tumor, might find protection beyond the BBB ("sanctuary site") or be more prone to develop resistance due to sub-therapeutic drug concentrations. The BBB has low passive permeability and expresses high levels of efflux transporters, which together

limit the penetration of drugs and their ability to reach therapeutic concentrations in the brain[140,141]. Examples of drugs with limited ability to cross the BBB include trastuzumab with its high molecular weight, and paclitaxel and doxorubicin, which are excluded from the brain by efflux transporters[142,143]. The BBB and the BBB around brain tumors – the blood-tumor-barrier (BTB) – is discussed in further detail in section 4.4.2.4.

Corticosteroids are an integral part of the clinical management of brain metastases, and dexamethasone is the drug of choice due to its limited mineralocorticoid effects[144,145]. Dexamethasone effectively reduces peritumoral edema within 24-72 hours in up to 75% of patients[144], and is recommended to provide temporary symptomatic relief from increased intracranial pressure and focal mass effect[145]. Corticosteroids should be tapered slowly over two weeks or more in symptomatic patients.

Lung cancer, breast cancer and melanoma are the most common causes of brain metastases, but also the cancers that have seen the greatest advances in targeted therapies over the last decade[17,127]. The main findings from some of the most influential clinical studies of systemic therapies for patients with brain metastases from lung cancer, breast cancer and melanoma over the last 10 years are summarized in **Tables 2-4**. At present, there are 557 open studies on brain metastasis at ClinicalTrials.gov (U.S. National Institutes of Health). Most of these studies involve novel systemic therapies or combinatorial regimens.

4.2.3.4.1. Lung cancer brain metastases

Lung cancer is the most common cause of brain metastases, and approximately 40% of patients with NSCLC develop brain metastases[146]. Chemotherapeutic regimens with platinum-based drugs as up-front therapy of brain metastases have shown response rates between 28% and 45%[147-152]. Two small patient series of recurrent or progressive NSCLC brain metastases reported objective responses of TMZ in 2/22[153] and 3/30 patients[154]. The multitarget antifolate pemetrexed alone showed a 38.4% response rate in patients with recurrent disease[155], and first-line therapy with pemetrexed and cisplatin showed a 41.9% response rate[156].

SCLC represents 13% of lung cancer cases and more than 90% of patients are elderly smokers[157]. The treatment of choice is chemo- and radiotherapy, including consideration of prophylactic cranial irradiation; 24% of SCLC patients have brain metastases at diagnosis. In contrast to NSCLC, SCLC is not associated with a specific somatic mutation.

Targeted therapy of NSCLC has become increasingly important over the last 10 years. *EGFR* mutations are present in 10-60% of patients; non-smokers, adenocarcinomas, females and Asian individuals have the highest mutation frequencies[158-160]. The presence of *EGFR* mutations in tumors and cell lines are predictive of sensitivity to the EGFR inhibitors gefitinib and erlotinib[158,161]. However, the mutation status of a primary tumor does not necessarily reflect that of the corresponding metastasis, and this can have important implications for both diagnostics (new biopsy?) and treatment (new round or different drug?). For example, in a comparative analysis of *EGFR* mutation status in NSCLC primary lung tumors and metastases, Gow et al. reported that 9/18 patients had lost the mutation in their metastasis, whereas 17/26 had gained the mutation in their metastases; 7/17 patients that had transformed from *EGFR* wild-type to *EGFR* mutation positive had brain metastases[162].

EGFR inhibitors have been tested in both naïve and recurrent brain metastases from NSCLC with findings that reflect the underlying genetic makeup (**Tab. 2**). Ceresoli et al. reported a 10% response rate of gefitinib in heavily pretreated and unselected Italian patients[163]. In contrast, Hotta et al. found a 43% response rate in a Japanese population of 50% non-smokers[164], and Wu et al. found a 32% response rate in Chinese non-smokers[165], both with recurrent brain disease and undetermined *EGFR* mutation status. Small prospective studies of gefitinib and erlotinib in unselected Asian patients with newly diagnosed brain metastases have also shown encouraging response rates of 50%[166] and 73.9%[167]. An 81% response rate was observed in Chinese patients with unknown *EGFR* mutation status when WBRT was added to gefitinib[168]. In another study, an 82.4% response rate was noted from WBRT and erlotinib in *EGFR* mutation positive patients; notably, this study also featured 36 patients without *EGFR* mutations and none of these patients were responders[169]. Two other small series have also shown promising responses of erlotinib monotherapy in mutated patients[170,171]. In a recent phase II study of WBRT + erlotinib in both

pretreated and untreated US patients, Welsh et al. reported an 86% response rate; subgroups of patients with and without *EGFR* mutations had response rates of 89% and 63%, respectively[109]. In contrast, in a recent RCT of WBRT versus WBRT + erlotinib in English patients with treatment-naïve NSCLC brain metastases and undetermined *EGFR* status, Lee et al. failed to demonstrate an improvement in PFS or OS[172]. Interestingly, in a RCT of WBRT + SRS with or without TMZ or erlotinib in unselected, newly diagnosed patients, Sperduto et al. observed a reduction in survival with the addition of either systemic agent, which could possibly reflect deleterious toxicity[110]. Taken together, these studies suggest that EGFR inhibitors should be reserved for patients with *EGFR* mutations.

Rearrangements in the *ALK* gene are present in 2-7% of patients with NSCLC and predict response to the ALK inhibitors crizotinib[135,173,174], ceritinib[134] and alectinib[175]. *ALK* rearrangements are more frequently seen in young patients, non-smokers and adenocarcinomas[174]. In a study by Preusser et al., *ALK* translocations were found to be constant between 16 matched primary tumors and brain metastases[176]. In a randomized trial of crizotinib versus chemotherapy (permetrexed/docetaxel) in *ALK*-positive patients with advanced disease, 35% and 34% of patients had brain metastases, respectively[174]. Overall response rates were significantly better with crizotinib (65% versus 20%), but there was no difference in OS. Costa et al. recently presented a retrospective review of 888 crizotinib-treated *ALK*-positive patients of which 275 patients had brain metastases at enrolment[177]. Crizotinib was effective in both newly diagnosed (response rate 18%) and pretreated (radiotherapy; response rate 33%) patients. Twenty percent of patients without brain metastases at inclusion were diagnosed with brain metastases while on crizotinib.

4.2.3.4.2. Breast cancer brain metastases

Historical series show that 10-30% of patients with breast cancer develop brain metastases[178]. Advances in systemic therapy for breast cancer have resulted in improved survival[179], and as patients are living longer, more patients eventually develop brain metastases during the course of their disease[19]. Younger age, *HER2* mutation status, hormone receptor status (estrogen receptor (ER) and progesterone receptor (PR)), and presence of lung metastases are associated with an increased risk

of developing brain metastases[179,180]. Conventional chemotherapeutic regimens using cyclophosphamide, 5-fluorouracil, methotrexate, vincristine and/or doxorubicin have shown intracranial response rates between 17% and 76%[181,182]. Combinatorial treatment with cisplatin and etoposide has induced response rates of 38-55%[151,183]. Case reports and small patient series have shown some efficacy of capecitabine[184] or topotecan[185] monotherapy.

Targeted agents have become key elements in the contemporary management of advanced breast cancer. Brain metastases develop in 29-37% of patients with *HER2*-positive breast cancer[179,186,187]. Breast cancer patients who overexpress HER2 benefit from targeted treatment with trastuzumab[188], but trastuzumab has poor CNS penetration and its survival advantages have largely been ascribed to control of extracranial disease[189]. However, positron emission tomography (PET) imaging studies of isotope-labeled trastuzumab in patients with metastatic breast cancer have shown a higher uptake than previously appreciated in brain metastases[190]. Furthermore, and as discussed for *EGFR*-mutated NSCLC, the mutation status of primary tumors and brain metastases is not always concordant and can have important implications for clinical management and prognosis. Duchnowska et al. investigated HER2, ER and PR expression of 120 matched primary breast cancers and brain metastases, and HER2 expression was lost in 12% and gained in 16% of brain metastases, whereas ER and PR was lost in 43% and 56% and gained in 19% and 14% of brain metastases, respectively[191].

Kirsch et al. showed that trastuzumab treatment more than doubled the OS of patients with HER2-overexpressing brain metastases[189]. However, the OS of patients with *HER2*-negative tumors was similar to that of patients with *HER2*-positive tumors that did not receive trastuzumab (**Tab. 3**). Two recent case reports have shown regression of *HER2*-positive brain metastases with trastuzumab emtansine (T-DM1)[192,193], an antibody-drug conjugate of trastuzumab and the cytotoxic agent mertansine (DM1); T-DM1 is currently being evaluated in clinical trials.

Lapatinib, an inhibitor of HER2 and EGFR, combined with capecitabine is used for advanced *HER2*-positive breast cancer that has progressed on trastuzumab. Lapatinib was the first HER2-directed drug to be validated in a preclinical brain metastasis

model[194], but has shown a rather discouraging 6% response rate as monotherapy in patients with recurrent *HER2*-positive breast cancer brain metastases[195]. Better intracranial responses have been seen when lapatinib is combined with capecitabine in patients with *HER2*-positive brain metastases: 20% in recurrent[195] and 65% in treatment-naïve patients[196]. An OS of 11.3-17 months has been reported with this combined therapy[196,197], though with a 49% frequency of grade 3 and 4 adverse events[196]. Lin et al. recently reported a 79% response rate of lapatinib and WBRT in *HER2*-positive brain metastases[198]. In a study of Asian *HER2*-positive breast cancer patients with brain metastases, OS was 10.5, 21.4 and 25.9 months with trastuzumab alone, lapatinib alone and trastuzumab + lapatinib, respectively[199].

In a study by Lin et al. of 116 patients with metastatic triple-negative breast cancer (HER2-, ER- and PR-negative), almost half of the patients developed brain metastases and median OS was only 4.9 months[200]. In contrast to *HER2*-positive disease, triple-negative patients with brain metastases usually succumb to progressive extracranial disease regardless of the frequent CNS involvement. Therapeutic advances in triple-negative breast cancer have been unsuccessful and there is great need for targeted agents that can control both intracranial and extracranial disease[200]. Phosphatase and tensin homolog (PTEN) loss is present in up to 60% of triple-negative brain metastases and has been associated with a more aggressive disease course[201,202]. Ongoing studies are looking at agents that target the PTEN-Phosphoinositide 3-kinase (PI3K)-Protein kinase B (AKT) pathway in breast cancer.

In a recent prospective study of bevacizumab (inhibitor of vascular endothelial growth factor (VEGF)) + WBRT treatment of 19 patients (13 with breast cancer) with newly diagnosed brain metastases, Lévy et al. reported intracranial responses in 10 patients at three months; there was a trend towards better responses with higher doses of bevacizumab[203]. Combination treatment with trastuzumab, lapatinib and bevacizumab has also shown intracranial efficacy in heavily pretreated *HER2*-positive patients[204]. In a small study of four patients with breast cancer brain metastases, all patients responded to treatment with paclitaxel + bevacizumab[205]. Several other studies have reported substantial responses with various combinatorial regimens that include bevacizumab, but reduced contrast enhancement and/or edema may not be true surrogates of tumor response[206].

4.2.3.4.3. Melanoma brain metastases

Brain metastases are diagnosed in 10-40% and found in up to 75% of melanoma patients on autopsy[207-217]. Multiple brain metastases are present in 50-70% of patients[23,215-217]. Other organ metastases are seen in 50-80% of patients[210,216,218]. An increasing number of patients are diagnosed with asymptomatic brain metastases; 30-60% of patients in clinical and autopsy series[210,215,219,220]. Spontaneous hemorrhage occurs in 10-40% of lesions[212,214,218,221]. The median time from the diagnosis of melanoma to the diagnosis of brain metastases was 3.7 years in two large patient series[216,222]. Treatment is the major determinant of survival and patient selection is the major determinant of treatment[31,216,222].

Importantly, although a number of clinical trials have been conducted in patients with brain metastases, no prospective RCTs of local therapies (SRS, WBRT or surgery) have been conducted in the melanoma population[223].

For many years, no conventional chemotherapy or targeted agent were shown to improve OS in patients with metastatic melanoma in phase III RCTs[224,225]. Traditional chemotherapy regimens with dacarbazine were associated with an overall response rate of only 15%[226], and its efficacy in patients with brain metastases was even lower[227]. TMZ, an oral analogue of dacarbazine with excellent brain penetration, was widely used over the first decade of the 21st century. However, therapeutic responses of TMZ were modest at best[228-230].

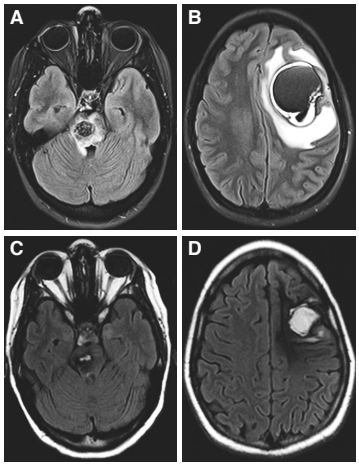

Figure 6 Vemurafenib for melanoma brain metastases. Brain MRI (**A**,**B**) before and (**C**,**D**) after six months of treatment with vemurafenib. Modified with permission from Rochet et al. 2011[231]. Copyright Massachusetts Medical Society.

In 2002, Davies et al. reported that 66% of melanomas harbor mutations in the *BRAF* oncogene, which results in activation of the mitogen-activated protein kinase (MAPK) pathway[232]. This discovery initiated a hunt for pertinent therapeutics as well as other molecular aberrations in metastatic melanoma, and since 2011 we have seen a therapeutic revolution with the clinical development of MAPK-targeted therapies (BRAF inhibitors vemurafenib and dabrafenib, and mitogen-activated protein kinase kinase (MEK) inhibitor trametinib)[233] (**Fig. 6**). In parallel, we have witnessed considerable advances in immunotherapy with the introduction of immune checkpoint inhibitors (antibodies against cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4; ipilimumab) and programmed cell death protein 1 (anti-PD-1; pembrolizumab and nivolumab)). In brief, MAPK-targeted treatments and immune checkpoint inhibitors have shown impressive antitumor effects in subgroups of patients with metastatic melanoma, but gains in OS are generally modest (**Tab. 7**); the survival benefits for patients with brain metastases are even more discouraging (**Tab. 4**). Treatment responses are usually short-lived due to resistance development, and many of these therapies are also significantly hampered by side effects both as monotherapies and as combinatorial regimens[233,234]. We still have a long way to go, as we consider this genetically heterogeneous disease as a whole, and particularly the unmet needs of patients with melanoma brain metastases[47,224,225].

Melanoma therapy and the biology of melanoma brain metastasis are discussed in further detail in sections 4.3 and 4.4.

Table 2 Selected studies of systemic therapies in non-small cell lung cancer brain metastases.

Study	*New/recurrent disease*	*Treatment*	*Number of patients (mutation status)*	*RR/PFS/OS*
Ceresoli 2004[163]	Recurrent	Gefitinib	41 (*EGFR* ND)	10/3/5
Hotta 2004[164]	Recurrent	Gefitinib	14 (*EGFR* ND)	43/8.8/NR
Chiu 2005[166]	Both	Gefitinib	21 (*EGFR* ND)	50/4.8/9.9
Wu 2007[165]	Recurrent	Gefinitib	40 (*EGFR* ND)	32/9/15
Kim 2009[167]	New	Gefitinib/Erlotinib	23 (*EGFR* ND)	73.9/7.1/18.8
Ma 2009[168]	New	WBRT + Gefitinib	21 (*EGFR* ND)	81/10/13
Katayama 2009[170]	Recurrent	Erlotinib	7 (*EGFR*+)	43/NR/2.9
Grommes 2011[171]	Both	Erlotinib	9 (*EGFR*+)	67/2.7/12
Porta 2011[169]	Both	Erlotinib ± WBRT	17 (*EGFR*+)	82.4/11.7/12.9
	Both	Erlotinib ± WBRT	36 (*EGFR* wt)	0/5.8/3.1
Sperduto 2013[110]	New	WBRT + SRS	44 (*EGFR* ND)	NR/8.1/13.4
	New	WBRT + SRS + Temozolomide	40 (*EGFR* ND)	NR/4.6/6.3
	New	WBRT + SRS + Erlotinib	41 (*EGFR* ND)	NR/4.8/6.1
Welsh 2013[109]	Both	WBRT + Erlotinib	40	86/8/11.8
	Both	WBRT + Erlotinib	9 (*EGFR*+)	89/12.3/19.1
	Both	WBRT + Erlotinib	8 (*EGFR* wt)	63/5.2/9.3
Lee 2014[172]	New	WBRT	40 (*EGFR* wt)	NR/1.6/2.9
	New	WBRT + Erlotinib	40 (*EGFR* wt)	NR/1.6/3.4
Gadgeel 2014[175]	Both	Alectinib	21 (*ALK*-rearranged)	52/NR/NR
Costa 2015[177]	New	Crizotinib	109 (*ALK*-rearranged)	18/7/NR
	Recurrent	Crizotinib	166 (*ALK*-rearranged)	33/13.2/NR

Abbreviations: RR, complete + partial response rate (%). PFS, progression-free survival (months). OS, overall survival (months). NR, not reported. *EGFR* ND, *EGFR* mutation status not determined.

Table 3 Selected studies of systemic therapies in breast cancer brain metastases.

Study	*New/recurrent disease*	*Treatment*	*Number of patients (mutation status)*	*RR/PFS/OS*
Kirsch 2005[189#]	Recurrent	Trastuzumab	36 (*HER2*+)	NR/NR/26.6
	Recurrent	No trastuzumab	11 (*HER2*+)	NR/NR/8.6
	Recurrent	No trastuzumab	48 (*HER2*-)	NR/NR/9.4
Lin 2009[195]	Recurrent	Lapatinib	237 (*HER2*+)	6/2.4/6.4
	Recurrent	Lapatinib + Capecitabine	50 (*HER2*+)	20/3.65/NR
Siena 2010[235]	Recurrent	Temozolomide	51	4/1.9/NR
Brufsky 2011[179§]	Both	Trastuzumab	258 (*HER2*+)	NR/NR/17.5
	Both	Chemotherapy	262 (*HER2*+)	NR/NR/16.4
	Both	Radiotherapy	269 (*HER2*+)	NR/NR/13.9
	Both	Surgery	29 (*HER2*+)	NR/NR/20.3
Yap 2012[199]	Both	Trastuzumab	58 (*HER2*+)	NR/NR/10.5
	Both	Lapatinib	30 (*HER2*+)	NR/NR/21.4
	Both	Lapatinib + Trastuzumab	28 (*HER2*+)	NR/NR/25.9
Ro 2012[197]	Recurrent	Lapatinib + Capecitabine	58 (*HER2*+)	NR/4.3/11.3
Bachelot 2013[196]	Both	Lapatinib + Capecitabine	45 (*HER2*+)	65.9/5.5/17
Lin 2013[198]	Recurrent	WBRT + Lapatinib	28 (*HER2*+)	79/NR/NR

Abbreviations: RR, complete + partial response rate (%). PFS, progression-free survival (months). OS, overall survival (months). NR, not reported. [#]No significant differences in WBRT, SRS or surgery between groups; 17% of *HER2*- patients received trastuzumab. [§]Multivariate analysis of prospectively collected data where trastuzumab and chemotherapy were significant predictors of survival; no trastuzumab OS 3.8 months, no chemotherapy OS 3.7 months, no surgery OS 11.3 months, no radiotherapy OS 8.4 months.

Table 4 Selected studies of systemic therapies in melanoma brain metastases.

Study	*New/recurrent disease*	*Treatment*	*Number of patients (mutation status)*	*RR/PFS/OS*
Margolin 2002[228]	Both	WBRT + Temozolomide	31	9.7/2/6
Avril 2004[227]	Both	Fotemustine	112	15.2/1.8/7.3
	Both	Dacarbazine	117	6.8/1.9/5.6
Agarwala 2004[230]	New	Temozolomide	117	7/1.2/3.5
	Recurrent	Temozolomide	34	1/1/2.2
Hofmann 2006[229]	Both	Temozolomide	13	7/NR/5
	Both	SRS + Temozolomide	12	8/NR/9
	Both	WBRT + Temozolomide	10	10/NR/7
Atkins 2008[236]	Recurrent	WBRT + Temozolomide + Thalidomide	39	7.6/1.6/4
Hong 2010[237]	New	Adoptive cell transfer therapy	26 (asymptomatic)	35[#]/NR/10.8
Margolin 2012[238]	Both	Ipilimumab	51 (asymptomatic)	16/1.4/7
	Both	Ipilimumab + Steroids	21 (symptomatic)	5/1.2/3.7
Di Giacomo 2012[239]	Both	Ipilimumab + Fotemustine	20	25/3/13.4
Long 2012[240]	New	Dabrafenib	74 (*BRAFV600E*+)	39.2/3.7/7.6
	New	Dabrafenib	15 (*BRAFV600K*+)	6.7/1.9/3.8
	Recurrent	Dabrafenib	65 (*BRAFV600E*+)	30.8/3.8/7.2
	Recurrent	Dabrafenib	18 (*BRAFV600K*+)	22.2/3.6/5
Falchook 2012[241]	Recurrent	Dabrafenib + Trametinib	26 (*BRAFV600*+)	15/3.6/10
	New[§]	Dabrafenib + Trametinib	45 (*BRAFV600*+)	13/3.6/11.8
Konstantinou 2013[242]	Recurrent	Ipilimumab ± WBRT/SRS	38	5.3/NR/3.3
Queirolo 2014[243]	Recurrent	Ipilimumab	146 (asymptomatic)	12/3.1/4.3
Dummer 2014[244]	Recurrent	Vemurafenib	19 (*BRAFV600*+)	16/3.9/5.3
Dzienis 2014[245]	Both	Vemurafenib	22 (*BRAFV600E*+)	50/4/7.7
Larkin 2014[246]	Both	Vemurafenib	750 (*BRAFV600*+)	24/3.8/7.5

Abbreviations: RR, complete + partial response rate (%). PFS, progression-free survival (months). OS, overall survival (months). NR, not reported. [#]Only complete responses. [§]Crossover from dabrafenib monotherapy to combination therapy.

4.2.4. Prognosis

Patients with brain metastases generally have a dismal prognosis. Left untreated, patients survive on average 1-2 months after diagnosis[216,222,247-249]. Patient survival is dependent on multiple variables: the brain metastases *per se* (size, number, location), but also the cancer (histology, disease stage, treatment response), the patient (age, performance status, co-morbidity), the doctor (diagnostics, treatment, follow-up) and the goals of care (patient and physician preferences). Several prognostic indices have been published; the most influential have been the RTOG recursive partitioning analysis (RPA)[95] and the graded prognostic assessment (GPA)[250]. Rodrigues et al. recently published a systematic review of prognostic systems for patients with brain metastases, and concluded that the ideal prognostic index had yet to be defined[251]. However, in contrast to the GPA index, the RTOG RPA is not diagnosis-specific and does not reflect current advances in systemic therapy[252].

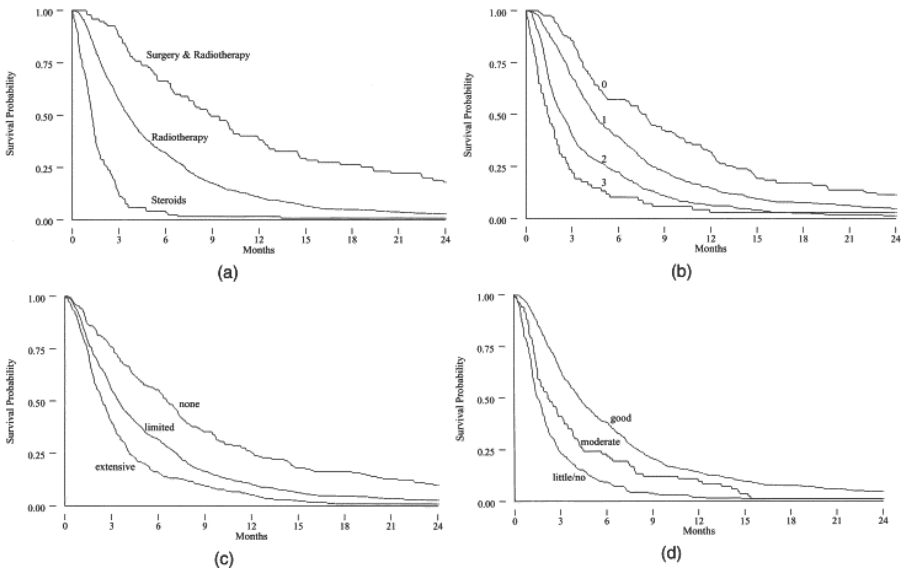

Figure 7 Historical survival curves for prognostic factors in patients with brain metastases. (**A**) Treatment modality (surgery + radiotherapy, radiotherapy and steroids). (**B**) Performance status at diagnosis. Eastern Cooperative Oncology Group (ECOG) performance status: 0 = asymptomatic; 1 = symptomatic, but completely ambulatory; 2 = symptomatic, <50% in bed during the day; 3 = symptomatic, >50% in bed, but not bedbound. (**C**) Systemic tumor burden (none, limited and extensive). (**D**) Response to steroid treatment (good, moderate and little/no). Reprinted with permission from Lagerwaard et al.[31].

Lagerwaard et al. investigated prognostic factors in 1,296 patients with brain metastases, treated in a single institution in the Netherlands between 1981 and 1990[31]. Treatment modality, performance status at diagnosis, systemic tumor burden and response to steroid treatment had the strongest impact on survival (**Fig. 7**). Gaspar et al. reported similar findings in a series of 1,200 patients with brain metastases[95]. Both studies also confirmed a significantly negative impact of higher age. Interestingly, 19% of patients in Lagerwaard et al.'s study were ≥70 years of age[31], and this subset of patients is increasing. Taken together, although these patients were treated in the 1980s and before the era of molecularly targeted agents, these data are still highly relevant today. They provide a good overview of what can be achieved with the different treatments that are available and which patients are most likely to benefit from aggressive therapies. Unfortunately, they also suggest that even though new systemic therapy has induced substantial intracranial responses and improved PFS, OS is largely the same now as in the 1980s and 90s. Diagnosis-specific prognostic factors and median survival with different treatment combinations for NSCLC, SCLC, melanoma, renal cell carcinoma, breast cancer and gastrointestinal cancer, adapted from a large retrospective study of 3,809 patients with brain metastases are provided[253] (**Tab. 5**).

In a recent patient series from a multi-disciplinary brain metastasis clinic, 114 patients with oligometastatic disease and good performance status showed a median survival of 16 months (two-year survival 31.5%)[254]. The median survival was 12 months for surgery, 16 months for surgery + WBRT, 13 months for SRS and 23 months for WBRT. Patients were initially treated with surgery (52%), WBRT (23%), SRS (14%), surgery + WBRT (9%) and supportive care (2%). Twenty-five percent of patients developed local relapse, 11% developed distant relapse and 15% developed both local and distant relapse. Second-line treatment was WBRT (21%), SRS (13%) and surgery (9%). This study shows what can be achieved with careful patient selection and multi-disciplinary management within a dedicated joint neurosurgical/neuro-oncology clinic.

Table 5 Prognostic factors and median survival for 3,809 patients with newly diagnosed brain metastases treated between 1985 and 2007.

			Median survival						
	Patients	*Prognostic factors*	*Overall*	*WBRT*	*SRS*	*WBRT+SRS*	*S+SRS*	*S+WBRT*	*S+WBRT+SRS*
NSCLC	1888	Age KPS ECM No	7.00	3.42	9.92	12.59	11.86	11.66	12.06
SCLC	299	KPS ECM No	4.90	3.87	6.90	15.23	12.02	14.66	14.95
Melanoma	483	KPS No	6.74	2.86	7.26	6.67	12.78	11.10	13.11
Renal cell carcinoma	286	KPS No	9.63	5.08	10.78	12.12	12.91	15.52	8.80
Breast cancer	642	KPS	11.93	5.55	13.80	15.47	21.68	18.23	15.80
GI cancer	211	KPS	5.36	2.92	7.33	7.13	9.76	10.37	7.92

Abbreviations: NSCLC, non-small cell lung cancer. SCLC, small cell lung cancer. GI cancer, gastrointestinal cancer. KPS, Karnofsky performance status. ECM, extracranial metastases. No, number of brain metastases. WBRT, whole brain radiotherapy. SRS, stereotactic radiosurgery. S, surgery. Prognostic factors: Multivariate analysis of diagnosis-specific factors ($P < 0.05$). Adapted from Sperduto et al. 2010[253].

4.3. MELANOMA

4.3.1. Melanoma: a poster child for personalized medicine

Melanoma is the most deadly form of skin cancer. Over the last decade, major progress has been made in our biologic understanding of melanoma and this has been directly translated into new therapies. Melanoma has become a poster child for personalized medicine with the parallel clinical development of molecularly targeted therapies and immunotherapies.

For localized melanoma and regional lymph node metastases, surgery remains the standard of care[234]. Precise disease staging can be achieved with sentinel-node biopsy and non-sentinel lymph node dissection, but this has not been shown to affect survival in prospective series[255-257].

4.3.2. Epidemiology and risk factors

The incidence of melanoma is increasing and death rates continue to rise[258-260]. In the US, melanoma was the fifth most common cancer in 2014, accounting for 4.6% of all new cancer cases and 1.7% of all cancer deaths (**Tab. 6**). The rates for new melanoma cases in the US have been rising on average 1.8% each year over the last 10 years[a].

Table 6 Melanoma epidemiology in the United States.

Number of new cases per 100,000 per year (total)[#]	21.3 (76,100)
Men	27.7
Women	16.7
Median age in years at diagnosis[#]	62
Number of deaths per 100,000 per year (total)[#]	2.7 (9,710)
Men	4.1
Women	1.7
Median age in years at death[#]	69
5-year survival[§]	91.3%
Localized – confined to primary site (84%[$])	98.1%
Regional – spread to regional lymph nodes (9%[$])	62.6%
Distant – metastasized (4%[$])	16.1%
Unknown – unstaged (3%[$])	78.3%
Lifetime risk of developing melanoma[&]	2.1%
Prevalence of melanoma[@]	960,231

[#]Age-adjusted rates based on 2007-2011 cases and deaths. [§]Based on 2004-2010 data. [$]Percent of all melanoma patients. [&]Based on 2009-1011 data. [@]2011 data. Adapted from[a].

[a]http://www.seer.cancer.gov/statfacts/html/melan.html

Over the last decade in Norway, incidence rates have increased annually by 4.6% for men and 3.9% for women, and mortality rates have increased by 1.8% for men and decreased by 0.4% for women[a]. Five-year survival rates were 81% for men and 90% for women in the period 2009-2012. The recorded and predicted numbers of new cases and deaths per year in Norway are illustrated in **Figure 8**.

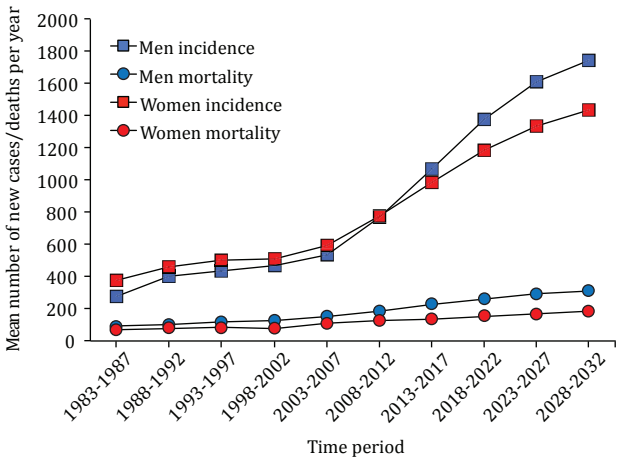

Figure 8 Recorded and predicted number of annual new melanoma cases and deaths in Norway. Illustration by T. Sundstrøm based on incidence and mortality data from the Association of the Nordic Cancer Registries (NORDCAN project)[b].

