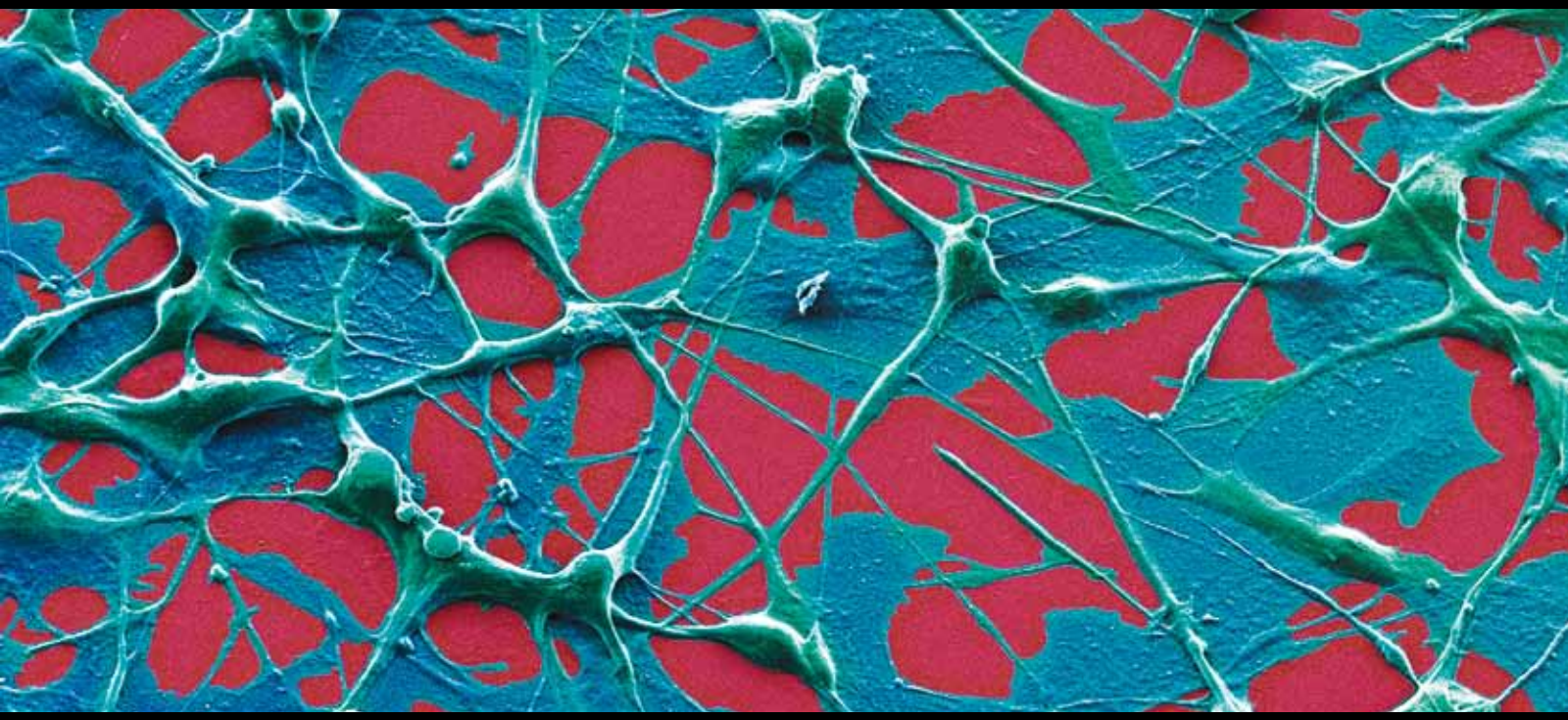
Journal of Skin Cancer

Melanoma: From Research to Treatment

Guest Editors: Paolo A. Ascierto, John M. Kirkwood, Francesco M. Marincola, and Giuseppe Palmieri



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Editorial Board

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Editorial

Melanoma: From Research to Treatment

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The last two decades have deepened our understanding of the biology of melanoma in significant new directions. This progress has resulted in the development of therapeutics with unprecedented clinical potential. Ipilimumab and tremelimumab are anti-CTLA4 monoclonal antibodies that alter the regulatory immune network responsible for damping anticancer immune responses, and this class of agent has shown the first significant impact upon survival of metastatic melanoma. Meanwhile, agents that inhibit activating mutations that drive the oncogenic processes such as B-RAF or c-KIT have shown clear effectiveness and almost immediate responses. It remains uncertain how the current individual treatments work at the level of the tumor during treatment. Moreover, the weight that the genetic background of the host may have in relation to treatment response has yet to be explored. A lot still needs to be done to improve the durability of treatment benefits, and cure remains an elusive goal. The topics presented here are not an exhaustive representation of the field of melanoma research, but a sampling of the large and many-faceted agenda of current interest that we have the pleasure of sharing with the readers. We would like to thank the authors for their excellent contributions and patience in this venture. Finally, the fundamental work of reviewers of the papers is also very warmly acknowledged.

This special issue contains seven papers, three related to epidemiology, diagnosis, risk factors, and characteristics of melanoma. Two papers focus on specific disease treatments, one using the isolated limb infusion (ILI) and the second based on the adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL) for therapy of locally or systemically advanced melanoma, respectively. Also two papers address the management of brain metastases and uveal melanoma.

In the paper entitled "*The contribution of electron paramagnetic resonance to melanoma research*," Q. Godechal and B. Gallez showed how electron paramagnetic resonance (EPR), a method able to detect free radicals trapped in melanin pigments, has recently provided insight to basic features of melanoma that may improve its diagnosis. These advances may improve the diagnosis of melanoma, but the limitations of the method are also detailed.

The paper entitled "*Nonsteroidal anti-inflammatory drugs and risk of melanoma*" presents a pilot study of the association between NSAID usage and melanoma incidence, to determine whether epidemiologic evidence of a chemopreventive effect of these agents is compelling. On the basis of the conflicting reports in the literature, it is suggested that meta-analysis may better establish this possible association.

In the paper entitled "*What is really risky in melanoma? prognostic parameters for the primary care of melanoma patients*," D. Göppner and M. Leverkus reviewed the literature data and summarized current understanding of carcinogenesis in melanoma giving a detailed overview of known morphologic and potentially future genetic prognostic parameters in malignant melanoma.

The paper entitled "*Treatment of locally advanced melanoma by isolated limb infusion with cytotoxic drugs*" overviews isolated limb infusion (ILI), as a less invasive technique than the classical isolated limb perfusion (ILP) which may be preferred for locally advanced melanoma confined to a limb. The minimally invasive character of ILI may replace ILP in the future as palliation for locally advanced limb tumors.

In the paper entitled "*Characterization of ex vivo expanded tumor infiltrating lymphocytes from patients with malignant melanoma for clinical application*," M. H. Andersen et al. report a different method for expanding tumor infiltrating lymphocytes (TIL) to clinically relevant quantities in two steps within 8 weeks. This method may be utilized for new clinical trials, where adoptive transfer of autologous tumor infiltrating lymphocytes (TIL) has shown objective clinical responses in up to 50% of treated patients.

In the paper entitled "*Management of melanoma brain metastases in the era of targeted therapy*," D. G. Shapiro and W. E. Samlowski discuss approaches for melanoma brain metastases using targeted agents combined with classical surgery and radiosurgery, along with the possibility of improving survival in at least a subset of melanoma patients with brain metastases.

In the paper entitled "*Uveal melanoma*," V. M. L. Cohen and V. P. Papastefanou give a 360°C overview uveal melanoma from genetic alterations to diagnosis and treatment.

These studies span the range of etiology and regional as well as systemic therapy for melanoma, to provide a background that may be useful for readers in relation to regional and systemic metastasis, including the most ominous phase of brain metastasis, which punctuate the course of disease. The clear differences between uveal melanoma, a disease nominally included among "the melanomas" are now biologically recognized as diverse in histogenesis and relevant mutational oncogenic pathways. These papers are timely in this year of regulatory approvals for several new agents, and the pending likelihood that new agents will join this armamentarium for melanoma. An area not touched upon is that of adjuvant therapy, which may provide insights to further roles of therapy and ultimately, with chemoprevention, may lessen the burden of advanced disease in the future.

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Review Article

Management of Melanoma Brain Metastases in the Era of Targeted Therapy

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Disseminated metastatic disease, including brain metastases, is commonly encountered in malignant melanoma. The classical treatment approach for melanoma brain metastases has been neurosurgical resection followed by whole brain radiotherapy. Traditionally, if lesions were either too numerous or surgical intervention would cause substantial neurologic deficits, patients were either treated with whole brain radiotherapy or referred to hospice and supportive care. Chemotherapy has not proven effective in treating brain metastases. Improvements in surgery, radiosurgery, and new drug discoveries have provided a wider range of treatment options. Additionally, recently discovered mutations in the melanoma genome have led to the development of "targeted therapy." These vastly improved options are resulting in novel treatment paradigms for approaching melanoma brain metastases in patients with and without systemic metastatic disease. It is therefore likely that improved survival can currently be achieved in at least a subset of melanoma patients with brain metastases.

1. Introduction

It is estimated that metastatic melanoma was responsible for more than 8700 caner-related deaths in the United States in 2010 [1]. Melanoma ranks fourth in the incidence of brain metastases, behind lung, breast, and unknown primary cancers [2]. In addition, metastatic melanoma patients overall have a median survival of only 6–10 months and a 5-year survival of less than 10% [3]. There has been virtually no improvement in survival of those patients in the past several decades [3]. The trend toward targeted therapies [4], novel immunotherapeutic agents [5], stereotactic radiosurgery, and improved neurosurgical interventions give great hope to improving this trend in the coming years.

2. Screening

In our own institutional experience, the risk of brain metastasis in malignant melanoma is approximately 30% at presentation of metastatic disease and may rise to 60% over the next two years in surviving patients [6]. This risk increases substantially with disease duration, as up to 75% of Stage IV melanoma patients are found to have brain metastases at autopsy [7–10]. The implication of this finding is that brain metastases are an almost inevitable part of the disease process, if patients survive long enough. Therefore, the potential development of brain metastases needs to be anticipated in both staging and follow-up strategies. Further evidence of the high risk of brain metastases even in earlier stage disease can be drawn from the recently completed Southwest Oncology Group S0008 adjuvant therapy study. In these Stage IIIb and IIIc patients, there was a 16% isolated CNS failure rate within the first 2 years as the initial site of relapse (Samlowski et al., manuscript in preparation). Even with such a high and alarming incidence, no standard screening recommendations currently exist for Stage III or IV melanoma patients to detect presymptomatic disease. This is in part because of the increasingly outdated perception

that brain metastases represent a terminal event. This has discouraged physicians from attempts at early detection.

Patients presenting with neurologic symptoms, such as seizure or hemiplegia, are commonly found to have either large (greater than 4 cm) or numerous (greater than 7) lesions. These clinical presentations are very difficult to treat and generally become palliative situations.

In contrast to this, less numerous and smaller (<2 cm) lesions encountered in asymptomatic patients are much more readily and effectively treated. It has been shown that MRI scans with gadolinium contrast are superior to CT scans when investigating for brain metastases [11]. Unfortunately, there are no randomized clinical trials, to date, to define the optimal screening strategy (e.g., timing of scans and duration of followup) that would lead to efficient and cost-effective detection. Another caveat is a need for these studies to demonstrate that earlier detection actually leads to improved functional outcome and survival, rather than apparent differences induced by lead-time bias. With current improvements in therapy, it is time, in our opinion, to identify high-risk patients and begin such studies.

3. Palliative Therapy

When dealing with metastatic brain lesion, neurologic symptoms often present suddenly, including devastating headaches, dizziness, and seizures. This may be due to the development of peritumoral edema or bleeding into previously silent metastases. The initial treatment is generally oral or intravenous glucocorticoids. Their anti-inflammatory activity helps to reduce peritumoral edema and swelling and prevent further neurological deficits [12]. Antiepileptic drugs are indicated for treatment of patients who have experienced seizures secondary to brain metastasis [13]. However, studies have shown no benefit in prophylactic treatment of patients with antiepileptic medications. These medications can produce both side effects and potential interactions with chemotherapeutic agents [14].

4. Surgical Resection

Treatment of brain metastases has historically been based on the use of surgical resection, when possible. The consideration of surgical resection is dependent upon the number of lesions, the overall state of systemic disease and symptoms at the time of diagnosis. In general, patients with minimal or no systemic disease, one or two superficial metastases, and excellent functional capacity will benefit from surgery. In this very small group of patients (perhaps 5% overall), surgical intervention has been shown to improve quality of life, as well as survival [10, 15]. Factors adversely affecting the decision to perform surgery include the size of the tumor (greater than 2 cm) and the location, (the ability to resect lesions without significant neurologic sequelae) [16, 17]. This surgical decision-making process is aided by current technology, using stereotactic localization of the lesion at the time of surgery [15]. Additionally, intraoperative functional mapping may help to decrease the risk of neurologic sequelae at the time of resection [18, 19]. It should be noted that surgical resection may also benefit the more severely symptomatic patients, as excision of lesions causing significant edema or herniation can have a valuable palliative effect. After surgery, postoperative WBRT has been shown to lead to longer functional independence for the patients, with decreasing the risk of relapsing at the CNS resection site [20].

5. Whole Brain Radiotherapy (WBRT)

Traditionally, whole brain radiotherapy (e.g., 30 Gy in 10 fractions over 2 weeks elapsed time) has become a *de facto* (but not evidence-based) standard treatment for brain metastasis. Some patients may have significant palliation of symptoms following treatment. Melanoma has traditionally been considered radioresistant to these treatment doses, however [21]. A number of series have established a median survival of 3-4 months. For example, a large recent series from the Sydney Melanoma Unit, encompassing close to 700 patients, showed that resected patients, with or without WBRT had a median survival of 8.7 to 8.9 months, where as patients treated solely with WBRT had a median survival of only 3.4 months. This is clearly a poor outcome, although the patient selection process for surgery versus radiotherapy was not described. As has been seen with all treatment modalities, a patient with excellent performance status and no systemic metastatic disease, and/or a single lesion relapse, WBRT have a somewhat better outcome (termed Recursive Partition Class 1) [22]. In reality, these patients are very uncommon in a medical oncology clinic, where the vast majority of patients have active systemic metastatic disease (Class II or III). Surgery may add survival benefit in selected WBRT-treated patients, such as those with a solitary brain metastasis [23, 24].

6. Radiosurgery

In contrast to large (>4 cm) lesions, smaller metastatic lesions have become easily treated via radiosurgery. In these situations solitary or multiple lesions can be treated with either stereotactic radiosurgery (SRS) or Gamma-knife (GK) treatment. At present, no head-to-head comparisons exists randomizing patient to SRS versus GK. Based on radiobiology studies, it is currently believed that patient outcomes should be comparable between these approaches. The major benefit of radiosurgery is that it allows for treatment of brain lesions that would otherwise be inoperable, including lesions in deep structures and close to functionally critical brain structures. This can be accomplished due to the rapid drop-off of radiation dose at the margin of the treatment volume and the capability of computerized MRI-based dosimetry planning. In small case series local control, survival following radiosurgery (either SRS or GK) has been quite encouraging [25–39].

As with all modalities there are preferred parameters such as lesion size (ideally under 3 cm) and a total number of lesions (7 or less) that make radiosurgery most effective. The maximum number of lesions that can be effectively

treated is still evolving, but is generally functionally defined as the number that can be effectively treated in about 1 hour of patient immobilization. There may be an important biological issue, to consider as well, as patients with oligometastatic disease (1–3 metastases) may have a different biology and outcome to those with many (10–20) metastases. The optimal break point in deciding whether to employ radiosurgery or WBRT as the primary treatment modality remains to be better defined as radiosurgery technology evolves to allow more rapid treatment of increasing numbers of lesions.

7. Should WBRT Automatically Be Added after SRS?

Historically, WBRT was used to treat brain metastases and after the development of SRS/GK, these were initially added to boost the radiation dose to larger lesions, which were unlikely to be controlled by WBRT alone. It soon became apparent that primary SRS/GK of smaller lesions provided excellent long-term lesion control as a primary treatment, and that many of these patients did not appear to require additional CNS therapy (either WBRT or surgery) [40]. This has led to an ongoing debate about whether GK/SRS should be followed by immediate WBRT for treatment of patients with melanoma brain metastases, or whether WBRT could be deferred. There have now been four randomized clinical trials including patients with 1–3 brain metastases from a variety of cancers. These studies have generally shown that local control at the SRS-/GK-treated site is not improved by WBRT, but that the development of new brain metastases is significantly decreased by immediate addition of WBRT [38, 41–43]. Overall survival does not appear to have been affected by immediate WBRT. This is because delayed salvage with additional SRS/GK [44, 45], or delayed WBRT, is possible (in the <50% of patients who progress in the CNS) [46]. Thus, quite a few investigators have concluded that delaying WBRT may be appropriate for some patients, with the caveat that these patients need to be followed closely with the intent of early retreatment at CNS progression [46, 47]. This has the potential to decrease neuropsychiatric sequelae and memory loss from radiotherapy in the increasing number of long-term survivors of brain metastases [48], as in our experience 50% of patients never require salvage therapy [6]. In addition, the only patients who developed radiation necrosis as a treatment complication in our series had received both SRS and WBRT [6].

8. Systemic Therapy

Systemic therapy has historically not been effective in melanoma therapy and, therefore, has rarely been utilized as primary treatment for metastatic brain lesions [49]. However, most patients with brain metastases also have active systemic metastases. Once brain metastases are controlled (e.g., with surgery, or SRS/GK), failure to treat systemic disease results in invariable progression and death of the patient (both due to reseeding of the CNS and systemic progression). This was particularly true in the era of ineffective chemotherapy. Remarkably, if even modestly effective IL-2-based immunotherapy was added after control of CNS metastases, prolonged survival appeared to be achievable in some patients. We initially demonstrated this principle by using biochemotherapy after SRS treatment of brain metastases and showed that median survival of over 1 year could be achieved, with 15% of patients alive at 3 years [50]. This observation is currently being recapitulated in clinical trials of the CTLA-4 monoclonal antibody ipilimumab. In these studies, patients with SRS/GK controlled brain metastases have been included and have shown significant survival prolongation. These trials have included patients with apparent CNS responses to ipilimumab therapy [5, 51, 52]. What appears most interesting is that if the CNS lesions can be effectively controlled for an extended period (perhaps 18–24 months), eventual long-term CNS control appears to be achievable, as was previously seen with biochemotherapy [50].

9. Targeted Therapies

A series of somatic genetic mutations has been identified in melanoma cells, leading to the research in specific targeted therapies. These include mutations in NRAS, BRAF, C-KIT (in cutaneous melanoma) [53], GNAQ, and GNA11 (in ocular melanoma) [54, 55], as well as in tumor suppressor genes such as PTEN and p16 [56]. A single mutation in BRAF (V600E) is present in about 50–60% of human cutaneous melanomas [57]. This substitution triggers a kinase-signaling cascade that can lead to cell growth, tissue invasion, and ultimately metastasis [57, 58]. While inhibitors of the wild-type B-RAF are not clinically active in melanoma [59], inhibitors of the V600E mutation are highly active in patients whose tumor expresses this mutation [4].

Unfortunately, most early B-RAF V600E inhibitor trials were designed to specifically exclude patients with brain metastases. In October of 2010, at the 35th Congress of the European Society for Medical Oncology (ESMO), a phase I/II clinical trial involving the newly developed GSK2118436 V600E BRAF inhibitor was presented. This trial allowed enrollment of a cohort of 10 patients with V600 BRAF positive melanomas and brain metastases. All patients experienced control of their brain metastases, with 90% experiencing a significant decrease in lesions size. This response was seen in brain metastases measuring 3 mm or larger in diameter, with an estimated reduction of between 20 and 100%. This is the first targeted agent that has shown objective responses in the CNS. This observation will be followed up with larger clinical trials in the United States and internationally.

10. Conclusions

Treatment of brain lesions in metastatic melanoma patients remains an ongoing challenge. As the incidence of melanoma has increased, so has the need to confront this problem. We have begun to rethink our approaches, including the screening of appropriate patient populations (overviewed

Table 1: Potential management strategies for melanoma patients with brain metastases.