Melanoma incidence rates have large geographical, ethnic/racial and socioeconomic variations[258-260]. These variations are tightly connected to skin type, recreational exposure to sunlight and indoor tanning patterns; exposure to solar ultraviolet (UV) radiation is the only established modifiable cause of melanoma[261,262]. Incidence rates are generally highest in white Caucasians from the more affluent parts of the world. Australia and New Zealand have the highest incidence rates, two to three orders of magnitude higher than in Norway and the US[258,260]. Incidence rates are two to four times lower in eastern European countries as compared to western European countries[258,260]. Over the last decades in the US, melanoma incidence rates have increased by 6.1% per year in white women younger than 44 years of age[259].

[a] http://www-dep.iarc.fr/NORDCAN/English/StatsFact.asp?cancer=310&country=578
[b] http://www-dep.iarc.fr/NORDCAN/English/frame.asp

Patients with a previous history of melanoma have an increased risk of developing new primary melanomas[263]. Other established risk factors for melanoma are dysplastic nevus syndrome, familial history of melanoma and certain predisposing genetic mutations where *CDKN2A* and *CDK4* mutations have the highest penetrance[264]. As early identification is the most important intervention to reduce melanoma mortality, risk-stratified screening should be adopted to detect melanoma at its earliest and most curable stages[264,265].

4.3.3. Pediatric, uveal and amelanotic melanomas

Pediatric melanoma is rare, but its incidence is increasing, particularly among adolescents[266]. Pediatric and adult melanomas have a very similar UV-induced mutational spectrum[267], which emphasizes the protective role of sun protection, but also the potential applicability of novel therapeutics explored in adult populations.

Uveal melanoma is rare, but it is the most common primary malignancy of the eye[268]. Metastatic disease occurs in up to 50% of patients, of which 90% develop liver metastases. Uveal melanomas frequently display activating mutations in *GNAQ* or *GNA11* with subsequent MEK-extracellular signal-regulated kinase (ERK) pathway activation, and are possibly susceptible to MEK inhibition[225] (currently under investigation in the trial NCT01143402). Uveal melanomas are not characterized by activating mutations in *BRAF* or *NRAS*[269].

Approximately 2-8% of melanomas are amelanotic, i.e. they lack pigmentation[270]. Amelanotic melanomas are frequently associated with diagnostic delay and have a higher mortality than pigmented melanomas[271]; brain metastasis is independent of pigmentation[216].

4.3.4. Tumor progression and staging

Melanomas arise from skin melanocytes, either from a pre-existing nevus (20-30%) or with no visible precursor lesion (60-70%)[272] (**Fig. 9**). A primary cutaneous lesion cannot be identified in up to 12% of patients with metastatic melanoma. Approximately 80% of melanocytic nevi have an activating mutation in $BRAF^{V600E}$ (Val → Glu in codon 600)[273], and the constitutive activation of BRAF is thought to

drive the initial steps of nevus formation[272]. Subsequent tumor progression is driven by the accumulation of genetic and epigenetic events (e.g. *CDKN2A* mutations, PTEN loss).

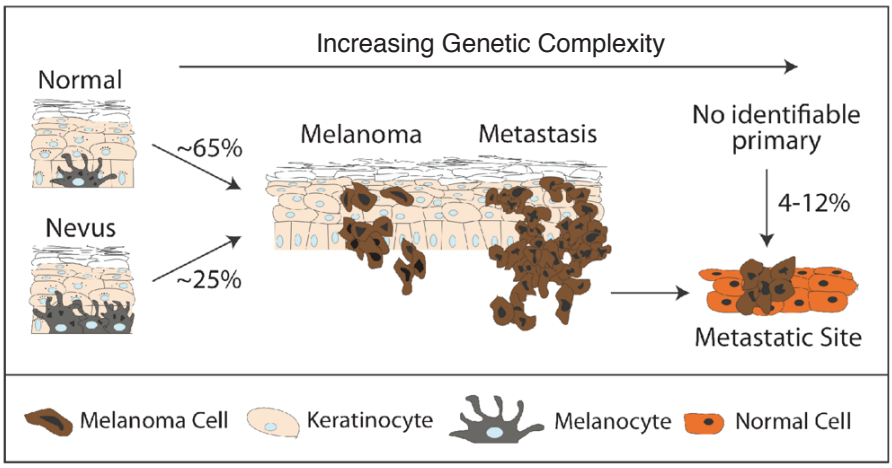

Figure 9 Melanoma development and progression. The tumor arises within the epidermis (melanoma *in situ*), grows into the dermis and invades lymph and blood vessels to form regional and distant metastases. Modified with permission from Damsky et al.[272].

Cutaneous melanomas are most common (91.2%), whereas acral (2.3%), mucosal (1.3%) and ocular/uveal melanomas (5.2) are more rare[274]. Different types of melanomas are characterized by different mutational spectra, e.g. with higher frequencies of *BRAF* mutations in areas that receive intermittent UV-exposure (e.g. trunk) and higher frequencies of *KIT* mutations in non-exposed areas (acral, mucosal)[234].

Most melanomas are diagnosed when thin (Breslow thickness ≤1 mm) and have a favorable prognosis with surgery alone. Nonetheless, in a study of 2,243 patients with thin melanomas, Maurichi et al. reported a 12-year survival of 85.3%; age, mitotic rate, ulceration, lymphovascular invasion, regression and sentinel node status were found to be independent predictors of survival[275]. Most recurrences occurred more than five years after the initial diagnosis and more than 10% developed regional or distant metastatic disease as the first event.

Melanoma has a proclivity to metastasize to certain organs, primarily lung, skin, lymph nodes, brain and liver; however, metastases can occur anywhere and in an unpredictable fashion[276]. Superficially spreading and nodular melanomas metastasize more frequently to the brain, whereas acrolentiginous and mucosal melanomas more often spread to the skeleton[277]. Head and neck melanomas have a higher incidence of brain metastases[278] and the highest incidence is seen with scalp melanomas[279]. Together, metastatic dissemination from very small tumors and widespread metastasis to any organ site are characteristic features of melanoma, and constitute great challenges for both research and clinical management.

The 7th edition of the American Joint Committee on Cancer (AJCC) melanoma staging recommendations was published in 2009[280]. In brief, staging criteria include (T) tumor thickness, ulceration status and mitoses, (N) number of metastatic lymph nodes and nodal metastatic burden, and (M) site of distant metastasis and serum lactate dehydrogenase (LDH) status. Localized melanoma is stages I and II, regional metastatic melanoma is stage III, and distant metastatic melanoma is stage IV (**Tab. 6**).

4.3.5. Genomic landscape of melanoma

Since the landmark publication by Davies et al. in 2002, which described a high frequency of *BRAF* mutations in melanomas[232], a number of investigations have helped to define the genomic landscape of melanoma. The most important and clinically relevant alterations are summarized in **Figure 10**. The Cancer Genome Atlas (TCGA) study[a] on melanoma is not yet published; this study will primarily focus on metastatic melanoma and currently aims to collect 500 patient samples.

Approximately 65% of melanomas harbor mutations in the MAPK (RAS-RAF-MEK-ERK) pathway[281-283]. About 43% and 15% of melanomas have *BRAF* and *NRAS* mutations, respectively[284]. The most prevalent *BRAF* mutations are $BRAF^{V600E}$ (80%) and $BRAF^{V600K}$ (5-30%)[285,286]. *BRAF* (48%) and *NRAS* (15%) mutations occur with similar frequencies in metastatic tumors[284], and mutation status is not associated with outcome or site of distant metastasis[287,288]. Concurrent *NRAS* and $BRAF^{V600}$ mutations

[a] http://cancergenome.nih.gov/cancersselected/melanoma

are rare (1.6%), whereas *NRAS* and *BRAF*$^{Non\text{-}V600}$ mutations are more frequent (18%)[289].

More than 50% of melanomas have genetic alterations (*CCND1*, *CDK4* or *CDKN2A*) that confer cyclin-dependent kinase 4 (CDK4) activation[290,291]. About 30% of melanomas have deletions or inactivating mutations in *PTEN* (40% of *BRAF*-mutant melanomas)[291,292]. Mutations or amplifications of other constituents of the PTEN-PI3K-AKT-mammalian target of rapamycin (mTOR) pathway are infrequent[293,294]. PI3K inhibition blocks downstream signaling better than AKT inhibition[295]. Mutations or deletions in *TP53*, or amplifications of the cellular tumor antigen p53 (p53) inhibitor mouse double minute 2 homolog (MDM2), are rare in melanomas[296-298]. On the other hand, the p53 inhibitor protein Mdm4 (MDM4) is upregulated in approximately 65% of melanomas, and promotes melanoma cell survival by antagonizing the proapoptotic function of p53[299].

Next-generation sequencing studies have identified several recurrent mutations in melanomas, including *EPHA3* and *ERBB4*[300], *MAP3K5* and *MAPK3K9*[301], *PREX2*[302], *RAC1*[303,304], *GRIN2A*[305], *GRM3*[306], *BAP1*[307], *PP6C* and *STK19*[303], *TERT* promoter[267,308,309] and *TMEM216*[310]. Importantly, most of these genetic alterations occur with relatively low frequencies (<15%; "long tail") and few genes are validated across different studies.

A myriad of putative mediators of metastasis have been identified, and include: Apolipoprotein-E (APOE)[311,312], β-Catenin[313], breast cancer metastasis-suppressor 1 (BRMS1)[314], CD44 splicing variant 6 (CD44v6)[315], *CDH13*[316], *CDKN2A/B*[316], *GRIA2*[311], *HOXD9*[317], *KISS-1*[318], liver X receptor β (LXRβ)[312], *MDA-9/syntenin*[319], *NEDD9*[320], *NM23*[321], *PLEKHA5*[322], *PRRX1*[323], Rho family of guanosine triphosphate hydrolases (GTPases) and Rho-associated protein kinase (ROCK)[324,325], STAT3[326], among others[327].

Several large-scale attempts have been made to identify metastasis regulators in melanoma by comparing messenger ribonucleic acid (mRNA) expression[328-330], deoxyribonucleic acid (DNA) copy number changes[331-333] and DNA methylation[317] of primary and metastatic melanomas, but these analyses have shown little overlap.

Indeed, in a comparative analysis of 14 gene expression profiling studies, Tremante et al. found negligible overlap in molecular signatures between studies[334]. Importantly, melanoma is characterized by a profound and dynamic heterogeneity; in fact, melanoma is the most heterogeneous of all cancers[335]. Hence, we are faced with considerable obstacles in our attempts to clinically translate vast amounts of genetic information[336] into meaningful clinical benefit for patients. Paramount in this regard is the development and extension of integrated platforms of accumulated knowledge of tumor genetics and pharmacological data[337] (see **Paper IV**). Furthermore, to improve translational success rates from preclinical research, there is great need for more reproducible, predictive and representative animal models[338,339] (see **Papers I** and **II**).

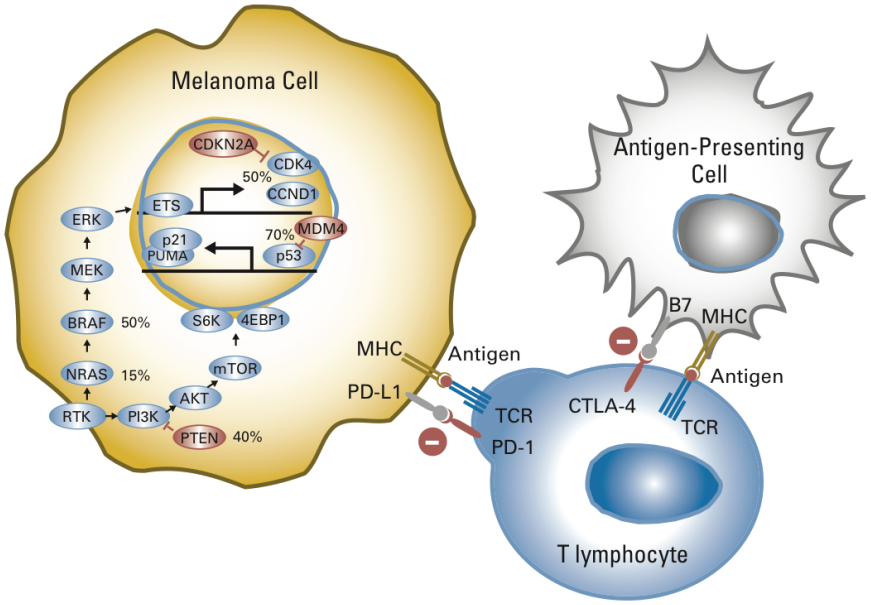

Figure 10 Overview of the therapeutic biology of melanoma. The most frequent genomic changes in melanoma (percentages of patients with mutations or altered protein expression; see text for details). T lymphocytes (programmed cell death protein 1, PD-1) interact with melanoma cells (programmed death-ligand 1, PD-L1). Antigen-presenting cells (B7) interact with T lymphocytes (cytotoxic T-cell lymphocyte-associated antigen 4, CTLA-4). CCND1, cyclin D1; CDK4, cyclin-dependent kinase 4; CDKN2A, p16INK4A inhibitor of CDK4; MHC, major histocompatibility molecule; TCR, T-cell receptor. Reproduced with permission from McArthur and Ribas[340].

4.3.6. Melanoma immunotherapy: past, present and future

Melanoma is an immunogenic cancer[340] with its high mutation rate and many point mutations[335,341]. Neoantigens from mutated proteins can be recognized by the immune system and can be exploited therapeutically to activate the immune system. Historical reports have indeed described an increased occurrence of melanomas in immunosuppressed patients[342] as well as spontaneous regressions of melanoma[343]. Moreover, tumor-specific antibodies and immune infiltrates have been positively associated with survival[344,345].

Immunotherapy has been an active area of research for many years with the use of melanoma vaccines (e.g. inactivated tumor cells) and non-specific immune stimulants (e.g. Bacillus Calmette-Guerin; BCG)[346]. High-dose interlukin-2 (IL-2) was approved for the treatment of advanced melanoma in 1998, and was actually the first approved treatment since the introduction of dacarbizine in 1976. Although these approaches have failed to provide predictable clinical benefit for patients and often been associated with severe toxicities, there are occasional responders (usually <10% of patients) with profound and durable responses[347,348].

All immunotherapy approaches aim to induce intratumoral infiltration of activated T cells, which posses cancer-specific cytotoxic activity[340]. This also applies to adoptive cell transfer (ACT) with autologous T cells, which is another promising and rapidly evolving technology where tumor-infiltrating lymphocytes (TILs) are harvested from patients, expanded and activated *ex vivo*, and reinfused into the patient following chemotherapy-induced depletion of endogenous lymphocytes[349]. In a study of ACT therapy in heavily pretreated patients with metastatic melanoma, Rosenberg et al. reported objective cancer regression in 56% of patients[350]. Notably, complete regression was seen in 22% of patients, of which all but one patient had an ongoing complete response beyond three years. Indeed, TIL-based ACT is an effective therapy for metastatic melanoma, and represents the ultimate form of personalized medicine, since a new "drug" is developed for each patient[349]. In the future, ACT therapy can probably gain further momentum with simplified and automated expansion of TILs within the confines of blood banks or hospital laboratories. Modified ACT approaches and combined regimens encompassing ACT therapy are currently under investigation.

Immune checkpoint inhibition is the most successful immunotherapy approach to date[346] (**Fig. 10**). Activation of a T lymphocyte requires (1) T-cell receptor (TCR) recognition of an antigenic peptide/major histocompatibility complex (MHC) on an antigen-presenting cell (APC), and (2) a coordinated interaction between the T lymphocyte and the APC through receptor-ligand immune checkpoints[351]. The most clinically relevant receptors on T lymphocytes are both inhibitory and mediate immune tolerance: CTLA-4 and PD-1. In 2011, the US Food and Drug Administration (FDA) approved the monoclonal anti-CTLA-4 antibody ipilimumab and, in 2014, the anti-PD-1 antibodies pembrolizumab and nivolumab.

In a recent pooled analysis of long-term survival data of 1,891 ipilimumab-treated patients with advanced melanoma, Schadendorf et al. reported an OS of 11.4 months and a 22% three-year survival rate[352]. Survival curves plateaued around three years, which further substantiates the durability of ipilimumab in subgroups of patients. Furthermore, Maio et al. recently published long-term results of ipilimumab + dacarbizine in 250 patients versus placebo + dacarbizine in 252 patients with advanced melanoma; for patients receiving ipilimumab, the same three-year plateau was described and the five-year survival rate was 18.2% as compared to 8.8% for patients on placebo[353]. Pembrolizumab[354], nivolumab[355,356], lambrolizumab (anti-PD-1 antibody)[357] and BMS-936559 (anti-PD-L1 antibody)[358] have shown even higher response rates and less toxicity in clinical trials than ipilimumab. Several new immune checkpoint blockers are in the pipeline and more regulatory approvals are expected in the years to come.

In 2013, ipilimumab accounted for nearly 2/3 ($577 million) of total US sales of therapies for melanoma[359]. The costs and benefits associated with ipilimumab have been subjected to much debate[360]. Pre-treatment identification of patients that are likely to benefit from ipilimumab therapy is necessary[361]. Research is now focused on patient selection, potential synergistic effects of combinatorial regimens and development of novel therapies with less toxicity. Co-inhibition of CTLA-4 and PD-1/PD-L1 is currently under investigation[362]. The ipilimumab + vemurafenib trial was stopped due to severe hepatotoxicity[363], but other studies are ongoing to explore the potentially synergistic effects of MAPK pathway inhibition and immune checkpoint blockade. Conclusively, the combination of MAPK-targeted therapies with *rapid*

tumor responses and immunotherapies with *durable* tumor responses brings together the best of both worlds and holds great promise for the future.

4.3.7. Current management of metastatic melanoma

Patients with advanced melanoma should be assessed for the presence of a $BRAF^{V600}$ driver mutation, and considered for treatment with a BRAF inhibitor (vemurafenib or dabrafenib) and/or a MEK inhibitor (trametinib). Patients with acral or mucosal melanomas that are $BRAF^{V600}$ negative should be examined for a *KIT* driver mutation[364-368]. Patients with other MAPK pathway alterations (e.g. *NRAS* mutation) often respond better to high-dose IL-2 therapy[369].

Phase III trials have shown a median time to tumor response with ipilimumab of 3.18 months[370] and vemurafenib of 1.45 months[129]. The most common (≥30%) adverse effects associated with vemurafenib are rash, alopecia, arthralgia, fatigue, nausea and photosensitivity reaction[371]. Keratoacanthomas and cutaneous squamous cell carcinomas develop in approximately 24% of patients. Ipilimumab therapy is typically associated with immune-related adverse events due to general immunological enhancement (61% total; 10-20% grade 3-4)[372,373]. The most clinically relevant immune-related immune adverse events are exanthemas, hepatic transaminitis and diarrhea/colitis; the latter has resulted in treatment-related deaths.

The main findings from the most influential clinical trials of systemic therapies for patients with metastatic melanoma over the last five years are summarized in **Table 7**. These studies form the basis of our current standards of care (**Fig. 11**), but, notably, the treatment of metastatic melanoma is a rapidly transforming field with active preclinical and clinical research. At present, there are 423 open studies on melanoma at ClinicalTrials.gov (U.S. National Institutes of Health); most of these deal with metastatic melanoma and involve molecularly targeted therapies, immunotherapies or combinations thereof.

Table 7 Selected clinical trials of systemic therapies in metastatic melanoma from 2010-2015.

Study	*New/recurrent disease*	*Treatment*	*Patients (mutations)*	*RR/PFS/OS*
Topalian 2014[356]	Recurrent	Nivolumab	107	31/3.7/16.8
Topalian 2012[358]	Recurrent	BMS-936559	52	17/42%[a]/NR
Sosman 2012[374]	Recurrent	Vemurafenib	132 (*BRAFV600E*+)	53/6.8/15.9
Robert 2013[375]	Both	Selumetinib + Dacarbizine	44 (*BRAFV600*+)	40/5.6/13.9
	Both	Placebo + Dacarbizine	45 (*BRAFV600*+)	26/3/10.5
Robert 2011[373]	Both	Ipilimumab + Dacarbizine	250	15.2/3/11.2
	Both	Placebo + Dacarbizine	252	10.3/3/9.1
Robert 2014[355]	Both	Nivolumab	210	40/5.1/72.9%[b]
	Both	Dacarbizine	208	13.9/2.2/10.8
Robert 2014[354]	Recurrent	Pembrolizumab	173	26/3.8/60%[b]
Robert 2015[376]	Both	Dabrafenib + Trametinib	352 (*BRAFV600*+)	64/11.4/72%[b]
	Both	Vemurafenib	352 (*BRAFV600*+)	52/7.3/17.2
Ribas 2014[377]	New	Vemurafenib + Cobimetinib	63 (*BRAFV600*+)	88/13.7/83%[b]
	Recurrent	Vemurafenib + Cobimetinib	66 (*BRAFV600*+)	15/2.8/8.3
Ribas 2013[378]	Both	Tremelimumab	328	11/20.3%[c]/12.6
	Both	Dacarbizine / Temozolomide	327	10/18.1%[c]/10.7
McArthur 2014[379]	New	Vemurafenib	337 (*BRAFV600*+)	57/6.9/13.6
	New	Dacarbizine	338 (*BRAFV600*+)	29/1.6/9.7
Long 2014[380]	Both	Dabrafenib + Trametinib	211 (*BRAFV600*+)	67/9.3/93%[d]
	Both	Dabrafenib	212 (*BRAFV600*+)	51/8.8/85%[d]
Larkin 2014[246]	Both	Vemurafenib	2708 (*BRAFV600*+)	34/5.6/12
Larkin 2014[381]	Both	Vemurafenib + Placebo	248 (*BRAFV600*+)	44/6.2/73%[e]
	Both	Vemurafenib + Cobimetinib	247 (*BRAFV600*+)	67/9.9/81%[e]
Johnson 2014[382]	Recurrent[f]	Dabrafenib + Trametinib	26 (*BRAFV600*+)	15/3.6/10
	Recurrent[g]	Dabrafenib + Trametinib	45 (*BRAFV600*+)	13/3.6/11.8
Hodi 2010[370]	Recurrent	Ipilimumab	137	10.9/2.86/10.1
	Recurrent	Ipilimumab + gp100	403	5.7/2.76/10
	Recurrent	gp100	136	1.5/2.76/6.4
Hodi 2013[366]	Both	Imatinib	13 (*KIT*+)	53.8/3.9/12.9

Hodi 2014[383]	Recurrent	Ipilimumab	122	14.8/3.1/12.7
	Recurrent	Ipilimumab + Sargramostim	123	15.5/3.1/17.5
Hauschild 2012[130]	Both	Dabrafenib	187 (*BRAFV600*+)	50/5.1/NR
	Both	Dacarbizine	63 (*BRAFV600*+)	6/2.7/NR
Hamid 2013[357]	Both[h]	Lambrolizumab	78	37/NR/NR
	Both[f]	Lambrolizumab	39	38/NR/NR
Flaherty 2012[384]	Both	Trametinib	214 (*BRAFV600*+)	22/4.8/81%[d]
	Both	Dacarbizine / Paclitaxel	108 (*BRAFV600*+)	8/1.5/67%[d]
Flaherty 2012[385]	Both	Dabrafenib	54 (*BRAFV600*+)	54/5.8/NR
	Both	Dabrafenib + Trametinib[i]	54 (*BRAFV600*+)	76/9.4/NR
Flaherty 2012[386]	Both	Carboplatin + Paclitaxel	413	18.2/4.2/11.3
	Both	Carboplatin + Paclitaxel + Sorafenib	410	20.5/4.9/11.1
Flaherty 2010[387]	Both	Vemurafenib	32 (*BRAFV600*+)	81/>7/NR
Chiarion-Sileni 2014[388]	Recurrent	Ipilimumab	51	12/NR/21
Chapman 2011[129]	Both	Vemurafenib	336 (*BRAFV600E*+)	48/5.3/84%[d]
	Both	Dacarbizine	336 (*BRAFV600E*+)	5/1.6/64%[d]
Ascierto 2013[389]	Both	MEK162	30 (*NRAS*+)	20/3.7/NR
	Both	MEK162	41 (*BRAFV600*+)	20/3.6/NR
Ascierto 2013[390]	Both	Dabrafenib	76 (*BRAFV600E*+)	59/6.3/13.1
	Both	Dabrafenib	16 (*BRAFV600K*+)	13/4.5/12.9
Carvajal 2011[364]	Both	Imatinib	25 (*KIT*+)	24/2.8/10.7

Abbreviations: RR, partial + complete response rate (%); PFS, progression-free survival (months); OS, overall survival (months).

Treatments: BRAF inhibitors: vemurafenib, dabrafenib. MEK inhibitors: trametinib, cobimetinib, selumetinib, MEK162. Anti-PD-1 antibody: pembrolizumab, nivolumab, lambrolizumab. Anti-PD-L1 antibody: BMS-936559. Anti-CTLA-4 antibody: ipilimumab, tremelimumab. Glycoprotein 100 peptide vaccine: gp100. Granulocyte-macrophage colony-stimulating factor (GM-CSF): sargramostim. Chemotherapy: dacarbizine, paclitaxel, carboplatin, temozolomide.

Annotations: [a]Rate of PFS at 24 weeks. [b]Rate of OS at 1 year. [c]Rate of PFS at 6 months. [d]Rate of OS at 6 months. [e]Rate of OS at 9 months. [f]All patients were previously treated with BRAF inhibitor. [g]All patients were previously treated with dabrafenib. [h]No prior or [f]prior ipilimumab therapy with lambrolizumab 10 mg/kg every 2 weeks. [i]Dabrafenib 150 mg/day + Trametinib 2 mg/day. [j]Retreatment with ipilimumab in pretreated patients.

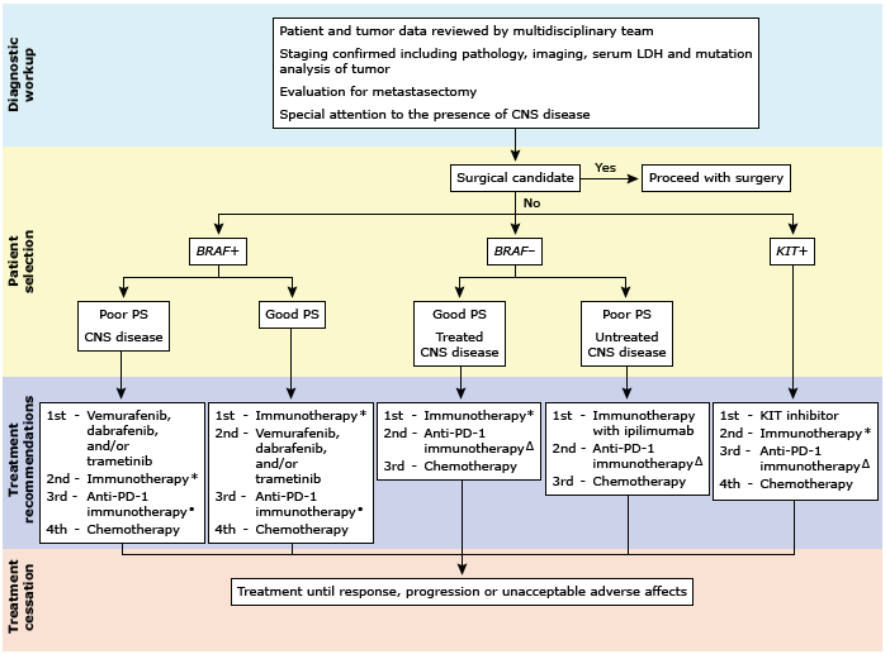

Figure 11 Treatment of metastatic melanoma. The possibility of clinical trial participation or palliative radiation therapy should be considered at all stages. (*) Ipilimumab or interleukin-2 (IL-2): Consider ipilimumab or IL-2 for patients without brain metastases, good organ function, physiologic age < 70 years and normal serum level of lactate dehydrogenase (LDH). Consider ipilimumab alone for all other patients without autoimmune conditions. (•/Δ) Pembrolizumab or nivolumab: for patients with progressive disease. See also **Figure 2**. CNS, central nervous system; PS, performance status. Reproduced with permission from Kaufman et al.[391] and UpToDate[a].

4.3.8. Resistance mechanisms to MAPK-targeted therapies

Only around 50% of melanoma patients have *BRAF*^{V600} mutations, and targeted therapies are limited for the remaining half. Moreover, close to 10% of patients with mutations display primary resistance to BRAF inhibitors and progress during initiation of therapy[387]. Most responders have partial and short-lived responses, e.g. vemurafenib has shown a PFS of just 5.3-7.3 months[129,246,374,376,379,381]. Furthermore, gains in OS are modest; vemurafenib trials have shown an OS of 12-17.2 months[246,374,376,379] as compared to 9.1-10.5 months in contemporary dacarbizine

[a] http://www.uptodate.com

series[373,375,379]. Most of the published trials of MAPK-targeted therapies (**Tab. 7**) show similar limitations in therapeutic efficacy and durability, and suggest that there are both intrinsic and adaptive mechanisms that need to be overcome (see **Paper IV**).

Acquired drug resistance is a major issue with the new MAPK-targeted therapies and resistance mechanisms usually involve reactivation of the MAPK pathway[392-395] (**Fig. 12**). Recent insights have been achieved through a wide-range of preclinical investigations using drug-resistant *BRAF*^{V600E}-mutated cell lines.

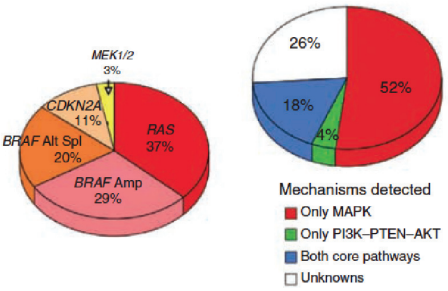

Figure 12 Mechanisms of acquired resistance to BRAF inhibitor therapy. MAPK-reactivating mechanisms (**left**) and distribution of core pathways among progressive melanomas (**right**). Adapted and reproduced with permission from Shi et al.[393].

Mechanisms of resistance to MAPK-targeted therapies:

(1) ERK activation can be restored through several bypass mechanisms within the MAPK pathway regardless of ongoing BRAF inhibition: activating mutations in *NRAS*[394,396,397] or *MEK*[138,394,398], upregulation of RAF proto-oncogene serine/threonine-protein kinase (CRAF)[399,400], activation of serine/threonine kinase Cot (COT)/*MAP3K8*[399], overexpression of mutant BRAF[401], upregulation of platelet-derived growth factor β (PDGFRβ)[396] or receptor tyrosine-protein kinase erbB-2 (ERBB2)[399], and inactivation of neurofibromin (NF1)[402].

(2) Modified forms (splicing variants) of the BRAF protein that are insensitive to BRAF inhibitors have been found in a significant subset of patients with acquired resistance[403].

(3) Compensatory activation of the PI3K-AKT pathway can sustain cell growth and survival through adaptive upregulation (e.g. increased expression of insulin-like growth factor 1 receptor (IGF-1R))[404] and activating mutations (e.g. *AKT1 Q79K* mutant)[405].

(4) Intrinsic[406] or acquired[407] induction of the microphthalmia-associated transcription factor (MITF) and the mitochondrial master regulator peroxisome

proliferator-activated receptor γ coactivator 1-α (PGC1α) result in enhanced mitochondrial oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) detoxification capacities. Melanoma cells become addicted to mitochondrial respiration and show resistance to BRAF inhibitors (see **Paper IV**).

(5) Increased CDK4 activity due to elevated levels of CRAF[400], *CCND1* (cyclin D1) amplification[408,409], activating mutations in *CDK4*[409] or loss of its inhibitor p16INK4A inhibitor of CDK4 (CDKN2A)[408] increases cell proliferation, and may both confer baseline and acquired resistance to MAPK-targeted therapies.

(6) Increased expression of the antiapoptotic Bcl-2-related protein A1 (BCL2A1)[410], elevated levels of the hepatocyte growth factor (HGF)[411,412] or loss of the tumor suppressor PTEN[408] may confer intrinsic resistance to BRAF inhibitors.

4.3.9. Challenges and future directions of melanoma therapy

Contemporary therapy for metastatic melanoma is hampered by limited efficacy and durability, partly due to intrinsic and adaptive resistance mechanisms[413,414]. Moreover, there are significant concerns with regards to drug-related adverse effects[233,234], patient selection criteria[225,361] and cost-benefit[359,360]. Most importantly, melanoma is characterized by a high genomic complexity and variability, and a high metastatic potential. This section will focus on molecularly targeted therapies. Future directions of immunotherapy are outlined above (section 4.3.6.).