Brain metastases	Largest lesion	Symptomatic*	Suggested CNS treatment	Systemic metastases	Systemic therapy§
1	<3 cm	yes or no	surgery†GK or SRS•	no	not suggested
1	>4 cm	yes or no	surgery†	no	not suggested
2–5	<4 cm	yes or no	GK or SRS	no	no
2–5	<4 cm	yes or no	GK or SRS	yes	yes¶
>5	<4 cm	yes or no	WBRT¥	no	no
>5	<4 cm	yes or no	WBRT¥	yes	yes¶

* Palliative glucocorticosteroid administration should be considered to decrease symptomatic edema, if present.
† Resectability may depend on location related to critical brain structures.
• GK and SRS are probably equivalent to surgical resection for lesion control if <2 cm.
§ The majority of these patients do not progress with systemic disease and there is little evidence that early systemic treatment improves either the risk of systemic relapse or helps control CNS metastases.
¶ Patients should have CNS lesions treated and controlled first, potentially effective agents include immunotherapy (ipilimumab, possibly IL-2) and targeted therapy (B-RAF inhibitor, etc.), if the appropriate activating mutation is present in tumors.
¥ Stereotactic boost to dominant lesions > 1 cm after WBRT may increase local lesion control and survival in patients with early CNS control and controlled systemic disease based on randomized trials.

in Table 1). There have been refinements in traditional modalities, such as neurosurgery, as well as development of stereotactic guidance and functional mapping techniques that have enhanced their effectiveness. The evolution of computer-image-guided radiosurgery has further improved treatment options in radioresistant cancers and limited collateral injury to normal tissue structures. Effective sequential or concurrent therapy of CNS metastases and systemic disease has proven to be possible, especially with the development of new and active immunotherapy and targeted therapeutic agents. There remains more work to be done, especially with improving local CNS control at the treated sites (the eventual cause of about 1/3 of mortality), development of new CNS metastases (resulting in another 1/3 of patient deaths), and systemic treatment failures (the remaining 1/3 of patient deaths). Close followup of treated patients is essential, as retreatment of the CNS is frequently possible, with long-term salvage as a potential goal in some patients. With the continuing evolution of therapy and continued clinical trials, hope of an entire new spectrum of therapeutic options is within our grasp. We must remain steadfast in our pursuit of these attainable goals through continued high-quality patient-centered research.

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Review Article

Prognostic Parameters for the Primary Care of Melanoma Patients: What Is Really Risky in Melanoma?

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Due to intensified research in recent years, the understanding of the molecular mechanisms involved in the development of melanoma has dramatically improved. The discovery of specific, causal mutations such as BRAF or KIT oncogenes not only renders a targeted and thus more effective therapeutic approach possible, but also gives rise to a new genetic-based classification. Targeting just a few out of several potential mutations, BRAF-Inhibitors such as PLX 4032 achieved already tremendous results in the therapy of metastatic melanoma. Up to now, the correlation of clinical, histomorphologic, and genetic features is, however, not understood. Even more, is it not well known precisely what kind of molecular changes predispose the primary melanoma for metastasis. The identification of morphological surrogates and prognostic parameters in tumors with such genetic alteration seems therefore crucial when differentiating and classifying this heterogeneous tumor entity in more detail and thus facilitates the stratification of prognosis as well as therapy. This review summarizes the current understanding of carcinogenesis and gives a detailed overview of known morphologic and potentially future genetic prognostic parameters in malignant melanoma.

1. Introduction

Despite all preventive and therapeutical efforts, melanoma is still the most aggressive and deadliest skin cancer especially in persons of fair complexion. To a certain extent, primary prevention campaigns already achieved an earlier diagnosis of thinner tumors with a better prognosis [1]. Incidence rates are nonetheless increasing worldwide mainly due to unreasonable sun exposure habits, especially in young adults [2]. Once diagnosed, prognosis and therapy is stratified so far by several clinicopathological risk factors such as tumor thickness, sentinel lymph node status, ulceration, and the recently added mitotic rate [3]. In view of an often unpredictable rather heterogeneous biological behavior mainly in >4 mm thick (Stage IIC) or locally advanced melanoma (Stage III), the AJCC classification remains of limited clinical relevance in particular for these high risk patients [4]. Moreover, we currently do not have reliable tissue biomarkers that mark the disease of the individual patient for progression or complete remission [5]. At the same time, an enormous amount of basic research within the last decade has dramatically changed the molecular understanding of melanoma. Proof of several specific genomic key mutations such that BRAF could not only be causally linked to disease progression [6] but also gave rise to new, highly effective therapies targeted specifically at those mutated molecules [7]. While the multistep carcinogenesis of melanoma is still too little understood in its complexity in order to foresee when, how, and what kind of mutation develops in an invasive or metastatic tumor, genome-wide genetic analysis of primary or metastatic tumors will undoubtedly change future classifications and subsequent treatment algorithms.

But are standard clinical prognostic parameters such as age, location, and metastasis already outdated? Could dermatopathology, the current cost-efficient diagnostic gold standard, consequently be redundant? Will we possibly be able to correlate certain histomorphologic features to specific genetic aberrations and their consecutive pathological or

compensatory molecular cascades in order to recognize, treat, or even prevent the systemic metastasic impact of this tumor in our patients? These important questions arise and may contribute to a better classification of melanoma patients. With the focus on their metastatic potential, our review summarizes the current knowledge of genetic, as well as molecular features of malignant melanoma and examines their possible correlation. Moreover, we discuss the clinical implications as well as current therapies that may target these new hallmarks of melanoma.

2. Epidemiology of Malignant Melanoma

A growing body of evidence has already addressed melanoma as an "umbrella term" for several biological distinct subtypes as a result of multiple causative genetic aberrations, impaired pathways, or epigenetic changes. Epidemiology, in contrast, strongly indicates that UV-induced DNA damage is the primary cause of melanoma development [8], even though certain regions in which melanoma subtypes occur, such as mucosal or acral tumours, are not typically exposed to ultraviolet light. Numerous studies about phenotypic risks such as age, gender, and skin type favour sun exposure as the major cause for thinner tumors of less incidence in young patients (<35 years) on minimally exposed sites and thicker tumours in elderly patients and UV-exposed locations such as the head and neck [9, 10]. Searching for the underlying causes of initiation and progression in these melanomas, it was demonstrated that cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (PP) photoproducts are the most abundant DNA lesions in those UV-exposed tumors [11]. A well-determined repair system of minimal necessary factors such as XPA, RPA, XPC, and so forth, is, however, sufficient to remove those photoproducts from DNA [12]. Although there is clear evidence linking a deficient repair system in Xeroderma pigmentosum to a higher susceptibility of cutaneous melanoma, a presumably impaired altered expression of repair genes may also contribute to the development of melanoma but was thus far not detected [13, 14]. On the contrary, as recently shown by Gaddameedhi et al., melanoma cell lines and melanocytes have displayed an equally efficient DNA repair system in primary tumours as well as in metastasis [15]. Even in NRAS or BRAF mutant melanomas, no reduced function or expression of the DNA repair system could be found [15]. p53 mutations are only found in 1% of primary melanomas and only 5% of metastasis. Nonetheless, it was suggested that the p53-mediated repair system and well as other aberrations such as MCR1, MITF, or CDKN2A influence UV-induced expression of this potent tumour suppressor. However, it is still not known how the different p53 functions ultimately manipulate the cell fate in melanoma [15, 16]. Recent numerous molecular genetic studies, strongly support that melanomas of the trunk of younger patients with multiple nevi differ enormously from those in elderly patients with cumulatively sun-damaged skin [17, 18]. Despite the evidence for causal factors such as age, phenotype, pattern, and dose of sun exposure, the underlying genetic propensities in subentities such as desmoplastic melanoma, uvea melanoma, or melanoma in childhood are not really understood. Genome-wide studies will, however, help to identify these constitutional factors as likely heritable contributors to melanoma risk and to propose possible new target-oriented therapies in the future [19].

3. Clinicopathological Parameters in Malignant Melanoma

Measurable diagnostic prognostic indicators and prognostic biomarkers are needed to refine the risk and assess the outcome in patients with malignant melanoma. As much effort as has been made by the AJCC in identifying reliable risk factors, the current classification still allows only a limited stratification of this rather heterogeneous tumour [4]. Apart from the classic clinical adverse parameters such as gender, age, location, and metastasis, histopathological parameters included so far are Breslow thickness, Clark Level, ulceration, sentinel status, and the recently added mitotic rate [3]. Yet, the new forthcoming genetic features of primary tumours, for example, the BRAF or KIT mutation, are not taken into account up to now within the classifications but certainly merit reflection in the future. Although their consideration would certainly be premature, several approaches already propose to integrate those molecular markers and thereby refine distinct subcategories of malignant melanoma [18, 20, 21]. In order to identify homogeneous disease groups in greater detail and implement an improved patient management, phenotypic consequences of those genetic alterations must be better understood [22]. But in virtually all well-established, time-tested, clinicohistopathological standard factors, the underlying biological mechanisms are, as shown below, completely unknown.

3.1. Breslow's Thickness. First introduced by Breslow in 1970 and later named after him, "Breslow thickness" is the eldest and one of the most important tissue biomarkers of the AJCC classification [23]. In association with horizontal enlargement, it was originally viewed and, thereafter, rectified as a parameter of tumour burden. Breslow's thickness nonetheless accurately predicts the risk of lymph node metastasis, with deeper tumours being more likely to involve the nodes [24]. Compared to Breslow's depth, Clark's level which describes the depth of tumoral penetration according to the anatomical skin layer (epidermis, dermis, and subcutis) has been proven to be less reproducible, more operator dependent, and of lower predictive value [25]. Its prognostic significance has, therefore, been limited to patients with very thin tumors in the current AJCC staging system [3]. The biological relevance of Breslow's depth's is, however, still almost unknown. Several potential molecular contributors to proliferation and, therefore, tumor thickness are currently under investigation. In particular, basic fibroblast growth factor (bFGF) is characterized as a highly mitogenic factor in melanoma especially when combined with UV [26] FGF receptor 4 (FGFR4) and its Arg388 genotype [27], cell cycle regulator proteins, or genes such as p53 and others [28, 29] as well as Bcl-2 oncoprotein [28], cell adhesion

defects, or cell-cell signaling mutations [29] have proven to be correlated with increased tumor thickness. Especially for the FGFR4Arg388 allele, there was convincing evidence of intensified cell motility and invasiveness [30, 31] but also increased vertical growth and risk of metastasis in nodular and superficial spreading melanoma [27]. Even though no correlation between decreased survival rate and outcome could so far be provided and the precise mechanism is not understood, FGFR4 Arg388 polymorphism predicts a more aggressive phenotype in terms of progression in melanoma as well as breast cancer [27, 31]. As the largest genomic structure in the FGFR family, loss-of-function mutations in FGFR2 have lately also been shown to occur in subsets of melanomas [32]. Neither mutations in FGFR4 nor in FGFR2 as a possible contribution to an inherited predisposition to skin cancer, could, however, be detected in healthy caucasian women [33]. Genetic variants of FGFR4 and FGFR2 seem, therefore, to function as potential biomarkers for progression rather than as a risk factor of skin cancer development [33].

3.2. Ulceration. In contrast to an ulcer due to trauma, ulceration in melanoma is defined as "a consumption of the epidermis" with a thinned epidermis to the side of the defect [34]. Initially identified as an adverse prognostic parameter by Allen and Spitz in 1953 [35], subsequently validated by Balch et al. [36], and later on by numerous other studies [37–39], ulceration has been convincingly shown to be an independent predictor of sentinel status and overall survival even in high-risk thick melanomas >4 mm [40, 41]. Despite its inclusion in the AJCC classification already in 2001 [42], the knowledge about why, when, and for what reason ulceration occurs and how it favours tumor progression is at best theoretical. Studies concentrated on width [36], depth [43], and proportion of ulceration [44], its association with mitotic rate [45] or vascular involvement, and tumor vascularity [46]. The results were, however, often inconclusive. The most plausible hypothesis that considered ulceration as a consequence of tumor proliferation, and therefore secondary epidermal thinning and contact ulceration, has been reevaluated. A recent study has demonstrated an independent prognostic association of ulceration and mitotic activity [47]. In addition, a direct influence on the local tumor environment seems nonetheless possible. Hence, ulceration challenges the control functions of keratinocytes, melanoma cells are enabled to transform more easily, therefore favoring tumor progression [48].

3.3. Regression. More common in melanoma than in any other neoplasia [49], regression is defined as a partial or complete disappearance of the tumor without treatment [50]. Due to the loss in pigmentation in terms of a blue or grey-whitish discoloration, it is clinically highly apparent in this particular tumor entity. With an incidence of approximately 10–35% of patients with primary malignant melanoma [51], regression arises specifically in thinner tumors but hardly ever in nodular melanoma [52]. Associated with variable degrees of inflammatory and stromal changes, this particular phenomenon proceeds from an early dense lichenoid infiltrate of lymphocytes and dermal edema to a late fibrosis and a usual melanosis within a thickened papillary dermis [53]. Especially when the tumor is pigmented, melanophages as the histopathological telltale sign are often present. Although the current understanding of regression is clearly that of an immune-mediated, cancer-autonomous process [21], neither its biological significance nor the underlying molecular or genomic aberrations are so far recognized. Possible explanations vary from an increased T-cell response [54], an inhibited angiogenesis [53], to a forced apoptosis of tumor cells [53, 55]. Consequently there are different therapeutical implications of regression. While a positive host immune response may supersede wider excision margins or sentinel lymph node biopsy [56, 57], regression may, however, on the other side indicate a formerly deeper infiltrating tumor and thus a lower threshold for sentinel lymph node biopsy [58]. Especially in thin melanomas <1 mm, regression as a left-over of a presumably thicker tumor therefore still leads to wider surgical margins and a lower threshold for SLN biopsy [58]. The most convincing, although unproven, hypotheses for a regression-driven tumor progression so far are the Hammon's effect, which postulates a natural selection of aggressive residual tumor clones as a result of regression [59, 60] and Bastian's telomere crisis, which argues that a massive senescence and cellular apoptosis equally favor the selection of genomic aberrations and therefore progression [55]. Future epidemiologic studies investigating the impact of regression of the primary tumor for the prognosis of melanoma are certainly required to further investigate those intriguing details.

3.4. Mitotic Rate. Tumor proliferation as defined by mitotic rate has been confirmed as an independent adverse prognostic parameter in many solid neoplasia including melanoma [61–64]. Due to the fact that its increase is significantly correlated with reduced survival rates primarily within melanoma of less than 1 mm tumor thickness, it has recently replaced Clark's level as the primary criteria for defining the subcategory of T1b in AJCC classification 2009 [3, 65]. The lack of a universally agreed approach of how to document mitotic figures led to many studies that did not include mitotic rate in their analyses up to now [66]. As recently detailed by the AJCC manual, starting with dermal areas that contain most mitoses (so-called hot spots), and extending the approach later to adjacent fields up to 1 mm^2, now allows for the first time a reproducible assessment [3] although this approach is time consuming to the dermatopathologist. So far, only two sorts of genes and their pathways are identified to be overrepresented in melanoma with higher mitotic activity. Replication Origins Firing (ROF) genes such as MCM4 and MCM6 as well as the oncogene securin are strongly correlated with metastases and therefore poorer prognosis even after considering other prognostic parameters such as sex, age, location of the primary, thickness, and ulceration [29, 67]. As much effort has been made in defining the biological relevance of these dermatohistopathological parameters, they cannot reliably distinguish the metastatic behaviour of certain subgroups such as Stage IIC melanoma.

Moreover, the exact diagnosis in some cases of melanoma might be problematic altogether as the individual assessment of these criteria differs among pathologists [68]. In addition, benign melanocytic proliferations such as atypical nevi can also display a number of those features, given that routinely performed immunohistochemical markers, for example, S100B and HMB-45 are of little help in distinguishing nevi from melanoma [69]. Taken these reflections into account, a more molecular understanding of melanoma might therefore be desirable. Inevitably, the understanding of the molecular basis of malignant melanoma has to be further improved to identify the critical "drivers" and "passengers" during oncogenesis of melanoma [70, 71].

4. Current Knowledge about Oncogenesis of Malignant Melanoma

The core issue obscuring the best possible treatment of malignant melanoma is still its unpredictable pattern of progression and metastasis. Well-established prognostic parameters alone or in combination are so far not effective enough to accurately predict the outcome for every individual patient. Biologically distinct as malignant melanoma is, the greatest therapeutical potential lies without doubt in the understanding of what key indicators influence the course of the disease most, regardless whether they may be genetic, possibly molecular, least likely clinical, or even combined, and therefore predispose for the risk of systemic disease. The multistep process of carcinogenesis in malignant melanoma is, however, complex and at best only in part understood. A number of excellent reviews have summarized the exciting developments in the understanding of this tumor in depth [72]. To date, four pivotal, nonlinear, and rather netlike interwoven defective signaling pathways have been implicated. These are MAP kinase, PI3K/AKT, MITF, and WNT. The following scheme gives a simplified overview of these pathways with their most common aberration and the percentage of mutations detected within these signaling pathways. Certain rare subtypes such as uveal melanoma also have been found to have mutations in GNAQ [73] or GNA11 [74] that also lead to constitutive activation of these signaling pathways (Figure 1).

Proven to be one of the most frequently mutated cascades in melanoma, the mitogen-activated protein (MAP) kinase pathway shows several pathologically activated mutations that may contribute to malignant transformation. The most common mutations or cytogenetic amplifications occur in the BRAF, the KIT, the NRAS, or the CDKN2A genes. In 8–12% of familiar malignant melanoma alone, mutations of CDKN2A gene that are linked to chromosome 9p21 arise [75, 76].

Unlike regular sites of cutaneous melanoma, uncommon subsets of melanocytic neoplasia such as uveal melanoma or malignant blue nevus lack frequent oncogenetic mutations in cKIT, NRAS, or BRAF [77–79]. Notwithstanding other oncogenes such as the alpha subunit of a class of heterotrimeric GTP-binding proteins (Gq), namely GNAQ and GNA11, are activated. Hypermorphic mutations in those genes were found to contribute to skin darkening and therefore melanocyte biology in mice [80]. Proven to occur early in progression, they seem, however, not to be related to clinical outcome so far [81, 82]. When active, GNAQ and GNA11 alternatively upregulate the MAP kinase pathway [73]. Operating downstream of several G-protein coupled receptors, GNA11 has presumably a more potent adverse effect than GNAQ in locally advanced or metastasized tumors although overall survival did not differ [74]. GNAQ mutations are, however, considered to be more sensitive to the upcoming therapeutical MEK inhibition [73].

Cross-linked via NRAS, the MAP kinase cascade also initiates the PI3K and thereby the PI3K signaling pathway, another defective cascade found in a large percentage of melanomas. Apart from NRAS, either deletion of PTEN or overexpression of AKT mainly lead to the stimulation of mTOR, a central regulator of cell growth and proliferation that has raised substantial interest in this signaling pathway in melanoma [83].

Of central importance for benign as well as malignant melanocytes, MITF and its cascade were found to represent a central transcription factor that regulates differentiation in the pigment cell system [84]. In addition to α-MSH and ACTH that activate MITF via the MC1R, it is also physiologically regulated by MAP kinase and PI3K signaling pathway [85, 86]. In the development of melanoma, however, an optimized level of MITF as an oncogene for proliferation and survival of tumor cells needs to be maintained by BRAF [87]. Insufficiently high or low expression of MITF results in tumor-protective differentiation, cell cycle arrest, and subsequent apoptosis [88]. MITF amplification, single based MITF substitution and even mutation of its regulator SOX10 have all been proven lately to be causative for altered MITF function in both primary and metastatic melanoma [89, 90] underscoring the involvement of MITF in melanomagenesis.

Although mutations of the β-catenin gene and APC have already been detected, the WNT signaling pathway has not been extensively implicated in melanoma development this far, due to the fact that defective β-catenin is rarely identified although it clearly acts as a melanoma-specific antigen [91, 92]. Under physiological conditions, WNT-signaling proteins bind to Frizzeled receptors, thereby stabilizing β-catenin with subsequent release from a multiprotein complex. It then accumulates in the nucleus and initiates as a coactivator the transcription of a multitude of target genes. In case of genetic mutations of β-catenin, such as in malignant melanoma, it forms a complex with LEF-1 (lymphoid enhancer-binding protein), which in turn leads to malignant transformation of the cell [93, 94]. In particular Wnt-2, a survival factor in human carcinogenesis, [95] has lately become focus of intensified research as a biomarker and a potential target to subclassify and treat malignant melanoma [96, 97]. Besides the main canonical WNT signaling pathway, a variation of the so-called noncanonical pathway with altered receptors and enzymes, and even a signal regulated in a paracrine manner (the so-called "notch" cascade), diversify and complicate the WNT signaling pathway considerably [98]. Specific inhibitors in terms of small molecular antagonists or RNA aptamers

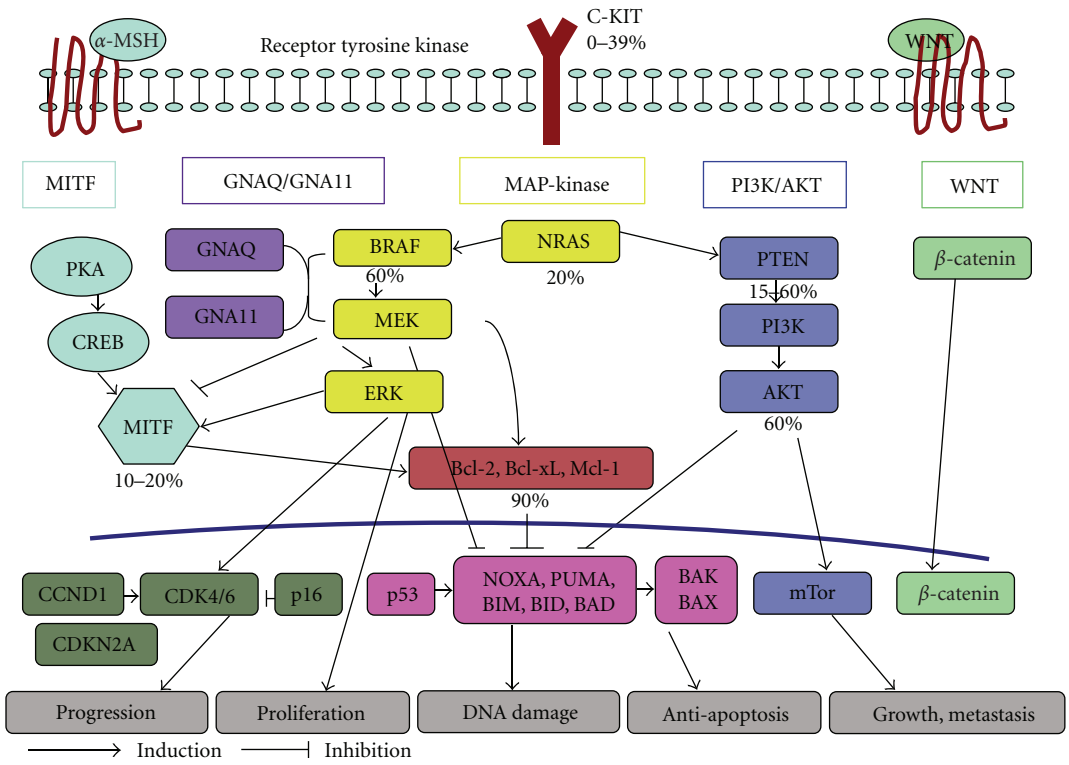

Figure 1: Signaling pathways in malignant melanoma (modified according to http://www.cancercommons.org/).

have nonetheless been developed to potentially target this pathway, an intriguing possibility given the important role of this pathway in the so-called "tumor-initiating cells" in other tumor entities [99]. WNT2 has also been found to be overexpressed in malignant melanoma [97]. Of therapeutic interest, a specific anti-WNT-2 monoclonal antibody has been proven to inhibit WNT signaling and subsequently induce apoptosis [96].