Preclinical investigations suggest that discontinuous dosing schedules might forestall BRAF inhibitor resistance and sustain drug sensitivity[415]. Recent clinical trials indicate that newly diagnosed *BRAF*-mutated patients benefit from combined BRAF + MEK inhibition as opposed to BRAF or MEK monotherapy[376,377,380,381,385] (**Tab. 7**). Preclinical evidence suggests that the ERK inhibitor SCH772984 can overcome acquired resistance to BRAF and MEK inhibitors[416]. Clinical trials of ERK inhibitors are currently ongoing, but information thus far is scarce. Efficient ERK phosphorylation is dependent of the interaction between copper and MEK1, and recent work has found copper chelation therapy to decrease proliferation of naïve and drug resistant human and murine $BRAF^{V600E}$-mutated melanoma cells[417]. Many

components of the MAPK pathway are reliant on the chaperone protein heat shock protein 90 (HSP90), including BRAF, CRAF and COT proteins, and preclinical work have demonstrated that HSP90 inhibitors can abrogate BRAF inhibitor resistance[418]; a clinical trial combining vemurafenib and the HSP90 inhibitor XL888 is currently ongoing (NCT01657591).

Combinations of BRAF-MEK-ERK pathway inhibitors and PI3K-AKT-mTOR pathway inhibitors could prevent therapeutic escape through enhanced PI3K-AKT-mTOR signaling[393,404,419]. This combination will probably have a narrow therapeutic window as both pathways are implicated in multiple cellular processes[340,414]; nevertheless, several clinical trials are underway (e.g. NCT01616199, NCT01519427).

Direct targeting of the neuroblastoma RAS viral oncogene homolog (NRAS) has proven to be difficult, but combination therapies that block downstream signaling may provide benefit to *NRAS*-mutated patients (e.g. BRAF, ERK)[420]. In a recent meta-analysis of somatic mutations from next generation sequencing of 241 melanomas, 69 tumors were found to be $BRAF^{WT}$, $NRAS^{WT}$ and KIT^{WT} ("pan-negative") and 12 potential driver mutations were identified (*ADAMTS18*, *ALK*, *DGKI*, *EPHA4*, *EPHA7*, *ERBB4*, *KDR*, *NF1*, *RAC1*, *STK31*, *SYK* and *TAF1L*), each in a small percentage of patients[421]. Strategies to target these "pan-negative" melanomas have proven even more elusive than *NRAS*-mutant melanomas[413].

Early treatment responses should be evaluated so that combination therapies can be personalized or modified before resistance ensues[413]. For this, better biomarkers are needed; proteomic methods and analyses of circulating tumor cells/DNA are currently being explored.

The majority of actionable driver mutations in melanoma have probably been identified and a vast amount of knowledge has been generated about the molecular biology of melanoma[336]. Still, we have a limited grasp of the complexity of this intricate genomic network[337] as well as the associated intra- and intertumoral heterogeneity[303,335]. As our knowledge evolves, significant improvements should be expected in both durability and efficacy of targeted therapies (see also section 4.3.5.).

There is also a prevailing need for novel drugs with more broad-spectrum efficacy against metastatic melanoma, including drugs that can prevent the emergence of metastatic disease[5,17,312,414,422-425]. A rapidly evolving field of research involves decoding and therapeutic interference of the rewired metabolic network in cancers, including melanomas[426,427]. This is further discussed in **Papers III** and **IV**, and in section 4.4.2.6.

4.4. MELANOMA BRAIN METASTASIS

4.4.1. Contemporary clinical and preclinical landscape

Melanoma is the fifth most common cancer[a], but the third most common cause of brain metastases[3,6,18,32]. Hence, melanoma patients carry a high risk of developing brain metastases[5,428-430]. Recent advances in systemic therapies for metastatic melanoma offer promise (**Tab. 7**), but have thus far provided limited benefit for patients with brain metastases (**Tab. 4;** section 4.2.3.4.3).

Melanomas are characterized by a high metastatic capacity[431] and unprecedented genetic heterogeneity[303,335]. Brain metastases find protection and alliance beyond the BBB/BTB and within the brain microenvironment[5,44,432]. These features require robust and representative preclinical model systems (see **Papers I** and **II**) to elucidate the biology and assess new therapies in preventive and established scenarios (see **Papers III** and **IV**).

4.4.2. Biology of melanoma brain metastasis

Pioneering studies from Isaiah Fidler and others have provided important insights into the molecular biology of melanoma brain metastasis[429]. However, the regulatory mechanisms are still relatively poorly understood and knowledge is fragmented[272]. The causal mechanisms of brain-specific tropism, increased BBB permeability and enhanced cell survival in the brain are not fully characterized or understood. A myriad of proposed mechanisms underscore the complexity of this process, and reflect the profound and dynamic intra- and intertumoral heterogeneity of melanomas. A better and more integrated understanding of the molecular biology is critical to the development of new preventatives and therapeutics.

4.4.2.1. Animal models

Animal models involving hematogenous dissemination of cancer cells have been important tools of brain metastasis research for many years, despite their inherent methodological flaws[433-437]. The values and limitations of past and present pan-cancer animal models of brain metastasis are reviewed in **Paper I**. In brief, we can apply the

[a] http://www.seer.cancer.gov/statfacts/html/melan.html

well-worn dictum "*all models are wrong, some models are useful*"[438]. A further discussion of reproducibility and predictivity of brain metastasis models is provided in **Paper II** and animal models are employed in **Papers II-IV**.

4.4.2.2. Preclinical imaging

MRI, PET and bioluminescence imaging (BLI) are complementary, noninvasive imaging platforms that enable exceptional temporal and spatial tracking of multiple tumors in a single mouse[439]. This is clearly advantageous for metastasis models, which typically involve many tumors. Not only can we follow the total tumor burden over time, we can also visualize and differentiate tissue specific effects, and evaluate both growth inhibition and targeting efficacy. MRI is the best modality for brain imaging due to its high spatial resolution and excellent tissue contrast, whereas PET and BLI provide high sensitivity and overview of systemic tumor involvement[439]. PET imaging suffers from limited availability and throughput, but is rapidly evolving, both in the preclinical and clinical setting[440,441].

MRI alone cannot identify single tumor cells or micrometastases in the brain. Nanoparticle-based contrast agents for MRI have thus seen an increasing use in preclinical research, clinical diagnostics and therapeutics[439]. Superparamagnetic iron oxide nanoparticles (SPIONs) have dominated the field of MRI-based cell tracking[439,442]. MRI coupled with cellular SPION labeling provides the opportunity to visualize and quantify cancer cells and tumors in the brain. Utilities and caveats of SPION labeling in brain metastasis models are specifically discussed in **Paper II**. The value of multimodal imaging is discussed in **Paper I** and its applied in **Papers II-IV** to facilitate reproducible and predictive *in vivo* modeling of melanoma brain metastasis.

Early detection of brain metastases is critical and several techniques are currently in preclinical development. These include a method that specifically permeabilize the BBB at sites of brain metastases using recombinant human tumor necrosis factor (TNF) and that enables detection of micrometastases not visible using standard imaging modalities[443]. Another promising technique utilizes a targeted MRI contrast agent where microparticles of iron oxide are conjugated to antibodies against vascular

cell adhesion molecule-1 (VCAM-1). These complexes may be detected when they bind to the endothelium of developing tumor-associated blood vessels[444]. At clinical imaging resolutions, this technique could translate to metastasis detection at volumes two to three orders of magnitude smaller than currently possible ($0.3\text{-}3 \times 10^5$ cells versus $10^7\text{-}10^8$ cells).

4.4.2.3. The metastatic process: seed, soil and climate

In 1889, Stephen Paget presented the "seed-and-soil" theory where he suggested that inherent qualities of different seeds (=tumor cells) make them more prone to grow in different soils (=organ microenvironments)[445]. This organ specificity of various cancers has been experimentally confirmed in numerous studies, including melanomas[446-448]. It seems the metastatic pattern of melanomas is not explained by the anatomy of circulation alone; however, head and neck melanomas do have a higher incidence of brain metastases[278,279]. Evidence also suggests that the "climate" (=the host) is an important determinant of tumor growth in distant organs[449]; Chen et al. showed that the same cancer cell line could be bone-tropic in one mouse strain and liver-tropic in another host strain[450].

There is no lymphatic system in the brain. Circulating tumor cells are lodged in the brain microvasculature and traverse the BBB to form brain metastases (**Fig. 13**). These unique steps of tumor formation from the single-cell level have been elegantly characterized by real-time imaging with multiphoton laser scanning microscopy in mice[451]. In this study, Kienast et al. also demonstrated the inefficiency of the metastatic process as well as the presence of long-term dormancy. Following capillary arrest, some tumor cells adhere to the vessel endothelium and extravasate into the brain parenchyma, a process dependent on close interaction with the vascular basement membrane[451-453]. Melanoma cells that later give rise to brain metastases extravasate within 3-7 days after inoculation[441,451]. Melanoma cells remain in close contact with microvessels and co-opt these for nutrients, as opposed to lung cancer cells who induce neoangiogenesis. Correspondingly, Kienast et al. showed that VEGF-A inhibition induced co-option and prevented tumor formation in lung cancer brain metastases, whereas melanoma brain metastasis was not influenced by VEGF-A inhibition[451].

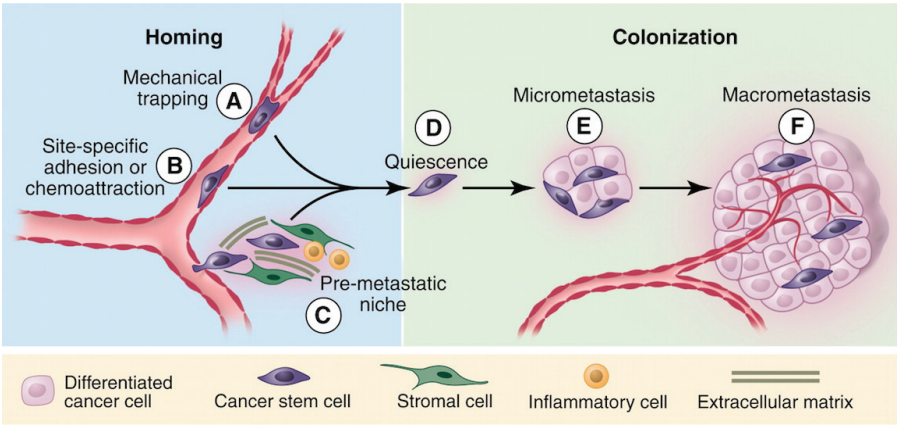

Figure 13 Homing and colonization of cancer cells to a distant organ. Circulating tumor cells can become lodged in the capillary bed of distant organs. Organ-specificity may be dependent of site-specific adhesion, chemoattractants or seeding to pre-metastatic niches (i.e. fertilized microenvironments). Cancer cells that extravasate may remain quiescent (dormant) or step-wise evolve into a metastatic tumor, processes that rely on stimulatory/inhibitory interactions with the organ environment and recruitment of adequate blood supply. Successful colonization requires stem-like properties (e.g. enhanced tumorigenicity, self-renewal potential). Mechanisms governing brain-specific homing and colonization of melanomas are discussed in the text. From Chaffer et al.[2]. Reprinted with permission from the American Association for the Advancement of Science (AAAS).

4.4.2.4. The blood-brain barrier

The BBB protects the brain from endogenous and exogenous toxins[139]. Capillary endothelial cells in the brain lack fenestrations, have low pinocytosis, high electrical resistance, are inter-connected by tight junctions and express high levels of drug efflux transporters. Moreover, a basal membrane, extracellular matrix, astrocytic end-feet and pericytes surround the outer surface of brain endothelial cells. All these factors comprise the BBB, which limit the penetration of drugs into the brain.

Drugs that effectively traverse the BBB by means of passive diffusion have low molecular weights (<400 Da) and they are nonpolar and lipophilic[454,455]. Hydrophilic, polar and large molecules are reliant of active transport systems. There is major research activity in the reengineering of drugs to access carrier-mediated or receptor-

mediated transport systems within the BBB[455], and in the development of strategies to circumvent the BBB[44].

The BBB around tumors – the BTB – is often compromised[456], but the BTB is still a significant obstacle to drug delivery and efficacy. In an analysis of more than 2,000 experimental brain metastases from breast cancer, Lockman et al. reported a higher uptake of chemotherapeutic agents in metastases than in normal brain[457]. However, there was less than 15% uptake in brain metastases compared to that of extracranial tissues/metastases, and cytotoxic concentrations were only achieved in about 10% of brain metastases.

4.4.2.5. Molecular biology

Paper I features a short review of the molecular determinants of brain metastasis across different cancers, including melanomas. Importantly, although several genes and proteins have been found selectively expressed in brain-metastatic cells, there is little overlap between studies, models and cancers. In the following, I will elaborate on some of the molecular factors that are specifically important in melanoma and/or reported to be specifically associated with melanoma brain metastasis in the literature. Metabolic pathways in melanoma brain metastasis are discussed in the next section.

MAPK pathway

Molecular profiling of matched brain and extracranial metastases of melanoma recently revealed full concordance for 156 known hotspot mutations (including driver mutations in *BRAF* and *NRAS*) as well as similar overall patterns of copy number variations, mRNA expression and protein expression[458]. Hamilton et al.'s study of matched extracranial metastases and brain metastases confirmed this similarity in gene expression profiles; there were no significantly enriched pathways between the two groups[328]. Niessner et al.'s study of matched extracranial metastases and brain metastases found no differences in *BRAF* or *NRAS* mutation status and identical ERK and pERK immunohistochemical staining patterns[459]. Capper et al. found no relationship between $BRAF^{V600E}$ status and survival in matched primary metastases and brain metastases from melanoma patients, but $BRAF^{V600E}$-positive patients were younger than $BRAF^{V600E}$-negative patients[460].

PTEN-PI3K-AKT pathway

Overexpression of AKT enhances the invasiveness and growth of primary melanomas through increased VEGF expression, ROS production and switch to a glycolytic phenotype[461]. Analyses of human melanoma metastases and human melanoma cell lines have shown higher levels of phosphorylated AKT and lower PTEN protein levels in *BRAF*-mutant melanomas compared to *NRAS*-mutant melanomas, and in melanoma brain metastases compared to lung and liver metastases[462]. However, the levels of AKT or PTEN did not predict survival. In contrast, Bucheit et al. recently showed that PTEN loss in melanoma lymph node metastases correlated with decreased OS and shorter time to brain metastasis[463]. Studies of matched melanoma brain metastases and extracranial metastases have confirmed increased PI3K-AKT activation and PTEN loss in brain metastases[458,459]. Inhibition of PI3K-AKT signaling has been proposed to enhance and/or prolong the effects of BRAF inhibitors in patients with melanoma brain metastases[459]; PI3K inhibition blocks downstream signaling better than AKT inhibition[295].

JAK-STAT pathway

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway promotes survival, growth and angiogenesis. Experimental studies have shown that STAT3 activation via phosphorylation[326] or downregulation of its inhibitor suppressor of cytokine signaling 1 (SOCS-1)[464] increase the expression of matrix metalloproteinase-2 (MMP-2), basic fibroblast growth factor (bFGF) and VEGF with consequent melanoma invasion and angiogenesis. Silencing of the immunoregulatory protein B7 homolog 3 (B7-H3) has been found to decrease STAT3 and metalloproteinase activation, and reduce melanoma brain metastasis *in vivo*[465]. Yet, STAT3 activation is generally associated with a pro-metastatic phenotype, and might not be a brain-specific phenomenon[466].

Migration/Adhesion

Cellular adhesion molecules like VCAM-1 have been shown to play important roles in the early steps of breast cancer brain metastasis[444,467]. Melanoma-bearing mice with negligible levels of stimulatory cytokines (tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β)) also display upregulated endothelial expression of VCAM-1 in

the brain, suggesting a direct interaction between tumor cells and the endothelium[468]. Interestingly, *VCAM-1* gene expression was found to be positively associated with survival in metastatic melanomas along with a cluster of immune response-related genes, indicating a benefit of an existent immune presence in melanomas[469]. Prolonged patient survival in patients with melanoma brain metastases was also associated with tumor immune infiltrates and several immune-related gene sets in a study of matched primary melanomas, extracranial and brain metastases[328].

Chemokine receptor/ligand interactions might be involved in organ-specific metastasis of melanoma through regulation of chemoattraction, adhesion and survival[470]. The C-C chemokine receptor type 4 (CCR4) has been found overexpressed in a melanoma brain metastasis cell line compared to its corresponding cutaneous variant[471]. Brain-derived soluble factors have been shown to upregulate CCR4 in matched cutaneous and brain-metastatic cells, but only brain-metastatic cells displayed increased migration[472]. This divergent ability to respond to motility-enhancing signals (e.g. chemokine (C-C motif) ligand 22 (CC22)) could be explained by either an acquired ability in the brain or an inherent ability that attracted these cells to the brain in the first place. The C-X-C chemokine receptor type 4 (CXCR4)/stromal cell-derived factor 1α (SDF-1α) receptor/ligand interaction has been shown to facilitate directed migration of breast cancer cells through human brain microvascular endothelial cells[473].

Melanoma cells and platelets interact with endothelial selectins in the brain microvasculature to facilitate adhesion to the vessel wall[474]. Preclinical studies suggest that heparin in clinically relevant doses can inhibit adhesion and attenuate melanoma brain metastasis formation.

Invasion/Colonization

Both melanoma cells and their conditioned media have been found to compromise junctional integrity by reducing transendothelial electrical resistance and disrupt tight junction molecules like claudin-5 and tight junction protein ZO-1 (ZO-1)[475]. This process is incompletely understood, but proteolytic enzymes are probably involved.

Melanoma cells colonize the brain while in close contact with the vasculature[451,452], and might utilize connexin gap junction proteins to initiate tumor formation within the vascular niche[476]. Connexin 26 was shown to mediate extravasation and vessel co-option using transparent zebrafish and chicken embryo models of melanoma brain metastasis[476]. Other studies have shown that activated astrocytes protect brain-metastatic melanoma cells from chemotherapeutic drugs, and this effect was dependent on physical contact and gap junctional communication between tumor cells and astrocytes[477]. Whether this chemoprotection could be abrogated by connexin inhibition remains to be determined.

Endothelin receptor B (EDNRB) overexpression induced overall metastasis and brain metastasis in a spontaneous brain metastasis model of melanoma[478]. The interaction between EDNRB and its endothelin ligands, which are highly expressed in the brain relative to other organs, was proposed to mediate the increased incidence of brain metastases and promote intracranial tumor growth. Previous work has also implicated EDNRB in melanoma progression and shown that EDNRB activation mediates cell proliferation, adhesion, migration and matrix metalloproteinase-dependent invasion[479]. EDNRB might be facilitator of metastatic spread in general, but particularly important in the brain where its ligands (especially endothelin-3 (ET3)) are abundant[478].

Heparanase (HPSE) cleaves heparin sulfate chains of proteoglycans in the extracellular matrix and has been linked to tumor growth, invasion and angiogenesis. Elevated levels of HPSE have been shown to augment invasion of brain-metastatic melanoma cells in a brain slice model[480]. Co-incubation of astrocytes with brain-metastatic melanoma cells further increased HPSE activity and invasion *in vitro*[481]. Neurotrophins and neurotrophin receptors have been proposed to mediate brain-specificity in melanoma metastasis through ligand/receptor interactions like nerve growth factor (NGF)/p75 neurotrophin receptor and neurotrophin-3 (NT-3)/tropomyosin receptor kinase C (TrkC), but also to promote brain colonization through enhanced HPSE production[482]. Moreover, microRNA (miR)-1258 has been found to suppress breast cancer brain metastasis *in vivo* through direct targeting of HPSE[483]. Both active and latent forms of HPSE can modulate the invasive phenotype

driven by GTPases such as Ras-related C3 botulinum toxin substrate 1 (Rac1) and Ras homolog gene family, member A (RhoA) in brain-metastatic melanoma cells[484].

Overexpression of the antiapoptotic protein BCL2A1 in melanomas did not increase brain metastasis in a spontaneous brain metastasis model, but appeared to facilitate tumor growth in an orthotopic model[478]. BCL2A1 has also been associated with intrinsic resistance to BRAF inhibitors[410].

The transforming growth factor-β (TGF-β) cytokine family is involved in a range of biologic processes. TGF-β2 has been found to be a molecular determinant of parenchymal brain metastases, but not meningeal or ventricular metastases[485]. TGF-β2 has also been shown to promote the growth of GBMs[486]; suggesting that there are common microenvironmental factors that might facilitate tumor progression in specific organs. Moreover, TGF-β signaling has been implicated in acquired BRAF and MEK inhibitor resistance via induction of *EGFR* and *PDGFRβ* expression[487].

Melanotransferrin, a surface antigen of melanoma cells, has been found to stimulate plasmin formation and subsequent invasion via cleavage of extracellular matrix proteins and growth factor precursors. Inhibition of melanotransferrin reduced the ability of melanoma cells to cross the BBB and form brain metastases in mice[488]. Direct plasmin inhibition has also resulted in reduced brain metastasis in mouse models of melanoma[489]. Moreover, tumor-expressed inhibitors of plasminogen activator (PA), serpins, have been found to inhibit melanoma lung metastasis[490]. Joan Massagué's group recently described a different mechanism[491]. They found that metastasis-associated astrocytes released PA in the presence of extravasated breast and lung cancer cells. Plasmin subsequently stimulated FasL-mediated apoptosis of cancer cells and inhibited L1 cell adhesion molecule (L1CAM)-mediated vascular co-option. Brain-metastatic cells could block these effects by releasing anti-PA serpins and thus promote cell survival and growth. Thus, plasmin might have both pro- and anti-metastatic effects in the brain and the role of serpins in melanoma brain metastasis is unclear.

MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNAs that negatively regulate gene expression at the post-transcriptional level, and have been implicated in brain metastasis from several cancers[492]. Co-culture with astrocytes downregulated miR-768-3p in brain-metastatic melanoma cells; miR-768-3p drives *KRAS* expression and the downstream effectors ERK and BRAF[493]. Exosomes of matched breast cancer and melanoma brain metastases and their primary tumors showed upregulated miR-210 and downregulated miR-19a and miR-29c, which are implicated in adhesion and invasion[494]. Overexpression of miR-146a suppressed migratory and invasive capacity in brain-metastatic melanoma cells via upregulation of β-Catenin and downregulation of matrix metalloproteinases[495]. MiR-1258 was discussed above[483]. Given the ability of miRNAs to control multiple targets, they are promising candidates to regulate such a complex process as metastasis.

4.4.2.6. Metabolic pathways

In recent years, it has been shown that several genetic and molecular drivers of melanoma modulate cellular metabolism in ways that are critical to tumor development, metastasis and drug resistance (reviewed in[426,427]). Little is known about the metabolic rearrangements in melanoma brain metastases, but in breast cancer it has been found that metastatic cells adapt their energy production to facilitate growth and survival in the brain[496,497]. Breast cancer cells in the brain can for example proliferate independent of glucose[496] and have been shown to utilize mitochondrial respiration for energy production and antioxidant defense[497]. Whether these changes reflect intrinsic or adaptive properties of tumor cells to thrive in the neural niche remains to be determined. However, brain-metastatic cells from breast cancer have been found to display neuron-like characteristics in the brain microenvironment[311,498,499]. Moreover, the brain interstitium is a low glucose environment[496], and when cancer cells are deprived of glucose they switch from glycolysis to OXPHOS[500]. Melanomas display significant intra- and intertumoral heterogeneity in their expression of genetic drivers[303,335] and in mitochondrial versus glycolytic function[501-503]. Still, cell lines derived from metastatic melanomas and melanoma metastases (none from brain) have revealed elevated levels of OXPHOS compared to primary melanomas[503,504]. The balance between glycolysis and OXPHOS

is probably skewed towards glycolysis in the hypoxic tumor core and towards OXPHOS in the oxygenated tumor periphery of metastatic melanomas. The former might be mediated by increased hypoxia-inducible factor 1α (HIF1α)-dependent expression of LDHA and conversion of pyruvate to lactate[504,505]. Hence, the contribution of the Warburg effect[506,507] – aerobic glycolysis instead of OXPHOS in cancer cells and consequent lactate production – can be different in metastatic and primary melanomas, and in different organ microenvironments.

The spatial, temporal and functional features of LDHA in melanoma brain metastasis are discussed in **Paper III**. Inhibition of mitochondrial respiration is discussed in **Paper IV**.

The MAPK pathway is a key regulator of metabolism in melanomas[426,427] (**Fig. 14**). Signaling through the MAPK pathway increases glycolytic activity[508] and reduces mitochondrial respiration[406,407,509]. BRAF inhibitors confer the opposite effect[406,407,508,510,511]. One of the mediators of MAPK signaling is pyruvate dehydrogenase kinase, isoenzyme 1 (PDK1), which inhibits pyruvate dehydrogenase (PDH)-mediated entry of acetyl-CoA into the citric acid (TCA) cycle[509]. PDK1 is suppressed in *BRAF*-mutated melanomas, and treatment with BRAF inhibitors restores PDK1 activity. PDK1 inhibitors (e.g. dichroacetate) have been found to synergize with BRAF inhibitors and abrogate BRAF inhibitor resistance[508,509]. The MITF-PGC1α axis is another important regulator of mitochondrial activity in melanomas, and induction of MITF and PGC1α increases OXPHOS and ROS scavenging capabilities[406,407]. A subset of melanomas overexpresses PGC1α[406], and treatment of *BRAF*-mutated melanomas with BRAF inhibitors upregulates PGC1α[407]; both of these groups display increased OXPHOS and ROS detoxification capacities. Notably, these mechanisms can provide intrinsic and acquired survival advantages, but the resultant dependence of OXPHOS also opens up the possibility of targeting OXPHOS in melanomas[406,407,510,511].

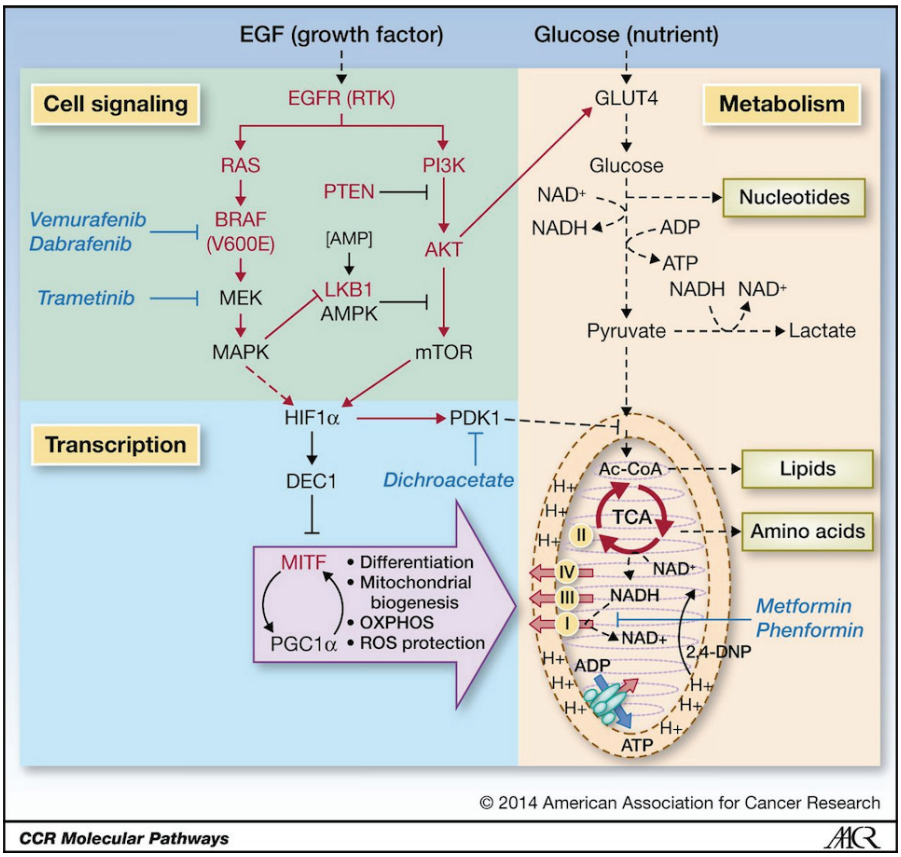

Figure 14 Regulatory network of cell signaling, transcription and metabolism in melanoma. Growth factor-mediated activation of receptor tyrosine kinases (RTKs) leads to downstream activation of the RAS-RAF-MEK-MAPK and PI3K-AKT-mTOR pathways. MAPK suppresses LKB1/AMPK energy sensing and AKT triggers increased glucose import via the glucose transporter GLUT4. MAPK stabilizes and mTOR increases translation of HIF1α. Increased HIF1α activity (1) decreases MITF and PGC1α levels and subsequent mitochondrial bioactivity, and (2) increases PDK1 activity, which in turn inhibits PDH-mediated entry of acetyl-CoA into the TCA cycle. The combined result of these regulatory interactions is increased glycolysis and decreased mitochondrial respiration. Frequently mutated components in human cancers are indicated in red and known inhibitors are indicated in blue. See text for more details. Reproduced with permission from Haq et al.[427].

Corazao-Rozas et al. found *in vitro* and *in vivo* support of adding elesclomol, which targets the mitochondrial electron transport chain, to vemurafenib-resistant melanomas[510]. The antidiabetic biguanides metformin and phenformin inhibit

mitochondrial Complex I (CI), and increase AMPK-dependent mTOR inhibition[426]. Niehr et al. found synergistic effects *in vitro* of vemurafenib and metformin in *BRAF*- and *NRAS*-mutated melanomas[512]. It has been argued that the effective dose levels of metformin *in vitro* were too high to be clinically relevant[426,513], but observations also suggest that metformin accumulate in target organs *in vivo* and in humans to concentrations that are much higher than plasma concentrations[514]. Yuan et al. found enhanced therapeutic benefit of $BRAF^{V600E}$ inhibition in melanoma xenografts with the more potent biguanide phenformin[513]. These studies suggest a therapeutic potential for mitochondrial inhibition, but further studies are needed to determine the clinical utility of these findings.

Many metabolic modulators like natural compounds and drugs used for other conditions than cancer have favorable cost and toxicity profiles, and might offer additional therapeutic benefit in metastatic melanoma. The abovementioned importance of mitochondrial respiration in melanoma brain metastases might suggest even greater advantage of mitochondrial inhibition in preventing and treating brain-metastatic lesions (see **Paper IV**).

5. AIMS

The overall aim of this thesis was to study the biology of melanoma brain metastasis and find novel therapeutic strategies *in vivo*.

The main aims of each study were:

Paper I

To review the current literature on animal models of brain metastasis and critically address their pros and cons.

Paper II

To develop a reproducible and predictive mouse model of melanoma brain metastasis.

Paper III

To examine the mechanistic importance of LDHA in melanoma brain metastasis *in vivo*.

Paper IV

To identify potential therapeutic compounds against melanoma brain metastases based on genomics-based drug repositioning and functional assessment *in vivo*.

6. DISCUSSION

Papers I-IV are cited in relevant sections throughout the general introduction. Here, I will discuss the timeliness, key results and methodological considerations of each study.

6.1. Paper I

We reviewed the literature on the various animal models used to study brain metastasis, and sought to attain an overview of their strengths and weaknesses. Our review was placed in the context of the multi-step metastatic process and the limited overlap of molecular signatures between studies (**Fig. 15**). Taken together, models are just models, and none of them fully reflect the complexity or biology of brain metastasis. Thus, experimental findings should be interpreted with caution, examined across different models and human validation is essential.