The complexity of these crosstalking circuits is increased even more by the fact that one genetic alteration is not enough to make a melanoma. Several additional changes are needed in a multistep process to result in malignant transformation [100]. Cumulative genetic instability gradually induces arbitrary genomic aberrations that lead to uncontrolled replication and growth, inhibition of apoptosis, and finally the ability to invade and metastasize due to a Darwinian-like selection process of the tumor cells [101–103]. Considering further stem cell-determined, epigenetic, tumor-environmental, or immunologic changes, the variety of possible influencing factors on the classic hallmarks of cancer is multiplied beyond measure [104], and the knowledge of critical constitutional and somatic genetic parameters is not yet complete [22].

5. Epigenetics

Recent progress in the understanding of genetic aberrations in malignant melanoma has likewise prompted significant efforts in defining so called "epigenetic" changes that accompany the malignant transformation in melanocytes. Defined as any changes in gene expression that are not achieved through alterations in the primary sequence of the genomic DNA, epigenetics influence a wide range of alternative gene functions such as cell cycle regulation, cell signaling, differentiation, DNA repair, apoptosis, invasion, metastasis, angiogenesis, and immune recognition [105]. Although their precise contribution to tumor progression is still unknown, they were proven to efficiently restore the expression of aberrantly silenced genes and thereby to reestablish silenced signaling pathway function [106]. The most clearly identified epigenetic mediators so far are the methylation of DNA in the context of CpG dinucleotides, the posttranslational changes of histone proteins and, though less characterized, the influence of microRNAs (miRNAs). The reactivation of "sleeper" genes and the maintenance of these epigenetics aberrations requires functioning enzymes such as DNA methyltransferases (DNMT) or histone deacetylases (HDAC), and histone methyl transferases (HMT), respectively. In case of DNA methylation, three different DNMTs are implicated in new methylation patterns with gene-specific hypermethylation on the one hand as well as genome-wide hypomethylation on the other [107]. In addition to genetic alterations, epigenetic DNA hypermethylation is, therefore, a complementary, frequent, and important mechanism to inactivate tumor suppressor genes such as CDKN2A [108]. While hypermethylation silences tumor suppressor genes, global hypomethylation might, however, activate the expression of oncogenes. This could lead to a diversified and significantly impaired methylation disbalance of multiple genes that eventually initiates genomic

instability, tumorigenesis, and cancer progression [106, 107]. As common as this phenomenon of hypomethylation is in many tumors, little is known so far about target genes regulated by this event in melanoma [109]. Similar to the discussion of driver and passenger mutations in genetic aberrations, the biological significance of several identified aberrantly hypomethylated epigenetic genes, for example, cancer-testis antigen (CTAs), PRAME, and MAGE continue to be poorly understood. Nonetheless, given the broad relevance of these pathways in almost every tumor entity, substances have already been developed for therapeutical approaches, and the epigenetic status of certain genes may potentially predict the biological function and could serve as a biomarker [110, 111].

Along with DNA methylation patterns, initial studies about histone acetylation have addressed a possible role in melanoma development and progression [112]. In particular hypoacetylation-mediated downregulation of CDKN1A and, similarly, proapoptotic proteins such as BAX, BAK, BID, and BIM may profoundly influence cell cycle and apoptosis of the cell and thereby lead to tumor progression or therapeutic resistance [113, 114]. In the demanding packing and outpacking machinery of genomic DNA into nucleosomes and chromatine, respectively, at least three groups of histone acetyltransferases (HAT) and 18 identified histone deacetylases (HDAC) are involved thus far [115]. Complicating this picture, histone methyl transferases (HMTs) modulate the chromatin compaction grade of the DNA that finally determines the transcriptional status of target genes [116]. In contrast to DNA methylation, the knowledge of the posttranslational aberration of histones is altogether scarce and mainly gathered indirectly by treatment results of HDACs thus far. Promising results of multiple HDAC inhibitors concerning vascular endothelial growth factor (VEGF), generation of reactive oxygen species (ROS), cell death, senescence, and especially intrinsic as well as extrinsic apoptosis in the transformed cells have already been described in various solid tumor entities [117–119]. Proapoptotic stimuli are, however, known to be less effective in human melanoma cell lines. Recently discovered key mediators such as the cleavage of Poly-ADP ribose protein (PARP) [113] and HDAC inhibitors like the short fatty acid VPA [120] have led already to promising results with antitumor activity in combination therapy with anthracyclines in melanoma [121]. The level of understanding of the molecular mechanism in histone posttranslational modifications has yet to become more refined to predict the outcome of such promising therapies in subgroups or individual melanoma patients.

The most recently discovered players in epigenetic regulation have been noncoding microRNA (miRNA). Once transcribed in the nucleus and further processed by several intermediate stages, they are finally incorporated into a RNA-induced silencing complex that recognizes their target miRNA. This either inhibits their translation or (less frequent) causes their degradation [122]. Each miRNA has several target RNAs and vice versa. In addition to more than a hundred currently confirmed miRNAs, more than 1000 miRNA have been predicted by bioinformatics [123]. Despite the limited data available so far, miRNAs are proven to play pivotal roles in the epigenetic pathogenesis of human cancer. As proof of principle, several key miRNAs have already been identified in driving tumorigenesis and progression in malignant melanoma [124]. Especially the lack of an inhibition by miR-137 and miR-182 was found to result in an overexpression of MITF, a master regulator in benign melanocytes as well as melanoma [124]. On the other hand, overexpression of miR-182 contributes likewise to progression and metastasis by repressing MITF [124]. In a similar way, miR-34b, miR34c, miR199a, and miRNAs involved in the expression of the oncogene MET modify target gene expression in accordance with the stage of cancer development [125]. Considering the fact that miRNAs themselves are also targets of epigenetic regulations as, for example, miR-34a, which is proven to be silenced by a CpG-mediated methylation in up to 60% of primary melanomas [126], further studies are mandatory to define their role in melanoma biology more precisely.

6. Oncogene-Defined Targeted Therapy in the Era of BRAF Inhibitors

As one of the most devastating forms of cancer in terms of life expectancy and outcome, metastatic melanoma was until recently an almost intractable disease. This was largely explained by the fact that mono- or polychemotherapy, the standard of care for over 30 years, only benefits a very small subset of patients. With the discovery of an activating mutation of BRAF in 50–60% of all melanoma, with 90% of these tumors carrying a substitution at V600, a first tumor-specific target for a treatment was identified in 2002 [6]. Sorafenib, a multikinase inhibitor and one of the first targeted therapies in clinical testing, has unfortunately shown little efficacy in patients with activated MAP kinase pathway (and therefore BRAF positive) patients [127]. Consequently, more selective BRAF inhibitors were subsequently tested in clinical trials, which in case of vemurafenib (also known as PLX 4032) and GSK2118436 have demonstrated unprecedented clinical results in metastatic malignant melanoma harboring BRAF mutation [7, 128, 129]. Within two weeks, the majority of patients stated a symptomatic improvement, and approximately 60% showed an objective response according to response evalutation criteria in solid tumors (RECISTs). Overall about 80% of all patients with metastatic tumors experienced some degree of regression [7]. In the subsequent extension phase of the trial, 81% patients demonstrated tumor regression, and the progression-free survival was at an average of 7 months [7]. Dose-dependant adverse events like rash, photosensitivity, fatigue, and arthralgia were well managed by either dose reduction or by the termination of the treatment if necessary. GSK2118436 has proven to be even of higher potency at a lower concentration [129]. Apart from pyrexia, rash, fatigue, headache, nausea, and vomiting, severe adverse events such as squamous cell carcinoma and keratoacanthoma were reported. A series of publications, however, quickly discovered novel mechanisms that paradoxically activate the MAP kinase pathway in the presence of

Table 1: Genetic mutations and corresponding current and future targeted therapies.

Pathway	Target	Therapy
MAP-kinase	Receptor tyrosine kinase	Imatinib
		Dasatinib
		Nilotinib
		Masitinib
	BRAF	GSK2118436
		Vemurafenib
	NRAS	Sorafenib
		Tipifamib
	CRAS	Lonafamib
		RAF265
PI3AK	mTor	Sirolimus
		Temsirolimus
		Everolimus
	PI3, AKT	GDC0941, GSK2126458, BEZ235, BKM120, XL765, MK2206, GSK 690693
MITF	CDK2, HDAC	SCH727965, panobinostat
WNT	B catenin	Small molecular antagonists
		RNA aptamers

BRAF inhibitors [130, 131]. Due to three isoenzymes of RAF (A-RAF, B-RAF, and C-RAF), the inhibition of one of them such as B-RAF can induce a compensatory transactivation of C-RAF, which in turn activates downstream MEK and the subsequent pathway [130, 132]. As a consequence of "gatekeeper" mutations that sterically prevent the inhibitor binding to the active side in RAF, the crossactivation of C-RAF is not always initiated and even to a certain extent inhibited by the given drug [132]. ATP competitive inhibitors for instance are supposed to stabilize the interaction between B-RAF and C-RAF [133]. Besides C-RAF as a paradoxical bypass of B-RAF, other ERK-dependent mechanisms such as N-RAS mutation, COX overexpression, or MEK1 mutations contribute to an acquired resistance to B-RAF [134]. Complicating the picture, even ERK-independent alterations like PDGFRβ overexpression, IGF1R activation and PTEN loss have been identified to reactivate ERK signaling in B-RAF mutant tumors [134, 135]. Although the benefit of B-RAF inhibition as monotherapy has been sufficiently confirmed, rapidly occuring secondary resistance mechanisms in tumors will most likely favor combination therapies targeting other genetic "hot spots" in melanoma such as MEK, RAS, and KIT.

RAS, in particular N-RAS mutations, occur in approximately 15–25% of malignant melanoma. They inhibit the GTPase-mediated activity of RAS and thus keep it in an continuously active state [136]. Demanding as task to develop an agent is that would rival GTP, several interacting pathways such as MAP kinase or PI3 kinase seem to play an important role in the N-RAS mutant subset of melanoma [137, 138]. Mutually exclusive to B-RAF V600E mutation [136], NRAS mutations have been shown to be sensitive to MEK-targeted therapies particularly in combination with PI3K, AKT, or mTOR inhibitors [137].

KIT mutations have so far been found in a small subgroup of melanomas, in particular acral or mucosal tumors that are not related to sun exposure [20]. According to the results in gastrointestinal stroma tumors (GISTs), KIT inhibitors such as imatinib and sunitinib, and newer inhibitors such as nilotinib or dasatinib have been described, however, to be less responsive [139, 140]. Encouraging to this subgroup of patients, anecdotal reports have shown complete remission lasting up to one year [141].

Despite several promising new agents (Table 1), there are, however, still no therapeutic strategies that would reliably conquer the complexity of pathways resulting in a highly aggressive malignancy in melanoma. Considering several multimarker assays using in vivo samples and cell culture of primary melanomas and metastasis together, melanoma development itemizes to several hundreds of involved genes that seem too plentiful to be individualized for a targeted therapy in a single patient, even though new, potentially essential, marker genes have been identified and are currently tested [142]. The very view of resistance, unwanted side effects, and rapid progression after initial responsiveness clearly emphasize the importance of a thorough, genotypical stratification, and a "driver-focused" synergistic therapy. The development of an oncogene hierarchy with differentiation into important drivers and bystanding passengers seems therefore necessary.

7. Conclusion

The recently gained knowledge about the functional importance of muted genes in a high proportion of malignant melanoma has fundamentally changed the diagnostic and therapeutic approach. In view of the focus on BRAF, NRAS, KIT, and PTEN, four key genomic defective alterations and their corresponding pathways are identified that without any doubt refine and extend the understanding of its bewildering biological complexity. Although an improved classification [4, 18, 22] and corresponding risk stratifications and target-oriented therapies (Table 1) are within reach, or in case of the latter even under effective investigation, a restriction to some precious few control factors seems to be a too easy answer. The serious question remains, how do the highly relevant histopathological parameters translate in a benefit for distinct subsets of the melanoma patients?

The answer probably lies in the identification of the biological "Achilles heel" of individual tumors. As convincingly shown, molecular analysis of subsets of melanoma has

at first revealed mutations in cKIT. This knowledge was then rapidly translated into a successful targeted therapy [18]. Other positive examples are the more recent successful translation of the knowledge of the BRAF mutational status (e.g., V600E) into elegant mutation specific, and at least short-term successful therapy in these patients [7]. However it is not surprising that in a large number of melanoma patients such single mutations do not precisely delineate the biological behaviour of the tumor at the time of primary melanoma diagnosis. In fact, there appear to be a multitude of biologically distinct melanoma entities. Thus, it is likely that this straightforward approach is too narrow, given that in a considerable fraction of melanomas so far unknown oncogenes or tumor suppressors, or combinations thereof may control tumor cell fate [143]. Most likely unbiased approaches to melanoma using 21st century technology of genetic profiling will yield intriguing results [144]. As much as the classic hallmarks of cancer withstood the test of time [102]: recently discovered characteristics such as antiapoptotic parameters [145], the role of tumor stem cells [146], telomerases [147], or circulating tumor cells [148], as well as other tumor-environmental and epigenetic phenomena [106, 115] have also to be taken into account and may translate into successful therapy [104]. But hopefully, as Hanahan and Weinberg lately stated, this phenotypic myriad in melanoma [19, 149] may portray just a few of the causal principles of distinct tumor cell types that need to be clarified in order to improve the treatment and outcome in our melanoma patients [104]. So, in the era of molecular profiling, the gist of the matter "what's really risky in melanoma" seems within reach.

List of Abbreviations to Figure 1

α-MSH:	Ligand of melanocyte-stimulating hormone
β-Catenin:	Tumor-oncogene ind Wnt pathway
BAK/BAX:	Proapoptotic effectors of Bcl-2 gene family
Bcl-2/Bcl-xL/Mcl-1:	Proapoptotic members of Bcl-2 gene family
BIM, BID, BAD:	Proapoptotic members of Bcl-2 gene family
BRAF:	Serine/threonine-protein kinase B-Raf protooncogene
CCND1/CDK4/6/CDKN2A:	Cyclin-dependent kinases (CDKs)
c-Kit:	Protooncogene
ERK:	Extracellular-signal regulated kinase
GNAQ:	Guanine nucleotide-binding protein G(q) subunit alpha
GNA11:	Guanine nucleotide-binding protein subunit alpha-11
MAP/MEK:	Mitogen-activated proteins
MITF:	Microphthalmia-associated transcription factor
mTOR:	Mammalian target of rapamycin
NOXA/PUMA:	p53-inducible proapoptotic members of the Bcl-2 family
NRAS:	Neuroblastoma RAS viral oncogene homolog
P16:	Cell cycle regulator
P53:	Tumor suppressor gene
PI3K:	Phosphoinositid-3-kinase
PTEN:	Phosphatase tensin homolog, tumor suppressor
WNT:	Ligand of WNT-pathway.

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Review Article

The Contribution of Electron Paramagnetic Resonance to Melanoma Research

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The incidence of malignant melanoma, the most dangerous form of skin cancer, is rising each year. However, some aspects of the tumor initiation and development are still unclear, and the current method of diagnosis, based on the visual aspect of the tumor, shows limitations. For these reasons, developments of new techniques are ongoing to improve basic knowledge on the disease and diagnosis of tumors in individual patients. This paper shows how electron paramagnetic resonance (EPR), a method able to detect free radicals trapped in melanin pigments, has recently brought its unique value to this specific field. The general principles of the method and the convenience of melanin as an endogenous substrate for EPR measurements are explained. Then, the way by which EPR has recently helped to assess the contribution of ultraviolet rays (UVA and UVB) to the initiation of melanoma is described. Finally, we describe the improvements of EPR spectrometry and imaging in the detection and mapping of melanin pigments inside *ex vivo* and *in vivo* melanomas. We discuss how these advances might improve the diagnosis of this skin cancer and point out the present capabilities and limitations of the method.

1. Introduction

Malignant melanoma is a skin tumor characterized by the uncontrolled proliferation of melanocytes. This tumor is the most dangerous form of skin cancer and is responsible of 3 of 4 deaths linked to a skin cancer. The incidence of malignant melanoma is rising each year [1, 2], and, nowadays, the cumulative lifetime risk for an invasive melanoma is estimated at 1/59 in the USA A link between sunlight exposure and melanoma causation has been for a long time established [3–5]; however, it is still not clear how the different ultraviolet wavelengths (UVA and UVB) are implicated [6]. The role of melanin as a possible endogenous photosensitizer is also subject to discussion. This discussion and the way that a new measurement method based on electron paramagnetic resonance (EPR) might answer to it will be explained and debated.

The abilities and perspectives of EPR spectrometry and imaging as a detection method for melanin pigments, and consequently for pigmented melanomas, will be also discussed. Indeed, for now the only method used by dermatologists for suspecting melanoma is an optical examination of the lesion based on the detection of five factors: Asymmetry, irregular Borders, nonhomogenous Color, large Diameter, and Evolving (also known as the ABCDE rule). This optical examination, even if effective, shows limitations: it does not provide any information about the penetration of the tumor in the skin, which is a crucial factor to determine the growth state of the potential melanoma and to provide an adapted treatment. The use of EPR imaging for the mapping of free radicals trapped in melanin might allow filling in this lack of spatial information in a noninvasive way.

2. A Few Words about Melanin(s)

Melanin is the most widespread pigment in the epithelia of vertebrates. This molecule is responsible of the pigmentation of skin, hair, and eye. Historically, its name comes from the

Greek word *melanos* that means "dark". The origin of this name is generally attributed to the Swedish chemist Berzelius [7]. As explained by Riley in his review about melanin [8], the term "melanin" has been used fairly indiscriminately to mean any dark pigment but the nomenclature has been refined in the case of mammalian melanin. Indeed, later, two major different kinds of melanin have been described: eumelanin and pheomelanin. Eumelanin is a brown-black pigment derived from the enzymatic oxidation of tyrosine through 3,4-dihydroxyphenylalanine (dopa) [9], while pheomelanin is a yellow reddish-brown pigment following a similar synthesis pathway but including cysteine [10]. However, it is generally accepted that pure eumelanin or pure pheomelanin is rare in normal tissues. Most of the time, eumelanin and pheomelanin monomers are linked in various proportions to create the melanin macromolecules [11, 12].

The melanin polymer has some interesting properties among which light absorbance, redox properties, and chelating properties [8]. The wide spectral absorbance of light is linked to the high degree of conjugation in the molecule. The redox properties, especially of eumelanins, are caused by the delocalization of an electron between orthoquinone and catecholic moieties, which give rise to semiquinone free radicals (Figure 1). Due to these radicals, melanins can take part in one-electron and two-electron redox reactions. This property is responsible of a photooxidation reaction when the pigment absorbs light and of the photosensitizer role of melanin, role that will be explained in a dedicated paragraph of this paper. Moreover, the semiquinone free radicals trapped in melanin are detectable using EPR. As we will see later, this makes EPR a unique tool to assess the extension of melanoma.

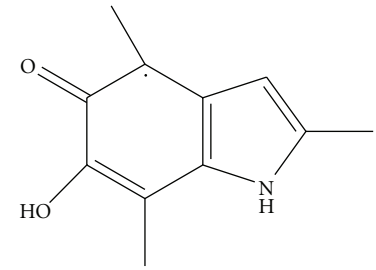

Figure 1: Semiquinone radical trapped in melanin, responsible of the paramagnetic properties of the molecule.

3. Paramagnetism and Electron Paramagnetic Resonance (EPR)

Electron paramagnetic resonance (EPR) is a magnetic resonance method similar to nuclear magnetic resonance (NMR) which focuses on the detection of paramagnetic materials. The paramagnetism notion refers to the behavior of a material substrate that does not possess spontaneous magnetization but, under an external magnetic field, acquires a magnetization parallel to this magnetic field. This phenomenon, explained by the quantum physics principles [13], results from the properties of the electron spin [14]. It is important to note that only the unpaired electrons might move from a low energy level to a higher (and vice versa) and enter into resonance. EPR spectrometry is a method that detects the absorption of energy linked to the resonance phenomenon. The quantity of this energy differs in function of the kind of radicals and their environment. Consequently, this technique is able to detect and characterize free radicals by providing spectra which are specific to the radicals detected.

Although the EPR technique is similar to NMR, there are two notable differences between them [16]. First, the gyromagnetic ratio of an unpaired electron is largely higher than that of a proton. This means that the standard EPR spectrometers have to operate at much higher frequencies and lower fields than conventional NMR spectrometers. When working at such high frequencies (typically, 9 GHz, which is a standard frequency used in the most usual EPR spectrometer), the nonresonant absorption of the electromagnetic radiation by the liquid water of the biological samples presents a serious problem. At 9 GHz (X-Band mode), the microwaves will only penetrate up to 1 mm inside a biological water-containing sample. Larger aqueous samples or animals can be studied only by reducing the operating frequency to 1 GHz (L-Band mode) or less. At this frequency, the penetration of microwaves is about 1 cm, which can be convenient for studies on small samples, small animals, or superficial tissues such as skin. However, this results in an important loss of sensitivity.

The second major difference between EPR and NMR comes from the difference of their relaxation time, which is in the range of nanoseconds for electron and in the range of milliseconds for proton. This has two consequences: first, *in vivo* EPR spectra are mostly obtained through continuous wave (CW) experiments, whereas, in clinical MRI, results are always obtained with the use of pulsed radiowave. Second, compared to MRI, EPR imaging requires a gradient field at least one order of magnitude greater.

During the last 60 years, EPR spectrometry has been successfully used in many parts of the scientific research, especially in biology where it contributed to the understanding of a large number of physiological processes. The fields where EPR has been the most used and useful already possess their own review(s), such as the study of iron-containing molecules [17], the assessment of oxygen concentration in tumors [16, 18], the detection of reactive nitrogen and oxygen species *in vivo* [19, 20], or the assessment of redox state in biological tissues *in vivo* [21].

A more recent method derived from EPR spectroscopy, called EPR imaging, consists in adding an external gradient field that modulates the resonance frequency of the paramagnetic species in function of their position in space [22, 23]. As a consequence, it is possible to get 2D and 3D maps of the distribution of free radicals. This ability has, for example, been used, alone or coupled, with another technology, to improve our knowledge of the brain redox state [24], measure venous and arterial oxygenation [25], monitor the evolution of tumor oxygenation after treatment [26, 27], or map the repartition of radio-induced free radicals after irradiation [28].

Because in 1954, melanin was found to contain stable free radicals detectable by EPR [29] and that these radicals provided a specific EPR spectrum (Figure 2), this molecule became a very interesting substrate for further spectrometry and imaging studies. For a long time, these two methods, and especially spectrometry, have been used to try to determine the structure of melanin, to understand the mechanisms behind its protective role against sunlight aggressions, and to improve our knowledge of melanoma.

4. Melanin, UV Rays, and EPR

An excellent extensive review of this topic (putting together melanin pigments, UV irradiation, and EPR spectrometry) has been published by Lund and Timmins in 2007 [6]. We briefly summarize here the research advances in this field. The role of melanin in the protection of skin from light radiations has been generally accepted for a long time. Indeed, a link between melanoma causation and exposure to sunlight has been observed [3–5]. However, it is unclear how ultraviolet (UV) rays of sunlight might cause melanoma. The two major types of UVs, UVA and UVB, seem to have different effects on the causation of the development on this tumor, but the role of each of them on the initiation of the tumor is still debated. The resolution of this question could have important consequences, both in term of recommendations of sanitary authorities and development of effective sunscreens. The discussion is so controversial that few studies even brought the benefits of sunscreens into question and explained that, due to a nonprotection against UVA, they might be ineffective [30, 31] and, as a indirect consequence, contribute to increase melanoma incidence [32, 33]. Some other epidemiological studies suggested that both UVA and UVB were involved in melanoma causation [34–37], whereas nonmelanoma skin cancers have primarily linked to UVB [38]. These results suggest that melanin could play a role in the melanoma sensitization to UVA.

This role of melanin was put in evidence when Sarna and Sealy demonstrated that the exposition of melanin to UV and blue light generates reactive melanin radicals (RMRs). Moreover, it was demonstrated that RMR could react with biomolecules and molecular oxygen [39, 40] to lead to the formation of oxygen reactive species, such as superoxide, leading to hydrogen peroxide and hydroxyl radical.

An EPR technique was recently developed by Wood et al. [41] to enable accurate measurement of RMR *in situ* in skin. This method was used to assess quantitatively the RMR formation in function of the exposition to different wavelength in the skin of a mouse model for which the action spectrum was already known [30]. They observed that the 2 action spectra were identical from 303 to 434 nm, a range spanning both UVB and UVA. This result demonstrated that the EPR measurement of reactive melanin radicals could act as good indicator to determine the contribution of UVA and UVB in melanoma causation. In this study, it was shown that over 95% of measured RMR were caused by UVA, with less than 5% by UVB. Consequently, a sunscreen that would block the major part of UVB, but would be ineffective versus UVA, would offer very limited protection against RMR formation.

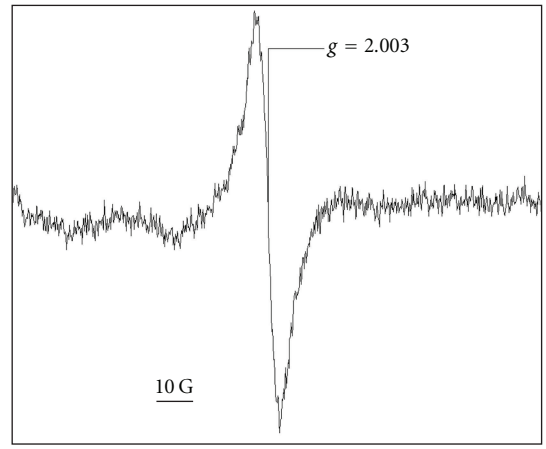

Figure 2: Typical EPR spectrum of melanin. This spectrum was obtained for measurement of 5 μg of synthetic melanin.

These results still have to be confirmed on other animal species (including human), and we can predict that the controversy about UVA, UVB, and melanoma is far away from the end, but these preliminary results could bring the outcome faster than expected.

5. The Detection and Growth State Assessment of Melanoma by EPR

The first important work concerning the quantitative detection of melanin by EPR spectrometry inside melanoma samples was published by Elek et al. in 1980. When measuring ocular melanomas embedded in paraffin, they observed a free-radical signal situated at g-factor = 2.003, corresponding to the EPR signal of melanin [42]. When comparing the amplitude of this signal with the number of melanin granules observed in histological sections, they observed a straight positive correlation. They concluded from this experiment that EPR spectrometry might be suitable for estimating the melanin content inside melanoma samples.

This study was followed in 1990 by Katsuda et al. who adapted a self-made EPR cavity to an EPR imaging device and managed to get the first EPR image of endogenous pigments inside *ex vivo* melanoma [43]. However, an EPR image of melanoma had been obtained *in vivo* three years before by injecting a paramagnetic nitroxide contrast agent [44] near the tumor. This image was actually the first EPR image of melanoma but was not the reproduction of the repartition of melanin, but of the nitroxide contrast agent.

In 2005, after many years of work using EPR to study the properties of melanin and the effects of UV's on melanin and melanoma development, Timmins had the premention that EPR imaging could be helpful in the melanoma diagnosis and submitted a patent for the general concept of "detecting melanin by EPR" [45].

In 2008, Vanea et al. [15] assessed the potential of EPR to image freeze-dried mouse cutaneous melanomas and lung

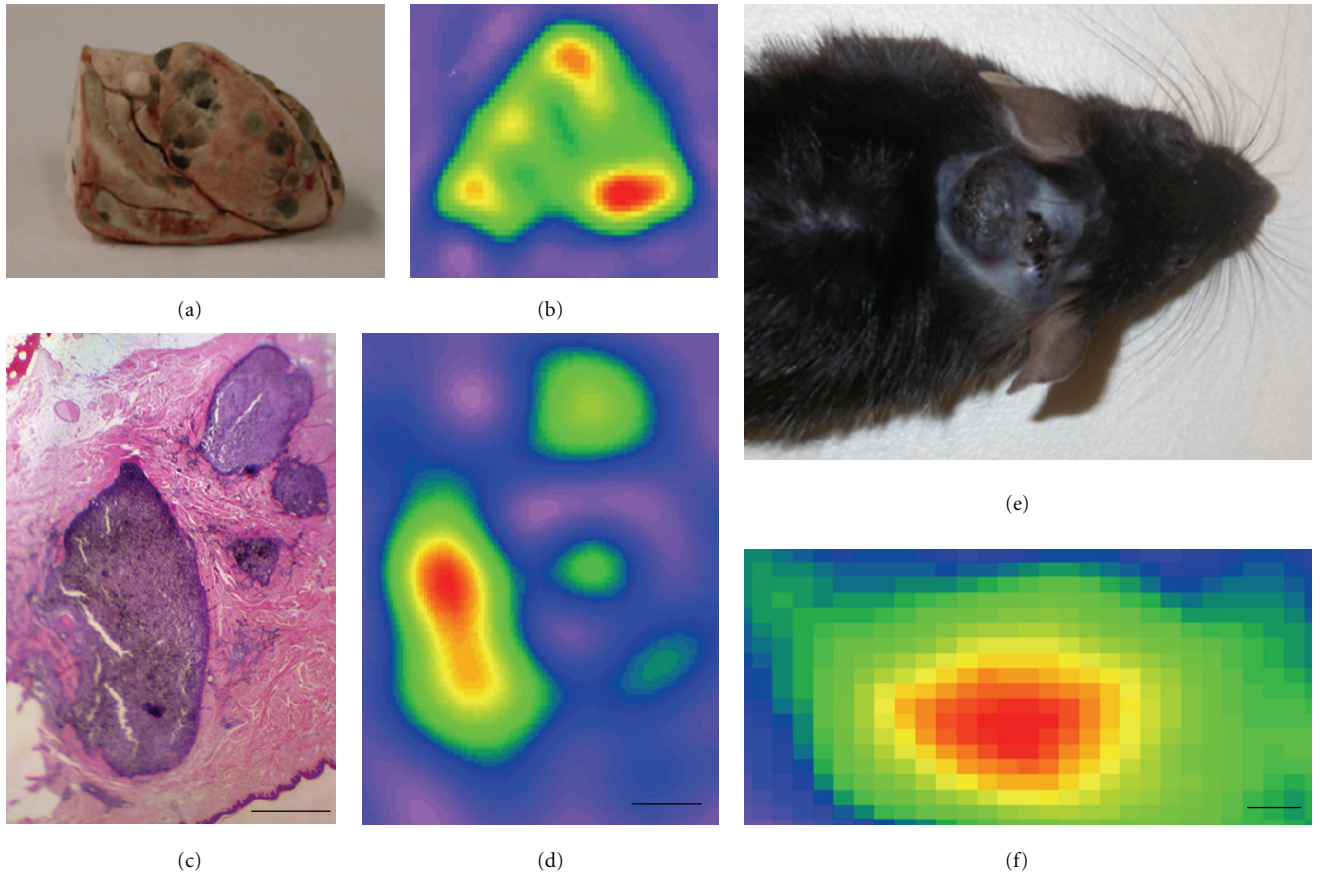

Figure 3: (a, b) Melanoma B16 metastases in the lungs of mice: picture of freeze-dried lungs with metastases (a) and the respective 2D transversal EPR image (b). (c, d) 2D EPR image through a section of thickness 500 mm of a paraffin-embedded human melanoma (d) and the histological section (5 mm thick) from a contiguous slice (c). Scale bars: 1 mm. (e, f) *In vivo* studies on B16 melanoma in mice. Melanoma grown in subcutaneous tissue (e) and *in vivo* EPR image obtained using a low-frequency EPR spectrometer with a head-coil loop-gap resonator. Scale bar: 2 mm. Pictures coming from Vanea et al. [15].

metastases. The freeze-drying process was required to avoid a nonresonant absorption of the microwaves by the water in the 9 GHz EPR system. They observed that the 2D and 3D EPR images were fitting very well with the visual aspect of the melanoma samples and invaded lungs (Figures 3(a), and 3(b)).

In the same study, they adapted their method to the measurement of paraffin-embedded human melanomas. As a result, they were able to correlate EPR images of these human melanomas with contiguous histological sections coming from the same tumors (Figures 3(c), and 3(d)) and demonstrate fair accuracy of EPR imaging.

In the final part of their work, they were able to obtain an EPR image *in vivo* of a large mouse melanoma B16 model, using a low microwave frequency EPR imager. This image was the first EPR image of the endogenous melanin pigments inside of an *in vivo* melanoma (Figures 3(e), and 3(f)).

Consecutively to the work of Vanea et al., it was decided to investigate the relationship between the growth state of mouse B16 cutaneous melanomas or metastases expressing luciferase, measured by the bioluminescence imaging (BLI) technique and their EPR spectra [15, 46]. In this study, we showed that there was a straight correlation between the EPR intensity of the signal, reflecting the melanin content of the tumor or invaded lung, and the bioluminescence intensity corresponding to the same sample. The same study was made on KHT fibrosarcomas (nonpigmented) so that the predominant role of the presence of melanin on the spectrum was confirmed.

By comparing the two methods, it was moreover demonstrated that EPR spectrometry was more accurate than BLI in terms of assessment of the tumoral growth level. These results, even if very encouraging, might however be moderated as BLI measurements were performed *in vivo*, while EPR measurements were performed *ex vivo*.

Finally, the limit of detection of the method in the present configuration (EPR Bruker E540 Elexsys equipped with a super high-sensitivity probe for X-Band (9 GHz) measurements and equipped with an E540R23 L-B and EPR head-coil resonator for L-Band (1.1 GHz) was assessed. This experiment, made in parallel with synthetic melanin and melanoma powder, allowed us to determine that such a tiny quantity like 2 μg of melanin could be detected *ex vivo* in favorable conditions using X-Band EPR spectrometry

(system dedicated to *in vitro* or *ex vivo* studies; large freeze-dried samples, or aqueous samples with a thickness of les than 1 mm). The detection threshold was however around 10^3 times higher for L-Band and measurements (adapted for *in vivo* studies; 1 cm depth penetration). Consequently, the performances of the method should still be improved to allow accurate and sensitive measurements *in vivo*.

6. Discussion and Conclusion

For approximately fifty years, considerable progresses were performed in the field of melanin and melanoma knowledge. These progresses are notably due to advances in biochemistry, genetics, and molecular biology. Parallel to these advances, technological developments in the field of magnetic resonance were achieved so that, nowadays, it is possible to apply the electron paramagnetic resonance spectroscopy and imaging to the detection of biological-free radicals, including those trapped in melanins, with a high sensitivity.

The application of EPR to melanins has led to many discoveries concerning melanin structure and melanoma development. As shown by Lund and Timmins [6], EPR spectrometry appeared as a unique tool to identify the ultraviolet range responsible of melanoma causation, and, by the way, to help to resolve a thirty-year-old controversy. We can expect that the consequences of this demonstration will have important consequences in the field of sunscreen development, which should undoubtedly contribute to a reduction of melanoma prevalence.

On the other hand, the improvements achieved in the field of EPR spectrometry during these last years allowed us to measure accurately and sensitively the presence of melanin pigments inside melanoma samples. As a straight correlation was found between the intensity of the EPR signal of melanin and the tumor growth state, the signal of melanin appears as a good indicator for melanoma development. Moreover, due to a continuous improvement of EPR imager performances, the first *in vivo* mapping of endogenous melanin pigments inside melanoma could recently be achieved by EPR imaging. Ongoing researches are now focusing on the characterization of human melanoma samples by this technique. Indeed, the diagnosis of melanoma in human by the ABCDE optic rule, even if effective, does not refer to any objective quantifiable standard. Moreover, an important limitation of the technique is the impossibility to obtain information about the tumor penetration (Clark's index) without performing a biopsy. The use of EPR imaging could fill in these lacks, that is why this method is at present tested on *ex vivo* human melanoma samples with different Clark's index. Applied *in vivo*, it would help the surgeon to define more precisely the margins for lesions resections, based on a noninvasive method. Preliminary results are encouraging, but it seems that early melanomas could provide an EPR signal that is insufficient for the reconstruction of EPR images. If these results are confirmed, new technical improvements in terms of magnetic gradient field or EPR cavity should be required before adapting the method to *in vivo* measurements.

Acknowledgments

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Review Article

Treatment of Locally Advanced Melanoma by Isolated Limb Infusion with Cytotoxic Drugs

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Since its introduction in the late 1950s, isolated limb perfusion (ILP) has been the preferred treatment option for locally advanced melanoma and sarcoma confined to a limb. This treatment results in high response rates with a satisfying duration of response in both tumor types. A drawback of ILP, however, is the invasive and complex character of the procedure. Isolated limb infusion (ILI) has been designed in the early 1990s as a minimally invasive alternative to ILP. Results of this simple procedure, reported by various centers around the world, show comparable response rates for melanoma and sarcoma when compared to ILP. Due to its minimally invasive character, ILI may replace ILP in the future as the preferred treatment for these locally advanced limb tumors.

1. Introduction

Patients who suffer from advanced melanoma or sarcoma confined to a limb are often challenging to treat due to the size and number of the satellite and/or in-transit metastasis in melanoma or the invasion of the tumor in sarcoma. In the past, results of systemic therapies in these tumours have often been disappointing. Although promising results have been published using ipilimumab and RG 7204 for metastatic melanoma, little is known about the effect of these agents when metastases are limited to a limb [1–4]. In the past an amputation was often inevitable but since the late 1950s this mutilating procedure can be avoided in the majority of patients by performing an isolated limb perfusion (ILP).

During ILP the blood circulation of the limb is temporarily discontinued from the systemic circulation. With a surgical procedure the femoral artery and vein (when treating a leg) or the axillary artery and vein (when treating an arm) are clamped and connected to an extracorporeal circuit containing a heart-lung machine in order to preserve physiological circumstances in the isolated limb. To achieve optimal isolation, minor vessels of subcutaneous tissues and muscles are compressed by a tourniquet. In this isolated circuit the dose of the cytotoxic drug, normally melphalan, can be safely applied up to a tenfold higher than systemically tolerated without compromising locally irreversible adverse effects [4–6]. Since flow of the cytotoxic drug to the systemic circulation could result in a life-threatening situation, potential leakage is continuously monitored during the procedure [7]. The cytotoxic drug circulates typically 60 to 90 minutes after which the limb is flushed to discard the remaining drugs in the isolated limb. The procedure is finalized by surgically disconnecting the tubes of the heart-lung machine, closing the vessels with sutures or a patch and deflating the tourniquet to restore the normal circulation in the limb.

Following ILP for melanoma complete response (CR) percentages of 7–91 (median 46) and partial response (PR) percentages of 0–44 (median 34) are reported. The median recurrence-free survival is 14 months, which is 23 months (range 8– > 72) following a CR. Overall survival following ILP is 24 months and 44 months (IQR 22– > 120) after a CR [4, 8, 9]. In patients with locally advanced sarcoma response percentages of 63–91 are reported [5, 10]. In both patient groups ILP can prevent amputation of the affected limb in 90% of the cases.

Despite these excellent results, ILP has some major disadvantages. It is an invasive and technically complex

Table 1: Mean blood gas values of the isolated limb after 30 minutes in 185 patients [17].

pO_2	8.4 mmHg
pCO_2	54.3 mmHg
pH	7.11
BE	−10.8 mmol/L
SO_2	6.9%

procedure in which not only a surgeon is involved but also a perfusionist and a large number of supporting staff are needed [11]. Although some have reported that ILP can be performed safely in elderly and frail patients, it is only performed in carefully selected patients [12–14]. Finally, a repeat ILP, after disease recurrence, is complex and can result in major complications due to scar tissue from the previous surgical approach of the vessels.

2. Isolated Limb Infusion

In the early 1990s the isolated limb infusion (ILI) technique was developed by Thompson and colleagues at the Sydney Melanoma Unit (currently Melanoma Institute Australia; MIA) as a simplified and minimally invasive alternative to ILP [25]. In contrast to ILP, during ILI no invasive surgical approach is needed. Radiological catheters with additional side holes near their tips are inserted percutaneously into the axial artery (6 French) and vein (8 French) of the disease-bearing limb via the contralateral groin using the Seldinger technique. Their tips are positioned in such a way that they are at the level of the knee or elbow joint. Tissues more proximally located in the limb but distal to the level of the tourniquet were perfused in a retrograde fashion via collateral vascular channels. The patient is then given a general anesthetic, and heparin (3 mg/kg) is infused to achieve full systemic heparinization. The catheters are connected to an extracorporeal circuit filled with saline solution incorporating a blood-warming coil but without a heart-lung machine. A pneumatic tourniquet is inflated around the root of the to be treated limb, and the cytotoxic agents are infused into the isolated circuit via the arterial catheter. In this isolated circuit a low-flow circulation can be realized without oxygenating the circulated blood resulting in a hypoxic and acidotic environment (Table 1) [17]. The cytotoxic drugs that are used are melphalan 5–10 mg/L of tissue (mostly 7.5 mg/L) and actinomycin-D 50–100 μg/L of tissue (mostly 75 μg/L) in 400 mL warmed, heparinized normal saline. Actinomycin-D is used because of the good response rates (CR 73%) of the melphalan/actinomycin-D combination when administered by conventional ILP, without excessive limb toxicity [26].