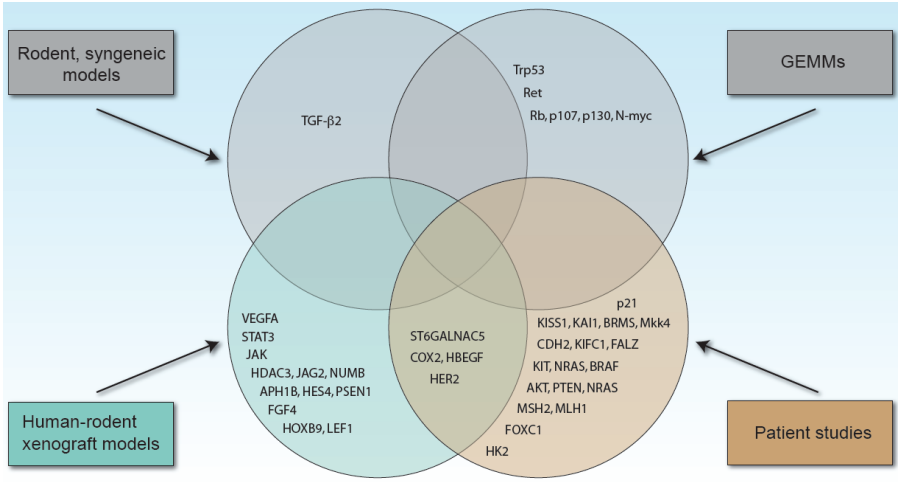

Figure 15 Genes implicated in brain metastasis. These genes are derived from preclinical and clinical studies, and the missing overlap between different models is evident. However, there is also significant discordance within model systems. For further details of molecular and genetic mechanisms, see previous sections on melanoma and melanoma brain metastasis and reviews[5,17,44,429]. GEMMs, genetically engineered mouse models. Reproduced with permission from Daphu et al.[515].

Many different models are available, but mouse or rat models are mostly used. These models typically rely on inoculation of cancer cell lines from rodents into rodents (syngeneic), or inoculation of cancer cell lines or tissues from humans into rodents (xenogeneic). The inoculation route is orthotopic (into the organ of origin) or ectopic (into another organ/site). Genetically engineered mouse models (GEMMs) are genetically modified by for example insertion of an oncogene or deletion of a tumor suppressor gene, and have so far had limited impact in brain metastasis research. Human-to-rodent xenograft models rely on the use of immuno-compromised hosts, whereas syngeneic models and GEMMs enable study of the interaction between immuno-competent hosts and tumor cells.

Some of the most valuable, available and least expensive animal models for brain metastasis research are those using human tumor xenografts and immunodeficient mice[436] (see **Paper II**). However, they are also some of the most criticized models[516]. Moreover, data from cancer cell lines grown *in vitro* and *in vivo* should be cautiously evaluated. Gillet et al. found established cell lines from six different cancers to be genetically more similar to each other than to the clinical samples they were supposed to model[517]. Domcke et al. compared the genetic similarity between 47 cancer cell lines and 316 tumor samples; the commonly used cell lines were most different and the least used cell lines were most similar to the tumors[518]. Integration with human tissue biobanks and clinical outcome data add clinical relevance to these model systems[519], and cellular characterization and authentication is instrumental in model development (see previous work from our group[520,521] and **Papers II-IV**). Conclusively, standardized and reproducible animal models are needed to uncover the biology of melanoma brain metastasis and to improve clinical translation (see **Paper II**).

I would like to draw attention to two important, but different, animal models of melanoma brain metastasis. First is the model by Kienast et al. using multiphoton laser scanning microscopy through cranial windows to image the single steps of metastasis formation[451]. This model allows high-resolution, real-time tracking of cancer cells in relation to blood vessels within the live brain over months, and enables the observation of metastasis as a process rather than a simple endpoint (see also section 4.4.2.3.). Second is the model by Cruz-Munoz et al., which is a unique

orthotopic human melanoma xenograft model with spontaneous brain metastasis[522]. In contrast to models based on intracardiac or intracarotid injections (commonly referred to as experimental models), this model enables examination of all steps in the metastatic cascade and closely resembles clinical disease. However, metastases develop slowly and appear in only approximately 50% of mice; hence, therapeutic experiments might be laborious and require large numbers of mice[523]. Using this model system, Cruz-Munoz et al. identified the significance of EDNRB and BCL2A1 in promoting melanoma brain metastasis[478] (see also section 4.4.3.5.).

6.2. Paper II

We here report on the development and validation of a novel and reproducible brain metastasis model that routinely incorporates automated quantification of nanoparticle-labeled melanoma cells in the brain. We show that brain metastasis formation is dependent on the brain cell load, recapitulates the spread and growth seen in humans and is unaffected by nanoparticle labeling. This model enables early homogenization of study animals. This is essential to draw reliable conclusions of biologic differences and therapeutic efficacy (**Fig. 16**), and the model can readily be tailored to other cancer cell lines. We propose that it can help increase the poor success rates of anti-cancer agents in clinical trials, which currently display 95% drug attrition rates[524].

This project was inspired by the difficulties associated with intracardiac injections in mice, which is one of the most used techniques to study brain metastasis. We were also motivated by the heterogenous metastatic potential of cancers and the inefficiency of the metastatic process. Not surprisingly, we found the tumor cell load in the brain at baseline to be strongly correlated to the formation of brain metastases. Importantly, MRI-based quantification of SPION-labeled cells was superior to standard BLI methods in evaluating injection success or failure. Not only could we discriminate hits and misses with more certainty than BLI, but everything in between, and importantly, we showed that this is related to metastasis formation. Following this publication, we have extended our model to include US-guidance using a custom-made needle-holder (see **Papers III** and **IV**). US-guided intracardiac injection does improve reproducibility, but it does not make MRI-based quantification superfluous.

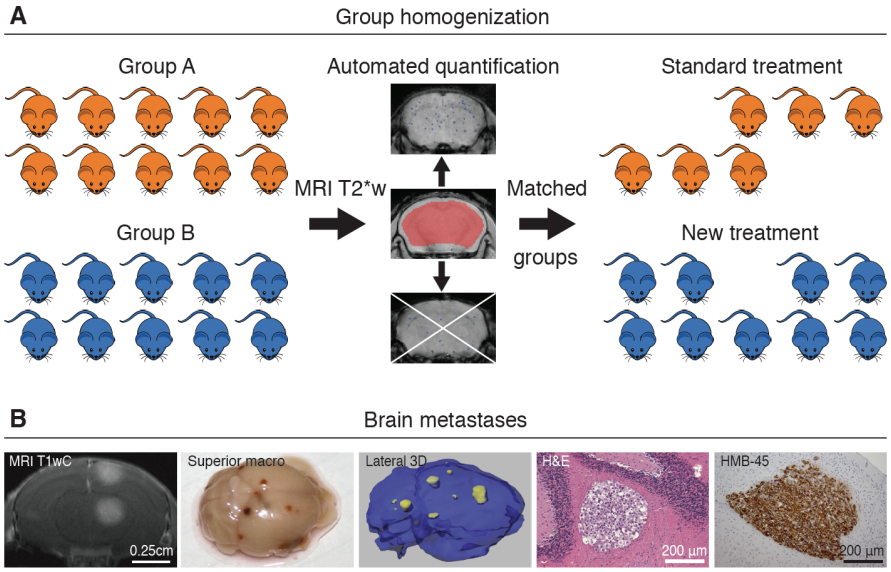

Figure 16 Quantification of SPION-labeled melanoma cells improves the predictive power of an experimental brain metastasis model. (**A**) Mice in groups A and B are injected intracardially at time zero. T2*-weighted brain MRI is analyzed 24 hours after injection to determine the tumor cell load in the brain (signals are only included within a pre-defined 3D brain mask (red) to avoid artifacts along the brain periphery). Only animals that have comparable tumor cell exposure are routed to further follow-up, e.g. comparing two treatments. (**B**) Imaging and morphological characteristics of metastatic spread and growth in mice resemble human melanoma brain metastasis; T1-weighted brain MRI, macroscopic image with corresponding 3D model, and H&E and HMB-45 (melanocytic marker) stainings. Adapted with permission from Sundstrøm et al.[525].

In **Papers II-IV**, we predominantly used the H1 cell line. This cell line was developed in our laboratory from a resected melanoma brain metastasis from a female patient[521]. The H1 cell line and its derivatives have been extensively characterized genetically and across multiple *in vitro* and *in vivo* assays (see **Papers II-IV** and other reports from our group[520,521]). The genetic profile of the H1 cell includes: $BRAF^{V600E}$ mutation, *PTEN* deletion, $NRAS^{wt}$, *MITF* amplification, *CDKN2A/B* deletion and $LDHA^{wt}$. As described previously, this mutational profile is common in melanomas and in brain metastases. The cell line is highly tumorigenic in immunodeficient mice and rats when injected into the bloodstream (intracardiac or tail vein) or into the skin or brain, but it only forms brain metastases via the intracardiac route. Systemic

metastases consistently develop in the brain, adrenals, ovaries and bones. Interestingly, there is some shared ectodermal ancestry (brain, adrenal medulla and melanocytes), but circulatory explanations are probably more important determinants of this organ-selectivity. Akin to Cruz-Munoz et al.[522], we have also done experiments with serial passaging in mouse brains to increase H1's brain-tropic potential (not published). More brain metastases did arise with intracardiac injections of sequential generations, but protracted *in vivo* assays with subcutaneous injections of these cell lines did not result in systemic metastases, including to the brain.

T2*-weighted MRI sequences are commonly applied to image SPIONs, which appear as local hypointensive spots[526]. Although SPION labeling has been reported to allow single-cell tracking[527], we (and others) found that it is difficult to identify individual signal voids as single or multiple cells due to artifacts and partial volume effects[528]. To detect signals within the brain in a reproducible manner, we used a 3D brain mask (avoided artifacts along the brain periphery) and machine-learning tools (trained to identify signals). Automated signal detection was strongly correlated to manual signal registrations. The automated capacity substantially strengthens the applicability and throughput of the model, but it comes with a necessary trade-off between sensitivity and specificity. Therefore, our model does not provide an absolute number of cells in the brain, but a relative number that is proportional to the injected quantity. Most importantly, the model is predictive of brain metastasis formation. Taken together, quantitative analyses of SPION-labeled cells are model-specific and reliant on the SPIONs and cells that are used, as well as on imaging hardware and software. With the advent of stable MRI reporter genes and improved MRI technology, we can in the future envision the long-term tracking of cells and tumors through all stages of progression[439].

This model was streamlined towards comparative therapeutic *in vivo* assays; qualitative aspects of the early steps of brain metastasis are better studied using other methods, such as multiphoton laser scanning microscopy through cranial windows[451], targeted MRI contrast agents[444] or histopathological techniques[453].

This animal model was used in **Papers III** and **IV**.

6.3. Paper III

The rewired metabolic network has emerged as an attractive venue for the development of novel anticancer drugs[529]. The metabolic enzyme LDHA plays a key role in the Warburg effect[506,507] and is overexpressed in many cancers[530-533]. Preclinical studies have shown promising results of LDHA inhibition in several cancers[533-542], but not in melanoma or brain metastasis. Metabolic networks are also complex and heterogeneous between and within various cancers[543] and different organ environments can significantly influence tumor growth and metastasis, especially in the brain[497-499]. Furthermore, it remains unclear whether the Warburg effect is a contributor to or a consequence of cancer. With these perspectives, we explored the spatial, temporal and functional features of LDHA expression in melanoma brain metastasis across multiple *in vitro* assays, using our animal model (see **Paper II**) with brain MRI and PET imaging, and in a large patient cohort. We further assessed the contemporary genomic and proteomic landscapes of LDHA in different cancers, particularly melanomas, and associations to OS and brain metastasis-free survival in patients.

We first investigated the temporal trends of LDHA protein expression during metastasis formation, and found a biphasic pattern over time and with tumor size: a strong expression in small tumors, reduced expression in enlarging tumors and regionally increased expression in the largest tumors (**Fig. 17A**). This prompted the hypothesis that LDHA was important during the early stages of metastasis formation, as well as later, when the tumors outgrow their blood supply. We thus explored LDHA protein expression in 80 operated human melanoma brain metastases and found it to be micromilieu-dependent and associated with larger tumors, but not with tumor number or survival. Hence, regionally increased LDHA protein expression in large tumors was seemingly without clinical consequence. Motivated by the contrasting preclinical findings with high LDHA expression in microscopic tumors, which are difficult to interrogate in patients, we developed an effective and stable LDHA knockdown cell line (short hairpin (sh)RNA interference) with significantly reduced glycolytic capacity.

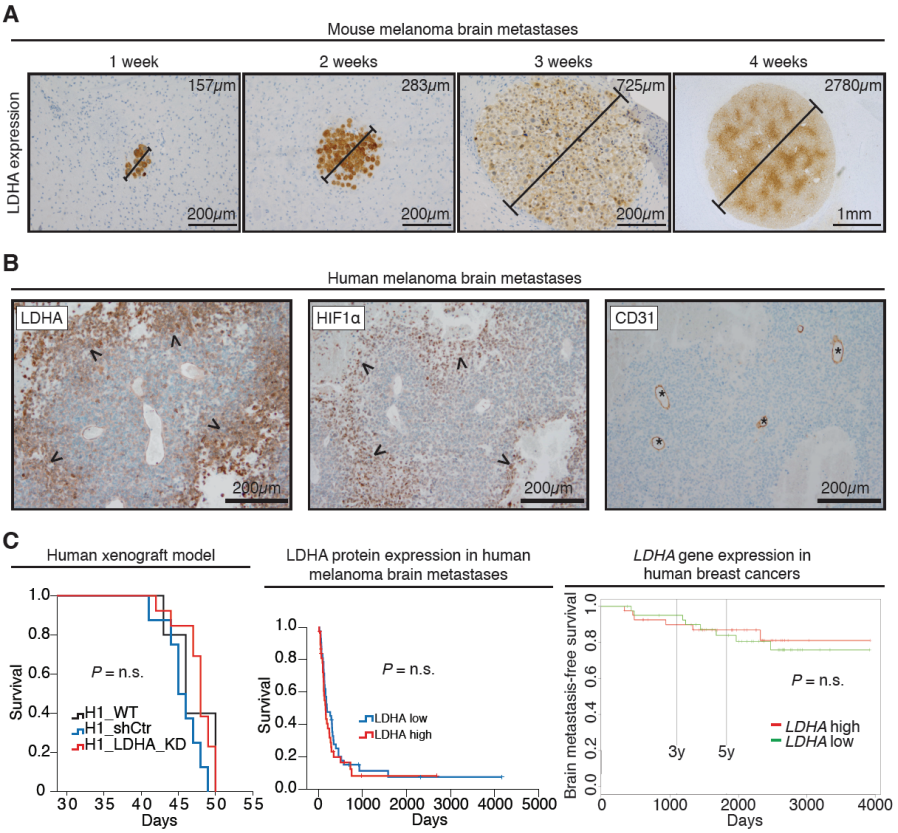

Figure 17 LDHA expression displays a biphasic pattern over time, is hypoxia-dependent and does not influence survival. (**A**) LDHA protein expression in mouse melanoma brain metastases was high in small tumors and regionally distributed in large tumors. (**B**) LDHA protein expression in operated human melanoma brain metastases showed a spatial overlap of LDHA and HIF1α expression away from CD31-positive vessels. (**C**) LDHA knockdown by shRNA interference did not affect survival in mice. LDHA protein expression in 80 human melanoma brain metastases was not predictive of survival. *LDHA* gene expression in 82 breast cancers did not predict brain metastasis-free survival (GSE2603 was the only dataset with this survival measure). H1_WT, naïve H1 cells; H1_LDHA_KD, H1 LDHA knockdown cells; H1_shCtr, H1 empty vector control; n.s., not significant. Adapted with permission from Sundstrøm et al.[588].

LDHA depletion did not affect cell proliferation or 3D tumorsphere growth *in vitro*, or the number and volume of brain metastases or survival *in vivo*. However, we confirmed that LDHA protein expression was strongly associated with hypoxia, both

in vivo and in patients (**Fig. 17B**). Moderate degrees of LDHA staining were present in most melanoma samples featured in the Human Protein Atlas, but there were no samples from brain metastases. *LDHA* was genetically altered in only 5% of 375 available TCGA samples and all of these were primary tumors or extracranial metastases. *LDHA* aberrations were generally infrequent in other cancer studies and melanoma series. Finally, *LDHA* expression levels did not predict OS in TCGA melanoma patients or brain metastasis-free survival in 82 breast cancer patients (only cancer series with this survival measure) (**Fig. 17C**). Together, these integrated analyses of independent genomic and proteomic data indicated that LDHA is not a driver of human melanoma brain metastasis or associated with survival. In summary, our findings show that LDHA expression varies with tumor size, but that tumor progression and survival seem to be functionally independent of LDHA expression. Thus, it is possible that the Warburg effect, observations of increased LDHA expression levels and increased serum levels of LDH, are more likely consequences of, rather than contributors to, melanoma brain metastasis.

LDHA knockdown resulted in decreased glycolysis. Metabolic assays did not reveal any compensatory increase in LDHB or PDK1 expression with LDHA knockdown or hypoxia. We did however observe a slight increase of respiratory capacity in LDHA knockdown cells, but the mechanistic explanation for this was not explored further (e.g. induction of MITF and PGC1α). It is possible that the brain microenvironment abrogated the effect of LDHA knockdown by inducing a metabolic shift towards more oxidative respiration. Then again, we should probably have observed increased tumorigenicity in controls, but there were no differences in their *in vivo* phenotype. Other members of our group have also failed to demonstrate an effect of LDHA knockdown in an orthotopic glioma model (H. Espedal, personal communication). For these reasons, we did not pursue further investigations of migratory or invasive capabilities.

Conclusively, our *in vitro* and *in vivo* results established that hypoxia is a key determinant of LDHA expression, but we were not able to detect any pro-metastatic capacity of LDHA in melanoma brain metastasis *in vivo* or in humans. The missing effect could be related to the limitations of single-targeting in cancer (intrinsic/adaptive compensation/resistance) or that LDHA is not a critical factor in

melanoma brain metastasis *per se*. Based on our results and currently available evidence, there seems to be more attractive targets or processes to pursue in brain-metastatic cancer than LDHA.

6.4. Paper IV

In this study, we addressed several key hurdles to translational advances in brain metastasis research[338] and described a potential new avenue of treatment for patients with melanoma brain metastases. We leveraged the merits of our brain metastasis model to define a brain metastasis gene signature and took advantage of the comprehensive Connectivity Map (cMap) pharmacogenomic database to identify compounds with the ability to invert this signature (**Fig. 18A**). Using this approach, we identified β-sitosterol, a natural compound and cholesterol analogue, which is well tolerated and used as a drug to treat hypercholesterolemia and benign prostatic hyperplasia. Moreover, β-sitosterol had shown anti-cancer potential in previous preclinical studies and been found to readily cross the BBB. We found that β-sitosterol effectively reduced the growth of brain metastases and improved survival in established and preventive scenarios in tailored human xenograft models of melanoma and lung cancer brain metastasis (**Fig. 18B-E**). Of particular importance for metastatic melanoma, we found that β-sitosterol not only extensively suppressed the important MAPK pathway (**Fig. 18F**), but also inhibited mitochondrial respiration (**Fig. 18G**), a major facilitator of resistance to MAPK-targeted therapies. Furthermore, we provided evidence of the clinical relevance and prognostic utility of our findings at several independent levels. Together, this study strongly encourages further assessment of β-sitosterol as an adjuvant to established MAPK-targeted therapies for patients at risk for, or that already have, melanoma brain metastases.

We developed a 108-gene brain metastasis signature using a combined workflow of several independent comparative analyses of gene expression profiles in human melanoma xenograft brain metastases versus other organ metastases. Many of the signature genes were altered in TCGA melanoma patients, demonstrated significant individual prognostic utility, and were associated with a number of cancer-related signaling pathways. However, given the challenges of single-targeting in subgroups of

patients with metastatic disease, we opted for a multi-targeting approach to achieve more broad-spectrum efficacy and durability.

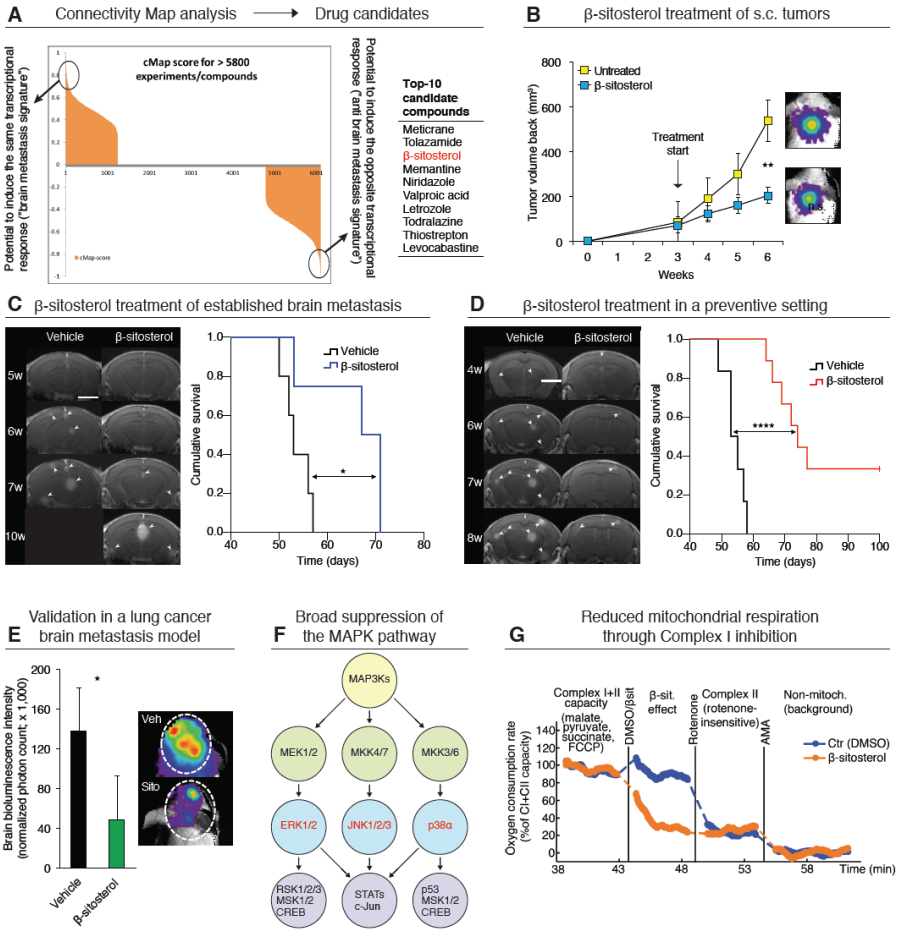

Figure 18 β-sitosterol provides broad-spectrum suppression of melanoma brain metastasis. (**A**) Query results from the Connectivity Map (cMap) database using the 108-gene signature and the top 10 list of anti-brain metastasis compounds. *In vivo* assessments of β-sitosterol treatment: (**B**) subcutaneous macroscopic melanoma tumors, (**C**) established melanoma brain metastasis, (**D**) prevention of melanoma brain metastasis, and (**E**) prevention of lung cancer brain metastasis. β-sitosterol's mechanisms of action: (**F**) broad suppression of the MAPK pathway through simultaneous targeting of its converging downstream regulators (ERK1/2, JNK1/2/3 and p38α) and corresponding transcription factors, and (**G**) high-resolution respirometry showing reduction of mitochondrial respiration through selective Complex I (CI) inhibition. Illustration by T. Sundstrøm.

Basically, we wanted to identify compounds that could counteract the entire brain metastasis signature and act in an anti-brain-metastatic fashion. We therefore turned to genomics-based drug repositioning and leveraged the cMap framework. We identified 1313 candidate compounds, carried out comprehensive *in vitro* screening of the top 10 candidates (all with cMap scores <-0.90, i.e. >90% of the expression profiles could potentially be reversed), and *in vivo* assessments of the four most potent compounds revealed β-sitosterol as a potential therapeutic agent. The therapeutic efficacy of β-sitosterol was confirmed using the H1 cell line in both established and preventive scenarios of brain metastasis, the H1 cell line in a macroscopic subcutaneous tumor model, and the aggressive PC14-PE6 lung cancer cell line in a preventive scenario of brain metastasis.

The cMap database, first launched in 2006, is a powerful and freely available tool to find new uses for existing drugs[544]. cMap contains gene expression data from thousands of treatment versus control experiments independently performed on human cancer cell lines from breast, prostate, leukemia and melanomas. Drug repositioning has indeed been viewed as one of the most promising venues of translational medicine[545], and a recent makeover and 1000-fold expansion of cMap will probably fast-track research and our understanding of drug repositioning.

β-sitosterol: clinical applicability and anti-cancer potential

The phytosterol β-sitosterol is structurally very similar to cholesterol, and is abundant in certain plants and foodstuffs, especially peanuts, tree nuts and avocados. β-sitosterol competes with cholesterol for uptake via the intestinal Niemann-Pick C1 Like 1 (NPC1L1) transporter[546]. The majority of β-sitosterol is re-secreted into the intestine via adenosine triphosphate (ATP) binding cassette proteins (ATP-binding cassette sub-family G members 5 and 8; ABCG5 and ABCG8)[547], but some is transported by lipoproteins (mostly high-density lipoprotein (HDL)) and incorporated into cell membranes[548,549]. Less than 5% of β-sitosterol is absorbed, whereas approximately 50% of cholesterol is absorbed[550]. Intriguingly, β-sitosterol can cross the BBB[551-553], whereas cholesterol cannot[554,555]. Additionally, β-sitosterol is to a higher degree incorporated into glial than neuronal cells, and brain accumulation in healthy mice has been found irreversible over a six-month period[553].

RCTs in humans have found beneficial effects of β-sitosterol (and its ester sitostanol) on hypercholesterolemia[556], benign prostatic hyperplasia[557], androgenic alopecia[558] and as an adjuvant in the treatment of tuberculosis[559] and anogenital warts[560]. Phytosterols are classified as "Generally Recognized As Safe" (GRAS) by the FDA, and the European Foods Safety Authority (EFSA) have concluded that a daily phytosterol and/or phytostanol intake of 1.5-2.4 grams can reduce blood cholesterol by 7-10.5% and sustain this effect for up to 85 weeks[561]. Notably, we used a daily dose of 5 mg/kg in our experimental studies of brain metastasis, which translates into 375 mg for a person weighing 75 kg. To our knowledge, no clinical trials have examined the effect of β-sitosterol on cancer. However, a number of epidemiological studies have suggested that increased consumption of phytosterols can reduce the risk of breast[562,563], lung[564], stomach[565] and colon cancer[566]. Furthermore, a wide range of studies have suggested several health benefits of nut consumption; recently, a dose-dependent reduction in mortality from a number of diseases including heart disease and cancer was reported[567]. Interestingly, 100 g of roasted peanuts contain 61-114 mg of phytosterols (78-83% β-sitosterol)[548].

Experimental data have shown that β-sitosterol can reduce cell proliferation in prostate[568], breast[569], colon[570], melanoma[571] and lung[572] cancer cell lines. Induced apoptosis has been observed in breast[573], stomach[574], colon[575], myeloma[576], hepatoma[577], fibrosarcoma[578] and prostate[579] cancer cell lines. In an *in vitro* study with MDA-MB-231 breast cancer cells the authors found reduced proliferation, adhesion and invasion[580].

In vivo data have shown that β-sitosterol treatment can reduce the growth of breast cancer xenografts[581], and that berry extracts with β-sitosterol can reduce tumorigenesis and progression in carcinogen-induced esophagus cancer[582]. Liposomal β-sitosterol treatment has been found to prevent metastatic lung colonization after tail vein injection of murine B16BL6 melanoma cells[586]. Oral treatment with phytosterols has been found to inhibit the growth of xenografted tumors and reduce lymph node and lung metastasis in a model using human PC-3 prostate cancer cells[568].

In summary, phytosterols have been shown to exhibit a number of different anticancer effects *in vitro* and *in vivo*, but the mechanisms of action remain somewhat elusive

(reviewed in[583]). They include effects on membrane structure and function, signal transduction pathways, apoptosis, cell cycle, antioxidant enzymes, free radical generation, immune function and cholesterol metabolism. Recently, it was suggested that phytochemicals (a long list of plant chemicals which includes phytosterols) might be applicable to melanoma therapy due to their low toxicity and ability to compromise several key pathways in melanomagenesis[424].

Putative mechanisms of action

We found that β-sitosterol treatment massively reduced phosphorylation of multiple oncogenic kinases and transcription factors. Of particular importance for metastatic melanoma, we found extensive suppression of the MAPK pathway mediated by downregulation of ERK1/2, c-Jun N-terminal kinases (JNK1/2/3) and mitogen-activated protein kinase 14 (p38α) as well as their corresponding transcription factors. Furthermore, *in silico* analyses associated major regulators of cell homeostasis to the therapeutic potential of β-sitosterol. From this, and previous work by us (see **Paper III**) and others[496,497], we wondered if β-sitosterol could interfere with basic cellular functions such as energy metabolism. Intriguingly, we found β-sitosterol to substantially reduce mitochondrial respiration and respiratory capacity, and induce cellular ROS production and apoptosis. Further mechanistic studies revealed that β-sitosterol exerted its effect by selective inhibition of respiratory CI. Taken together, our findings revealed a timely and potentially synergistic effect with particular relevance for patients with metastatic melanoma, as increased mitochondrial oxidative capacity has been shown to facilitate melanoma cell survival and growth, both as an intrinsic[406] and acquired[407] resistance mechanism to MAPK-targeted therapies. Furthermore, to survive and grow in the brain, cancer cells might be more reliant of functional mitochondria and mitochondrial respiration than glycolysis[496,497,500].

The abovementioned mechanisms are particularly relevant to melanoma and melanoma brain metastases, but other processes are also involved and might be important determinants of β-sitosterol efficacy. To name a few, we are currently studying membrane composition and protein-ligand interactions using atomic force microscopy, functional responses to fabricated microenvironments using combinatorial microarrays, blood levels of β-sitosterol, therapy in a canine melanoma

model, early metastasis/extravasation, migration assays, invasion assays and sequencing of treated and untreated cells and tumors.

I would like to mention two other potential mechanisms of action that are not discussed in the paper. First, β-sitosterol has been found to inhibit the expression of endothelial VCAM-1[584]. Interestingly, a marked upregulation of VCAM-1 has been observed in the early stages of breast cancer brain metastasis in both mice and humans[444]. Second, β-sitosterol might activate LXR-mediated induction of APOE[312,585]. LXR agonism has been found to suppress melanoma tumor growth and metastasis, including brain-metastatic colonization, through transcriptional induction of tumoral and stromal APOE[312]. Moreover, APOE has been found upregulated in intermediate stages of melanoma brain metastases[311] and β-sitosterol has also been shown to induce the expression of APOE in astrocytomas[552]. We investigated if β-sitosterol treatment increased APOE levels and observed a modest increase in APOE levels over a course of three days, however a striking increase in particularly one APOE fragment. We are currently exploring these mechanisms in more detail.

7. CONCLUSIONS

Paper I

Animal models of human brain metastasis are useful, but do not reflect the complex biology of malignant disease in humans. Valuable insights have been attained, but there is a continued requirement of new and more representative animal models. Technical and biologic limitations of established animal models should be acknowledged and addressed.

Paper II

Automated quantification of nanoparticle-labeled melanoma cells in the mouse brain can improve the reproducibility and predictivity of an experimental human xenograft model of melanoma brain metastasis.

Paper III

LDHA expression in melanoma brain metastases was hypoxia-dependent, but did not seem to have a functional bearing on tumor progression or survival *in vivo* or in patients.

Paper IV

β-sitosterol inhibited melanoma brain metastasis and improved survival *in vivo* through suppression of the MAPK pathway and inhibition of mitochondrial CI. Genomics-based drug repositioning was feasible in a human xenograft model of melanoma brain metastasis.

8. FUTURE PROSPECTS

Future prospects in melanoma and brain metastasis research are discussed throughout this thesis. Our group has built up considerable experience in experimental brain metastasis research and developed key relationships in Norway and abroad that provide great opportunities for the future. We are continuously seeking to improve our experimental set-ups and apply new methodologies. Our most recent work with β-sitosterol is rapidly evolving; we are currently extending our preclinical understanding of its mechanism(s) of action and also exploring the possibilities of conducting a clinical trial. Besides β-sitosterol itself, our most interesting finding could be the apparent significance of mitochondrial respiration as opposed to glycolysis in melanoma brain metastasis. This relative insignificance of glycolysis for brain metastasis has previously been proposed for breast cancer brain metastasis. Whether therapeutic targeting of mitochondrial respiration has implications for brain metastasis in general and perhaps primary malignant brain tumors remains to be determined. Furthermore, the mechanistic links described in this thesis may reflect an even wider role in cancer, such as a synergistic coupling of mitochondrial biogenesis and respiration to migratory and invasive capabilities of cancer cells[587].