After infusion of the drugs in the isolated circuit the infusate is continually circulated for 30 minutes by repeated aspiration from the venous catheter and reinjection into the arterial catheter using a syringe attached to a three-way tap in the external circuit.

Since the half time of melphalan is 15 to 20 minutes and both melphalan and actinomycin-D are quickly absorbed by the tissues of the isolated limb, a relative short circulation time of 30 minutes is sufficient [27, 28].

Great care is given to the limb temperature since cooling of the extremity has a negative effect on the efficacy of the cytotoxic drugs. Heating of the limb is achieved by the aforementioned blood-warming coil in the extracorporeal circuit and by encasing the limb in a hot-air blanket, with a radiant heater placed over it [6]. Subcutaneous and intramuscular limb temperatures are monitored continuously during the ILI procedure. If these precautions are taken into account it is possible to achieve limb temperatures just above 40°C. Blood samples are taken at regular intervals to measure the melphalan concentrations and blood gases (Table 1). The drug leakage rate from the isolated limb is assessed retrospectively on the basis of systemic melphalan concentrations that are measured routinely during each procedure. Intraoperative systemic leakage monitoring is not performed because systemic leakage is negligibly low due to the low-flow and low-pressure circuit of the isolated limb and the effective isolation using the tourniquet.

After 30 minutes, the limb is flushed with one liter of Hartmann's solution via the arterial catheter, and the venous effluent was discarded. The tourniquet is then deflated to restore normal limb circulation, the heparin is reversed with protamine, and the catheters are removed [29]. Figures 1 and 2 provide an overview of the procedure [15, 16].

Postoperatively the serum creatine phosphokinase (CK) level is measured daily as an indicator for muscle and tissue breakdown, and limb toxicity, systemic toxicity, and tumor response are assessed regularly.

3. Results of Isolated Limb Infusion

Since 1992 over 400 ILIs have been performed in the MIA, mostly for melanoma but also for patients with locally advanced sarcoma. Following ILI a CR rate of 38% and a PR of 46% are seen in patients suffering from melanoma (Figure 3) [17]. The median LRFI in these patients was 13 months and 22 months (range 5 to >72: $P = .012$) for those experiencing a CR. The median survival following a CR was 53 months (range 28 to >120), 26 months (range 14 to >120) following a PR, and only 6 months for the small group of patients who had stable or progressive disease following the procedure ($P = .004$). These results are comparable to those reported after ILP [30, 31]. To date only one multicenter retrospective analysis for ILI has been published [18]. In this analysis 31% of the patients experienced a CR, 33% a PR, and 36% showed no response to the treatment. In addition to these institutions a number of institutions around the world have reported their experiences. These are listed in Table 2 [18–22]. The wide range of the results in these studies is possibly caused by the low number of patients in some of the studies and possibly by the lack of experience with this new technique. Furthermore, all institutes have used a protocol that is different in very small, but potentially essential, ways. The impact of these differences in protocol and the effect of increased experience have recently been investigated by Huismans et al. [32]. They showed that increased experience

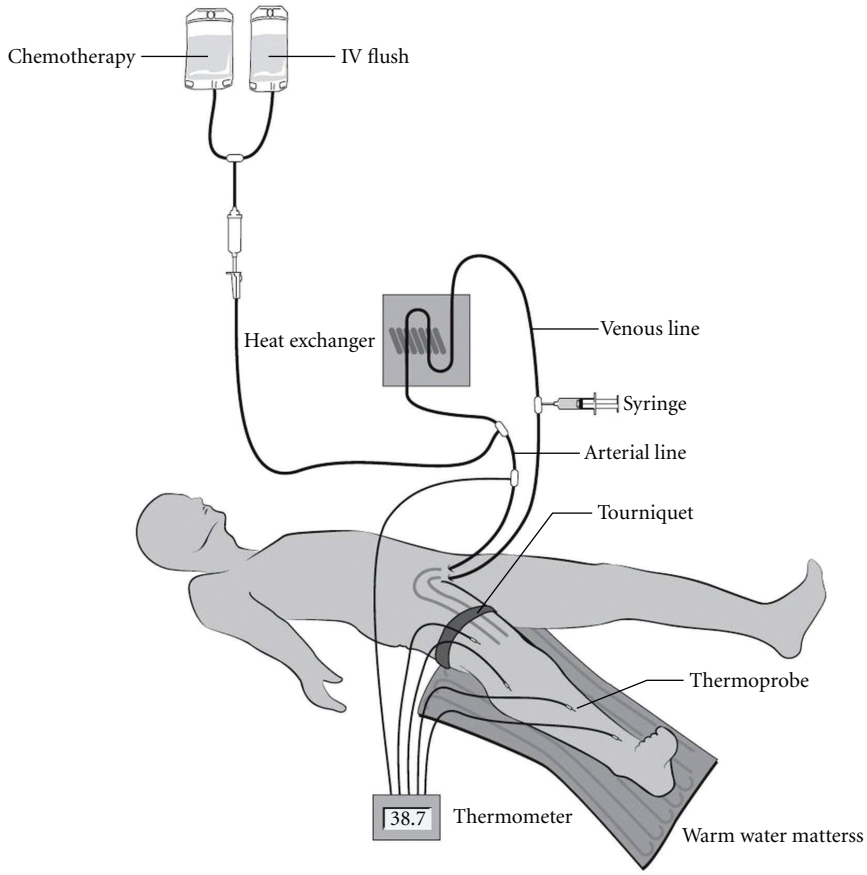


Figure 1: Schematic illustration of the circuit used for isolated infusion of a lower limb [15].

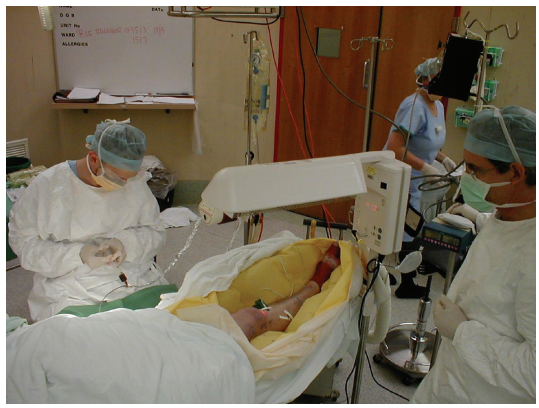
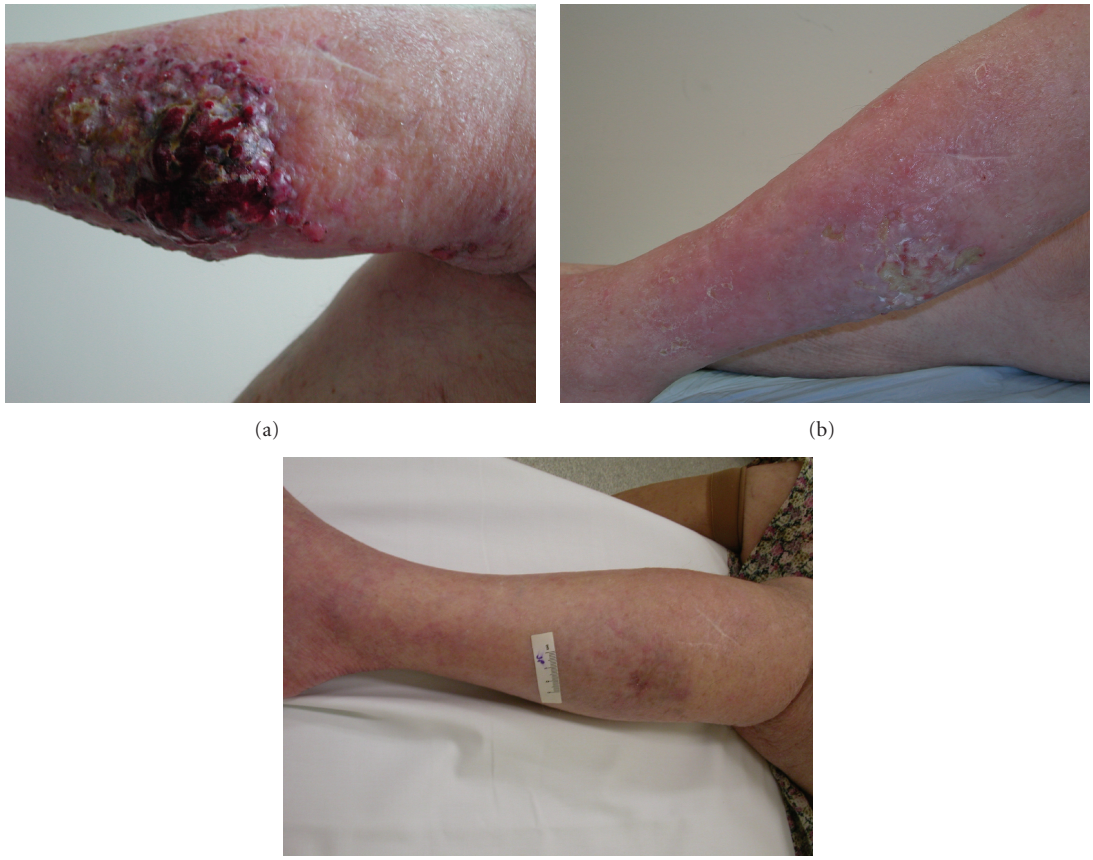


Figure 2: Photograph of an isolated limb infusion procedure in progress in the operating theatre [16].

and small modifications that were made to the ILI protocol at the MIA over the years resulted in a positive effect on the outcome. Another explanation for the reported range in results could be the point in time when the response of the procedure was investigated. Beasley et al., for instance, took the response after exactly 3 months while others took the best response at any time after ILI [20]. Despite the differences in experience, protocol, and outcome, most investigators have reported that patients who obtain a CR have significantly improved survival compared with nonresponders [20, 22].

The experience in using ILI for inoperable sarcoma is still limited, to two studies. However, the results reported



(a) (b)

(c)

Figure 3: (a) Extensive in-transit melanoma metastases of the left lower leg before ILI. (b) Remission 4 weeks post-ILI. (c) Complete response 4 months post-ILI.

Table 2: Isolated limb infusion studies using melphalan and actinomyocin-D [17–23].

Author, year	No. of patients	Response criteria	CR	PR	SD	PD
Mian et al., 2001 [19]	9*	Best response	44%	56%	0%	0%
Lindnér et al., 2002 [21]	128	Best response	41%	43%	12%	4%
Brady et al., 2006 [23]	22**	3 months	23%	27%	0%	50%
Kroon et al., 2008 [17]	185	Best response	38%	46%	10%	6%
Beasley et al., 2008 [20]	50	3 months	30%	14%	10%	46%
Marsden, 2008 [24]	16***	Unknown	26%	58%	—	16%
Barbour et al., 2009 [22]	74	Best response	24%	30%	37%	7%
Beasley et al., 2009 [18]	128	3 months	31%	33%	7%	29%

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.
*3 patients had >1 ILIs.
**1 patient had advanced sarcoma.
***3 patients had >1 ILIs, 4 patients had squamous cell carcinoma, and 2 patients had Merkel cell carcinoma.

are comparable to those seen after ILP [16, 23]. In these two separate studies CR rates of 23 and 57% and PR rates of 27 and 33% were reported with a median LRFI of 15 months. Following ILI amputation of the affected limb could be avoided in 76–94% of the sarcoma patients.

Following ILI the regional toxicity due to the cytotoxic drug is low [20, 25, 33]. Slight erythema and oedema are seen in 41–57% of the patients, and in 39–53% this is accompanied with the forming of blisters. In most cases a conservative treatment involving bed rest, elevation, and sometimes administering steroids is sufficient. In 3% of the patients the deeper tissues are involved, and in order to prevent a compartment syndrome a fasciotomy is sometimes carried out. To date at the MIA, it has not been necessary to amputate a limb due to severe tissue damage following ILI with melphalan and actinomycin-D. A study focusing

Table 3: Differences between isolated limb perfusion and isolated limb infusion.

Isolated limb perfusion	Isolated limb infusion
Technically complex	Technically simple
Open surgical exposure of vessels for catheter insertion	Percutaneous vascular catheter insertion in radiology department
4 to 6 hours duration	Approximately 1 hour
Perfusionist and large number of staff required	No perfusionist required and fewer total staff
Complex and expensive equipment needed	Equipment requirements modest
Magnitude of procedure excludes patients	Well tolerated by medically compromised, frail, and elderly patients
Not possible in occlusive vascular disease	Can be performed in occlusive vascular disease
Technically challenging to perform a repeat procedure	Not difficult to perform a repeat procedure
Systemic metastases normally a contraindication	Systemic metastases not a contraindication
Higher perfusion pressures predispose to systemic leakage	Low pressure system, effective vascular isolation with tourniquet
Limb tissues oxygenated, with normal blood gases maintained	Progressive hypoxia and acidosis
Hyperthermia (>41°C can be achieved)	Usually not possible to raise limb temperature above 40°C
General anesthesia required	Possible with regional anesthesia

on toxicity showed that patients with larger limb volumes experience increased toxicity grades without receiving higher cytotoxic drug doses [34]. In order to decrease the toxicity rates in these patients Beasley et al. corrected the melphalan dose for ideal body weight (IBW) [35]. In their hands this decreased toxicity significantly ($P = .001$) with only a small insignificant decrease in response ($P = .345$). However, these results could not be reproduced in a study initiated by the MIA in which the ratio of IBW and actual body weight did not predict toxicity or outcome (unpublished data) [36].

4. Discussion

One of the main advantages of ILI is the minimally invasive character of the procedure.

Morbidity as a result of the surgical approach of the blood vessels as seen in ILP is not experienced, and normally patients can be discharged from the hospital 7 days after the procedure [32, 34]. ILI can also safely be performed in elderly and frail patients without risking severe adverse effects. No increase in toxicity or morbidity was seen in the MIA patients, despite the fact that their average age was considerably higher than those seen in most ILP studies [17, 37]. Even in patients who suffer from distant metastatic disease and concurrent symptomatic limb disease ILI can effectively be used as a palliative treatment to provide local tumor control and limb salvage [38]. Also, because scar tissue is hardly formed following ILI, the procedure can easily be repeated in case of recurrent disease. The response rates of these repeat procedures are comparable to those seen after a first ILI [39].

Another advantage of ILI is the hypoxemia and acidosis in the isolated limb. Animal studies have shown that a hypoxic and acidotic environment enhances the effect of melphalan by a factor 3 [40]. Clinically enhanced responses were observed when isolation of the limb lasted longer than 40 minutes [21].

Furthermore, ILI has a number of practical advantages over ILP. The time in the operating theatre is considerably shorter (on average one hour compared to 3–5 hours for ILP), no complex and expensive equipment is used, and less personnel is needed [29]. Because of this, ILI is a much cheaper procedure. Finally, ILI is often used in trial settings to provide insight for developing novel treatment strategies [41]. One of these studies used systemic ADH-1 in combination with melphalan. It was well tolerated and provided a CR of 50% [42]. In Table 3 differences between ILI and ILP are listed.

5. Conclusion

Over the last two decades ILI has become a serious alternative to the traditionally used ILP technique. Studies, most published in the recent years, have shown that, when performed correctly, response rates following ILI are comparable to those seen after ILP. ILI, however, results in less toxicity and morbidity. In the future more research and in particular a randomized controlled trial is needed to prove the effect of ILI, and it is not unthinkable that ILI will become the preferred treatment for patients who suffer from advanced melanoma or sarcoma confined to a limb.

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Review Article

Uveal Melanoma

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Uveal melanoma is the most common primary intraocular malignancy and the leading primary intraocular disease which can be fatal in adults. In this paper epidemiologic, pathogenetic, and clinical aspects of uveal melanoma are discussed. Despite the advance in local ocular treatments, there has been no change in patient survival for three decades. Development of metastases affects prognosis significantly. Current survival rates, factors predictive of metastatic potential and metastatic screening algorithms are discussed. Proposed and emerging treatments for uveal melanoma metastases are also overviewed. Current advances in genetics and cytogenetics have provided a significant insight in tumours with high metastatic potential and the molecular mechanisms that underlie their development. Biopsy of those lesions may prove to be important for prognostication and to allow further research into genetic mutations and potential new therapeutic targets in the future.

1. Epidemiology

1.1. Incidence. Uveal melanoma is the most common primary intraocular malignancy and the leading primary intraocular disease which can be fatal in adults. In the general population it is uncommon with an incidence of 5.3–10.9 cases per million population per years [1, 2]. There has been no change in the incidence of uveal melanoma over the past 30 years in the USA [2]. No change in incidence has been reported for Denmark or Finland. In Sweden, an annual relative decrease of 1% has been reported [3]. The incidence rate in black populations has been shown to be low whether for Africans or African-Americans [1, 4]. The risk is also low among Asian populations [5] and American populations of Asian descent [1]. In Europe a north to south decreasing gradient of melanoma incidence among European populations does support the protective role of pigmentation [6]. Uveal tract melanoma is usually diagnosed in the sixth decade of life with a median age of 55 in most series [7]. The incidence rate has been shown to progressively increase up to the age of 70 years in the USA [2]. In Europe, similar findings have been reported with incidence rates increasing with age, peaking at 75 and then reaching a plateau [6].

Most series indicate that both sexes are equally affected with a slight predominance of males [2, 6, 8]. In a review of systemic databases, the age of diagnosis is slightly increased in females (males 59.4, females 61.5) [2].

1.2. Risk Factors. Development of uveal melanoma has been associated with the presence of ocular or cutaneous melanocytic lesions. Ocular lesions include, primarily, choroidal naevi but also include ocular melanocytosis. The latter manifests as heterochromia, and a dark eye is due to a congenital unilateral hyperpigmentation of the episclera and the uveal tract. The cutaneous conditions associated with uveal melanoma are familial atypical mole and melanoma [9], cutaneous melanoma, and oculodermal melanocytosis (naevus of Ota).

Another host risk factor is a lightly coloured iris, though there was no definite association found in regards to prior nonocular malignancy or hormonal levels [10]. In regard to environmental factors, weak associations have been made with sun exposure [11]. Some occupations are believed to be associated with an increased risk of ocular melanoma such as arc welders and airline pilots, but this has not been

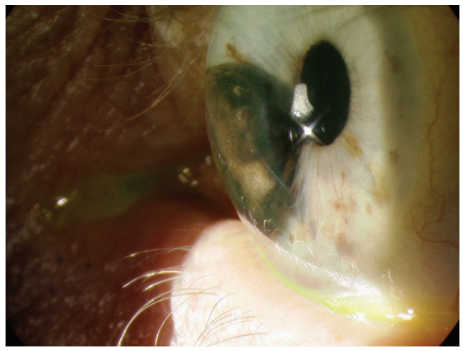


Figure 1: Large iris melanoma occupying a significant percentage of the anterior chamber with associated corectopia.

proven. No association has been found with any dietary habits, smoking, or alcohol consumption [9].

2. Pathogenesis

The development of uveal melanoma has been associated with early oncogenic mutations. These mutations affect pathways involved with the regulation of the cell cycle or the control of cell apoptosis.

2.1. Regulation of the Cell Cycle. The retinoblastoma protein inhibits cell cycle progression through the G1-S transition point, and its inactivation by hyperphosphorylation allows cells to reenter the cell cycle [12].

These mutations appear to involve the RAF/MEK/ERK pathway. It has been shown in the past that a target of this pathway, *CCND1,* responsible for encoding cyclin D1, is overexpressed in uveal melanomas leading to phosphorylation and inactivation of the retinoblastoma tumour suppressor gene in uveal melanomas [13].

An oncogene mutation affecting this pathway is a mutation of the genes *GNAQ* and GNA11 in codon 29. These genes encode GNAQ, the alpha subunit of a heterotrimeric GTP-binding protein that couples G-protein-coupled receptor signaling to the RAF/MEK/ERK and other intracellular pathways. These pathways are important for melanocyte homeostasis. In addition, GNAQ is involved in endothelin signaling which is essential for melanocyte survival early in development [14]. Activation of GNAQ mimics growth factor signaling in the RAF/MEK/ERK pathway leading to transcriptional activation of CCND1.

GNAQ mutation was noted at 49% of the examined uveal melanoma postenucleation samples [13] and 45% of biopsy samples [14, 15], a fact delineating that other mutations could also be responsible. *GNA11* mutations have been noted at 31.9% of uveal melanoma samples [15].

Another molecular event associated with dysfunction of the retinoblastoma protein is the inactivation of the INK4A gene [12].

2.2. Control of Cell Apoptosis. The molecular events that have been associated with inhibition of apoptosis in uveal melanoma include inactivation of the p53 pathway [16], defects in the Bcl-2 pathway [17], and activation of the prosurvival PI3K-AKT pathway [18].

3. Clinical Features

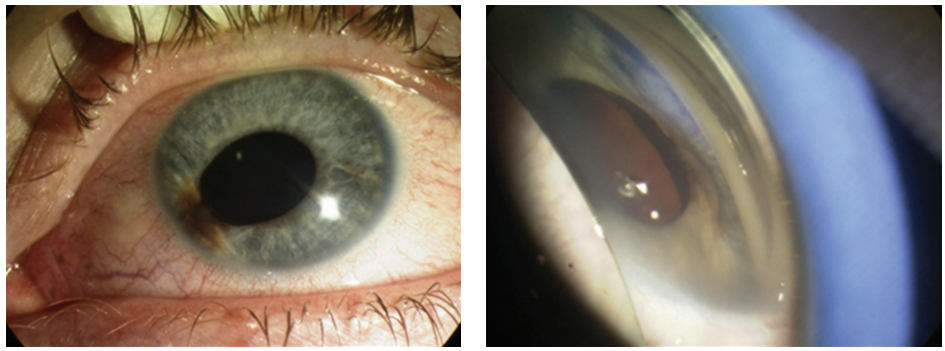
Iris melanoma appears as a variably pigmented, usually well-defined mass in the iris stroma and has an 80% predilection to appear in the inferior portion of the iris (Figure 1). Size and shape can also be variable. Less common variables of the iris melanoma are the *diffuse* melanoma, which causes hyperchromic heterochromia of the iris and is associated with infiltration of the trabecular meshwork, *tapioca* melanoma, which has a gelatinous nodular iris appearance, and the *melanoma* of the trabecular meshwork with a ring configuration (Figure 2) [19].

Ciliary body melanoma can attain a larger size before it is recognized clinically. The patient is often asymptomatic. However, a ciliary body mass can cause lens tilting or anterior displacement of the lens which results in an uncorrectable astigmatism. Signs include dilated episcleral vessels (sentinel vessels), dense cataract, and in the case of ring melanoma, raised intraocular pressure. Extrascleral extension is rarely seen at presentation (Figure 3). Usually the lesion has a dome-shaped configuration visualised after dilation of the pupil. Less frequently it can attain a circumferential ring pattern. It can extend toward the lens and cause localized cataract (Figure 4), toward the anterior chamber angle and iris (iridociliary melanoma), or posteriorly into the choroid (ciliochoroidal melanoma) [7].

Choroidal melanoma may present with visual symptoms if it is located at the macula; here it will produce micropsia or visual distortion. Peripheral choroidal melanoma tends to present with a visual field defect or a localised flickering light corresponding to the location of the mass. If an exudative retinal detachment is present, the patient may reach a retinal surgeon first before the diagnosis of melanoma is made. The tumour may be dome or mushroomed shaped. Diffuse choroidal melanoma is a rare aggressive variant. Small choroidal melanomas often have superficial orange pigment known as lipofuscin and associated subretinal fluid (Figure 5). Melanoma is frequently pigmented, and an amelanotic melanoma must be distinguished from other simulating lesions. If the tumour is amelanotic, blood vessels are visible through it and the classical double circulation described with fluorescein angiography may be seen (Figure 6).

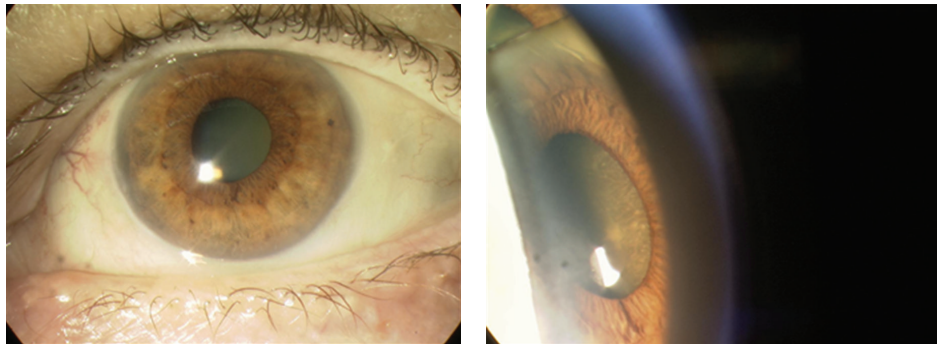
4. Diagnosis

Iris melanoma is usually diagnosed with slit lamp biomicroscopy. However ultrasound biomicroscopy can be used to detect extension of iris melanoma towards the ciliary body and to differentiate it from iris cysts [19]. If there is no evidence of documented growth of the lesion, fine needle aspiration biopsy (FNAB) or conventional iris biopsy is sometimes required to differentiate an iris melanoma from a suspicious iris naevus.



(a) (b)

Figure 2: Iris melanoma located in the inferonasal portion of the right eye. Of note the associated corectopia and an episcleral sentinel vessel were adjacent to the lesion. This lesion extended to the anterior chamber and acquired a ring configuration as shown in gonioscopy.



(a) (b)

Figure 3: Ciliary body melanoma (a) pigmented area with associated sentinel vessel nasally. (b) gonioscopy reveals the underlying lesion.

Ciliary body and choroidal melanoma are typically diagnosed with slit lamp biomicroscopy or indirect ophthalmoscopy. The clinical diagnosis can however be supported or confirmed with various ancillary studies.

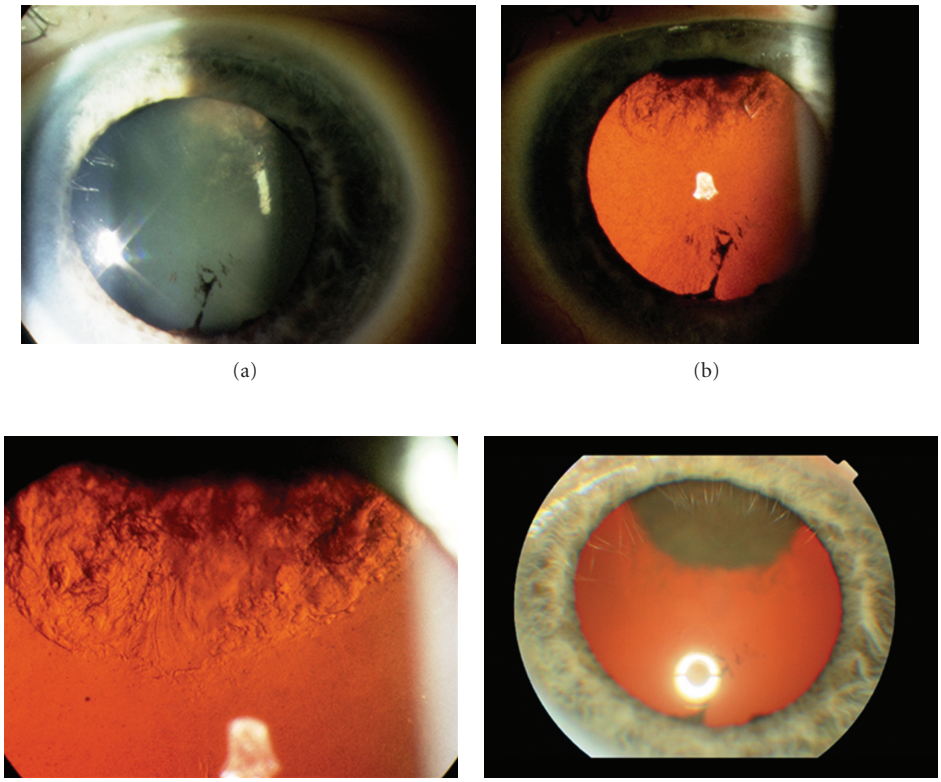
Transillumination is a useful technique to detect ciliary body and anterior choroidal melanomas. It is also used for the delineation of the tumour margins intraoperatively. This technique is performed by placing a fibre-optic point light source on the ocular surface and observing the eye that glows like a light bulb. In most cases the tumour shows up as a dark shadow with well-defined margins.

4.1. Fluorescein Angiography. Typical findings on the fluorescein angiogram include mottled hyperfluorescence and late staining of the lesion. In the case of an amelanotic melanoma or a large melanoma that has broken through Bruch's membrane, the double circulation sign is noted (Figure 7). In this sign, both retinal and choroidal circulation are visualized.

4.2. Ultrasonography. A mode ultrasonography demonstrates medium to low internal reflectivity. However most ocular oncologists use B mode ultrasound which demonstrates the presence of ultrasonographic hollowness and choroidal excavation. Not only is it useful for the examination of a lesion in the presence of a dense cataract or a vitreous hemorrhage but also it can be helpful in measuring the elevation of the tumour. This is important not only in determining the malignant potential of a suspicious lesion but also in determining the response to treatment of a malignant melanoma after radiotherapy or laser treatment [7, 20].

Computed tomography and magnetic resonance imaging can be used to determine the presence and degree of extraocular extension, although this is best visualised with orbital ultrasound. *Optical coherence tomography* allows the identification of subretinal fluid associated with a suspicious choroidal naevus [21]. However the clinical importance of fluid on OCT alone is yet to be determined.

4.3. Risk Factors of Melanocytic Choroidal Lesions. Eight risk factors for malignant behaviour of melanocytic choroidal lesions have been identified [22]. These include: tumour thickness more than 2 mm at initial diagnosis, presence of associated fluid with the lesion, presence of symptoms, presence of orange pigment on the surface of the lesion, location of the lesion close to the margin of the optic disc (closer than two disc diameters or 3 mm), presence of ultrasonographic hollowness of the lesion at B mode



(a) (b)

(c) (d)

Figure 4: Ciliary body melanoma extending to the lens causing localized cataract.

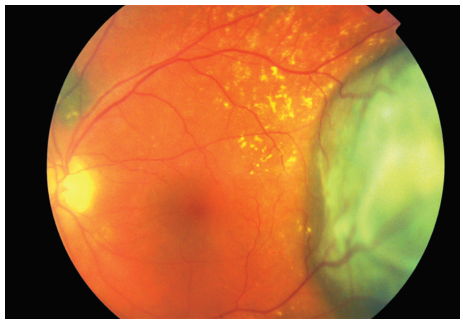


Figure 5: Typical choroidal melanoma with associated nonrhegmatogenous retinal detachment.

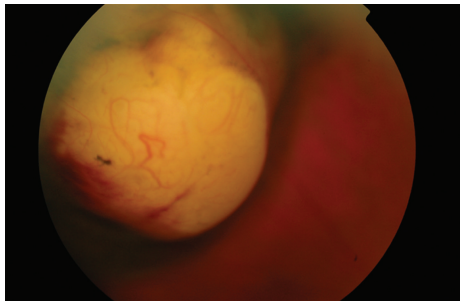


Figure 6: Amelanotic choroidal melanoma. Choroid vessels are visible through the tumor.

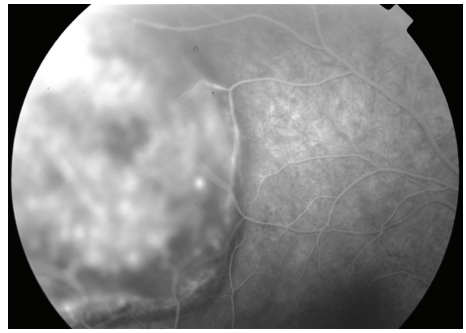


Figure 7: Fluorescein angiogram of a large amelanotic choroidal melanoma, notice the double circulation sign with visible leaking choroidal vessels at the tumor.

ultrasound, absence of a depigmented halo around the lesion, and absence of drusen. These factors determine the follow-up schedule of the patient or the initiation of treatment [21, 22].

5. Treatment

There are many options available for the treatment of uveal tract melanoma. The principal options are enucleation, plaque radiotherapy, proton beam radiotherapy, and transpupillary thermotherapy.

5.1. Enucleation. Enucleation is the traditional method of treating uveal melanomas. It is generally indicated for advanced melanomas that occupy most of the intraocular space or affected eyes with severe secondary glaucoma. It is also indicated for primary tumours that have invaded the optic nerve. Secondary enucleation is indicated if there is definite evidence of recurrence of a tumour initially treated with alternative treatment modalities (see below). Preenucleation radiotherapy involving the use of 2000 cGy of external beam radiotherapy for the affected eye and orbit is no longer advocated as it has not proven to be advantageous over standard enucleation [23].

In the western world enucleation tends to be performed with the placement of an orbital implant onto the socket. There are two major classes of implants: nonporous and porous implants. Nonporous implants are composed of silicone, acrylic or PMMA. The most commonly used porous implants are composed of hydroxyapatite. The porous surface allows fibrovascular growth into the implant, which prevents the extrusion or migration of the implant. Usual sizes are 18–22 mm in diameter.

5.2. Plaque Brachytherapy

5.2.1. Rationale. A radioisotope is a typically man-made element with an instable nucleus. The loss of an electron or a neutron is accompanied by the emission of ionising radiation as the radioisotope decays to a more stable element. When a radioactive source is placed in against the sclera or in close proximity to a tumour all structures close to that source are irradiated. Ionizing radiation is absorbed by the exposed tissue resulting in the formation of free radicals, DNA damage, and, eventually, loss of the cell division or cell death.

5.2.2. Technique. Radioactive plaques are typically round curvilinear-shaped episcleral discs of varying diameter. The convex inner surface contains the radioactive source (most commonly ruthenium-106, iodine-125, or palladium-103). The concave external surface consists of a heavy metal (e.g., silver and gold) to shield structures on the outer surface of the plaque. The plaque has two or more eyelets (lugs) to permit suturing to the sclera. Size of the plaque is selected to maintain a 2 mm safety margin around the base of the tumour. Radiation emitted to the apex of the lesion is between 80 and 100 Gy, which is considered to be the effective tumouricidal dose.

The radioactive plaque is removed 2–7 days after insertion when the calculated dose of radiation has been locally administered.

5.2.3. Complications. The complications of plaque brachytherapy include cataract, proliferative radiation retinopathy (Figure 8), radiation papillopathy (Figure 9), maculopathy, neovascular glaucoma [24], and an exudative tumour response [25].

Radiation-induced complications occur on average 18–24 months after plaque treatment [26, 27]. The incidence ranges from 18 to 43% in different series [26, 28]. Clinical signs of radiation maculopathy have been shown to occur to up to 75% of patients in the COMS study [29]. Risk factors for the development of radiation retinopathy include total radiation dose, proximity of the treated lesion to affected structures, diabetes mellitus, and younger age [30, 31]. Panretinal photocoagulation can be used for the treatment of proliferative radiation retinopathy causing regression of neovascularization in 66% of patients in a recent large series [31].

5.2.4. Indications. Plaque brachytherapy is indicated for small choroidal melanomas with evidence of growth, medium-sized uveal melanomas in eyes with useful or salvageable vision, and large melanomas or larger melanomas if in an only eye [32, 33].

5.2.5. Results. The COMS Medium-Sized Tumour Trial has indicated that mortality rates from iodine-125 brachytherapy and enucleation do not differ for up to 12 years after treatment proving the efficacy of radiation in the treatment of uveal melanoma [34]. Survival rates are 82% at 5 years for iodine-125 brachytherapy [25] and 84% for ruthenium-106 [35].

The overall tumour recurrence is 10% at 5 years [36]. Treatment failure has been associated with larger tumour size and posterior extension [36, 37]. The secondary enucleation rate is 12–17% at 3–5 years followup [35, 38] and is usually the result of local recurrence or neovascular glaucoma.

49–55% of patients treated with plaque brachytherapy maintain a best-corrected visual acuity of 6/60 or better and around 30% have 6/18 visual acuity or better in the treated eye [35, 38].

5.3. Proton-Beam Radiotherapy

5.3.1. Rationale. Tumour is exposed to a charged proton beam. Charged protons lose their energy in tissue with minimal scatter. The energy deposition occurs at the end of their range (Bragg peak). This property of photons allows a decreased entry chance through normal tissues. The proton beam is conformed to adjust to any tumour size [39].

5.3.2. Technique. Four radiopaque tantalum rings are sutured to the sclera at the border of the lesion to aid with tumour localisation with an ocular X-ray. During the treatment planning, a three-dimensional model of the tumour is superimposed on the normal eye and a face mask and collimator are custom designed for the patient. The fixation angle that will ensure minimal radiation exposure to lens, fovea, and optic disc and maximal exposure to the tumour is determined. Standard treatment is fractionated four times; total dose of 60–70 cobalt Gy equivalents (cGy) is administered.

5.3.3. Indications. Proton bean radiotherapy is indicated for all melanomas and in particular larger melanomas up to 24 mm in diameter and 14 mm in height. Tumours involving

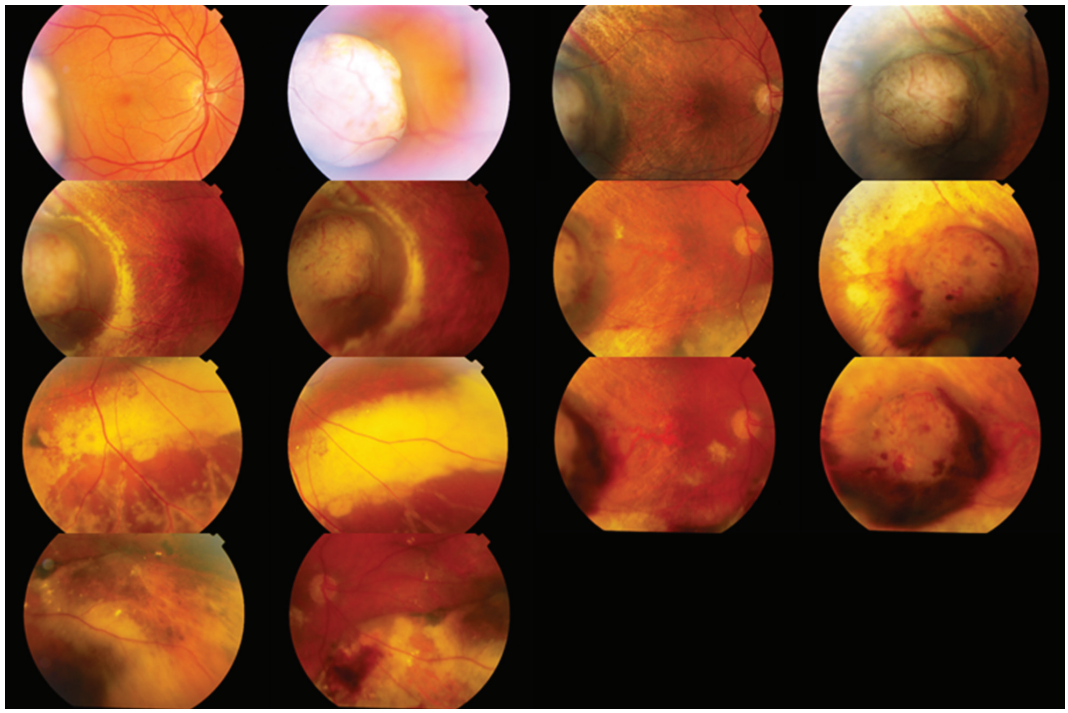
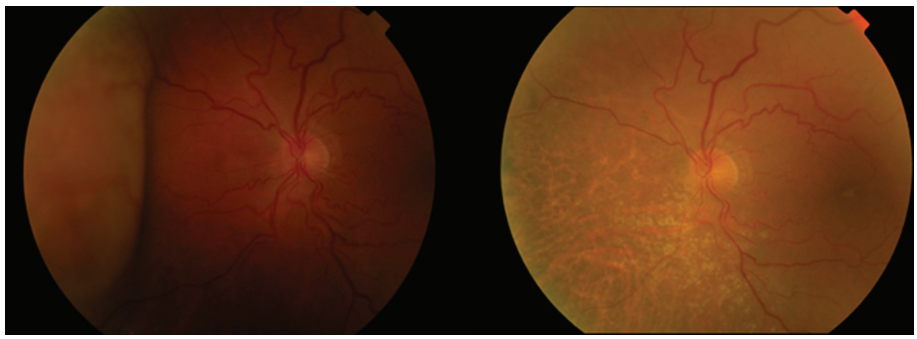
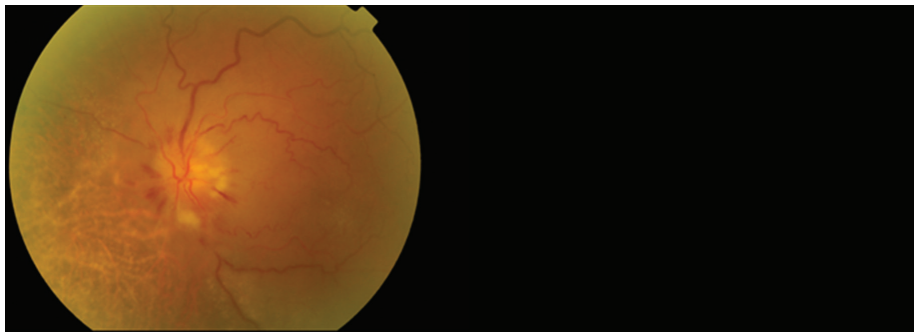


Figure 8: Amelanotic choroidal malignant melanoma treated with plaque brachytherapy. Follow-up images span a period of 7 years. Eventually extensive radiation retinopathy developed.