At present, we lack the necessary conceptual paradigms and computational strategies to make sense of all the information that is available, and to really understand what drives cancer in general and brain metastasis in particular[337]. Multidisciplinary efforts are needed to move beyond our fragmented understanding, and a stronger integration of preclinical and clinical knowledge is imperative for success.

9. REFERENCES

1. Valastyan, S. & Weinberg, R.A. Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147,** 275-292 (2011).

2. Chaffer, C.L. & Weinberg, R.A. A perspective on cancer cell metastasis. *Science* **331,** 1559-1564 (2011).

3. Nayak, L., Lee, E.Q. & Wen, P.Y. Epidemiology of brain metastases. *Curr Oncol Rep* **14,** 48-54 (2012).

4. Wen, P.Y. & Loeffler, J.S. Management of brain metastases. *Oncology (Williston Park)* **13,** 941-54, 957-61 (1999).

5. Steeg, P.S., Camphausen, K.A. & Smith, Q.R. Brain metastases as preventive and therapeutic targets. *Nat Rev Cancer* **11,** 352-363 (2011).

6. Barnholtz-Sloan, J.S., Sloan, A.E., Davis, F.G., Vigneau, F.D., *et al.* Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the Metropolitan Detroit Cancer Surveillance System. *J Clin Oncol* **22,** 2865-2872 (2004).

7. Schouten, L.J., Rutten, J., Huveneers, H.A. & Twijnstra, A. Incidence of brain metastases in a cohort of patients with carcinoma of the breast, colon, kidney, and lung and melanoma. *Cancer* **94,** 2698-2705 (2002).

8. Tabouret, E., Chinot, O., Metellus, P., Tallet, A., *et al.* Recent trends in epidemiology of brain metastases: an overview. *Anticancer Res* **32,** 4655-4662 (2012).

9. Tsukada, Y., Fouad, A., Pickren, J.W. & Lane, W.W. Central nervous system metastasis from breast carcinoma. Autopsy study. *Cancer* **52,** 2349-2354 (1983).

10. Percy, A.K., Elveback, L.R., Okazaki, H. & Kurland, L.T. Neoplasms of the central nervous system. Epidemiologic considerations. *Neurology* **22,** 40-48 (1972).

11. Posner, J.B. & Chernik, N.L. Intracranial metastases from systemic cancer. *Adv Neurol* **19,** 579-592 (1978).

12. Fox, B.D., Cheung, V.J., Patel, A.J., Suki, D. & Rao, G. Epidemiology of Metastatic Brain Tumors. *Neurosurg Clin N Am* **22,** 1-6 (2011).

13. Walker, A.E., Robins, M. & Weinfeld, F.D. Epidemiology of brain tumors: the national survey of intracranial neoplasms. *Neurology* **35,** 219-226 (1985).

14. Counsell, C.E., Collie, D.A. & Grant, R. Incidence of intracranial tumours in the Lothian region of Scotland, 1989-90. *J Neurol Neurosurg Psychiatry* **61,** 143-150 (1996).

15. Smedby, K.E., Brandt, L., Bäcklund, M.L. & Blomqvist, P. Brain metastases admissions in Sweden between 1987 and 2006. *Br J Cancer* **101,** 1919-1924 (2009).

16. Barker, F.G. Craniotomy for the resection of metastatic brain tumors in the U.S., 1988-2000: decreasing mortality and the effect of provider caseload. *Cancer* **100,** 999-1007 (2004).

17. Owonikoko, T.K., Arbiser, J., Zelnak, A., Shu, H.K., *et al.* Current approaches to the treatment of metastatic brain tumours. *Nat Rev Clin Oncol* **11,** 203-222 (2014).

18. Nieder, C., Spanne, O., Mehta, M.P., Grosu, A.L. & Geinitz, H. Presentation, patterns of care, and survival in patients with brain metastases: what has changed in the last 20 years? *Cancer* **117,** 2505-2512 (2011).

19. Frisk, G., Svensson, T., Bäcklund, L.M., Lidbrink, E., *et al.* Incidence and time trends of brain metastases admissions among breast cancer patients in Sweden. *Br J Cancer* **106,** 1850-1853 (2012).

20. Pestalozzi, B.C. & Brignoli, S. Trastuzumab in CSF. *J Clin Oncol* **18,** 2349-2351 (2000).

21. Stemmler, H.J., Schmitt, M., Willems, A., Bernhard, H., *et al.* Ratio of trastuzumab levels in serum and cerebrospinal fluid is altered in HER2-positive breast cancer patients with brain metastases and impairment of blood-brain barrier. *Anticancer Drugs* **18,** 23-28 (2007).

22. Stark, A.M., Stöhring, C., Hedderich, J., Held-Feindt, J. & Mehdorn, H.M. Surgical treatment for brain metastases: Prognostic factors and survival in 309 patients with regard to patient age. *J Clin Neurosci* **18,** 34-38 (2011).

23. Nussbaum, E.S., Djalilian, H.R., Cho, K.H. & Hall, W.A. Brain metastases. Histology, multiplicity, surgery, and survival. *Cancer* **78,** 1781-1788 (1996).

24. Fabi, A., Felici, A., Metro, G., Mirri, A., *et al.* Brain metastases from solid tumors: disease outcome according to type of treatment and therapeutic resources of the treating center. *J Exp Clin Cancer Res* **30,** 10 (2011).

25. Delattre, J.Y., Krol, G., Thaler, H.T. & Posner, J.B. Distribution of brain metastases. *Arch Neurol* **45,** 741-744 (1988).

26. Bender, E.T. & Tomé, W.A. Distribution of brain metastases: implications for non-uniform dose prescriptions. *Br J Radiol* **84,** 649-658 (2011).

27. Graf, A.H., Buchberger, W., Langmayr, H. & Schmid, K.W. Site preference of metastatic tumours of the brain. *Virchows Arch A Pathol Anat Histopathol* **412,** 493-498 (1988).

28. Madajewicz, S., Karakousis, C., West, C.R., Caracandas, J. & Avellanosa, A.M. Malignant melanoma brain metastases. Review of Roswell Park Memorial Institute experience. *Cancer* **53,** 2550-2552 (1984).

29. Vuong, D.A., Rades, D., Vo, S.Q. & Busse, R. Extracranial metastatic patterns on occurrence of brain metastases. *J Neurooncol* **105,** 83-90 (2011).

30. DeAngelis, L.M. & Posner, J.B. Neurologic complications of cancer, 2nd edition. *Oxford University Press, USA* (2008).

31. Lagerwaard, F.J., Levendag, P.C., Nowak, P.J., Eijkenboom, W.M., *et al.* Identification of prognostic factors in patients with brain metastases: a review of 1292 patients. *Int J Radiat Oncol Biol Phys* **43,** 795-803 (1999).

32. Davis, F.G., Dolecek, T.A., McCarthy, B.J. & Villano, J.L. Toward determining the lifetime occurrence of metastatic brain tumors estimated from 2007 United States cancer incidence data. *Neuro Oncol* **14,** 1171-1177 (2012).

33. Giordana, M.T., Cordera, S. & Boghi, A. Cerebral metastases as first symptom of cancer: a clinico-pathologic study. *J Neurooncol* **50,** 265-273 (2000).

34. Mavrakis, A.N., Halpern, E.F., Barker, F.G., Gonzalez, R.G. & Henson, J.W. Diagnostic evaluation of patients with a brain mass as the presenting manifestation of cancer. *Neurology* **65,** 908-911 (2005).

35. Rudà, R., Borgognone, M., Benech, F., Vasario, E. & Soffietti, R. Brain metastases from unknown primary tumour: a prospective study. *J Neurol* **248,** 394-398 (2001).

36. Patchell, R.A., Tibbs, P.A., Walsh, J.W., Dempsey, R.J., *et al.* A randomized trial of surgery in the treatment of single metastases to the brain. *N Engl J Med* **322,** 494-500 (1990).

37. Cha, S. Neuroimaging in neuro-oncology. *Neurotherapeutics* **6,** 465-477 (2009).

38. Law, M., Cha, S., Knopp, E.A., Johnson, G., *et al.* High-grade gliomas and solitary metastases: differentiation by using perfusion and proton spectroscopic MR imaging. *Radiology* **222,** 715-721 (2002).

39. Lai, P.H., Ho, J.T., Chen, W.L., Hsu, S.S., *et al.* Brain abscess and necrotic brain tumor: discrimination with proton MR spectroscopy and diffusion-weighted imaging. *AJNR Am J Neuroradiol* **23,** 1369-1377 (2002).

40. Grand, S., Laï, E. S., Estève, F., Rubin, C., *et al.* In vivo 1H MRS of brain abscesses versus necrotic brain tumors. *Neurology* **47,** 846-848 (1996).

41. Meier, R. Stereotactic radiosurgery for brain metastases. *Transl Cancer Res* **3,** 358-366 (2014).

42. Claus, E.B. Neurosurgical management of metastases in the central nervous system. *Nat Rev Clin Oncol* **9,** 79-86 (2011).

43. Suh, J.H. Stereotactic radiosurgery for the management of brain metastases. *N Engl J Med* **362,** 1119-1127 (2010).

44. Eichler, A.F., Chung, E., Kodack, D.P., Loeffler, J.S., *et al.* The biology of brain metastases-translation to new therapies. *Nat Rev Clin Oncol* **8,** 344-356 (2011).

45. Korn, E.L., Liu, P.Y., Lee, S.J., Chapman, J.A., *et al.* Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials. *J Clin Oncol* **26,** 527-534 (2008).

46. Tsimberidou, A.M., Letourneau, K., Wen, S., Wheler, J., *et al.* Phase I clinical trial outcomes in 93 patients with brain metastases: the MD Anderson Cancer Center experience. *Clin Cancer Res* **17,** 4110-4118 (2011).

47. Maher, E.A., Mietz, J., Arteaga, C.L., DePinho, R.A. & Mohla, S. Brain metastasis: opportunities in basic and translational research. *Cancer Res* **69,** 6015-6020 (2009).

48. Stortebecker, T.P. Metastatic tumors of the brain from a neurosurgical point of view; a follow-up study of 158 cases. *J Neurosurg* **11,** 84-111 (1954).

49. Grant, F.C. Concerning intracranial malignant metastases: their frequency and the value of surgery in their treatment. *Ann Surg* **84,** 635-646 (1926).

50. Mandell, L., Hilaris, B., Sullivan, M., Sundaresan, N., *et al.* The treatment of single brain metastasis from non-oat cell lung carcinoma. Surgery and radiation versus radiation therapy alone. *Cancer* **58,** 641-649 (1986).

51. Patchell, R.A., Cirrincione, C., Thaler, H.T., Galicich, J.H., *et al.* Single brain metastases: surgery plus radiation or radiation alone. *Neurology* **36,** 447-453 (1986).

52. Sundaresan, N., Galicich, J.H. & Beattie, E.J. Surgical treatment of brain metastases from lung cancer. *J Neurosurg* **58,** 666-671 (1983).

53. Hendrickson, F.R., Lee, M.S., Larson, M. & Gelber, R.D. The influence of surgery and radiation therapy on patients with brain metastases. *Int J Radiat Oncol Biol Phys* **9,** 623-627 (1983).

54. Katz, H.R. The relative effectiveness of radiation therapy, corticosteroids, and surgery in the management of melanoma metastatic to the central nervous system. *Int J Radiat Oncol Biol Phys* **7,** 897-906 (1981).

55. DiStefano, A., Yong Yap, Y., Hortobagyi, G.N. & Blumenschein, G.R. The natural history of breast cancer patients with brain metastases. *Cancer* **44,** 1913-1918 (1979).

56. Vieth, R.G. & Odom, G.L. Intracranial metastases and their neurosurgical treatment. *J Neurosurg* **23,** 375-383 (1965).

57. Posner, J.B. Diagnosis and treatment of metastases to the brain. *Clin Bull* **4,** 47-57 (1974).

58. Markesbery, W.R., Brooks, W.H., Gupta, G.D. & Young, A.B. Treatment for patients with cerebral metastases. *Arch Neurol* **35,** 754-756 (1978).

59. Berry, H.C., Parker, R.G. & Gerdes, A.J. Irradiation of brain metastases. *Acta Radiol Ther Phys Biol* **13,** 535-544 (1974).

60. Montana, G.S., Meacham, W.F. & Caldwell, W.L. Brain irradiation for metastatic disease of lung origin. *Cancer* **29,** 1477-1480 (1972).

61. Vecht, C.J., Haaxma-Reiche, H., Noordijk, E.M., Padberg, G.W., *et al.* Treatment of single brain metastasis: radiotherapy alone or combined with neurosurgery? *Ann Neurol* **33,** 583-590 (1993).

62. Noordijk, E.M., Vecht, C.J., Haaxma-Reiche, H., Padberg, G.W., *et al.* The choice of treatment of single brain metastasis should be based on extracranial tumor activity and age. *Int J Radiat Oncol Biol Phys* **29,** 711-717 (1994).

63. Patchell, R.A., Tibbs, P.A., Regine, W.F., Dempsey, R.J., *et al.* Postoperative radiotherapy in the treatment of single metastases to the brain: a randomized trial. *JAMA* **280,** 1485-1489 (1998).

64. Kalkanis, S.N., Kondziolka, D., Gaspar, L.E., Burri, S.H., *et al.* The role of surgical resection in the management of newly diagnosed brain metastases: a systematic review and evidence-based clinical practice guideline. *J Neurooncol* **96,** 33-43 (2010).

65. Bindal, R.K., Sawaya, R., Leavens, M.E. & Lee, J.J. Surgical treatment of multiple brain metastases. *J Neurosurg* **79,** 210-216 (1993).

66. Al-Zabin, M., Ullrich, W.O., Brawanski, A. & Proescholdt, M.A. Recurrent brain metastases from lung cancer: the impact of reoperation. *Acta Neurochir (Wien)* **152,** 1887-1892 (2010).

67. Bindal, R.K., Sawaya, R., Leavens, M.E., Hess, K.R. & Taylor, S.H. Reoperation for recurrent metastatic brain tumors. *J Neurosurg* **83,** 600-604 (1995).

68. McGirt, M.J., Mukherjee, D., Chaichana, K.L., Than, K.D., *et al.* Association of surgically acquired motor and language deficits on overall survival after resection of glioblastoma multiforme. *Neurosurgery* **65,** 463-469 (2009).

69. Gulati, S., Jakola, A.S., Nerland, U.S., Weber, C. & Solheim, O. The risk of getting worse: surgically acquired deficits, perioperative complications, and functional outcomes after primary resection of glioblastoma. *World Neurosurg* **76,** 572-579 (2011).

70. Sills, A.K. Current treatment approaches to surgery for brain metastases. *Neurosurgery* **57,** S24-S32 (2005).

71. Garber, S.T. & Jensen, R.L. Image guidance for brain metastases resection. *Surg Neurol Int* **3,** S111-S117 (2012).

72. Hatiboglu, M.A., Wildrick, D.M. & Sawaya, R. The role of surgical resection in patients with brain metastases. *Ecancermedicalscience* **7,** 308 (2013).

73. Kamp, M.A., Dibué, M., Santacroce, A., Zella, S.M., *et al.* The tumour is not enough or is it? Problems and new concepts in the surgery of cerebral metastases. *Ecancermedicalscience* **7,** 306 (2013).

74. Weil, R.J. & Lonser, R.R. Selective excision of metastatic brain tumors originating in the motor cortex with preservation of function. *Journal of Clinical Oncology* **23,** 1209-1217 (2005).

75. Kamp, M.A., Grosser, P., Felsberg, J., Slotty, P.J., *et al.* 5-aminolevulinic acid (5-ALA)-induced fluorescence in intracerebral metastases: a retrospective study. *Acta Neurochir (Wien)* **154,** 223-228 (2012).

76. Sanai, N., Eschbacher, J., Hattendorf, G., Coons, S.W., *et al.* Intraoperative confocal microscopy for brain tumors: a feasibility analysis in humans. *Neurosurgery* **68,** 282-290 (2011).

77. Kircher, M.F., de la Zerda, A., Jokerst, J.V., Zavaleta, C.L., *et al.* A brain tumor molecular imaging strategy using a new triple-modality MRI-photoacoustic-Raman nanoparticle. *Nat Med* **18,** 829-834 (2012).

78. Barone, D.G., Lawrie, T.A. & Hart, M.G. Image guided surgery for the resection of brain tumours. *Cochrane Database Syst Rev* **1,** CD009685 (2014).

79. Senft, C., Bink, A., Franz, K., Vatter, H., *et al.* Intraoperative MRI guidance and extent of resection in glioma surgery: a randomised, controlled trial. *Lancet Oncol* **12,** 997-1003 (2011).

80. Stummer, W., Pichlmeier, U., Meinel, T., Wiestler, O.D., *et al.* Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol* **7,** 392-401 (2006).

81. Wu, J.S., Zhou, L.F., Tang, W.J., Mao, Y., *et al.* Clinical evaluation and follow-up outcome of diffusion tensor imaging-based functional neuronavigation: a prospective, controlled study in patients with gliomas involving pyramidal tracts. *Neurosurgery* **61,** 935-948 (2007).

82. Willems, P.W., Taphoorn, M.J., Burger, H., Berkelbach van der Sprenkel, J.W. & Tulleken, C.A. Effectiveness of neuronavigation in resecting solitary intracerebral contrast-enhancing tumors: a randomized controlled trial. *J Neurosurg* **104,** 360-368 (2006).

83. Unsgaard, G., Rygh, O.M., Selbekk, T., Müller, T.B., *et al.* Intra-operative 3D ultrasound in neurosurgery. *Acta Neurochir (Wien)* **148,** 235-253 (2006).

84. Kocher, M., Soffietti, R., Abacioglu, U., Villà, S., *et al.* Adjuvant whole-brain radiotherapy versus observation after radiosurgery or surgical resection of one to three cerebral metastases: results of the EORTC 22952-26001 study. *J Clin Oncol* **29,** 134-141 (2011).

85. Yoo, H., Kim, Y.Z., Nam, B.H., Shin, S.H., *et al.* Reduced local recurrence of a single brain metastasis through microscopic total resection. *J Neurosurg* **110,** 730-736 (2009).

86. Baumert, B.G., Rutten, I., Dehing-Oberije, C., Twijnstra, A., *et al.* A pathology-based substrate for target definition in radiosurgery of brain metastases. *Int J Radiat Oncol Biol Phys* **66,** 187-194 (2006).

87. Sahgal, A., Soliman, H. & Larson, D.A. Whole-brain radiation therapy of brain metastasis. *Prog Neurol Surg* **25,** 82-95 (2012).

88. Chao, J.H., Phillips, R. & Nickson, J.J. Roentgen-ray therapy of cerebral metastases. *Cancer* **7,** 682-689 (1954).

89. Lokich, J.J. The management of cerebral metastasis. *JAMA* **234,** 748-751 (1975).

90. Posner, J.B. Management of central nervous system metastases. *Semin Oncol* **4,** 81-91 (1977).

91. McTyre, E., Scott, J. & Chinnaiyan, P. Whole brain radiotherapy for brain metastasis. *Surg Neurol Int* **4,** S236-S244 (2013).

92. Welzel, G., Fleckenstein, K., Schaefer, J., Hermann, B., *et al.* Memory function before and after whole brain radiotherapy in patients with and without brain metastases. *Int J Radiat Oncol Biol Phys* **72,** 1311-1318 (2008).

93. DeAngelis, L.M., Delattre, J.Y. & Posner, J.B. Radiation-induced dementia in patients cured of brain metastases. *Neurology* **39,** 789-796 (1989).

94. Li, J., Bentzen, S.M., Li, J., Renschler, M. & Mehta, M.P. Relationship between neurocognitive function and quality of life after whole-brain radiotherapy in patients with brain metastasis. *Int J Radiat Oncol Biol Phys* **71,** 64-70 (2008).

95. Gaspar, L., Scott, C., Rotman, M., Asbell, S., *et al.* Recursive partitioning analysis (RPA) of prognostic factors in three Radiation Therapy Oncology Group (RTOG) brain metastases trials. *Int J Radiat Oncol Biol Phys* **37,** 745-751 (1997).

96. Kohler, B.A., Ward, E., McCarthy, B.J., Schymura, M.J., *et al.* Annual report to the nation on the status of cancer, 1975-2007, featuring tumors of the brain and other nervous system. *J Natl Cancer Inst* **103,** 714-736 (2011).

97. Armstrong, C., Ruffer, J., Corn, B., DeVries, K. & Mollman, J. Biphasic patterns of memory deficits following moderate-dose partial-brain irradiation: neuropsychologic outcome and proposed mechanisms. *J Clin Oncol* **13,** 2263-2271 (1995).

98. Andrews, D.W., Scott, C.B., Sperduto, P.W., Flanders, A.E., *et al.* Whole brain radiation therapy with or without stereotactic radiosurgery boost for patients with one to three brain metastases: phase III results of the RTOG 9508 randomised trial. *Lancet* **363,** 1665-1672 (2004).

99. Kondziolka, D., Patel, A., Lunsford, L.D., Kassam, A. & Flickinger, J.C. Stereotactic radiosurgery plus whole brain radiotherapy versus radiotherapy alone for patients with multiple brain metastases. *Int J Radiat Oncol Biol Phys* **45,** 427-434 (1999).

100. Slotman, B., Faivre-Finn, C., Kramer, G., Rankin, E., *et al.* Prophylactic cranial irradiation in extensive small-cell lung cancer. *N Engl J Med* **357,** 664-672 (2007).

101. Gore, E.M., Bae, K., Wong, S.J., Sun, A., *et al.* Phase III comparison of prophylactic cranial irradiation versus observation in patients with locally advanced non-small-cell lung cancer: primary analysis of radiation therapy oncology group study RTOG 0214. *J Clin Oncol* **29,** 272-278 (2011).

102. Gondi, V., Pugh, S.L., Tome, W.A., Caine, C., *et al.* Preservation of memory with conformal avoidance of the hippocampal neural stem-cell compartment during whole-brain radiotherapy for brain metastases (RTOG 0933): a phase II multi-institutional trial. *J Clin Oncol* **32,** 3810-3816 (2014).

103. Gutiérrez, A.N., Westerly, D.C., Tomé, W.A., Jaradat, H.A., *et al.* Whole brain radiotherapy with hippocampal avoidance and simultaneously integrated brain metastases boost: a planning study. *Int J Radiat Oncol Biol Phys* **69,** 589-597 (2007).

104. Brown, P.D., Pugh, S., Laack, N.N., Wefel, J.S., *et al.* Memantine for the prevention of cognitive dysfunction in patients receiving whole-brain radiotherapy: a randomized, double-blind, placebo-controlled trial. *Neuro Oncol* **15,** 1429-1437 (2013).

105. Verger, E., Gil, M., Yaya, R., Viñolas, N., *et al.* Temozolomide and concomitant whole brain radiotherapy in patients with brain metastases: a phase II randomized trial. *Int J Radiat Oncol Biol Phys* **61,** 185-191 (2005).

106. Kouvaris, J.R., Miliadou, A., Kouloulias, V.E., Kolokouris, D., *et al.* Phase II study of temozolomide and concomitant whole-brain radiotherapy in patients with brain metastases from solid tumors. *Onkologie* **30,** 361-366 (2007).

107. Chua, D., Krzakowski, M., Chouaid, C., Pallotta, M.G., *et al.* Whole-brain radiation therapy plus concomitant temozolomide for the treatment of brain metastases from non-small-cell lung cancer: a randomized, open-label phase II study. *Clin Lung Cancer* **11,** 176-181 (2010).

108. Addeo, R., De Rosa, C., Faiola, V., Leo, L., *et al.* Phase 2 trial of temozolomide using protracted low-dose and whole-brain radiotherapy for nonsmall cell lung cancer and breast cancer patients with brain metastases. *Cancer* **113,** 2524-2531 (2008).

109. Welsh, J.W., Komaki, R., Amini, A., Munsell, M.F., *et al.* Phase II trial of erlotinib plus concurrent whole-brain radiation therapy for patients with brain metastases from non-small-cell lung cancer. *J Clin Oncol* **31,** 895-902 (2013).

110. Sperduto, P.W., Wang, M., Robins, H.I., Schell, M.C., *et al.* A phase 3 trial of whole brain radiation therapy and stereotactic radiosurgery alone versus WBRT and SRS with temozolomide or erlotinib for non-small cell lung cancer and 1 to 3 brain metastases: Radiation Therapy Oncology Group 0320. *Int J Radiat Oncol Biol Phys* **85,** 1312-1318 (2013).

111. Aoyama, H., Shirato, H., Tago, M., Nakagawa, K., *et al.* Stereotactic radiosurgery plus whole-brain radiation therapy vs stereotactic radiosurgery alone for treatment of brain metastases: a randomized controlled trial. *JAMA* **295,** 2483-2491 (2006).

112. Sneed, P.K., Suh, J.H., Goetsch, S.J., Sanghavi, S.N., *et al.* A multi-institutional review of radiosurgery alone vs. radiosurgery with whole brain radiotherapy as the initial management of brain metastases. *Int J Radiat Oncol Biol Phys* **53,** 519-526 (2002).

113. Muacevic, A., Wowra, B., Siefert, A., Tonn, J.C., *et al.* Microsurgery plus whole brain irradiation versus Gamma Knife surgery alone for treatment of single metastases to the brain: a randomized controlled multicentre phase III trial. *J Neurooncol* **87,** 299-307 (2008).

114. Soltys, S.G., Adler, J.R., Lipani, J.D., Jackson, P.S., *et al.* Stereotactic radiosurgery of the postoperative resection cavity for brain metastases. *Int J Radiat Oncol Biol Phys* **70,** 187-193 (2008).

115. Brennan, C., Yang, T.J., Hilden, P., Zhang, Z., *et al.* A phase 2 trial of stereotactic radiosurgery boost after surgical resection for brain metastases. *Int J Radiat Oncol Biol Phys* **88,** 130-136 (2014).

116. Serizawa, T., Hirai, T., Nagano, O., Higuchi, Y., *et al.* Gamma knife surgery for 1-10 brain metastases without prophylactic whole-brain radiation therapy: analysis of cases meeting the Japanese prospective multi-institute study (JLGK0901) inclusion criteria. *J Neurooncol* **98,** 163-167 (2010).

117. Kocher, M., Soffietti, R., Abacioglu, U., Villà, S., *et al.* Adjuvant whole-brain radiotherapy versus observation after radiosurgery or surgical resection of one to three cerebral metastases: results of the EORTC 22952-26001 study. *J Clin Oncol* **29,** 134-141 (2011).

118. Minniti, G., Esposito, V., Clarke, E., Scaringi, C., *et al.* Multidose stereotactic radiosurgery (9 Gy × 3) of the postoperative resection cavity for treatment of large brain metastases. *Int J Radiat Oncol Biol Phys* **86,** 623-629 (2013).

119. Sneed, P.K., Lamborn, K.R., Forstner, J.M., McDermott, M.W., *et al.* Radiosurgery for brain metastases: is whole brain radiotherapy necessary? *Int J Radiat Oncol Biol Phys* **43,** 549-558 (1999).

120. Chang, E.L., Wefel, J.S., Hess, K.R., Allen, P.K., *et al.* Neurocognition in patients with brain metastases treated with radiosurgery or radiosurgery plus whole-brain irradiation: a randomised controlled trial. *Lancet Oncol* **10,** 1037-1044 (2009).

121. Tsao, M., Xu, W. & Sahgal, A. A meta-analysis evaluating stereotactic radiosurgery, whole-brain radiotherapy, or both for patients presenting with a limited number of brain metastases. *Cancer* **118,** 2486-2493 (2012).

122. Soffietti, R., Kocher, M., Abacioglu, U.M., Villa, S., *et al.* A European Organisation for Research and Treatment of Cancer phase III trial of adjuvant whole-brain radiotherapy versus observation in patients with one to three brain metastases from solid tumors after surgical resection or radiosurgery: quality-of-life results. *J Clin Oncol* **31,** 65-72 (2013).

123. Yamamoto, M., Serizawa, T., Shuto, T., Akabane, A., *et al.* Stereotactic radiosurgery for patients with multiple brain metastases (JLGK0901): a multi-institutional prospective observational study. *The Lancet Oncology* **15,** 387-395 (2014).

124. Karlsson, B., Hanssens, P., Wolff, R., Söderman, M., *et al.* Thirty years' experience with Gamma Knife surgery for metastases to the brain. *J Neurosurg* **111,** 449-457 (2009).

125. Roos, D.E., Wirth, A., Burmeister, B.H., Spry, N.A., *et al.* Whole brain irradiation following surgery or radiosurgery for solitary brain metastases: mature results of a prematurely closed randomized Trans-Tasman Radiation Oncology Group trial (TROG 98.05). *Radiother Oncol* **80,** 318-322 (2006).

126. Soon, Y.Y., Tham, I.W., Lim, K.H., Koh, W.Y. & Lu, J.J. Surgery or radiosurgery plus whole brain radiotherapy versus surgery or radiosurgery alone for brain metastases. *Cochrane Database Syst Rev* **3,** CD009454 (2014).

127. Brastianos, H.C., Cahill, D.P. & Brastianos, P.K. Systemic therapy of brain metastases. *Curr Neurol Neurosci Rep* **15,** 518 (2015).

128. Zhu, W., Zhou, L., Qian, J.Q., Qiu, T.Z., *et al.* Temozolomide for treatment of brain metastases: A review of 21 clinical trials. *World J Clin Oncol* **5,** 19-27 (2014).

129. Chapman, P.B., Hauschild, A., Robert, C., Haanen, J.B., *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364,** 2507-2516 (2011).

130. Hauschild, A., Grob, J.J., Demidov, L.V., Jouary, T., *et al.* Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* **380,** 358-365 (2012).

131. Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* **344,** 783-792 (2001).

132. Shepherd, F.A., Rodrigues Pereira, J., Ciuleanu, T., Tan, E.H., *et al.* Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* **353,** 123-132 (2005).

133. Fukuoka, M., Wu, Y.L., Thongprasert, S., Sunpaweravong, P., *et al.* Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). *J Clin Oncol* **29,** 2866-2874 (2011).

134. Shaw, A.T., Kim, D.W., Mehra, R., Tan, D.S., *et al.* Ceritinib in ALK-rearranged non-small-cell lung cancer. *N Engl J Med* **370,** 1189-1197 (2014).

135. Solomon, B.J., Mok, T., Kim, D.W., Wu, Y.L., *et al.* First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med* **371,** 2167-2177 (2014).

136. Duchnowska, R., Jassem, J., Goswami, C.P., Dundar, M., *et al.* Predicting early brain metastases based on clinicopathological factors and gene expression analysis in advanced HER2-positive breast cancer patients. *J Neurooncol* **122,** 205-216 (2015).

137. Stemmler, H.J., Kahlert, S., Siekiera, W., Untch, M., *et al.* Characteristics of patients with brain metastases receiving trastuzumab for HER2 overexpressing metastatic breast cancer. *Breast* **15,** 219-225 (2006).

138. Wagle, N., Emery, C., Berger, M.F., Davis, M.J., *et al.* Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol* **29,** 3085-3096 (2011).

139. Deeken, J.F. & Löscher, W. The blood-brain barrier and cancer: transporters, treatment, and Trojan horses. *Clin Cancer Res* **13,** 1663-1674 (2007).

140. Ohtsuki, S. & Terasaki, T. Contribution of carrier-mediated transport systems to the blood-brain barrier as a supporting and protecting interface for the brain; importance for CNS drug discovery and development. *Pharm Res* **24,** 1745-1758 (2007).

141. Motl, S., Zhuang, Y., Waters, C.M. & Stewart, C.F. Pharmacokinetic considerations in the treatment of CNS tumours. *Clin Pharmacokinet* **45,** 871-903 (2006).

142. Kemper, E.M., van Zandbergen, A.E., Cleypool, C., Mos, H.A., *et al.* Increased penetration of paclitaxel into the brain by inhibition of P-Glycoprotein. *Clin Cancer Res* **9,** 2849-2855 (2003).

143. Colombo, T., Zucchetti, M. & D'Incalci, M. Cyclosporin A markedly changes the distribution of doxorubicin in mice and rats. *J Pharmacol Exp Ther* **269,** 22-27 (1994).