(a)



(b)

Figure 9: Amelanotic malignant choroidal melanoma treated with plaque brachytherapy. Development of radiation papillopathy and maculopathy one year after treatment.

the macula, the optic disc, or both are not a contraindication [40]. It is not recommended for very large melanomas that occupy greater than 30% of the ocular volume, or for tumours with extrascleral extension, large retinal detachment, or extensive neovascularisation [41].

5.3.4. Results. Most tumours regress for up to 2 years after treatment. Regression is complete in 15% of patients (Figure 9). Gragoudas et al. have reported vision loss to occur in 68% of patients at 5 years after treatment. This loss has been correlated with proximity of the tumour to the fovea and the optic disc, the elevation and diameter of the tumour, and baseline visual acuity [42]. Two additional risk factors were added in a more recent study [43], namely, diabetes and retinal detachment with percentages fluctuating from 16% for low-risk patients to 99% for high-risk patients. The probability of retaining the eye was 91% at 5 years, 88% at 10 years, and 84% at 15 years after irradiation as indicated by a large recent series [44]. Complications include iris neovascularization [41], posterior subcapsular cataract, radiation maculopathy, and papillopathy (Figures 10 and 11). Survival rates have been shown to be 86% at 5 years, 77% at 10 years, and 73% at 15 years after irradiation. Highest death rates were noted at 3–6 years after treatment [40].

5.4. Transpupillary Thermotherapy. Transpupillary thermotherapy (TTT) is a treatment method that utilizes a modified diode laser delivery system to induce hyperthermia to the tumour by delivering heat in the infrared range. Tumour is heated to a temperature of 60–65 degrees [45]. The sensory retina is not damaged as much as in laser photocoagulation. Despite initial results advocating a beneficial effect of TTT [46], high rates of tumour recurrence have been detected in 23–45% of cases [47, 48].

Recurrences have been attributed to the fact that the intrascleral tumour cells do not absorb the emitted heat [49]. Therefore, recurrences have been reported in the orbit due to extrascleral extension.

Complications of TTT include scotoma, macular traction, vascular occlusion, and hemorrhage [50].

TTT is currently combined with plaque radiotherapy [51] or is applied as secondary treatment to local tumour recurrence after radiotherapy or local resection [52].

6. Metastases

6.1. Survival Rates. Despite the availability of alternative treatment modalities, the survival rates of patients with uveal tract melanoma have not changed in 30 years. Cumulative rates of metastases in the Collaborative Ocular Melanoma Study at 5 and 10 years after treatment were 25% and 34%, respectively. Common sites of metastases include liver (90%), lung (24%), and bone (16%) [53, 54]. Patients with metastases confined to extrahepatic locations have longer survival (19–28 months) [55]. Median survival for a hepatic metastasis is 6 months with an estimated survival of 15–20% at 1 year and 10% at 2 years, irrespective of treatment [56, 57]. Asymptomatic patients at the time of diagnosis of metastases have a slightly longer survival in relation to symptomatic patients [57].

In the case of iris melanoma, distant metastasis to liver or other organs occurs in 5% of patients at 10 years of followup. The risk is higher if the tumour involves the iris root and angle and there is elevated intraocular pressure or extraocular extension [58].

6.2. Predictive Factors of Metastatic Potential

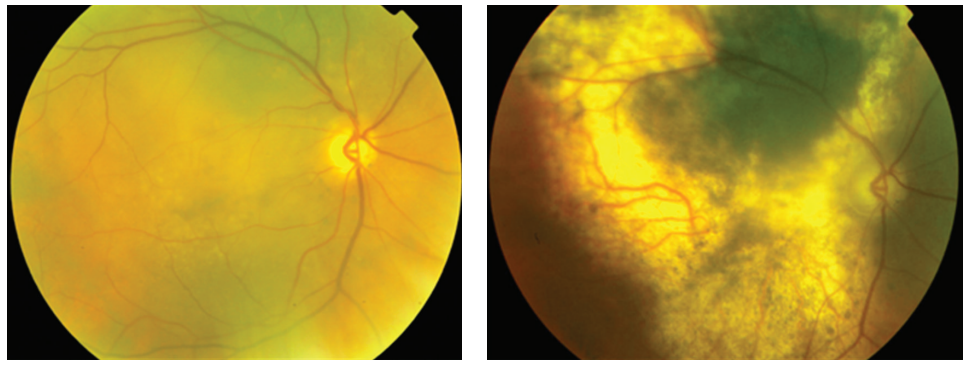
6.2.1. Tumour Size. Tumour size is one of the best parameters used to predict metastatic disease. According to the COMS classification, a tumour is defined as small if it measures 3 mm or less in thickness and less than 10 mm in diameter, as medium-sized if 3–5 mm in thickness and 10–15 mm in diameter, and as large if greater than 5 mm in thickness and more than 15 mm in diameter. A comparative analysis of uveal melanoma [59] has indicated that the 5-year survival rates after enucleation were 84% for small, 68% for medium-sized, and 47% for large tumours. Another study [60] indicated that increased tumour thickness increases the risk of metastasis.

6.2.2. Molecular Markers. Dissemination of tumour cells into the blood circulation occurs due to lack of lymphatics in the uveal tract. Haematological markers may, therefore, be useful for the detection of distant metastases. This rationale has prompted the research for determination of potential molecular markers for the early detection of disseminated tumour cells.

Tyrosinase is an enzyme involved in the synthesis of melanin by melanocytes and melanoma cells. Serum tyrosinase m-RNA levels have been shown to be increased in patients with primary uveal melanoma, and they correlate with metastatic disease. In addition, tyrosinase m-RNA can be used for the indirect quantification of circulating tumour cells and have been correlared with the dimensions of the primary tumour [61].

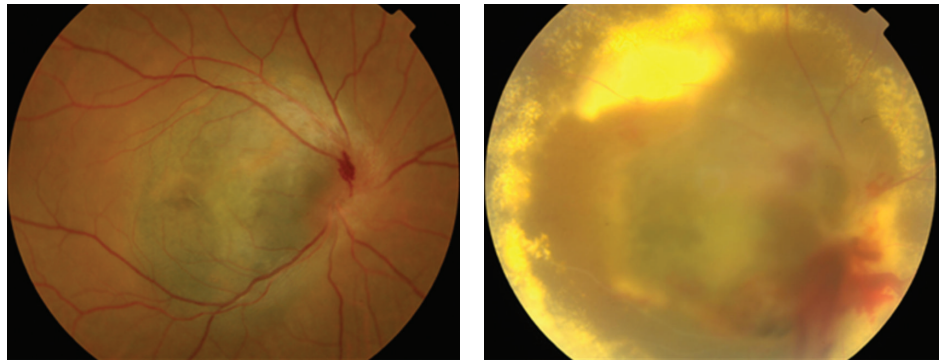
Vascular endothelial growth factor (VEGF) has been proven to overexpress in uveal melanoma cells. It has been suggested that this overexpression is indicative of an angiogenic switch of the uveal melanoma that is associated with a proliferative stage of the tumour and metastatic potential [62]. Overexpression of VEGF originates from abnormal new vessels within the tumour and hypoxia because of the irregular blood flow. VEGF has been traced in uveal melanoma cells and in the aqueous humor in eyes with uveal melanoma [63, 64]. Levels of VEGF have been associated with the metastatic potential of uveal melanoma [65], and serum levels are increased in the presence of micrometastases, and they parallel the extent of liver disease [62, 65].

Hepatocyte growth factor (HGF) and its receptor c-Met have been shown to have an important role in the growth of cells in the liver. Increased levels of c-Met in primary tumours have been associated with a high risk of metastatic potential [66]. Activation of HGF by c-Met has been shown to induce



(a) (b)

Figure 10: Large choroidal melanoma of the right eye treated with proton beam radiotherapy. Despite extensive atrophy the lesion is flat.



(a) (b)

Figure 11: Large juxtapapillary lesion treated with proton beam radiotherapy. Extensive radiation retinopathy and papillopathy developed postoperatively.

increased cell proliferation, downregulate apoptosis, and increase cell motility and invasive ability [67].

Insulin-like growth factor-1 (IGF-1) is also produced in the liver, as is HGF. IGF-1 binds to IGF-1R, a surface membrane glycoprotein. Expression of this molecule has been associated with worse prognosis in uveal melanoma [68]. Activation of IGF-1R by binding of circulating IGF-1 increases cell proliferation, prevents apoptosis, and is important for integrin adhesion to the extracellular matrix and invasion of basement membranes. These are essential steps in the formation of metastasis [69]. Therefore when metastatic disease is present, serum IGF-1 levels fall [70]. In a recent study, the coexpression of IGF-1 and c-met in uveal melanoma samples was highly predictive of metastasis [71]. Despite the promising role of serum molecular markers in determining metastatic disease at a subclinical level, their application in metastatic surveillance is limited as there is a wide variability in the normal range within the population. For instance, fluctuations of serum IGF-1 within an individual are more meaningful [70].

6.2.3. Genetic and Cytogenetic Aspects

Chromosomal Alterations. Prescher et al. [72] in Germany were the first to describe the chromosome changes seen in uveal tract melanoma which had not been discovered in cutaneous melanoma. The major chromosome alterations have been described in chromosomes 3, 6, 8, and 11. However the most important of these changes is seen in chromosome 3. In short, monosomy 3 (loss of whole of chromosome 3) tends to be found in large uveal tract melanoma in the ciliary body location. These chromosome changes have been strongly linked to patient survival. Monosomy 3 is associated with a 5-year survival of approximately 50%, whereas disomy 3 has been reported to predict 100% survival [73]. In a recent large series of 500 patients with uveal melanoma, those with monosomy 3 had a significantly worse 3-year prognosis in relation to patients with partial monosomy 3 or disomy 3 [74]. Interestingly, these chromosomal abnormalities are significantly correlated with the clinical high risk factors for metastasis in uveal melanoma (such as tumor size at diagnosis and epithelioid cell histology) [75].

Gene expression profiling has been shown to be more predictive of metastatic death than fluorescent in situ hybridization (FISH) analysis of chromosome alterations (12). Using gene expression profiling, melanomas have been categorized into two groups: Class I and Class II. Class I denotes tumours with two copies of chromosome 3 (disomy 3) and other beneficial chromosome changes including gain in chromosome 6p. Class II denoted tumours with only one

copy of chromosome 3 (monosomy 3) and other deleterious chromosome changes including gain of chromosome 8p and/ or isochromosome 8p. It is believed that as the tumor undergoes subsequent growth it either gains a fragment of chromosome 6p and becomes a less aggressive Class I melanoma or it loses a copy of chromosome 3 and develops into a Class II melanoma with high metastatic potential. Class II tumors have a greater chromosomal aneuploidy and a significantly different proliferative capacity as indicated by the expression of Ki-67 antigen [73].

This significant discovery has implications on the subsequent management of patients with uveal tract melanoma. For example, patients with Class II tumours are eligible for increased metastatic surveillance and entry into adjuvant treatment trials. At present, the primary surgical management of a uveal tract melanoma remains the same whether the melanoma falls into the category of Class I or Class II.

Gene Alterations. Mutations in genes *GNAQ* and *GNA11* have been associated with the development of uveal melanoma (see Pathogenesis). *GNAQ* and *GNA11* mutations at codon 209 were encountered in 21.7% and 56.5% of metastatic uveal melanoma samples, respectively [15]. In the same series, *GNA11* mutations were more common in locally advanced tumours and in tumours of the ciliochoroidal region. In a recent series of 75 patients [76], *GNAQ* mutations were not associated with disease-free survival despite an occurrence of 53.3%. In addition, no association was found with chromosome status reinforcing the notion that these mutations are an early pathogenetic event and probably are not associated with clinical outcome.

6.3. Metastatic Screening. Currently, here is no universally accepted algorithm for metastatic screening in patients with melanoma. In the COMS, chest radiographs and liver function tests were done every 6 months for at least 5 years. Despite high specificity (92%), liver function tests have a sensitivity of less than 15% in the diagnosis of metastatic uveal melanoma [54]. Serum markers indicative of metastatic disease have been shown to be alkaline phosphatase and lactate dehydrogenase [77].

Individual case series have demonstrated that whole-body F-18-fluoro-2-deoxyglucose positron emission tomography/computed tomography imaging is a sensitive modality in the followup of uveal melanoma patients [78, 79]. The advantage of this imaging modality is the depiction of metabolic activity as obtained by F-18-fluoro-2-deoxyglucose positron emission tomography with the combination of detailed morphologic characteristics from computed tomography.

Abdominal ultrasonography is also used for metastatic screening. In a recent study 602 treated patients were screened with biannual abdominal ultrasound. 63 patients developed liver metastases detected by ultrasound. 90% of those patients had metastases in both lobes of the liver, and 70% had more than 10 lesions. One-third of patients with liver metastases underwent complete surgical resection. However, not all metastases could be resected because of the presence of miliary metastases that were not detectable by ultrasound [80].

Computed tomography scan has also been used for staging of a malignancy and the detection of metastases. It has been demonstrated that the usage of abdominal CT as a screening tool is often confounded by the presence of benign lesions as cysts, fatty liver, or lesions that are too small in size to characterize [81]. In a recent retrospective study of 198 patients, 55% presented with benign lesions and only 3.3% were found to be metastatic lesions. The likelihood of malignancy increased in relation to the number of lesions detected.

6.4. Adjuvant Treatment to Prevent Metastatic Disease. Interferon-alfa-2a has been used as adjuvant treatment after the treatment of melanoma in an effort to prevent the development of metastases as it has been shown to alter the immune response and inhibit cell proliferation. A large recent series [82], however, indicated that the development of metastasis did not differ significantly between patients who received IFN and those who did not [67]. Intra-arterial hepatic fotemustine has shown promising results in the treatment of liver metastases from uveal melanoma (see next section). However, fotemustine did not have a statistically significant survival benefit when used as adjuvant treatment [83].

No adjuvant therapy is currently available for ocular melanoma. However, new phase 2 adjuvant treatment trials are underway in Europe for uveal tract melanoma. The London Ocular Oncology Service is collaborating with our European colleagues in Holland in the use of a dendritic cell melanoma vaccination (data not yet published) to prevent the development of metastatic disease in patients at high risk of metastases.

6.5. Treatment of Metastasis. A broad spectrum of management options are available for metastatic disease including systemic therapies, direct intra-arterial hepatic administration, and percutaneous hepatic perfusion.

In regard to systemic therapies, the BOLD regimen (bleomycin sulfate, vincristine sulfate (Oncovin), lomustine, and dacarbazine) combined with interferon has shown some response in a small percentage of patients [84].

Fotemustine is an alkylating agent with a high first pass liver extraction leading to hepatic concentrations of 8–47 times higher than in normal tissue. This agent has been evaluated with direct intra-arterial hepatic administration in 101 patients with liver metastases from uveal melanoma and has had promising results with a 36% overall response rate and a median overall survival of 15 months and a 2-year survival rate of 29% [85]. Efficacy of intra-arterial versus intravenous fotemustine is currently being evaluated in a Phase III trial by EORTC (European Organization for Research and Treatment of Cancer).

Another proposed management option is percutaneous hepatic perfusion with melphalan [50]. This has been shown to achieve progression-free disease or stabilisation of patients

with uveal melanoma metastases, but unfortunately there was no overall survival benefit [86].

6.5.1. Emerging Treatments for Metastases. In light of the molecular events associated with the pathogenesis of uveal melanoma, new therapeutic targets have emerged. Targeting of the effector molecule MEK in the RAF/MEK/ERK pathway with a small-molecule inhibitor, AZD6244, has shown promising results in a small subset of patients with metastatic uveal melanoma doubling the progression-free survival time at 114 days versus 50 days for temozolomide. AZD6244 is currently evaluated in a Phase II randomized trial with temozolomide in patients stratified with GNAQ/11 status [87]. Anti-VEGF treatment is currently under experimental investigation. Bevacizumab has been shown to suppress in vitro growth and in vivo development of micrometastasis of ocular melanoma cells in mice [88].

7. Conclusion

Uveal melanoma is the most common primary malignancy of the eye affecting approximately 500 patients each year in the UK. Detailed examination and ocular ultrasound invariably allow the clinician to make the diagnosis without the need for a diagnostic biopsy.

Successful local treatment options, such as plaque brachytherapy and proton beam radiotherapy, allow for the preservation of the eye and vision in some cases. Despite the advance in local ocular treatments, there has been no change in patient survival for three decades.

Once metastases have developed, prognosis is poor. However, advances in genetics and cytogenetics have helped discover more about the tumours with high metastatic potential and the molecular mechanisms that underlie their development. In that respect, FNAB or conventional biopsy may be important for prognostication and to allow further research into genetic mutations and potential new therapeutic targets.

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Research Article

Characterization of Ex Vivo Expanded Tumor Infiltrating Lymphocytes from Patients with Malignant Melanoma for Clinical Application

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Clinical trials of adoptive transfer of autologous tumor infiltrating lymphocytes (TILs) to patients with advanced malignant melanoma have shown remarkable results with objective clinical responses in 50% of the treated patients. In order to initiate a clinical trial in melanoma, we have established a method for expanding TILs to clinical relevant quantities in two steps with in 8 weeks. Further characterization of expanded TILs revealed an oligoclonal composition of T-cells with an effector memory like phenotype. When autologous tumor was available, TILs showed specific activity in all patients tested. TIL cultures contained specificity towards tumor cells as well as peptides derived from tumor-associated antigens (TAAs) during expansion procedures.

1. Introduction

The incidence of malignant melanoma is increasing worldwide, and upon dissemination has a very poor prognosis [1]. Only two systemic treatments are approved for disseminated disease and encompass IL-2 based immunotherapy (16% response rate and 6% complete responses) [2] and dacarbazine (6%–15% response rate with no improved survival) [3]. However, results from clinical trials of TIL-based immunotherapy conducted at two centres has shown 50% response rates in patients with advanced disease, and responses were long lasting [4, 5]. TILs were reported to be dominated by $CD8^+$ T-cells and mediate specific killing of autologous tumor in most patients [6]. Information on TAA-derived peptide specificities in TIL has mainly shown the occasional large frequency of MART-1 and gp100 specific T-cell populations. On the other hand, results on the clonotypic and phenotypic composition has been scarce; one publication has revealed a mixed clonal content of TIL by FACS analysis [7], and two recent studies report surface markers identical to memory like effector T-cells from a limited patient material [8, 9].

In our study, we have analysed TIL characteristics from 17 melanoma patients, whereof five have undergone lymphodepletion and TIL-based ACT with low-dose IL-2.

2. Materials and Methods

2.1. Patients. Patients referred to surgery for primary or recurrent stage III-IV malignant melanoma were eligible for the study. The study protocol was approved by the local ethics committee, and all patients were included after signing informed consent. Tumor material from the patients was obtained from the surgically removed tumour within 30 minutes after surgery.

2.2. TIL Bulk Cultures and Rapid Expansion. The TIL culturing method was adapted from Dudley et al. [10] constituting a two-step expansion process: (I) initiating bulk cultures and (II) rapid expansion of selected bulk cultures with a proliferative potential. Following surgical removal of tumor tissue from patients with MM the tumour sample were cut into 1-2 mm fragments. Fragments were subsequently placed

individually in 24-well culture plates (Nunc, Denmark) and maintained in 2 mL of culture medium (CM) containing RPMI1640 (Invitrogen), penicillin, streptomycin, fungizone (Bristol-Myers Squibb), 10% human serum (Sigma) and 7300 or 6000 IU/mL IL-2 (Aldesleukin, Novartis). Each fragment initiated an individual TIL culture which was maintained separately during subsequent expansion and activation.

Bulk cultures were selected for further expansion according to a rapid expansion protocol (REP). TIL were cocultured with irradiated (40 Gy) allogeneic PBMCs serving as feeder cells in a ratio of 1 : 200 in a 1 : 1 mixture of CM and AIM-V (Invitrogen) initially with 10% HS, and containing 30 ng/mL OKT-3 (Cilag AG, Suisse) and 7300 or 6000 IU/mL IL-2 (Aldesleukin, Novartis) in upright T-flasks. REPs for preclinical purposes generally were initiated from 1×10^5 TIL per flask, while 1×10^6 TIL were used per flask in clinical scale REPs. On day 5, half of the medium was replaced with fresh medium containing AIM-V, CM with 10% HS and 7300 IU/mL IL-2. From there on, the TIL concentration were maintained at 1×10^6 cells/mL by adding AIM-V supplemented with Fungizone and 7300 or 6000 IU/mL IL-2. Half of the patients TIL where cultured in 7300 IU/ml IL-2, while the other half received 6000 IU/mL IL-2 during culturing.

2.3. Viability. Cell counting and viability testing were performed by microscopy. Cells were stained with trypan blue followed by counting of live and dead cells in a haemocytometer.

2.4. Sterility Tests. Bulk and REP cultures were intermittently sampled for microbiological testing of fungal and bacterial contamination.

2.5. Peptides. We used the following HLA-A2 restricted peptides: SUR1M2 (LMLGEFLKL), HTERT P540 (ILAKFLHWL), Cyclin B1 204 (ILIDWLVQV), MART-1 27–35 (AAGIGILTV), and NY-ESO 1 157–165 (SLLMWITQC).