144. Soffietti, R., Cornu, P., Delattre, J.Y., Grant, R., *et al.* EFNS Guidelines on diagnosis and treatment of brain metastases: report of an EFNS Task Force. *Eur J Neurol* **13,** 674-681 (2006).

145. Ryken, T.C., McDermott, M., Robinson, P.D., Ammirati, M., *et al.* The role of steroids in the management of brain metastases: a systematic review and evidence-based clinical practice guideline. *J Neurooncol* **96,** 103-114 (2010).

146. Sørensen, J.B., Hansen, H.H., Hansen, M. & Dombernowsky, P. Brain metastases in adenocarcinoma of the lung: frequency, risk groups, and prognosis. *J Clin Oncol* **6,** 1474-1480 (1988).

147. Bernardo, G., Cuzzoni, Q., Strada, M.R., Bernardo, A., *et al.* First-line chemotherapy with vinorelbine, gemcitabine, and carboplatin in the treatment of brain metastases from non-small-cell lung cancer: a phase II study. *Cancer Invest* **20,** 293-302 (2002).

148. Cortes, J., Rodriguez, J., Aramendia, J.M., Salgado, E., *et al.* Front-line paclitaxel/cisplatin-based chemotherapy in brain metastases from non-small-cell lung cancer. *Oncology* **64,** 28-35 (2003).

149. Cotto, C., Berille, J., Souquet, P.J., Riou, R., *et al.* A phase II trial of fotemustine and cisplatin in central nervous system metastases from non-small cell lung cancer. *Eur J Cancer* **32A,** 69-71 (1996).

150. Minotti, V., Crinò, L., Meacci, M.L., Corgna, E., *et al.* Chemotherapy with cisplatin and teniposide for cerebral metastases in non-small cell lung cancer. *Lung Cancer* **20,** 93-98 (1998).

151. Franciosi, V., Cocconi, G., Michiara, M., Di Costanzo, F., *et al.* Front-line chemotherapy with cisplatin and etoposide for patients with brain metastases from breast carcinoma, nonsmall cell lung carcinoma, or malignant melanoma: a prospective study. *Cancer* **85,** 1599-1605 (1999).

152. Kleisbauer, J.P., Guerin, J.C., Arnaud, A., Poirier, R. & Vesco, D. [Chemotherapy with high-dose cisplatin in brain metastasis of lung cancers]. *Bull Cancer* **77,** 661-665 (1990).

153. Abrey, L.E., Olson, J.D., Raizer, J.J., Mack, M., *et al.* A phase II trial of temozolomide for patients with recurrent or progressive brain metastases. *J Neurooncol* **53,** 259-265 (2001).

154. Giorgio, C.G., Giuffrida, D., Pappalardo, A., Russo, A., *et al.* Oral temozolomide in heavily pre-treated brain metastases from non-small cell lung cancer: phase II study. *Lung Cancer* **50,** 247-254 (2005).

155. Bearz, A., Garassino, I., Tiseo, M., Caffo, O., *et al.* Activity of pemetrexed on brain metastases from non-small cell lung cancer. *Lung Cancer* **68,** 264-268 (2010).

156. Barlesi, F., Gervais, R., Lena, H., Hureaux, J., *et al.* Pemetrexed and cisplatin as first-line chemotherapy for advanced non-small-cell lung cancer (NSCLC) with asymptomatic inoperable brain metastases: a multicenter phase II trial (GFPC 07-01). *Ann Oncol* **22,** 2466-2470 (2011).

157. van Meerbeeck, J.P., Fennell, D.A. & De Ruysscher, D.K. Small-cell lung cancer. *Lancet* **378,** 1741-1755 (2011).

158. Paez, J.G., Jänne, P.A., Lee, J.C., Tracy, S., *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **304,** 1497-1500 (2004).

159. Wu, J.Y., Shih, J.Y., Chen, K.Y., Yang, C.H., *et al.* Gefitinib therapy in patients with advanced non-small cell lung cancer with or without testing for

epidermal growth factor receptor (EGFR) mutations. *Medicine (Baltimore)* **90,** 159-167 (2011).

160. Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350,** 2129-2139 (2004).

161. Jänne, P.A., Engelman, J.A. & Johnson, B.E. Epidermal growth factor receptor mutations in non-small-cell lung cancer: implications for treatment and tumor biology. *J Clin Oncol* **23,** 3227-3234 (2005).

162. Gow, C.H., Chang, Y.L., Hsu, Y.C., Tsai, M.F., *et al.* Comparison of epidermal growth factor receptor mutations between primary and corresponding metastatic tumors in tyrosine kinase inhibitor-naive non-small-cell lung cancer. *Ann Oncol* **20,** 696-702 (2009).

163. Ceresoli, G.L., Cappuzzo, F., Gregorc, V., Bartolini, S., *et al.* Gefitinib in patients with brain metastases from non-small-cell lung cancer: a prospective trial. *Ann Oncol* **15,** 1042-1047 (2004).

164. Hotta, K., Kiura, K., Ueoka, H., Tabata, M., *et al.* Effect of gefitinib ('Iressa', ZD1839) on brain metastases in patients with advanced non-small-cell lung cancer. *Lung Cancer* **46,** 255-261 (2004).

165. Wu, C., Li, Y.L., Wang, Z.M., Li, Z., *et al.* Gefitinib as palliative therapy for lung adenocarcinoma metastatic to the brain. *Lung Cancer* **57,** 359-364 (2007).

166. Chiu, C.H., Tsai, C.M., Chen, Y.M., Chiang, S.C., *et al.* Gefitinib is active in patients with brain metastases from non-small cell lung cancer and response is related to skin toxicity. *Lung Cancer* **47,** 129-138 (2005).

167. Kim, J.E., Lee, D.H., Choi, Y., Yoon, D.H., *et al.* Epidermal growth factor receptor tyrosine kinase inhibitors as a first-line therapy for never-smokers with adenocarcinoma of the lung having asymptomatic synchronous brain metastasis. *Lung Cancer* **65,** 351-354 (2009).

168. Ma, S., Xu, Y., Deng, Q. & Yu, X. Treatment of brain metastasis from non-small cell lung cancer with whole brain radiotherapy and gefitinib in a Chinese population. *Lung Cancer* **65,** 198-203 (2009).

169. Porta, R., Sánchez-Torres, J.M., Paz-Ares, L., Massutí, B., *et al.* Brain metastases from lung cancer responding to erlotinib: the importance of EGFR mutation. *Eur Respir J* **37,** 624-631 (2011).

170. Katayama, T., Shimizu, J., Suda, K., Onozato, R., *et al.* Efficacy of erlotinib for brain and leptomeningeal metastases in patients with lung adenocarcinoma who showed initial good response to gefitinib. *J Thorac Oncol* **4,** 1415-1419 (2009).

171. Grommes, C., Oxnard, G.R., Kris, M.G., Miller, V.A., *et al.* "Pulsatile" high-dose weekly erlotinib for CNS metastases from EGFR mutant non-small cell lung cancer. *Neuro Oncol* **13,** 1364-1369 (2011).

172. Lee, S.M., Lewanski, C.R., Counsell, N., Ottensmeier, C., *et al.* Randomized trial of erlotinib plus whole-brain radiotherapy for NSCLC patients with multiple brain metastases. *J Natl Cancer Inst* **106,** pii: dju151 (2014).

173. Kwak, E.L., Bang, Y.J., Camidge, D.R., Shaw, A.T., *et al.* Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* **363,** 1693-1703 (2010).

174. Shaw, A.T., Kim, D.W., Nakagawa, K., Seto, T., *et al.* Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med* **368,** 2385-2394 (2013).

175. Gadgeel, S.M., Gandhi, L., Riely, G.J., Chiappori, A.A., *et al.* Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose-finding portion of a phase 1/2 study. *Lancet Oncol* **15,** 1119-1128 (2014).

176. Preusser, M., Berghoff, A.S., Ilhan-Mutlu, A., Magerle, M., *et al.* ALK gene translocations and amplifications in brain metastases of non-small cell lung cancer. *Lung Cancer* **80,** 278-283 (2013).

177. Costa, D.B., Shaw, A.T., Ou, S-H.I., Solomon, B.J., *et al.* Clinical experience with crizotinib in patients with advanced ALK-rearranged non–small-cell lung cancer and brain metastases. *J Clin Oncol* pii: JCO.2014.59.0539 [Epub ahead of print] (2015).

178. Lin, N.U., Bellon, J.R. & Winer, E.P. CNS metastases in breast cancer. *J Clin Oncol* **22,** 3608-3617 (2004).

179. Brufsky, A.M., Mayer, M., Rugo, H.S., Kaufman, P.A., *et al.* Central nervous system metastases in patients with HER2-positive metastatic breast cancer: incidence, treatment, and survival in patients from registHER. *Clin Cancer Res* **17,** 4834-4843 (2011).

180. Slimane, K., Andre, F., Delaloge, S., Dunant, A., *et al.* Risk factors for brain relapse in patients with metastatic breast cancer. *Ann Oncol* **15,** 1640-1644 (2004).

181. Boogerd, W., Dalesio, O., Bais, E.M. & van der Sande, J.J. Response of brain metastases from breast cancer to systemic chemotherapy. *Cancer* **69,** 972-980 (1992).

182. Rosner, D., Nemoto, T. & Lane, W.W. Chemotherapy induces regression of brain metastases in breast carcinoma. *Cancer* **58,** 832-839 (1986).

183. Cocconi, G., Lottici, R., Bisagni, G., Bacchi, M., *et al.* Combination therapy with platinum and etoposide of brain metastases from breast carcinoma. *Cancer Invest* **8,** 327-334 (1990).

184. Hikino, H., Yamada, T., Johbara, K., Obayashi, N. & Ozaki, N. Potential role of chemo-radiation with oral capecitabine in a breast cancer patient with central nervous system relapse. *Breast* **15,** 97-99 (2006).

185. Oberhoff, C., Kieback, D.G., Würstlein, R., Deertz, H., *et al.* Topotecan chemotherapy in patients with breast cancer and brain metastases: results of a pilot study. *Onkologie* **24,** 256-260 (2001).

186. Kennecke, H., Yerushalmi, R., Woods, R., Cheang, M.C., *et al.* Metastatic behavior of breast cancer subtypes. *J Clin Oncol* **28,** 3271-3277 (2010).

187. Bendell, J.C., Domchek, S.M., Burstein, H.J., Harris, L., *et al.* Central nervous system metastases in women who receive trastuzumab-based therapy for metastatic breast carcinoma. *Cancer* **97,** 2972-2977 (2003).

188. Andre, F., Slimane, K., Bachelot, T., Dunant, A., *et al.* Breast cancer with synchronous metastases: trends in survival during a 14-year period. *J Clin Oncol* **22,** 3302-3308 (2004).

189. Kirsch, D.G., Ledezma, C.J., Mathews, C.S., Bhan, A.K., *et al*. Survival after brain metastases from breast cancer in the trastuzumab era. *J Clin Oncol* **23,** 2114-2116 (2005).

190. Dijkers, E.C., Oude Munnink, T.H., Kosterink, J.G., Brouwers, A.H., *et al.* Biodistribution of 89Zr-trastuzumab and PET imaging of HER2-positive lesions in patients with metastatic breast cancer. *Clin Pharmacol Ther* **87,** 586-592 (2010).

191. Duchnowska, R., Dziadziuszko, R., Trojanowski, T., Mandat, T., *et al.* Conversion of epidermal growth factor receptor 2 and hormone receptor

expression in breast cancer metastases to the brain. *Breast Cancer Res* **14,** R119 (2012).

192. Torres, S., Maralani, P. & Verma, S. Activity of T-DM1 in HER-2 positive central nervous system breast cancer metastases. *BMJ Case Rep* **2014,** pii: bcr2014205680 (2014).

193. Bartsch, R., Berghoff, A.S. & Preusser, M. Breast cancer brain metastases responding to primary systemic therapy with T-DM1. *J Neurooncol* **116,** 205-206 (2014).

194. Gril, B., Palmieri, D., Bronder, J.L., Herring, J.M., *et al.* Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain. *J Natl Cancer Inst* **100,** 1092-1103 (2008).

195. Lin, N.U., Diéras, V., Paul, D., Lossignol, D., *et al.* Multicenter phase II study of lapatinib in patients with brain metastases from HER2-positive breast cancer. *Clin Cancer Res* **15,** 1452-1459 (2009).

196. Bachelot, T., Romieu, G., Campone, M., Diéras, V., *et al.* Lapatinib plus capecitabine in patients with previously untreated brain metastases from HER2-positive metastatic breast cancer (LANDSCAPE): a single-group phase 2 study. *Lancet Oncol* **14,** 64-71 (2013).

197. Ro, J., Park, S., Kim, S., Kim, T.Y., *et al.* Clinical outcomes of HER2-positive metastatic breast cancer patients with brain metastasis treated with lapatinib and capecitabine: an open-label expanded access study in Korea. *BMC Cancer* **12,** 322 (2012).

198. Lin, N.U., Freedman, R.A., Ramakrishna, N., Younger, J., *et al.* A phase I study of lapatinib with whole brain radiotherapy in patients with human epidermal growth factor receptor 2 (HER2)-positive breast cancer brain metastases. *Breast Cancer Res Treat* **142,** 405-414 (2013).

199. Yap, Y.S., Cornelio, G.H., Devi, B.C., Khorprasert, C., *et al.* Brain metastases in Asian HER2-positive breast cancer patients: anti-HER2 treatments and their impact on survival. *Br J Cancer* **107,** 1075-1082 (2012).

200. Lin, N.U., Claus, E., Sohl, J., Razzak, A.R., *et al.* Sites of distant recurrence and clinical outcomes in patients with metastatic triple-negative breast cancer: high incidence of central nervous system metastases. *Cancer* **113,** 2638-2645 (2008).

201. Adamo, B., Deal, A.M., Burrows, E., Geradts, J., *et al.* Phosphatidylinositol 3-kinase pathway activation in breast cancer brain metastases. *Breast Cancer Res* **13,** R125 (2011).

202. Wikman, H., Lamszus, K., Detels, N., Uslar, L., *et al.* Relevance of PTEN loss in brain metastasis formation in breast cancer patients. *Breast Cancer Res* **14,** R49 (2012).

203. Lévy, C., Allouache, D., Lacroix, J., Dugué, A.E., *et al.* REBECA: a phase I study of bevacizumab and whole-brain radiation therapy for the treatment of brain metastasis from solid tumours. *Ann Oncol* **25,** 2351-2356 (2014).

204. Falchook, G.S., Moulder, S.L., Wheler, J.J., Jiang, Y., *et al.* Dual HER2 inhibition in combination with anti-VEGF treatment is active in heavily pretreated HER2-positive breast cancer. *Ann Oncol* **24,** 3004-3011 (2013).

205. Labidi, S.I., Bachelot, T., Ray-Coquard, I., Mosbah, K., *et al.* Bevacizumab and paclitaxel for breast cancer patients with central nervous system metastases: a case series. *Clin Breast Cancer* **9,** 118-121 (2009).

206. Wen, P.Y., Macdonald, D.R., Reardon, D.A., Cloughesy, T.F., *et al.* Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. *J Clin Oncol* **28,** 1963-1972 (2010).

207. Dasgupta, T. & Brasfield, R. Metastatic melanoma. A clinicopathological study. *Cancer* **17,** 1323-1339 (1964).

208. Davies, M.A., Liu, P., McIntyre, S., Kim, K.B., *et al.* Prognostic factors for survival in melanoma patients with brain metastases. *Cancer* **117,** 1687-1696 (2011).

209. de la Monte, S.M., Moore, G.W. & Hutchins, G.M. Patterned distribution of metastases from malignant melanoma in humans. *Cancer Res* **43,** 3427-3433 (1983).

210. Liew, D.N., Kano, H., Kondziolka, D., Mathieu, D., *et al.* Outcome predictors of Gamma Knife surgery for melanoma brain metastases. Clinical article. *J Neurosurg* **114,** 769-779 (2011).

211. Lonser, R.R., Song, D.K., Klapper, J., Hagan, M., *et al.* Surgical management of melanoma brain metastases in patients treated with immunotherapy. *J Neurosurg* **115,** 30-36 (2011).

212. Mathieu, D., Kondziolka, D., Cooper, P.B., Flickinger, J.C., *et al.* Gamma knife radiosurgery for malignant melanoma brain metastases. *Clin Neurosurg* **54,** 241-247 (2007).

213. Patel, J.K., Didolkar, M.S., Pickren, J.W. & Moore, R.H. Metastatic pattern of malignant melanoma. A study of 216 autopsy cases. *Am J Surg* **135,** 807-810 (1978).

214. Radbill, A.E., Fiveash, J.F., Falkenberg, E.T., Guthrie, B.L., *et al.* Initial treatment of melanoma brain metastases using gamma knife radiosurgery: an evaluation of efficacy and toxicity. *Cancer* **101,** 825-833 (2004).

215. Raizer, J.J., Hwu, W.J., Panageas, K.S., Wilton, A., *et al.* Brain and leptomeningeal metastases from cutaneous melanoma: survival outcomes based on clinical features. *Neuro Oncol* **10,** 199-207 (2008).

216. Sampson, J.H., Carter, J.H., Friedman, A.H. & Seigler, H.F. Demographics, prognosis, and therapy in 702 patients with brain metastases from malignant melanoma. *J Neurosurg* **88,** 11-20 (1998).

217. Zakrzewski, J., Geraghty, L.N., Rose, A.E., Christos, P.J., *et al.* Clinical variables and primary tumor characteristics predictive of the development of melanoma brain metastases and post-brain metastases survival. *Cancer* **117,** 1711-1720 (2011).

218. Wroński, M. & Arbit, E. Surgical treatment of brain metastases from melanoma: a retrospective study of 91 patients. *J Neurosurg* **93,** 9-18 (2000).

219. Amer, M.H., Al-Sarraf, M., Baker, L.H. & Vaitkevicius, V.K. Malignant melanoma and central nervous system metastases: incidence, diagnosis, treatment and survival. *Cancer* **42,** 660-668 (1978).

220. Staudt, M., Lasithiotakis, K., Leiter, U., Meier, F., *et al.* Determinants of survival in patients with brain metastases from cutaneous melanoma. *Br J Cancer* **102,** 1213-1218 (2010).

221. Redmond, A.J., Diluna, M.L., Hebert, R., Moliterno, J.A., *et al.* Gamma Knife surgery for the treatment of melanoma metastases: the effect of intratumoral hemorrhage on survival. *J Neurosurg* **109,** S99-S105 (2008).

222. Fife, K.M., Colman, M.H., Stevens, G.N., Firth, I.C., *et al.* Determinants of outcome in melanoma patients with cerebral metastases. *J Clin Oncol* **22,** 1293-1300 (2004).

223. Flanigan, J.C., Jilaveanu, L.B., Chiang, V.L. & Kluger, H.M. Advances in therapy for melanoma brain metastases. *Clin Dermatol* **31,** 264-281 (2013).

224. Nikolaou, V.A., Stratigos, A.J., Flaherty, K. T. & Tsao, H. Melanoma: New insights and new therapies. *J Invest Dermatol* **132,** 854-863 (2012).

225. Romano, E., Schwartz, G.K., Chapman, P.B., Wolchock, J.D. & Carvajal, R.D. Treatment implications of the emerging molecular classification system for melanoma. *Lancet Oncol* **12,** 913-922 (2011).

226. Lui, P., Cashin, R., Machado, M., Hemels, M., *et al.* Treatments for metastatic melanoma: synthesis of evidence from randomized trials. *Cancer Treat Rev* **33,** 665-680 (2007).

227. Avril, M.F., Aamdal, S., Grob, J.J., Hauschild, A., *et al.* Fotemustine compared with dacarbazine in patients with disseminated malignant melanoma: a phase III study. *J Clin Oncol* **22,** 1118-1125 (2004).

228. Margolin, K., Atkins, B., Thompson, A., Ernstoff, S., *et al.* Temozolomide and whole brain irradiation in melanoma metastatic to the brain: a phase II trial of the Cytokine Working Group. *J Cancer Res Clin Oncol* **128,** 214-218 (2002).

229. Hofmann, M., Kiecker, F., Wurm, R., Schlenger, L., *et al.* Temozolomide with or without radiotherapy in melanoma with unresectable brain metastases. *J Neurooncol* **76,** 59-64 (2006).

230. Agarwala, S.S., Kirkwood, J.M., Gore, M., Dreno, B., *et al.* Temozolomide for the treatment of brain metastases associated with metastatic melanoma: a phase II study. *J Clin Oncol* **22,** 2101-2107 (2004).

231. Rochet, N.M., Kottschade, L.A. & Markovic, S.N. Vemurafenib for melanoma metastases to the brain. *N Engl J Med* **365,** 2439-2441 (2011).

232. Davies, H., Bignell, G.R., Cox, C., Stephens, P., *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417,** 949-954 (2002).

233. Schadendorf, D. & Hauschild, A. Melanoma in 2013: Melanoma - the run of success continues. *Nat Rev Clin Oncol* **11,** 75-76 (2014).

234. Eggermont, A.M., Spatz, A. & Robert, C. Cutaneous melanoma. *Lancet* **383,** 816-827 (2014).

235. Siena, S., Crinò, L., Danova, M., Del Prete, S., *et al.* Dose-dense temozolomide regimen for the treatment of brain metastases from melanoma, breast cancer, or lung cancer not amenable to surgery or radiosurgery: a multicenter phase II study. *Ann Oncol* **21,** 655-661 (2010).

236. Atkins, M.B., Sosman, J.A., Agarwala, S., Logan, T., *et al.* Temozolomide, thalidomide, and whole brain radiation therapy for patients with brain metastasis from metastatic melanoma: a phase II Cytokine Working Group study. *Cancer* **113,** 2139-2145 (2008).

237. Hong, J.J., Rosenberg, S.A., Dudley, M.E., Yang, J.C., *et al.* Successful treatment of melanoma brain metastases with adoptive cell therapy. *Clin Cancer Res* **16,** 4892-4898 (2010).

238. Margolin, K., Ernstoff, M.S., Hamid, O., Lawrence, D., *et al.* Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *Lancet Oncol* **13,** 459-465 (2012).

239. Di Giacomo, A.M., Ascierto, P.A., Pilla, L., Santinami, M., *et al.* Ipilimumab and fotemustine in patients with advanced melanoma (NIBIT-M1): an open-label, single-arm phase 2 trial. *Lancet Oncol* **13,** 879-886 (2012).

240. Long, G.V., Trefzer, U., Davies, M.A., Kefford, R.F., *et al.* Dabrafenib in patients with Val600Glu or Val600Lys BRAF-mutant melanoma metastatic to the brain (BREAK-MB): a multicentre, open-label, phase 2 trial. *Lancet Oncol* **13,** 1087-1095 (2012).

241. Falchook, G.S., Long, G.V., Kurzrock, R., Kim, K.B., *et al.* Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *Lancet* **379,** 1893-1901 (2012).

242. Konstantinou, M.P., Dutriaux, C., Gaudy-Marqueste, C., Mortier, L., *et al.* Ipilimumab in melanoma patients with brain metastasis: A retrospective multicentre evaluation of thirty-eight patients. *Acta Derm Venereol* **94,** 45-49 (2013).

243. Queirolo, P., Spagnolo, F., Ascierto, P.A., Simeone, E., *et al.* Efficacy and safety of ipilimumab in patients with advanced melanoma and brain metastases. *J Neurooncol* **118,** 109-116 (2014).

244. Dummer, R., Goldinger, S.M., Turtschi, C.P., Eggmann, N.B., *et al.* Vemurafenib in patients with BRAF(V600) mutation-positive melanoma with symptomatic brain metastases: final results of an open-label pilot study. *Eur J Cancer* **50,** 611-621 (2014).

245. Dzienis, M.R. & Atkinson, V.G. Response rate to vemurafenib in patients with B-RAF-positive melanoma brain metastases: a retrospective review. *Melanoma Res* **24,** 349-353 (2014).

246. Larkin, J., Del Vecchio, M., Ascierto, P.A., Krajsova, I., *et al.* Vemurafenib in patients with BRAFV600 mutated metastatic melanoma: an open-label, multicentre, safety study. *Lancet Oncol* **15,** 436-444 (2014).

247. Manon, R., O'Neill, A., Knisely, J., Werner-Wasik, M., *et al.* Phase II trial of radiosurgery for one to three newly diagnosed brain metastases from renal cell carcinoma, melanoma, and sarcoma: an Eastern Cooperative Oncology Group study (E 6397). *J Clin Oncol* **23,** 8870-8876 (2005).

248. Peruzzi, P. & Chiocca, E.A. Prognosis and treatment of melanoma metastases to the central nervous system: lots of retrospective data, very few certainties. *World Neurosurg* **76,** 48-50 (2011).

249. Yu, C., Chen, J.C., Apuzzo, M.L., O'Day, S., *et al.* Metastatic melanoma to the brain: prognostic factors after gamma knife radiosurgery. *Int J Radiat Oncol Biol Phys* **52,** 1277-1287 (2002).

250. Sperduto, P.W., Berkey, B., Gaspar, L.E., Mehta, M. & Curran, W. A new prognostic index and comparison to three other indices for patients with brain metastases: an analysis of 1,960 patients in the RTOG database. *Int J Radiat Oncol Biol Phys* **70,** 510-514 (2008).

251. Rodrigues, G., Bauman, G., Palma, D., Louie, A.V., *et al.* Systematic review of brain metastases prognostic indices. *Pract Radiat Oncol* **3,** 101-106 (2013).

252. Sperduto, P.W., Kased, N., Roberge, D., Xu, Z., *et al.* Summary report on the graded prognostic assessment: an accurate and facile diagnosis-specific tool to estimate survival for patients with brain metastases. *J Clin Oncol* **30,** 419-425 (2012).

253. Sperduto, P.W., Chao, S.T., Sneed, P.K., Luo, X., *et al.* Diagnosis-specific prognostic factors, indexes, and treatment outcomes for patients with newly diagnosed brain metastases: a multi-institutional analysis of 4,259 patients. *Int J Radiat Oncol Biol Phys* **77,** 655-661 (2010).

254. Maclean, J., Fersht, N., Singhera, M., Mulholland, P., *et al.* Multi-disciplinary management for patients with oligometastases to the brain: results of a 5 year cohort study. *Radiat Oncol* **8,** 156 (2013).

255. Balch, C.M., Gershenwald, J.E., Soong, S.J., Thompson, J.F., *et al.* Multivariate analysis of prognostic factors among 2,313 patients with stage III melanoma: comparison of nodal micrometastases versus macrometastases. *J Clin Oncol* **28,** 2452-2459 (2010).

256. Morton, D.L., Thompson, J.F., Cochran, A.J., Mozzillo, N., *et al.* Final trial report of sentinel-node biopsy versus nodal observation in melanoma. *N Engl J Med* **370,** 599-609 (2014).

257. Pasquali, S., Mocellin, S., Mozzillo, N., Maurichi, A., *et al.* Nonsentinel lymph node status in patients with cutaneous melanoma: results from a multi-institution prognostic study. *J Clin Oncol* **32,** 935-941 (2014).

258. Lens, M.B. & Dawes, M. Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. *Br J Dermatol* **150,** 179-185 (2004).

259. Little, E.G. & Eide, M.J. Update on the current state of melanoma incidence. *Dermatol Clin* **30,** 355-361 (2012).

260. MacKie, R.M., Hauschild, A. & Eggermont, A.M. Epidemiology of invasive cutaneous melanoma. *Ann Oncol* **20 Suppl 6,** vi1-vi7 (2009).

261. Green, A.C., Williams, G.M., Logan, V. & Strutton, G.M. Reduced melanoma after regular sunscreen use: randomized trial follow-up. *J Clin Oncol* **29,** 257-263 (2011).

262. Viros, A., Sanchez-Laorden, B., Pedersen, M., Furney, S.J., *et al.* Ultraviolet radiation accelerates BRAF-driven melanomagenesis by targeting TP53. *Nature* **511,** 478-482 (2014).

263. Zimmer, L., Haydu, L.E., Menzies, A.M., Scolyer, R.A., *et al.* Incidence of new primary melanomas after diagnosis of stage III and IV melanoma. *J Clin Oncol* **32,** 816-823 (2014).

264. Moloney, F.J., Guitera, P., Coates, E., Haass, N.K., *et al.* Detection of primary melanoma in individuals at extreme high risk: a prospective 5-year follow-up study. *JAMA Dermatol* **150,** 819-827 (2014).

265. Gerami, P., Cook, R.W., Wilkinson, J., Russell, M.C., *et al.* Development of a prognostic genetic signature to predict the metastatic risk associated with cutaneous melanoma. *Clin Cancer Res* **21,** 175-183 (2015).

266. Wong, J.R., Harris, J.K., Rodriguez-Galindo, C. & Johnson, K.J. Incidence of childhood and adolescent melanoma in the United States: 1973-2009. *Pediatrics* **131,** 846-854 (2013).

267. Lu, C., Zhang, J., Nagahawatte, P., Easton, J., *et al.* The genomic landscape of childhood and adolescent melanoma. *J Invest Dermatol* **135,** 816-823 (2015).

268. Landreville, S., Agapova, O.A. & Harbour, J.W. Emerging insights into the molecular pathogenesis of uveal melanoma. *Future Oncol* **4,** 629-636 (2008).

269. Zuidervaart, W., van Nieuwpoort, F., Stark, M., Dijkman, R., *et al.* Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer* **92,** 2032-2038 (2005).

270. Adler, M.J. & White, C.R. Amelanotic malignant melanoma. *Semin Cutan Med Surg* **16,** 122-130 (1997).

271. Betti, R., Vergani, R., Tolomio, E., Santambrogio, R. & Crosti, C. Factors of delay in the diagnosis of melanoma. *Eur J Dermatol* **13,** 183-188 (2003).

272. Damsky, W.E., Rosenbaum, L.E. & Bosenberg, M. Decoding melanoma metastasis. *Cancers* **3,** 126-163 (2011).

273. Pollock, P.M., Harper, U.L., Hansen, K.S., Yudt, L.M., *et al.* High frequency of BRAF mutations in nevi. *Nat Genet* **33,** 19-20 (2003).

274. Vultur, A. & Herlyn, M. SnapShot: Melanoma. *Cancer Cell* **23,** 706-706.e1 (2013).

275. Maurichi, A., Miceli, R., Camerini, T., Mariani, L., *et al.* Prediction of survival in patients with thin melanoma: results from a multi-institution study. *J Clin Oncol* **32,** 2479-2485 (2014).

276. Leung, A.M., Hari, D.M. & Morton, D.L. Surgery for distant melanoma metastasis. *Cancer J* **18,** 176-184 (2012).

277. Schoenewolf, N.L., Belloni, B., Simcock, M., Tonolla, S., *et al.* Clinical implications of distinct metastasizing preferences of different melanoma subtypes. *Eur J Dermatol* **24,** 236-241 (2014).

278. Daryanani, D., Plukker, J.T., de Jong, M.A., Haaxma-Reiche, H., *et al.* Increased incidence of brain metastases in cutaneous head and neck melanoma. *Melanoma Res* **15,** 119-124 (2005).

279. Huismans, A.M., Haydu, L.E., Shannon, K.F., Quinn, M.J., *et al.* Primary melanoma location on the scalp is an important risk factor for brain metastasis: a study of 1,687 patients with cutaneous head and neck melanomas. *Ann Surg Oncol* **21,** 3985-3991 (2014).

280. Balch, C.M., Gershenwald, J.E., Soong, S.J., Thompson, J.F., *et al.* Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* **27,** 6199-6206 (2009).

281. Devitt, B., Liu, W., Salemi, R., Wolfe, R., *et al.* Clinical outcome and pathological features associated with NRAS mutation in cutaneous melanoma. *Pigment Cell Melanoma Res* **24,** 666-672 (2011).

282. Lee, J.H., Choi, J.W. & Kim, Y.S. Frequencies of BRAF and NRAS mutations are different in histological types and sites of origin of cutaneous melanoma: a meta-analysis. *Br J Dermatol* **164,** 776-784 (2011).

283. Omholt, K., Platz, A., Kanter, L., Ringborg, U. & Hansson, J. NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. *Clin Cancer Res* **9,** 6483-6488 (2003).

284. Colombino, M., Capone, M., Lissia, A., Cossu, A., *et al.* BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. *J Clin Oncol* **30,** 2522-2529 (2012).

285. Wan, P.T., Garnett, M.J., Roe, S.M., Lee, S., *et al.* Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **116,** 855-867 (2004).

286. Menzies, A.M., Haydu, L.E., Visintin, L., Carlino, M.S., *et al.* Distinguishing clinicopathologic features of patients with V600E and V600K BRAF-mutant metastatic melanoma. *Clin Cancer Res* **18,** 3242-3249 (2012).

287. Carlino, M.S., Haydu, L.E., Kakavand, H., Menzies, A.M., *et al.* Correlation of BRAF and NRAS mutation status with outcome, site of distant metastasis and response to chemotherapy in metastatic melanoma. *Br J Cancer* **111,** 292-299 (2014).

288. Meckbach, D., Keim, U., Richter, S., Leiter, U., *et al.* BRAF-V600 mutations have no prognostic impact in stage IV melanoma patients treated with monochemotherapy. *PLoS One* **9,** e89218 (2014).

289. Siroy, A.E., Boland, G.M., Milton, D.R., Roszik, J., *et al.* Beyond BRAF(V600): clinical mutation panel testing by next-generation sequencing in advanced melanoma. *J Invest Dermatol* **135,** 508-515 (2015).

290. Curtin, J.A., Fridlyand, J., Kageshita, T., Patel, H.N., *et al.* Distinct sets of genetic alterations in melanoma. *N Engl J Med* **353,** 2135-2147 (2005).

291. Gast, A., Scherer, D., Chen, B., Bloethner, S., *et al.* Somatic alterations in the melanoma genome: a high-resolution array-based comparative genomic hybridization study. *Genes Chromosomes Cancer* **49,** 733-745 (2010).

292. Tsao, H., Goel, V., Wu, H., Yang, G. & Haluska, F.G. Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. *J Invest Dermatol* **122,** 337-341 (2004).

293. Davies, M.A., Stemke-Hale, K., Tellez, C., Calderone, T.L., *et al.* A novel AKT3 mutation in melanoma tumours and cell lines. *Br J Cancer* **99,** 1265-1268 (2008).

294. Omholt, K., Kröckel, D., Ringborg, U. & Hansson, J. Mutations of PIK3CA are rare in cutaneous melanoma. *Melanoma Res* **16,** 197-200 (2006).

295. Silva, J.M., Bulman, C. & McMahon, M. BRAFV600E cooperates with PI3K signaling, independent of AKT, to regulate melanoma cell proliferation. *Mol Cancer Res* **12,** 447-463 (2014).

296. Muthusamy, V., Hobbs, C., Nogueira, C., Cordon-Cardo, C., *et al.* Amplification of CDK4 and MDM2 in malignant melanoma. *Genes Chromosomes Cancer* **45,** 447-454 (2006).

297. Castresana, J.S., Rubio, M.P., Vázquez, J.J., Idoate, M., *et al.* Lack of allelic deletion and point mutation as mechanisms of p53 activation in human malignant melanoma. *Int J Cancer* **55,** 562-565 (1993).

298. Albino, A.P., Vidal, M.J., McNutt, N.S., Shea, C.R., *et al.* Mutation and expression of the p53 gene in human malignant melanoma. *Melanoma Res* **4,** 35-45 (1994).

299. Gembarska, A., Luciani, F., Fedele, C., Russell, E.A., *et al.* MDM4 is a key therapeutic target in cutaneous melanoma. *Nat Med* **18,** 1239-1247 (2012).

300. Prickett, T.D., Agrawal, N.S., Wei, X., Yates, K.E., *et al.* Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. *Nat Genet* **41,** 1127-1132 (2009).

301. Stark, M.S., Woods, S.L., Gartside, M.G., Bonazzi, V.F., *et al.* Frequent somatic mutations in MAP3K5 and MAP3K9 in metastatic melanoma identified by exome sequencing. *Nat Genet* **44,** 165-169 (2012).

302. Berger, M.F., Hodis, E., Heffernan, T.P., Deribe, Y.L., *et al.* Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature* **485,** 502-506 (2012).

303. Hodis, E., Watson, I.R., Kryukov, G.V., Arold, S.T., *et al.* A landscape of driver mutations in melanoma. *Cell* **150,** 251-263 (2012).

304. Krauthammer, M., Kong, Y., Ha, B.H., Evans, P., *et al.* Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nat Genet* **44,** 1006-1014 (2012).

305. Wei, X., Walia, V., Lin, J.C., Teer, J.K., *et al.* Exome sequencing identifies GRIN2A as frequently mutated in melanoma. *Nat Genet* **43,** 442-446 (2011).

306. Prickett, T.D., Wei, X., Cardenas-Navia, I., Teer, J.K., *et al.* Exon capture analysis of G protein-coupled receptors identifies activating mutations in GRM3 in melanoma. *Nat Genet* **43,** 1119-1126 (2011).

307. Harbour, J.W., Onken, M.D., Roberson, E.D., Duan, S., *et al.* Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science* **330,** 1410-1413 (2010).

308. Horn, S., Figl, A., Rachakonda, P.S., Fischer, C., *et al.* TERT promoter mutations in familial and sporadic melanoma. *Science* **339,** 959-961 (2013).

309. Huang, F.W., Hodis, E., Xu, M.J., Kryukov, G.V., *et al.* Highly recurrent TERT promoter mutations in human melanoma. *Science* **339,** 957-959 (2013).

310. Guan, J., Gupta, R. & Filipp, F.V. Cancer systems biology of TCGA SKCM: Efficient detection of genomic drivers in melanoma. *Sci Rep* **5,** 7857 (2015).

311. Nygaard, V., Prasmickaite, L., Vasiliauskaite, K., Clancy, T. & Hovig, E. Melanoma brain colonization involves the emergence of a brain-adaptive phenotype. *Oncoscience* **1,** 82-94 (2014).

312. Pencheva, N., Buss, C.G., Posada, J., Merghoub, T. & Tavazoie, S.F. Broad-spectrum therapeutic suppression of metastatic melanoma through nuclear hormone receptor activation. *Cell* **156,** 986-1001 (2014).

313. Damsky, W.E., Curley, D.P., Santhanakrishnan, M., Rosenbaum, L.E., *et al.* β-Catenin signaling controls metastasis in BRAF-activated PTEN-deficient melanomas. *Cancer Cell* **20,** 741-754 (2011).

314. Shevde, L.A., Samant, R.S., Goldberg, S.F., Sikaneta, T., *et al.* Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1. *Exp Cell Res* **273,** 229-239 (2002).

315. Marzese, D.M., Liu, M., Huynh, J.L., Hirose, H., *et al.* Brain metastasis is predetermined in early-stages of cutaneous melanoma by CD44v6 expression through epigenetic regulation of the spliceosome. *Pigment Cell Melanoma Res* **28,** 82-93 (2014).

316. Marzese, D.M., Scolyer, R.A., Roqué, M., Vargas-Roig, L.M., *et al.* DNA methylation and gene deletion analysis of brain metastases in melanoma patients identifies mutually exclusive molecular alterations. *Neuro Oncol* **16,** 1499-1509 (2014).

317. Marzese, D.M., Scolyer, R.A., Huynh, J.L., Huang, S.K., *et al.* Epigenome-wide DNA methylation landscape of melanoma progression to brain metastasis reveals aberrations on homeobox D cluster associated with prognosis. *Hum Mol Genet* **23,** 226-238 (2013).

318. Lee, J.H., Miele, M.E., Hicks, D.J., Phillips, K.K., *et al.* KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst* **88,** 1731-1737 (1996).

319. Das, S.K., Bhutia, S.K., Kegelman, T.P., Peachy, L., *et al.* MDA-9/syntenin: a positive gatekeeper of melanoma metastasis. *Front Biosci (Landmark Ed)* **17,** 1-15 (2012).

320. Kim, M., Gans, J.D., Nogueira, C., Wang, A., *et al.* Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell* **125,** 1269-1281 (2006).

321. Jarrett, S.G., Novak, M., Harris, N., Merlino, G., *et al.* NM23 deficiency promotes metastasis in a UV radiation-induced mouse model of human melanoma. *Clin Exp Metastasis* **30,** 25-36 (2013).

322. Jilaveanu, L.B., Parisi, F., Barr, M.L., Zito, C.R., *et al.* PLEKHA5 as a biomarker and potential mediator of melanoma brain metastasis. *Clin Cancer Res* pii: clincanres.0861.2014. [Epub ahead of print] (2014).

323. Ocaña, O.H., Córcoles, R., Fabra, A., Moreno-Bueno, G., *et al.* Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer PRRX1. *Cancer Cell* **22,** 709-724 (2012).

324. Clark, E.A., Golub, T.R., Lander, E.S. & Hynes, R.O. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406,** 532-535 (2000).

325. Wilhelm, I., Fazakas, C., Molnár, J., Haskó, J., *et al.* Role of Rho/ROCK signaling in the interaction of melanoma cells with the blood-brain barrier. *Pigment Cell Melanoma Res* **27,** 113-123 (2013).

326. Xie, T.X., Huang, F.J., Aldape, K.D., Kang, S.H., *et al.* Activation of STAT3 in human melanoma promotes brain metastasis. *Cancer Res* **66,** 3188-3196 (2006).

327. Orgaz, J.L. & Sanz-Moreno, V. Emerging molecular targets in melanoma invasion and metastasis. *Pigment Cell Melanoma Res* **26,** 39-57 (2013).

328. Hamilton, R., Krauze, M., Romkes, M., Omolo, B., *et al.* Pathologic and gene expression features of metastatic melanomas to the brain. *Cancer* **119,** 1737-2746 (2013).

329. Riker, A.I., Enkemann, S.A., Fodstad, O., Liu, S., *et al.* The gene expression profiles of primary and metastatic melanoma yields a transition point of tumor progression and metastasis. *BMC Med Genomics* **1,** 13 (2008).

330. Tímár, J., Gyorffy, B. & Rásó, E. Gene signature of the metastatic potential of cutaneous melanoma: too much for too little? *Clin Exp Metastasis* **27,** 371-387 (2010).

331. Kabbarah, O., Nogueira, C., Feng, B., Nazarian, R.M., *et al.* Integrative genome comparison of primary and metastatic melanomas. *PLoS One* **5,** e10770 (2010).

332. Scott, K.L., Nogueira, C., Heffernan, T.P., van Doorn, R., *et al.* Proinvasion metastasis drivers in early-stage melanoma are oncogenes. *Cancer Cell* **20,** 92-103 (2011).

333. Valsesia, A., Rimoldi, D., Martinet, D., Ibberson, M., *et al.* Network-guided analysis of genes with altered somatic copy number and gene expression reveals pathways commonly perturbed in metastatic melanoma. *PLoS One* **6,** e18369 (2011).

334. Tremante, E., Ginebri, A., Lo Monaco, E., Frascione, P., *et al.* Melanoma molecular classes and prognosis in the postgenomic era. *Lancet Oncol* **13,** e205-e211 (2012).

335. Lawrence, M.S., Stojanov, P., Polak, P., Kryukov, G.V., *et al.* Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* **499,** 214-218 (2013).

336. Shtivelman, E., Davies, M.Q., Hwu, P., Yang, J., *et al.* Pathways and therapeutic targets in melanoma. *Oncotarget* **5,** 1701-1752 (2014).

337. Weinberg, R.A. Coming full circle-from endless complexity to simplicity and back again. *Cell* **157,** 267-271 (2014).

338. Brabletz, T., Lyden, D., Steeg, P.S. & Werb, Z. Roadblocks to translational advances on metastasis research. *Nat Med* **19,** 1104-1109 (2013).

339. Rubin, E.H. & Gilliland, D.G. Drug development and clinical trials - the path to an approved cancer drug. *Nat Rev Clin Oncol* **9,** 215-222 (2012).

340. McArthur, G.A. & Ribas, A. Targeting oncogenic drivers and the immune system in melanoma. *J Clin Oncol* **31,** 499-506 (2013).

341. Pleasance, E.D., Cheetham, R.K., Stephens, P.J., McBride, D.J., *et al.* A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* **463,** 191-196 (2010).

342. Greene, M.H., Young, T.I. & Clark, W.H. Malignant melanoma in renal-transplant recipients. *Lancet* **1,** 1196-1199 (1981).

343. Cole, W.H. & Everson, T.C. Spontaneous regression of cancer: preliminary report. *Ann Surg* **144,** 366-383 (1956).

344. Clemente, C.G., Mihm, M.C., Bufalino, R., Zurrida, S., *et al.* Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* **77,** 1303-1310 (1996).

345. Morton, D., Eilber, F.R., Malmgren, R.A. & Wood, W.C. Immunological factors which influence response to immunotherapy in malignant melanoma. *Surgery* **68,** 158-163 (1970).

346. Lo, J.A. & Fisher, D.E. The melanoma revolution: from UV carcinogenesis to a new era in therapeutics. *Science* **346,** 945-949 (2014).

347. Atkins, M.B., Lotze, M.T., Dutcher, J.P., Fisher, R.I., *et al.* High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol* **17,** 2105-2116 (1999).

348. Ribas, A., Butterfield, L.H., Glaspy, J.A. & Economou, J.S. Current developments in cancer vaccines and cellular immunotherapy. *J Clin Oncol* **21,** 2415-2432 (2003).

349. Rosenberg, S.A. Cell transfer immunotherapy for metastatic solid cancer - what clinicians need to know. *Nat Rev Clin Oncol* **8,** 577-585 (2011).

350. Rosenberg, S.A., Yang, J.C., Sherry, R.M., Kammula, U.S., *et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* **17,** 4550-4557 (2011).

351. Sharpe, A.H. Mechanisms of costimulation. *Immunol Rev* **229,** 5-11 (2009).

352. Schadendorf, D., Hodi, F.S., Robert, C., Weber, J.S., *et al.* Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in

unresectable or metastatic melanoma. *J Clin Oncol* pii: JCO.2014.56.2736. [Epub ahead of print] (2015).

353. Maio, M., Grob, J.J., Aamdal, S., Bondarenko, I., *et al.* Five-year survival rates for treatment-naive patients with advanced melanoma who received ipilimumab plus dacarbazine in a phase III trial. *J Clin Oncol* pii: JCO.2014.56.6018. [Epub ahead of print] (2015).

354. Robert, C., Ribas, A., Wolchok, J.D., Hodi, F.S., *et al.* Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *Lancet* **384,** 1109-1117 (2014).

355. Robert, C., Long, G.V., Brady, B., Dutriaux, C., *et al.* Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* **372,** 320-330 (2014).

356. Topalian, S.L., Sznol, M., McDermott, D.F., Kluger, H.M., *et al.* Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol* **32,** 1020-1030 (2014).

357. Hamid, O., Robert, C., Daud, A., Hodi, F.S., *et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* **369,** 134-144 (2013).

358. Topalian, S.L., Hodi, F.S., Brahmer, J.R., Gettinger, S.N., *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366,** 2443-2454 (2012).

359. Webster, R.M. & Mentzer, S.E. The malignant melanoma landscape. *Nat Rev Drug Discov* **13,** 491-492 (2014).

360. Wyller, T.B. [Politics or quasi-science?] *Tidsskr Nor Laegeforen* **134,** 320-322 (2014).

361. Snyder, A., Makarov, V., Merghoub, T., Yuan, J., *et al.* Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* **371,** 2189-2199 (2014).

362. Wolchok, J.D., Kluger, H., Callahan, M.K., Postow, M.A., *et al.* Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* **369,** 122-133 (2013).

363. Ribas, A., Hodi, F.S., Callahan, M., Konto, C. & Wolchok J, Hepatotoxicity with combination of vemurafenib and ipilimumab. *N Engl J Med* **368,** 1365-1366 (2013).

364. Carvajal, R.D., Antonescu, C.R., Wolchok, J.D., Chapman, P.B., *et al.* KIT as a therapeutic target in metastatic melanoma. *JAMA* **305,** 2327-2334 (2011).

365. Guo, J., Si, L., Kong, Y., Flaherty, K.T., *et al.* Phase II, open-label, single-arm trial of imatinib mesylate in patients with metastatic melanoma harboring c-KIT mutation or amplification. *J Clin Oncol* **29,** 2904-2909 (2011).

366. Hodi, F.S., Corless, C.L., Giobbie-Hurder, A., Fletcher, J.A., *et al.* Imatinib for melanomas harboring mutationally activated or amplified KIT arising on mucosal, acral, and chronically sun-damaged skin. *J Clin Oncol* **31,** 3182-3190 (2013).

367. Quintás-Cardama, A., Lazar, A.J., Woodman, S.E., Kim, K., *et al.* Complete response of stage IV anal mucosal melanoma expressing KIT Val560Asp to the multikinase inhibitor sorafenib. *Nat Clin Pract Oncol* **5,** 737-740 (2008).

368. Woodman, S.E., Trent, J.C., Stemke-Hale, K., Lazar, A.J., *et al.* Activity of dasatinib against L576P KIT mutant melanoma: molecular, cellular, and clinical correlates. *Mol Cancer Ther* **8,** 2079-2085 (2009).

369. Joseph, R.W., Sullivan, R.J., Harrell, R., Stemke-Hale, K., *et al.* Correlation of NRAS mutations with clinical response to high-dose IL-2 in patients with advanced melanoma. *J Immunother* **35,** 66-72 (2012).

370. Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363,** 711-723 (2010).

371. Kim, G., McKee, A.E., Ning, Y.M., Hazarika, M., *et al.* FDA Approval summary: vemurafenib for treatment of unresectable or metastatic melanoma with the BRAFV600E mutation. *Clin Cancer Res* **20,** 4994-5000 (2014).

372. Postow, M.A., Callahan, M.K. & Wolchok, J.D. Immune checkpoint blockade in cancer therapy. *J Clin Oncol* pii: JCO.2014.59.4358. [Epub ahead of print] (2015).

373. Robert, C., Thomas, L., Bondarenko, I., O'Day, S., *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* **364,** 2517-2526 (2011).

374. Sosman, J.A., Kim, K.B., Schuchter, L., Gonzalez, R., *et al.* Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med* **366,** 707-714 (2012).

375. Robert, C., Dummer, R., Gutzmer, R., Lorigan, P., *et al.* Selumetinib plus dacarbazine versus placebo plus dacarbazine as first-line treatment for BRAF-mutant metastatic melanoma: a phase 2 double-blind randomised study. *Lancet Oncol* **14,** 733-740 (2013).

376. Robert, C., Karaszewska, B., Schachter, J., Rutkowski, P., *et al.* Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med* **372,** 30-39 (2015).

377. Ribas, A., Gonzalez, R., Pavlick, A., Hamid, O., *et al.* Combination of vemurafenib and cobimetinib in patients with advanced BRAFV600-mutated melanoma: a phase 1b study. *Lancet Oncol* **15,** 954-965 (2014).

378. Ribas, A., Kefford, R., Marshall, M.A., Punt, C.J., *et al.* Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma. *J Clin Oncol* **31,** 616-622 (2013).

379. McArthur, G.A., Chapman, P.B., Robert, C., Larkin, J., *et al.* Safety and efficacy of vemurafenib in BRAFV600E and BRAFV600K mutation-positive melanoma (BRIM-3): extended follow-up of a phase 3, randomised, open-label study. *Lancet Oncol* **15,** 323-332 (2014).

380. Long, G.V., Stroyakovskiy, D., Gogas, H., Levchenko, E., *et al.* Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N Engl J Med* **371,** 1877-1888 (2014).

381. Larkin, J., Ascierto, P.A., Dréno, B., Atkinson, V., *et al.* Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N Engl J Med* **371,** 1867-1876 (2014).

382. Johnson, D.B., Flaherty, K.T., Weber, J.S., Infante, J.R., *et al.* Combined BRAF (dabrafenib) and MEK inhibition (trametinib) in patients with BRAFV600-mutant melanoma experiencing progression with single-agent BRAF inhibitor. *J Clin Oncol* **32,** 3697-3704 (2014).

383. Hodi, F.S., Lee, S., McDermott, D.F., Rao, U.N., *et al.* Ipilimumab plus sargramostim vs ipilimumab alone for treatment of metastatic melanoma: a randomized clinical trial. *JAMA* **312,** 1744-1753 (2014).

384. Flaherty, K.T., Robert, C., Hersey, P., Nathan, P., *et al.* Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med* **367,** 107-114 (2012).

385. Flaherty, K.T., Infante, J.R., Daud, A., Gonzalez, R., *et al.* Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* **367,** 1694-1703 (2012).

386. Flaherty, K.T., Lee, S.J., Zhao, F., Schuchter, L.M., *et al.* Phase III trial of carboplatin and paclitaxel with or without sorafenib in metastatic melanoma. *J Clin Oncol* **31,** 373-379 (2012).

387. Flaherty, K.T., Puzanov, I., Kim, K.B., Ribas, A., *et al.* Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* **363,** 809-819 (2010).

388. Chiarion-Sileni, V., Pigozzo, J., Ascierto, P.A., Simeone, E., *et al.* Ipilimumab retreatment in patients with pretreated advanced melanoma: the expanded access programme in Italy. *Br J Cancer* **110,** 1721-1726 (2014).

389. Ascierto, P.A., Schadendorf, D., Berking, C., Agarwala, S.S., *et al.* MEK162 for patients with advanced melanoma harbouring NRAS or Val600 BRAF mutations: a non-randomised, open-label phase 2 study. *Lancet Oncol* **14,** 249-256 (2013).

390. Ascierto, P.A., Minor, D., Ribas, A., Lebbe, C., *et al.* Phase II trial (BREAK-2) of the BRAF inhibitor dabrafenib (GSK2118436) in patients with metastatic melanoma. *J Clin Oncol* **31,** 3205-3211 (2013).

391. Kaufman, H.L., Kirkwood, J.M., Hodi, F.S., Agarwala, S., *et al.* The Society for Immunotherapy of Cancer consensus statement on tumour immunotherapy for the treatment of cutaneous melanoma. *Nat Rev Clin Oncol* **10,** 588-598 (2013).

392. Rizos, H., Menzies, A.M., Pupo, G.M., Carlino, M.S., *et al.* BRAF inhibitor resistance mechanisms in metastatic melanoma: spectrum and clinical impact. *Clin Cancer Res* **20,** 1965-1977 (2014).

393. Shi, H., Hugo, W., Kong, X., Hong, A., *et al.* Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. *Cancer Discov* **4,** 80-93 (2014).

394. Trunzer, K., Pavlick, A.C., Schuchter, L., Gonzalez, R., *et al.* Pharmacodynamic effects and mechanisms of resistance to vemurafenib in patients with metastatic melanoma. *J Clin Oncol* **31,** 1767-1774 (2013).

395. Van Allen, E.M., Wagle, N., Sucker, A., Treacy, D.J., *et al.* The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer Discov* **4,** 94-109 (2014).

396. Nazarian, R., Shi, H., Wang, Q., Kong, X., *et al.* Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* **468,** 973-977 (2010).

397. Tap, W.D., Gong, K.W., Dering, J., Tseng, Y., *et al.* Pharmacodynamic characterization of the efficacy signals due to selective BRAF inhibition with PLX4032 in malignant melanoma. *Neoplasia* **12,** 637-649 (2010).

398. Wang, H., Daouti, S., Li, W.H., Wen, Y., *et al.* Identification of the MEK1(F129L) activating mutation as a potential mechanism of acquired resistance to MEK inhibition in human cancers carrying the B-RafV600E mutation. *Cancer Res* **71,** 5535-5545 (2011).

399. Johannessen, C.M., Boehm, J.S., Kim, S.Y., Thomas, S R., *et al.* COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* **468,** 968-972 (2010).

400. Montagut, C., Sharma, S.V., Shioda, T., McDermott, U., *et al.* Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. *Cancer Res* **68,** 4853-4861 (2008).

401. Shi, H., Moriceau, G., Kong, X., Lee, M.K., *et al.* Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat Commun* **3,** 724 (2012).

402. Whittaker, S.R., Theurillat, J.P., Van Allen, E., Wagle, N., *et al.* A genome-scale RNA interference screen implicates NF1 loss in resistance to RAF inhibition. *Cancer Discov* **3,** 350-362 (2013).

403. Poulikakos, P.I., Persaud, Y., Janakiraman, M., Kong, X., *et al.* RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature* **480,** 387-390 (2011).

404. Villanueva, J., Vultur, A., Lee, J.T., Somasundaram, R., *et al.* Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* **18,** 683-695 (2010).

405. Shi, H., Hong, A., Kong, X., Koya, R.C., *et al.* A novel AKT1 mutant amplifies an adaptive melanoma response to BRAF inhibition. *Cancer Discov* **4,** 69-79 (2014).

406. Vazquez, F., Lim, J.H., Chim, H., Bhalla, K., *et al.* PGC1α expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress. *Cancer Cell* **23,** 287-301 (2013).

407. Haq, R., Shoag, J., Andreu-Perez, P., Yokoyama, S., *et al.* Oncogenic BRAF regulates oxidative metabolism via PGC1α and MITF. *Cancer Cell* **23,** 302-315 (2013).

408. Nathanson, K.L., Martin, A.M., Wubbenhorst, B., Greshock, J., *et al.* Tumor genetic analyses of patients with metastatic melanoma treated with the BRAF inhibitor dabrafenib (GSK2118436). *Clin Cancer Res* **19,** 4868-4878 (2013).

409. Smalley, K.S., Lioni, M., Dalla Palma, M., Xiao, M., *et al.* Increased cyclin D1 expression can mediate BRAF inhibitor resistance in BRAF V600E-mutated melanomas. *Mol Cancer Ther* **7,** 2876-2883 (2008).

410. Haq, R., Yokoyama, S., Hawryluk, E.B., Jönsson, G.B., *et al.* BCL2A1 is a lineage-specific antiapoptotic melanoma oncogene that confers resistance to BRAF inhibition. *Proc Natl Acad Sci U S A* **110,** 4321-4326 (2013).

411. Straussman, R., Morikawa, T., Shee, K., Barzily-Rokni, M., *et al.* Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* **487,** 500-504 (2012).

412. Wilson, T.R., Fridlyand, J., Yan, Y., Penuel, E., *et al.* Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature* **487,** 505-509 (2012).

413. Fedorenko, I.V., Gibney, G.T., Sondak, V.K. & Smalley, K.S. Beyond BRAF: where next for melanoma therapy? *Br J Cancer* **112,** 217-226 (2015).

414. Kwong, L.N. & Davies, M.A. Targeted therapy for melanoma: rational combinatorial approaches. *Oncogene* **33,** 1-9 (2014).

415. Das Thakur, M., Salangsang, F., Landman, A.S., Sellers, W.R., *et al.* Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. *Nature* **494,** 251-255 (2013).

416. Morris, E.J., Jha, S., Restaino, C.R., Dayananth, P., *et al.* Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discov* **3,** 742-750 (2013).

417. Brady, D.C., Crowe, M.S., Turski, M.L., Hobbs, G.A., *et al.* Copper is required for oncogenic BRAF signalling and tumorigenesis. *Nature* (2014).

418. Acquaviva, J., Smith, D.L., Jimenez, J.P., Zhang, C., *et al.* Overcoming acquired BRAF inhibitor resistance in melanoma via targeted inhibition of Hsp90 with ganetespib. *Mol Cancer Ther* **13,** 353-363 (2014).

419. Atefi, M., von Euw, E., Attar, N., Ng, C., *et al.* Reversing melanoma cross-resistance to BRAF and MEK inhibitors by co-targeting the AKT/mTOR pathway. *PLoS One* **6,** e28973 (2011).

420. Fedorenko, I.V., Gibney, G.T. & Smalley, K.S. NRAS mutant melanoma: biological behavior and future strategies for therapeutic management. *Oncogene* **32,** 3009-3018 (2013).

421. Xia, J., Jia, P., Hutchinson, K.E., Dahlman, K.B., *et al.* A meta-analysis of somatic mutations from next generation sequencing of 241 melanomas: a road map for the study of genes with potential clinical relevance. *Mol Cancer Ther* **13,** 1918-1928 (2014).

422. Flaherty, K.T., Hodi, F.S. & Fisher, D.E. From genes to drugs: targeted strategies for melanoma. *Nat Rev Cancer* **12,** 349-361 (2012).

423. Garbe, C., Eigentler, T.K., Keilholz, U., Hauschild, A. & Kirkwood, J.M. Systematic review of medical treatment in melanoma: current status and future prospects. *Oncologist* **16,** 5-24 (2011).

424. Strickland, L.R., Pal, H.C., Elmets, C.A. & Afaq, F. Targeting drivers of melanoma with synthetic small molecules and phytochemicals. *Cancer Lett* **359,** 20-35 (2015).

425. Villanueva, J., Vultur, A. & Herlyn, M. Resistance to BRAF inhibitors: unraveling mechanisms and future treatment options. *Cancer Res* **71,** 7137-7140 (2011).

426. Abildgaard, C. & Guldberg, P. Molecular drivers of cellular metabolic reprogramming in melanoma. *Trends Mol Med* **21,** 164-171 (2015).

427. Haq, R., Fisher, D.E. & Widlund, H.R. Molecular pathways: BRAF induces bioenergetic adaptation by attenuating oxidative phosphorylation. *Clin Cancer Res* **20,** 2257-2263 (2014).

428. Damsky, W.E., Theodosakis, N. & Bosenberg, M. Melanoma metastasis: new concepts and evolving paradigms. *Oncogene* **33,** 2413-2422 (2014).

429. Fidler, I.J., Schackert, G., Zhang, R.D., Radinsky, R. & Fujimaki, T. The biology of melanoma brain metastasis. *Cancer Metastasis Rev* **18,** 387-400 (1999).

430. Sul, J. & Posner, J.B. Brain metastases: epidemiology and pathophysiology. *Cancer Treat Res* **136,** 1-21 (2007).

431. Braeuer, R.R., Watson, I.R., Wu, C.J., Mobley, A.K., *et al.* Why is melanoma so metastatic? *Pigment Cell Melanoma Res* **27,** 19-36 (2013).

432. Palmieri, D., Chambers, A.F., Felding-Habermann, B., Huang, S. & Steeg, P.S. The biology of metastasis to a sanctuary site. *Clin Cancer Res* **13,** 1656-1662 (2007).

433. Bos, P.D., Zhang, X.H., Nadal, C., Shu, W., *et al.* Genes that mediate breast cancer metastasis to the brain. *Nature* **459,** 1005-1009 (2009).

434. Conley, F.K. Development of a metastatic brain tumor model in mice. *Cancer Res* **39,** 1001-1007 (1979).

435. Cranmer, L.D., Trevor, K.T., Bandlamuri, S. & Hersh, E.M. Rodent models of brain metastasis in melanoma. *Melanoma Res* **15,** 325-356 (2005).

436. Sausville, E.A. & Burger, A.M. Contributions of human tumor xenografts to anticancer drug development. *Cancer Res* **66,** 3351-3354 (2006).

437. Ushio, Y., Chernik, N.L., Shapiro, W.R. & Posner, J.B. Metastatic tumor of the brain: development of an experimental model. *Ann Neurol* **2,** 20-29 (1977).

438. Launer, R.L. & Wilkinson, G.N. Robustness in statistics. *Academic Press Inc., USA* (1974).

439. Kircher, M.F., Gambhir, S.S. & Grimm, J. Noninvasive cell-tracking methods. *Nat Rev Clin Oncol* **8,** 677-688 (2011).

440. Keunen, O., Taxt, T., Grüner, R., Lund-Johansen, M., *et al.* Multimodal imaging of gliomas in the context of evolving cellular and molecular therapies. *Adv Drug Deliv Rev* **76,** 98-115 (2014).

441. Thorsen, F., Fite, B., Mahakian, L.M., Seo, J.W., *et al.* Multimodal imaging enables early detection and characterization of changes in tumor permeability of brain metastases. *J Control Release* **172,** 812-822 (2013).

442. Rosen, J.E., Chan, L., Shieh, D.B. & Gu, F.X. Iron oxide nanoparticles for targeted cancer imaging and diagnostics. *Nanomedicine* **8,** 275-290 (2011).

443. Connell, J.J., Chatain, G., Cornelissen, B., Vallis, K.A., *et al.* Selective permeabilization of the blood-brain barrier at sites of metastasis. *J Natl Cancer Inst* **105,** 1634-1643 (2013).

444. Serres, S., Soto, M.S., Hamilton, A., McAteer, M.A., *et al.* Molecular MRI enables early and sensitive detection of brain metastases. *Proc Natl Acad Sci U S A* **109,** 6674-6679 (2012).

445. Paget, S. The distribution of secondary growths in cancer of the breast. *Lancet* **1,** 99-101 (1889).

446. Hart, I.R. & Fidler, I.J. Role of organ selectivity in the determination of metastatic patterns of B16 melanoma. *Cancer Res* **40,** 2281-2287 (1980).

447. Logan, P.T., Fernandes, B.F., Di Cesare, S., Marshall, J.C., *et al.* Single-cell tumor dormancy model of uveal melanoma. *Clin Exp Metastasis* **25,** 509-516 (2008).

448. Nicolson, G.L., Brunson, K.W. & Fidler, I.J. Specificity of arrest, survival, and growth of selected metastatic variant cell lines. *Cancer Res* **38,** 4105-4111 (1978).

449. Eccles, S.A. & Welch, D.R. Metastasis: recent discoveries and novel treatment strategies. *Lancet* **369,** 1742-1757 (2007).

450. Chen, Y. & Rittling, S.R. Novel murine mammary epithelial cell lines that form osteolytic bone metastases: effect of strain background on tumor homing. *Clin Exp Metastasis* **20,** 111-120 (2003).

451. Kienast, Y., von Baumgarten, L., Fuhrmann, M., Klinkert, W.E., *et al.* Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med* **16,** 116-122 (2010).

452. Carbonell, W.S., Ansorge, O., Sibson, N. & Muschel, R. The vascular basement membrane as "soil" in brain metastasis. *PLoS One* **4,** e5857 (2009).

453. Lorger, M. & Felding-Habermann, B. Capturing changes in the brain microenvironment during initial steps of breast cancer brain metastasis. *Am J Pathol* **176,** 2958-2971 (2010).

454. Mahar Doan, K.M., Humphreys, J.E., Webster, L.O., Wring, S.A., *et al.* Passive permeability and P-glycoprotein-mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. *J Pharmacol Exp Ther* **303,** 1029-1037 (2002).

455. Pardridge, W.M. Drug transport across the blood-brain barrier. *J Cereb Blood Flow Metab* **32,** 1959-1972 (2012).

456. Gerstner, E.R. & Fine, R.L. Increased permeability of the blood-brain barrier to chemotherapy in metastatic brain tumors: establishing a treatment paradigm. *J Clin Oncol* **25,** 2306-2312 (2007).

457. Lockman, P.R., Mittapalli, R.K., Taskar, K.S., Rudraraju, V., *et al.* Heterogeneous blood-tumor barrier permeability determines drug efficacy in experimental brain metastases of breast cancer. *Clin Cancer Res* **16,** 5664-5678 (2010).

458. Chen, G., Chakravarti, N., Aardalen, K., Lazar, A.J., *et al.* Molecular profiling of patient-matched brain and extracranial melanoma metastases implicates the PI3K pathway as a therapeutic target. *Clin Cancer Res* **20,** 5537-5546 (2014).

459. Niessner, H., Forschner, A., Klumpp, B., Honegger, J.B., *et al.* Targeting hyperactivation of the AKT survival pathway to overcome therapy resistance of melanoma brain metastases. *Cancer Med* **2,** 76-85 (2013).

460. Capper, D., Berghoff, A.S., Magerle, M., Ilhan, A., *et al.* Immunohistochemical testing of BRAF V600E status in 1,120 tumor tissue samples of patients with brain metastases. *Acta Neuropathol* **123,** 223-233 (2012).

461. Govindarajan, B., Sligh, J.E., Vincent, B.J., Li, M., *et al.* Overexpression of Akt converts radial growth melanoma to vertical growth melanoma. *J Clin Invest* **117,** 719-729 (2007).

462. Davies, M.A., Stemke-Hale, K., Lin, E., Tellez, C., *et al.* Integrated molecular and clinical analysis of AKT activation in metastatic melanoma. *Clin Cancer Res* **15,** 7538-7546 (2009).

463. Bucheit, A.D., Chen, G., Siroy, A.E., Tetzlaff, M., *et al.* Complete loss of PTEN protein expression correlates with shorter time to brain metastasis and survival in stage IIIB/C melanoma patients with BRAFV600 mutations. *Clin Cancer Res* **20,** 5527-5536 (2014).

464. Huang, F.J., Steeg, P.S., Price, J.E., Chiu, W.T., *et al.* Molecular basis for the critical role of suppressor of cytokine signaling-1 in melanoma brain metastasis. *Cancer Res* **68,** 9634-9642 (2008).

465. Tekle, C., Nygren, M.K., Chen, Y.W., Dybsjord, I., *et al.* B7-H3 contributes to the metastatic capacity of melanoma cells by modulation of known metastasis-associated genes. *Int J Cancer* **130,** 2282-2290 (2012).

466. Lee, I., Fox, P.S., Ferguson, S.D., Bassett, R., *et al.* The expression of p-STAT3 in stage IV melanoma: risk of CNS metastasis and survival. *Oncotarget* **3,** 336-344 (2012).

467. Soto, M.S., Serres, S., Anthony, D.C. & Sibson, N.R. Functional role of endothelial adhesion molecules in the early stages of brain metastasis. *Neuro Oncol* **16,** 540-551 (2013).

468. Langley, R.R., Carlisle, R., Ma, L., Specian, R.D., *et al.* Endothelial expression of vascular cell adhesion molecule-1 correlates with metastatic pattern in spontaneous melanoma. *Microcirculation* **8,** 335-345 (2001).

469. Bogunovic, D., O'Neill, D.W., Belitskaya-Levy, I., Vacic, V., *et al.* Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci U S A* **106,** 20429-20434 (2009).

470. Murakami, T., Cardones, A.R. & Hwang, S.T. Chemokine receptors and melanoma metastasis. *J Dermatol Sci* **36,** 71-78 (2004).

471. Izraely, S., Klein, A., Sagi-Assif, O., Meshel, T., *et al.* Chemokine-chemokine receptor axes in melanoma brain metastasis. *Immunol Lett* **130,** 107-114 (2010).

472. Klein, A., Sagi-Assif, O., Izraely, S., Meshel, T., *et al.* The metastatic microenvironment: Brain-derived soluble factors alter the malignant phenotype of cutaneous and brain-metastasizing melanoma cells. *Int J Cancer* **131,** 2509-2518 (2012).

473. Lee, B.C., Lee, T.H., Avraham, S. & Avraham, H.K. Involvement of the chemokine receptor CXCR4 and its ligand stromal cell-derived factor 1alpha in breast cancer cell migration through human brain microvascular endothelial cells. *Mol Cancer Res* **2,** 327-338 (2004).

474. Maraveyas, A., Johnson, M.J., Xiao, Y.P. & Noble, S. Malignant melanoma as a target malignancy for the study of the anti-metastatic properties of the heparins. *Cancer Metastasis Rev* **29,** 777-784 (2010).

475. Fazakas, C., Wilhelm, I., Nagyőszi, P., Farkas, A.E., *et al.* Transmigration of Melanoma Cells through the Blood-Brain Barrier: Role of Endothelial Tight Junctions and Melanoma-Released Serine Proteases. *PLoS One* **6,** e20758 (2011).

476. Stoletov, K., Strnadel, J., Zardouzian, E., Momiyama, M., *et al.* Role of connexins in metastatic breast cancer and melanoma brain colonization. *J Cell Sci* **126,** 904-913 (2013).

477. Lin, Q., Balasubramanian, K., Fan, D., Kim, S.J., *et al.* Reactive astrocytes protect melanoma cells from chemotherapy by sequestering intracellular calcium through gap junction communication channels. *Neoplasia* **12,** 748-754 (2010).

478. Cruz-Munoz, W., Jaramillo, M.L., Man, S., Xu, P., *et al.* Roles for endothelin receptor B and BCL2A1 in spontaneous CNS metastasis of melanoma. *Cancer Res* **72,** 4909-4919 (2012).

479. Bagnato, A., Rosanò, L., Spinella, F., Di Castro, V., *et al.* Endothelin B receptor blockade inhibits dynamics of cell interactions and communications in melanoma cell progression. *Cancer Res* **64,** 1436-1443 (2004).

480. Murry, B.P., Blust, B.E., Singh, A., Foster, T.P. & Marchetti, D. Heparanase mechanisms of melanoma metastasis to the brain: Development and use of a brain slice model. *J Cell Biochem* **97,** 217-225 (2006).

481. Marchetti, D., Li, J. & Shen, R. Astrocytes contribute to the brain-metastatic specificity of melanoma cells by producing heparanase. *Cancer Res* **60,** 4767-4770 (2000).

482. Denkins, Y., Reiland, J., Roy, M., Sinnappah-Kang, N.D., *et al.* Brain metastases in melanoma: roles of neurotrophins. *Neuro Oncol* **6,** 154-165 (2004).

483. Zhang, L., Sullivan, P.S., Goodman, J.C., Gunaratne, P.H. & Marchetti, D. MicroRNA-1258 suppresses breast cancer brain metastasis by targeting heparanase. *Cancer Res* **71,** 645-654 (2011).

484. Ridgway, L.D., Wetzel, M.D. & Marchetti, D. Modulation of GEF-H1 induced signaling by heparanase in brain metastatic melanoma cells. *J Cell Biochem* **111,** 1299-1309 (2010).

485. Zhang, C., Zhang, F., Tsan, R. & Fidler, I.J. Transforming growth factor-beta2 is a molecular determinant for site-specific melanoma metastasis in the brain. *Cancer Res* **69,** 828-835 (2009).

486. Fakhrai, H., Dorigo, O., Shawler, D.L., Lin, H., *et al.* Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci U S A* **93,** 2909-2914 (1996).

487. Sun, C., Wang, L., Huang, S., Heynen, G.J., *et al.* Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. *Nature* **508,** 118-122 (2014).

488. Rolland, Y., Demeule, M., Fenart, L. & Béliveau, R. Inhibition of melanoma brain metastasis by targeting melanotransferrin at the cell surface. *Pigment Cell Melanoma Res* **22,** 86-98 (2009).

489. Perides, G., Zhuge, Y., Lin, T., Stins, M.F., *et al.* The fibrinolytic system facilitates tumor cell migration across the blood-brain barrier in experimental melanoma brain metastasis. *BMC Cancer* **6,** 56 (2006).

490. Schroder, W.A., Major, L.D., Le, T.T., Gardner, J., *et al.* Tumor cell-expressed SerpinB2 is present on microparticles and inhibits metastasis. *Cancer Med* **3,** 500-513 (2014).

491. Valiente, M., Obenauf, A.C., Jin, X., Chen, Q., *et al.* Serpins promote cancer cell survival and vascular co-option in brain metastasis. *Cell* **156,** 1002-1016 (2014).

492. Alsidawi, S., Malek, E. & Driscoll, J.J. MicroRNAs in brain metastases: potential role as diagnostics and therapeutics. *Int J Mol Sci* **15,** 10508-10526 (2014).

493. Subramani, A., Alsidawi, S., Jagannathan, S., Sumita, K., *et al.* The brain microenvironment negatively regulates miRNA-768-3p to promote K-ras expression and lung cancer metastasis. *Sci Rep* **3,** 2392 (2013).

494. Camacho, L., Guerrero, P. & Marchetti, D. MicroRNA and protein profiling of brain metastasis competent cell-derived exosomes. *PLoS One* **8,** e73790 (2013).

495. Hwang, S.J., Seol, H.J., Park, Y.M., Kim, K.H., *et al.* MicroRNA-146a suppresses metastatic activity in brain metastasis. *Mol Cells* **34,** 329-334 (2012).

496. Chen, J., Lee, H.J., Wu, X., Huo, L., *et al.* Gain of glucose-independent growth upon metastasis of breast cancer cells to the brain. *Cancer Res* **75,** 554-565 (2014).

497. Chen, E.I., Hewel, J., Krueger, J.S., Tiraby, C., *et al.* Adaptation of energy metabolism in breast cancer brain metastases. *Cancer Res* **67,** 1472-1486 (2007).

498. Neman, J., Termini, J., Wilczynski, S., Vaidehi, N., *et al.* Human breast cancer metastases to the brain display GABAergic properties in the neural niche. *Proc Natl Acad Sci U S A* **111,** 984-989 (2014).

499. Park, E.S., Kim, S.J., Kim, S.W., Yoon, S.L., *et al.* Cross-species hybridization of microarrays for studying tumor transcriptome of brain metastasis. *Proc Natl Acad Sci U S A* **108,** 17456-17561 (2011).

500. Palorini, R., Simonetto, T., Cirulli, C. & Chiaradonna, F. Mitochondrial complex I inhibitors and forced oxidative phosphorylation synergize in inducing cancer cell death. *Int J Cell Biol* **2013,** 243876 (2013).

501. Hall, A., Meyle, K.D., Lange, M.K., Klima, M., *et al.* Dysfunctional oxidative phosphorylation makes malignant melanoma cells addicted to glycolysis driven by the (V600E)BRAF oncogene. *Oncotarget* **4,** 584-599 (2013).

502. Abildgaard, C., Dahl, C., Basse, A.L., Ma, T. & Guldberg, P. Bioenergetic modulation with dichloroacetate reduces the growth of melanoma cells and potentiates their response to BRAFV600E inhibition. *J Transl Med* **12,** 247 (2014).

503. Barbi de Moura, M., Vincent, G., Fayewicz, S.L., Bateman, N.W., *et al.* Mitochondrial respiration - an important therapeutic target in melanoma. *PLoS One* **7,** e40690 (2012).

504. Ho, J., de Moura, M.B., Lin, Y., Vincent, G., *et al.* Importance of glycolysis and oxidative phosphorylation in advanced melanoma. *Mol Cancer* **11,** 76 (2012).

505. Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., *et al.* Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* **271,** 32529-32537 (1996).

506. Vander Heiden, M.G., Cantley, L.C. & Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324,** 1029-1033 (2009).

507. Warburg, O. On the origin of cancer cells. *Science* **123,** 309-314 (1956).

508. Parmenter, T.J., Kleinschmidt, M., Kinross, K.M., Bond, S.T., *et al.* Response of BRAF-mutant melanoma to BRAF inhibition is mediated by a network of transcriptional regulators of glycolysis. *Cancer Discov* **4,** 423-433 (2014).

509. Kaplon, J., Zheng, L., Meissl, K., Chaneton, B., *et al.* A key role for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence. *Nature* **498,** 109-112 (2013).

510. Corazao-Rozas, P., Guerreschi, P., Jendoubi, M., André, F., *et al.* Mitochondrial oxidative stress is the Achille's heel of melanoma cells resistant to Braf-mutant inhibitor. *Oncotarget* **4,** 1986-1998 (2013).

511. Gopal, Y.N., Rizos, H., Chen, G., Deng, W., *et al.* Inhibition of mTORC1/2 overcomes resistance to MAPK pathway inhibitors mediated by PGC1α and oxidative phosphorylation in melanoma. *Cancer Res* **74,** 7037-7047 (2014).

512. Niehr, F., von Euw, E., Attar, N., Guo, D., *et al.* Combination therapy with vemurafenib (PLX4032/RG7204) and metformin in melanoma cell lines with distinct driver mutations. *J Transl Med* **9,** 76 (2011).

513. Yuan, P., Ito, K., Perez-Lorenzo, R., Del Guzzo, C., *et al.* Phenformin enhances the therapeutic benefit of BRAFV600E inhibition in melanoma. *Proc Natl Acad Sci U S A* 110, 18226-18231 (2013).

514. Wheaton, W.W., Weinberg, S.E., Hamanaka, R.B., Soberanes, S., *et al.* Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife* **3,** e02242 (2014).

515. Daphu, I., Sundstrøm, T., Horn, S., Huszthy, P. C., *et al.* In vivo animal models for studying brain metastasis: value and limitations. *Clin Exp Metastasis* **30,** 695-710 (2013).

516. Becher, O.J. & Holland, E.C. Genetically engineered models have advantages over xenografts for preclinical studies. *Cancer Res* **66,** 3355-3358 (2006).

517. Gillet, J.P., Calcagno, A.M., Varma, S., Marino, M., *et al.* Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proc Natl Acad Sci U S A* **108,** 18708-18713 (2011).

518. Domcke, S., Sinha, R., Levine, D.A., Sander, C. & Schultz, N. Evaluating cell lines as tumour models by comparison of genomic profiles. *Nat Commun* **4,** 2126 (2013).

519. de Bono, J.S. & Ashworth, A. Translating cancer research into targeted therapeutics. *Nature* **467,** 543-549 (2010).

520. Daphu, I., Horn, S., Stieber, D., Varughese, J. K., *et al.* in vitro treatment of melanoma brain metastasis by simultaneously targeting the MAPK and PI3K signaling pathways. *Int J Mol Sci* **15,** 8773-8794 (2014).

521. Wang, J., Daphu, I., Pedersen, P.H., Miletic, H., *et al.* A novel brain metastases model developed in immunodeficient rats closely mimics the growth of metastatic brain tumours in patients. *Neuropathol Appl Neurobiol* **37,** 189-205 (2011).

522. Cruz-Munoz, W., Man, S., Xu, P. & Kerbel, R.S. Development of a preclinical model of spontaneous human melanoma central nervous system metastasis. *Cancer Res* **68,** 4500-4505 (2008).

523. Cruz-Muñoz, W. & Kerbel, R.S. Preclinical approaches to study the biology and treatment of brain metastases. *Semin Cancer Biol* **21,** 123-130 (2011).

524. Hutchinson, L. & Kirk, R. High drug attrition rates - where are we going wrong? *Nat Rev Clin Oncol* **8,** 189-190 (2011).

525. Sundstrøm, T., Daphu, I., Wendelbo, I., Hodneland, E., *et al.* Automated tracking of nanoparticle-labeled melanoma cells improves the predictive power of a brain metastasis model. *Cancer Res* **73,** 2445-2456 (2013).

526. Huang, J., Zhong, X., Wang, L., Yang, L. & Mao, H. Improving the magnetic resonance imaging contrast and detection methods with engineered magnetic nanoparticles. *Theranostics* **2,** 86-102 (2012).

527. Heyn, C., Ronald, J.A., Mackenzie, L.T., MacDonald, I.C., *et al.* In vivo magnetic resonance imaging of single cells in mouse brain with optical validation. *Magn Reson Med* **55,** 23-29 (2006).

528. Liu, W. & Frank, J.A. Detection and quantification of magnetically labeled cells by cellular MRI. *Eur J Radiol* **70,** 258-264 (2009).

529. Chun, M.G. & Shaw, R.J. Cancer metabolism in breadth and depth. *Nat Biotechnol* **31,** 505-507 (2013).

530. Koukourakis, M.I., Giatromanolaki, A., Sivridis, E., Bougioukas, G., *et al.* Lactate dehydrogenase-5 (LDH-5) overexpression in non-small-cell lung cancer tissues is linked to tumour hypoxia, angiogenic factor production and poor prognosis. *Br J Cancer* **89,** 877-885 (2003).

531. Koukourakis, M.I., Giatromanolaki, A., Simopoulos, C., Polychronidis, A. & Sivridis, E. Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer. *Clin Exp Metastasis* **22,** 25-30 (2005).

532. Koukourakis, M.I., Giatromanolaki, A., Winter, S., Leek, R., *et al.* Lactate dehydrogenase 5 expression in squamous cell head and neck cancer relates to prognosis following radical or postoperative radiotherapy. *Oncology* **77,** 285-292 (2009).

533. Rizwan, A., Serganova, I., Khanin, R., Karabeber, H., *et al.* Relationships between LDH-A, lactate, and metastases in 4T1 breast tumors. *Clin Cancer Res* **19,** 5158-5169 (2013).

534. Fantin, V.R., St-Pierre, J. & Leder, P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* **9,** 425-434 (2006).

535. Langhammer, S., Najjar, M., Hess-Stumpp, H. & Thierauch, K.H. LDH-A influences hypoxia-inducible factor 1α (HIF1 α) and is critical for growth of HT29 colon carcinoma cells in vivo. *Target Oncol* **6,** 155-162 (2011).

536. Le, A., Cooper, C.R., Gouw, A.M., Dinavahi, R., *et al.* Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci U S A* **107,** 2037-2042 (2010).

537. Rong, Y., Wu, W., Ni, X., Kuang, T., *et al.* Lactate dehydrogenase A is overexpressed in pancreatic cancer and promotes the growth of pancreatic cancer cells. *Tumour Biol* **34,** 1523-1530 (2013).

538. Sheng, S.L., Liu, J.J., Dai, Y.H., Sun, X.G., *et al.* Knockdown of lactate dehydrogenase A suppresses tumor growth and metastasis of human hepatocellular carcinoma. *FEBS J* **279,** 3898-3910 (2012).

539. Xie, H., Valera, V.A., Merino, M.J., Amato, A.M., *et al.* LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer. *Mol Cancer Ther* **8,** 626-635 (2009).

540. Xie, H., Hanai, J., Ren, J. G., Kats, L., *et al.* Targeting lactate dehydrogenase-a inhibits tumorigenesis and tumor progression in mouse models of lung cancer and impacts tumor-initiating cells. *Cell Metab* **19,** 795-809 (2014).

541. Yao, F., Zhao, T., Zhong, C., Zhu, J. & Zhao, H. LDHA is necessary for the tumorigenicity of esophageal squamous cell carcinoma. *Tumour Biol* **34,** 25-31 (2013).

542. Zhang, Y., Zhang, X., Wang, X., Gan, L., *et al.* Inhibition of LDH-A by lentivirus-mediated small interfering RNA suppresses intestinal-type gastric cancer tumorigenicity through the downregulation of Oct4. *Cancer Lett* **321,** 45-54 (2012).

543. Hu, J., Locasale, J.W., Bielas, J.H., O'Sullivan, J., *et al.* Heterogeneity of tumor-induced gene expression changes in the human metabolic network. *Nat Biotechnol* **31,** 522-529 (2013).

544. Lamb, J., Crawford, E.D., Peck, D., Modell, J.W., *et al.* The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* **313,** 1929-1935 (2006).

545. Wang, Z.Y. & Zhang, H.Y. Rational drug repositioning by medical genetics. *Nat Biotechnol* **31,** 1080-1082 (2013).

546. Davis, H.R., Zhu, L.J., Hoos, L.M., Tetzloff, G., *et al.* Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J Biol Chem* **279,** 33586-33592 (2004).

547. Graf, G.A., Li, W.P., Gerard, R.D., Gelissen, I., *et al.* Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. *J Clin Invest* **110,** 659-669 (2002).

548. Awad, A.B. & Fink, C.S. Phytosterols as anticancer dietary components: evidence and mechanism of action. *J Nutr* **130,** 2127-2130 (2000).

549. Shi, C., Wu, F., Zhu, X.C. & Xu, J. Incorporation of beta-sitosterol into the membrane increases resistance to oxidative stress and lipid peroxidation via estrogen receptor-mediated PI3K/GSK3beta signaling. *Biochim Biophys Acta* **1830,** 2538-2544 (2013).

550. Salen, G., Ahrens, E.H. & Grundy, S.M. Metabolism of beta-sitosterol in man. *J Clin Invest* **49,** 952-967 (1970).

551. Fricke, C.B., Schrøder, M., Poulsen, M., von Bergmann, K., *et al.* Increased plant sterol and stanol levels in brain of Watanabe rabbits fed rapeseed oil derived plant sterol or stanol esters. *Br J Nutr* **98,** 890-899 (2007).

552. Jansen, P.J., Lütjohann, D., Abildayeva, K., Vanmierlo, T., *et al.* Dietary plant sterols accumulate in the brain. *Biochim Biophys Acta* **1761,** 445-453 (2006).

553. Vanmierlo, T., Weingärtner, O., van der Pol, S., Husche, C., *et al.* Dietary intake of plant sterols stably increases plant sterol levels in the murine brain. *J Lipid Res* **53,** 726-735 (2012).

554. Dietschy, J.M. & Turley, S.D. Cholesterol metabolism in the brain. *Curr Opin Lipidol* **12,** 105-112 (2001).

555. Turley, S.D., Burns, D.K., Rosenfeld, C.R. & Dietschy, J.M. Brain does not utilize low density lipoprotein-cholesterol during fetal and neonatal development in the sheep. *J Lipid Res* **37,** 1953-1961 (1996).

556. Miettinen, T.A., Puska, P., Gylling, H., Vanhanen, H. & Vartiainen, E. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *N Engl J Med* **333,** 1308-1312 (1995).

557. Berges, R.R., Windeler, J., Trampisch, H.J. & Senge, T. Randomised, placebo-controlled, double-blind clinical trial of beta-sitosterol in patients with

benign prostatic hyperplasia. Beta-sitosterol Study Group. *Lancet* **345,** 1529-1532 (1995).

558. Prager, N., Bickett, K., French, N. & Marcovici, G. A randomized, double-blind, placebo-controlled trial to determine the effectiveness of botanically derived inhibitors of 5-alpha-reductase in the treatment of androgenetic alopecia. *J Altern Complement Med* **8,** 143-152 (2002).

559. Donald, P.R., Lamprecht, J.H., Freestone, M., Albrecht, C.F., *et al.* A randomised placebo-controlled trial of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis. *Int J Tuberc Lung Dis* **1,** 518-522 (1997).

560. Stefanaki, C., Fasoulaki, X., Kouris, A., Caroni, C., *et al.* A randomized trial of efficacy of beta-sitosterol and its glucoside as adjuvant to cryotherapy in the treatment of anogenital warts. *J Dermatolog Treat* eo:1-4 [Epub ahead of print] (2014).

561. Bresson, J.-L., Flynn, A., Heinonen, M., Hulshof, K., *et al.* Plant sterols and blood cholesterol. *The EFSA Journal* **781,** 1-12 (2008).

562. Cui, X., Dai, Q., Tseng, M., Shu, X.O., *et al.* Dietary patterns and breast cancer risk in the shanghai breast cancer study. *Cancer Epidemiol Biomarkers Prev* **16,** 1443-1448 (2007).

563. Torres-Sanchez, L., Galvan-Portillo, M., Wolff, M.S. & Lopez-Carrillo, L. Dietary consumption of phytochemicals and breast cancer risk in Mexican women. *Public Health Nutr* **12,** 825-831 (2009).

564. Mendilaharsu, M., De Stefani, E., Deneo-Pellegrini, H., Carzoglio, J. & Ronco, A. Phytosterols and risk of lung cancer: a case-control study in Uruguay. *Lung Cancer* **21,** 37-45 (1998).

565. De Stefani, E., Boffetta, P., Ronco, A. L., Brennan, P., *et al.* Plant sterols and risk of stomach cancer: a case-control study in Uruguay. *Nutr Cancer* **37,** 140-144 (2000).

566. Nair, P.P., Turjman, N., Kessie, G., Calkins, B., *et al.* Diet, nutrition intake, and metabolism in populations at high and low risk for colon cancer. Dietary cholesterol, beta-sitosterol, and stigmasterol. *Am J Clin Nutr* **40,** 927-930 (1984).

567. Bao, Y., Han, J., Hu, F.B., Giovannucci, E.L., *et al.* Association of nut consumption with total and cause-specific mortality. *N Engl J Med* **369,** 2001-2011 (2013).

568. Awad, A.B., Fink, C.S., Williams, H. & Kim, U. In vitro and in vivo (SCID mice) effects of phytosterols on the growth and dissemination of human prostate cancer PC-3 cells. *Eur J Cancer Prev* **10,** 507-513 (2001).

569. Awad, A.B., Barta, S.L., Fink, C.S. & Bradford, P.G. Beta-sitosterol enhances tamoxifen effectiveness on breast cancer cells by affecting ceramide metabolism. *Mol Nutr Food Res* **52,** 419-426 (2008).

570. Awad, A.B., von Holtz, R.L., Cone, J.P., Fink, C.S. & Chen, Y.C. Beta-sitosterol inhibits growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle. *Anticancer Res* **18,** 471-473 (1998).

571. Choi, J.Y., Choi, E.H., Jung, H.W., Oh, J.S., *et al.* Melanogenesis inhibitory compounds from Saussureae Radix. *Arch Pharm Res* **31,** 294-299 (2008).

572. Vundru, S.S., Kale, R.K. & Singh, R.P. Beta-sitosterol induces G1 arrest and causes depolarization of mitochondrial membrane potential in breast carcinoma MDA-MB-231 cells. *BMC Complement Altern Med* **13,** 280 (2013).

573. Park, C., Moon, D.O., Ryu, C.H., Choi, B.T., *et al.* Beta-sitosterol sensitizes MDA-MB-231 cells to TRAIL-induced apoptosis. *Acta Pharmacol Sin* **29,** 341-348 (2008).

574. Zhao, Y., Chang, S.K., Qu, G., Li, T. & Cui, H. Beta-sitosterol inhibits cell growth and induces apoptosis in SGC-7901 human stomach cancer cells. *J Agric Food Chem* **57,** 5211-5218 (2009).

575. Baskar, A.A., Ignacimuthu, S., Paulraj, G.M. & Al Numair, K.S. Chemopreventive potential of beta-sitosterol in experimental colon cancer model - an in vitro and in vivo study. *BMC Complement Altern Med* **10,** 24 (2010).

576. Sook, S.H., Lee, H.J., Kim, J.H., Sohn, E.J., *et al.* Reactive oxygen species-mediated activation of AMP-activated protein kinase and c-Jun N-terminal kinase plays a critical role in beta-sitosterol-induced apoptosis in multiple myeloma U266 cells. *Phytother Res* **28,** 387-394 (2014).

577. Koschutnig, K., Heikkinen, S., Kemmo, S., Lampi, A.M., *et al.* Cytotoxic and apoptotic effects of single and mixed oxides of beta-sitosterol on HepG2-cells. *Toxicol In Vitro* **23,** 755-762 (2009).

578. Moon, D.O., Lee, K.J., Choi, Y.H. & Kim, G.Y. Beta-sitosterol-induced-apoptosis is mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells. *Int Immunopharmacol* **7,** 1044-1053 (2007).

579. von Holtz, R.L., Fink, C.S. & Awad, A.B. Beta-sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. *Nutr Cancer* **32,** 8-12 (1998).

580. Awad, A.B., Williams, H. & Fink, C.S. Phytosterols reduce in vitro metastatic ability of MDA-MB-231 human breast cancer cells. *Nutr Cancer* **40,** 157-164 (2001).

581. Ju, Y.H., Clausen, L.M., Allred, K.F., Almada, A.L. & Helferich, W.G. Beta-sitosterol, beta-sitosterol glucoside, and a mixture of beta-sitosterol and beta-sitosterol glucoside modulate the growth of estrogen-responsive breast cancer cells in vitro and in ovariectomized athymic mice. *J Nutr* **134,** 1145-1151 (2004).

582. Stoner, G.D., Chen, T., Kresty, L.A., Aziz, R.M., *et al.* Protection against esophageal cancer in rodents with lyophilized berries: potential mechanisms. *Nutr Cancer* **54,** 33-46 (2006).

583. Grattan, B.J. Plant sterols as anticancer nutrients: evidence for their role in breast cancer. *Nutrients* **5,** 359-387 (2013).

584. Koo, H.J., Park, H.J., Byeon, H.E., Kwak, J.H., *et al.* Chinese yam extracts containing β-sitosterol and ethyl linoleate protect against atherosclerosis in apolipoprotein E-deficient mice and inhibit muscular expression of VCAM-1 in vitro. *J Food Sci* **79,** H719-H729 (2014).

585. Plat, J., Nichols, J.A. & Mensink, R.P. Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. *J Lipid Res* **46,** 2468-2476 (2005).

586. Imanaka, H., Koide, H., Shimizu, K., Asai, T., *et al.* Chemoprevention of tumor metastasis by liposomal beta-sitosterol intake. *Biol Pharm Bull* **31,** 400-404 (2008).

587. LeBleu, V.S., O'Connell, J.T., Gonzalez Herrera, K.N., Wikman, H., *et al.* PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol* **16,** 992-1003, 1-15 (2014).

588. Sundstrøm, T., Espedal, H., Harter, P.N., Fasmer, K.E., *et al.* Melanoma brain metastasis is independent of lactate dehydrogenase A expression. *Neuro Oncol* Mar 19 pii: nov040. [Epub ahead of print] (2015)