2.6. Cell Lines. Autologous tumor cell lines were established from tumor fragments by outgrowth in 24 well or 6 well plates (Nunc) in medium consisting of RPMI1640 (Invitrogen), penicillin, streptomycin, fungizone, 10% fetal calf serum (Invitrogen), and SoluCortef (Pfizer).

Tumor cells were cryopreserved in 90% FCS and 10% DMSO (Hospital Pharmacy, RegionH, Copenhagen, Denmark) and stored at −140°C.

2.7. Flow Cytometry. Phenotyping were conducted using a FACS-Aria with Diva software (from BD) and fluorescence conjugated monoclonal antibodies (mAb) against CD3 APC-Cy7, CD4 APC, CD8 PerCP, CD25 PE, CD27 PE, CD45RA FITC, CD45RO PE, CD56 PE (all from BD), CCR7 FITC (R&D systems), CD16 FITC (Dako), CD28 FITC (Immunotech), CD62Ligand PE (BD Pharmingen), and CD57 FITC (BD Pharmingen) along with corresponding isotypes.

2.8. T-Cell Receptor (TCR) Clonotype Mapping by Denaturing Gradient Gel Electrophoresis (DGGE). RNA was extracted using the NucleoSpin RNA II (Macherey-Nagel, Germany). cDNA synthesis and quantitation of cDNA in each sample was carried out as previously described [11].

For TCR clonotype mapping, cDNA was amplified using a primer panel covering the 24 BV region families of the TCR. Resulting PCR products are suited for DGGE [12, 13]. Amplifications were carried out in a total volume of 45 μL containing 1xPCR buffer (50 mM KCl, 20 mM Tris pH 8.4, 2.0 mM $MgCl_2$, 0.2 mM cresol red, 12% sucrose, 0.005% (wt/v) BSA (Boehringer-Mannheim, Mannheim, Germany)), 2.5 pmol of each primer, 40 mM dNTPs (Pharmacia LKB, Uppsala, Sweden) and 1.25 units of AmpliTaq polymerase (Perkin Elmer Cetus Corporation, Emeryville, Calif, USA). Parameters and conditions used for amplification were 94°C for 30 sec., 60°C for 60 sec., and 72°C for 60 sec., as described, in [11, 12].

For DGGE 10 μL aliquots were loaded onto a denaturing gradient gel containing 6% polyacrylamide and a gradient of urea and formamide ranging from 20% to 80%. Gels were run at 160 V for 4.5 h in 1x TAE buffer kept at a constant temperature of 56°C. After electrophoresis, the gel was stained with SYBR Green I (Molecular Probes, Oregon, USA) and visualized using the FLA-3000 fluorescence detection system (FUJI film, Science Imaging Scandinavia, Sweden).

2.9. Elispot INFγ Measurement. Antitumor activity was assessed with Elispot INFγ quantification as described previously [14]. In brief, nitrocellulose bottomed 96 well plates (Multiscreen MAIP N45; Millipore, Denmark) were coated with INFγ capturing antibody (1-DIK; Mabtech, Sweden) and further washed and blocked with RPMI 1640. A maximum of 1×10^5 effector cells per well were either added alone when stimulated by peptides, or in coculture with target cells (1×10^4 cells per well) consisting of autologous tumor cells. After a four-hour or overnight incubation period, the medium was discarded and wells washed followed by application of secondary biotinylated antibody (7-B6-1-Biotin; Mabtech). The plates were incubated for one hour, further washed, and avidin-enzyme (Streptavidin; Mabtech) conjugate, were added to each well followed by one-hour incubation at room temperature. Succeedingly, the wells were washed and the enzyme substrate NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Mabtech) were added into each well. The plates incubated at room temperature for 2 to 10 minutes, while emerging purple spots developed. The reaction was terminated with tap water. Spots were counted with the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers) and the frequency of tumor specific TIL could be calculated from the numbers of spot forming cells. The assays were preferably done in triplets or in duplicates in case of low cell numbers.

2.10. Cr Release Assay. A standard Cr^{51}-release assay was used to quantify the specific cytotoxic ability of selected TIL cultures. In brief, 5×10^3 Cr^{51}-labeled tumor cells (duplicates or triplicates) were cocultured with TIL (maximum E : T

ratio of 100 : 1 and titrated) in RPMI containing 10% FCS for a 4 hour incubation period. Thereafter, Cr^{51}-release was measured and percentage of tumor lysis calculated as (#count − Min count)/(Max count −Min count) × 100%.

2.11. Statistical Analysis. We utilized Graphpad Prism statistical software to analyse for statistical differences, using a paired two-tailed t test. P values < .05 were considered significant.

3. Results

3.1. Patients. Tumor material were obtained from 17 patients with either locally advanced or advanced disease from metastasis localized either in lymph nodes (majority of specimens) or subcutaneously. A minimum requirement of 1 cm^3 of tumor was needed to ensure sufficient material for TIL expansion. The mean age was 62 years with an equal gender distribution. 12 of the patients had only been treated surgically prior to inclusion, while five patients who were included in our recent established clinical pilot trial had previously received IL-2 and/or DC vaccination based immunotherapies. Patients showed the following distribution of HLA-A types: one HLA-A1+, two HLA-A1/A3+, one HLA-A3+, two HLA-A3/A11+, one HLA-A11+, one HLA-A3+/A2+, four HLA-A2+, one HLA-A2/24+, and four non-HLA-A1/A2/A3/A11/A24.

3.2. TIL Expansion Kinetics. Lymphocytes migrated out from the fragments within two-to-five days and expanded into a confluent layer before splitting the wells. Each well initiated an individual bulk culture and were kept separated from other cultures. TIL bulk cultures expanded to at least 5×10^7 cells were considered sufficiently expanding. This was obtained in 15 out of 17 patients (88%) in 6% to 100% of the bulk cultures (mean 58%) within 3–5 weeks. We found that growth rates varied markedly even between cultures from the same patient, and there was no difference in success rate of TIL growth from LN or SC tumor material, nor between the IL-2 concentrations (data not shown).

We next tested the proliferative potential of a range of bulk cultures from 12 of the 15 patients with sufficiently growing TIL. This rapid expansion procedure (REP) involves the addition of allogeneic feeder cells and a CD3 antibody and has shown to increase TIL expansion rates considerably, in previously reports in both melanoma and head and neck squamous cell carcinoma. Again, the kinetics could vary between cultures from the same patient; however, the procedure could efficiently expand TIL bulk cultures to over a 1000-fold in more than half of the cultures in 2 weeks (Figure 1).

3.3. Phenotypes and Clonal Composition. TIL were visualized in the microscope, showing a blasted morphology related to actively dividing lymphocytes laying either as single cells or in clusters/clones.

In acquisition of cells by flow cytometry, gating of viable cells was performed on the basis of the forward and side scatter dot plots. T-cells ($CD3^+$) predominated the cultures,

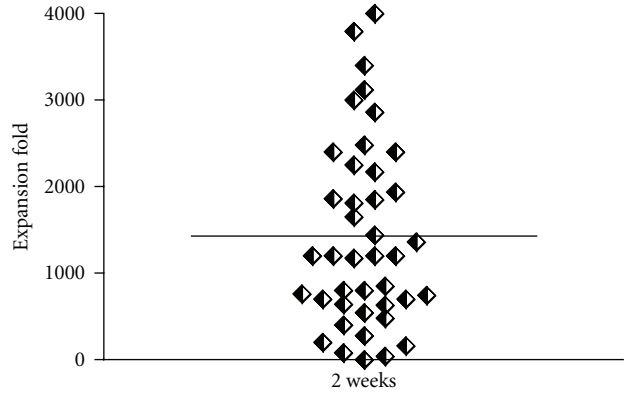

Figure 1: REP fold-expansion. During two weeks 41 TIL cultures (represented by single diamonds) reached a mean 1400 expansion fold.

while NK cells ($CD16/56^+$) (Figure 2) were consistently absent. In bulk cultures, we observed a heterogeneous $CD4^+$ and $CD8^+$ T-cell distribution among cultures inter- and intraindividually. There was, however, an overall skewing towards a $CD8^+$ (mean = 74% ± 24%, range 30%–94%) T-cell predominance in relation to $CD4^+$ T-cells (mean = 19.5% ± 23.5%, range 1%–64%). Next, we investigated the occurrence of surface markers identifying T-cell memory subsets, or alternatively, a differentiation path of effector cells, in the overall $CD3^+$ population, and among $CD4^+$ and $CD8^+$ T-cell subsets in comparison to TIL after two weeks of REP (Figure 2). Overall, there was a distinct predominance of $CD45RO^+$ and CCR-$7^{Lo/-}$ T-cell populations before and after REP identifying the cells as T effector memory like.

TIL were further characterized by surface markers according to a proposed model of effector $CD8^+$ T-cell differentiation stages by Gattinoni et al. [15]. Expression of the lymphoid homing marker CD62L was significantly reduced after REP in the $CD3^+$ population and the $CD8^+$ subsets and remained unchanged among the $CD4^+$ subsets. Concerning expression of costimulatory markers, we observed a significantly higher expression of CD27 among $CD8^+$ bulk TIL compared to $CD4^+$ cells, while $CD4^+$ cells had sustained higher CD28 expression in bulk cultures and after REP. Although there was a relatively high percentage of CD27/28 double positive cells in a few bulk TIL, they were downregulated after REP. Finally, there was a significant increase in the high-affinity IL-2 receptor (CD25) after REP in the $CD4^+$ population. In conclusion, the $CD8^+$ population express surface markers ($CD45RO^+$, CCR-7^{Lo}, $CD62L^{Lo}$, $CD27^{Lo}$, $CD28^{Lo}$, and $CD57^{Lo}$) resembling intermediate to late-stage effector cells as reported by other groups [8, 9, 16].

Selected expanded cultures were analyzed for the presence of clonally expanded T cells by RT-PCR/DGGE-based TCR clonotype mapping. Analysis revealed the presence of at least 10 different T-cell clonotypes in bulk cultures as well as in rapidly expanded cultures (data not shown). The results support our previous findings in expanded TIL from head and neck cancer patients, that expansion by high-dose IL-2 and CD3 antibody seems to support the continued expansion of bulk T-cell clones.

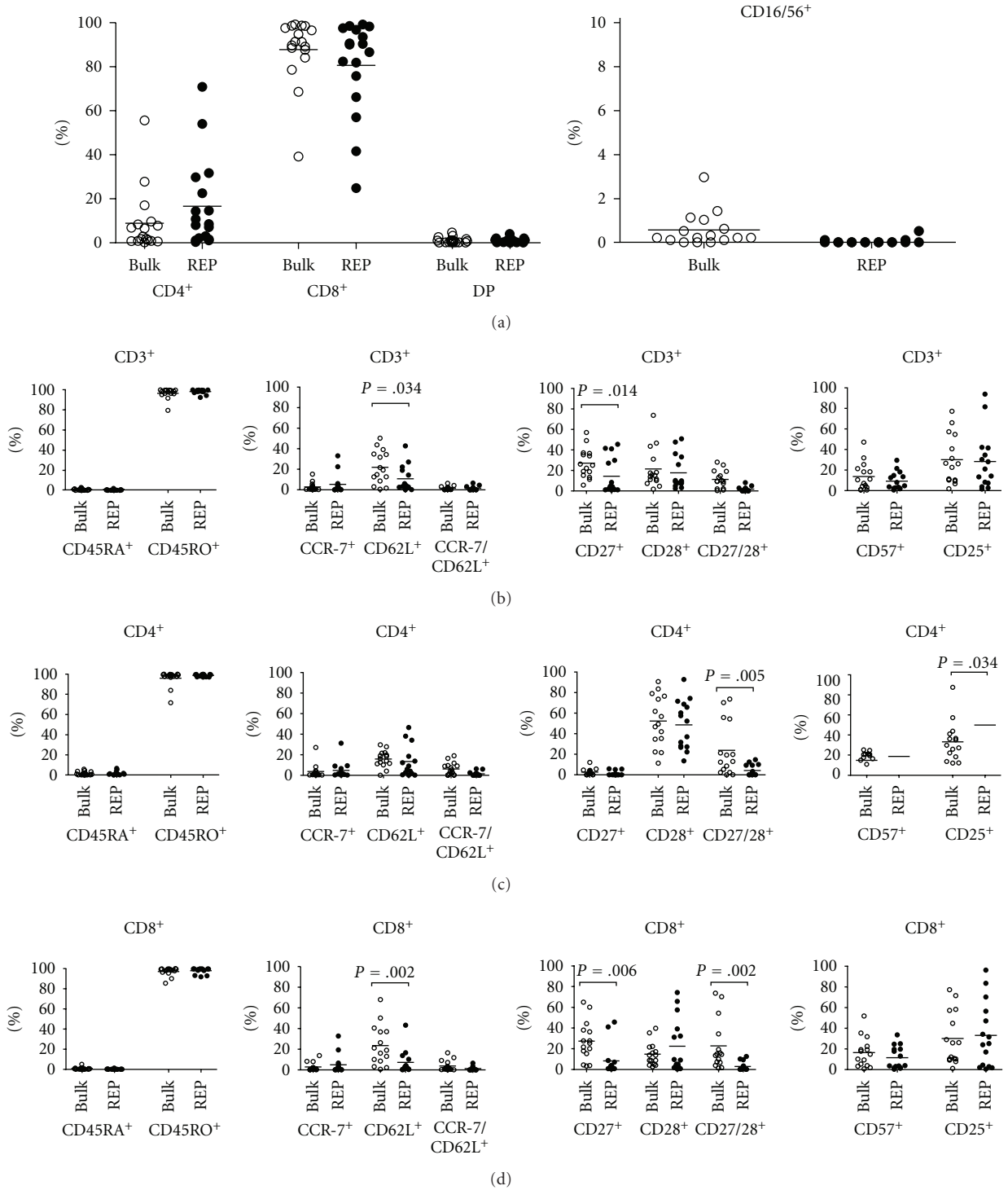

Figure 2: Phenotypes. FACS determination of phenotypes of TIL cultures pre- (open circles) and post-REP (closed circles) are represented from six patients. The overall T-cell effector memory like phenotype ($CD3^+CD45R0^+CCR\text{-}7^{Lo}$) is preserved after REP with a sustained low expression of CD57 and intermediate CD25 expression. CD28 remains unchanged, while CD62L and CD27 is downregulated, indicating a differentiation towards a later effector stage.

3.4. Sustained Functional Capacity during Expansion. TIL cultures from eight patients were selected to scrutinize the presence of specific T-cell populations in bulk cultures and after REP. Peptides derived from over expressed (Telomerase, Survivin, and Cyclin B1), differentiation (MART-1) and cancer testis antigens (NY-ESO-1) served as known targets, while autologous tumor cell lines presented a panel of unknown antigen specificities. Elispot detection of INFγ release upon antigenic stimulation revealed a sustained functionality of TIL after REP. Due to the high sensitivity of the assay, we

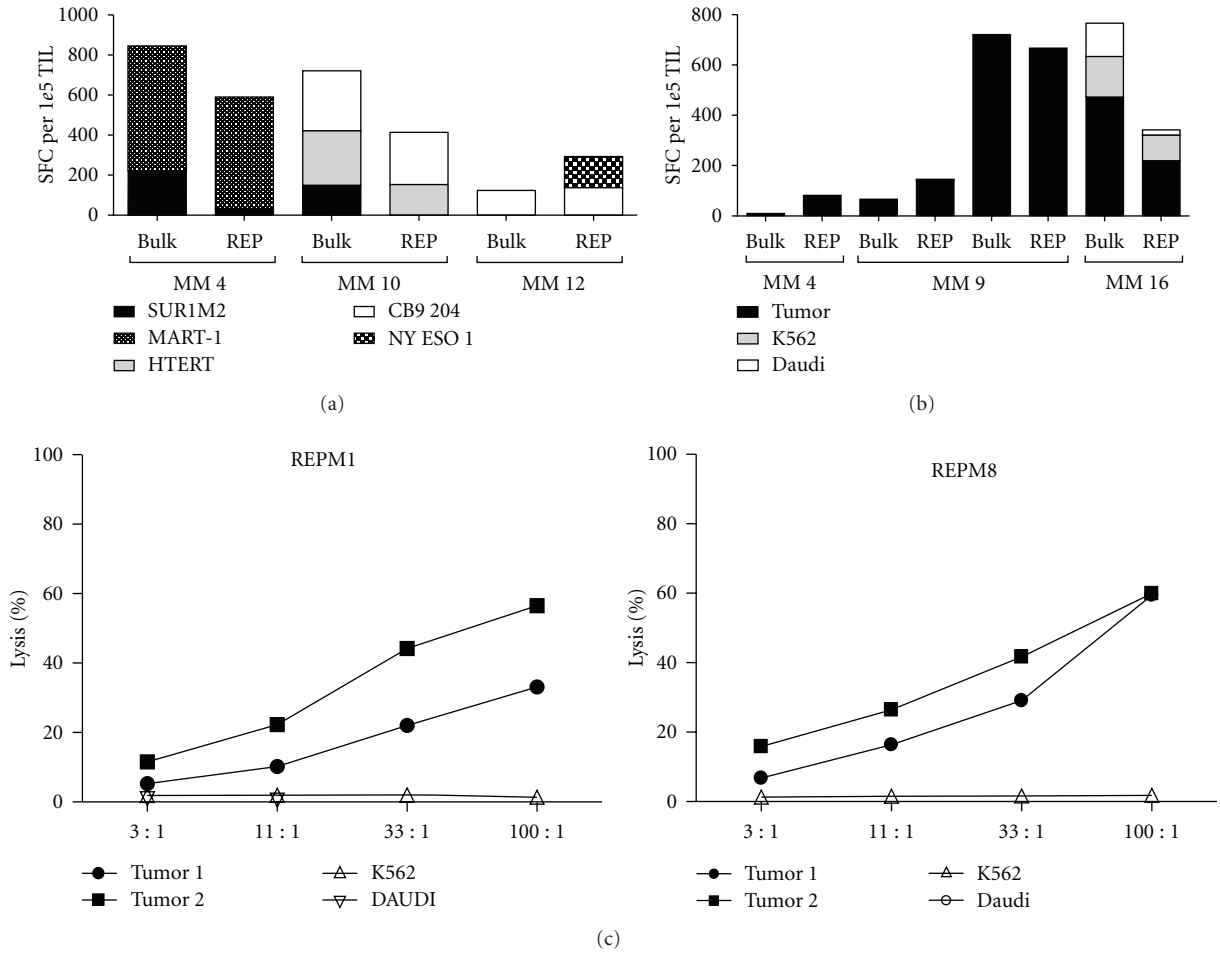

Figure 3: Functional capacity. Determination of TAA peptide-specific populations in TIL pre- and post-REP. Results from Elispot detection of INFγ in three patients exemplifies the general tendency of specificity retention, decline, and/or increase as a consequence of unspecific stimulation during expansion procedures, (a) Autologous tumor cell lines were available from four patients, and all showed TIL with antitumor activity by measuring INFγ in Elispot. Representative results of TIL from three patients show retained tumor-specific activity. However, in patient MM + 16 we found a component of unspecific NK/LAK cell activity, which seemed to decline after REP, (b). Example of preserved lytic capacity of TIL from MM 11 after REP. Both cultures show specific killing of two established autologous tumor cell lines. Interestingly, Tumor 1 seems more immunogenic than Tumor 2, (c).

could follow the presence and loss of low-frequency single-peptide-specific T-cell populations (Figure 3(a), occurring as a consequence of an increase or decrease in cell number of a given specific cell population, during the unspecific expansion procedures provided by IL-2 and anti-CD3. Autologous tumor cell lines were available in four patients, and all patients contained TIL showing antitumor activity in Elispot (Figure 3(b) and data not shown). The presence of autologous tumor-specific T-cell populations was more resistant during REP and showed a sustained functional capacity. Although T-cell-specific antitumor activity was predominating in TIL, we observed LAK/NK cell activity in a few cultures (Figure 3(b)) by unspecific engaging the cell lines K562 and Daudi. Finally, we confirmed a sustained tumoricidal capacity of TIL after REP (Figure 3(c)) indicating that TIL expanded to clinical relevant numbers (2400- and 4000-fold) can engage and kill autologous tumor.

4. Conclusion

We were able to establish sufficiently expanding TIL bulk cultures in five weeks from the majority of included melanoma patients. Further expansion by REP generated a mean expansion fold of 1400 in two weeks, ensuring the feasibility to reach clinical relevant quantities for clinical testing. Based on earlier studies of T-cell therapy of melanoma patients were as low as $1,3 \times 10^9$ infused cells containing 30% MART-1-specific $CD8^+$ T-cells mediated a complete clinical response [17], we estimate that a minimum of 3×10^9 cells are required to obtain a therapeutic effect. Cell-based analysis revealed an oligoclonal composition of T effector memory cells, predominated by $CD8^+$ cells showing an intermediate to late stage of differentiation after REP. TIL retained the functional capacity measured by INFγ release and lytic activity against autologous tumor. Notably, we did not find differences between the two doses of IL-2 used during TIL

culturing, and even further lowering of IL-2 dose to 3000 IU/mL is now the standard used in TIL expansion at other centres. Finally, there were no significant influence on TIL expansion kinetics or phenotypes by pretreatment, age or performance status of the patients.

5. Perspectives

In a recently initiated clinical trial of TIL-based ACT, low-dose IL-2, and lymphodepletion preconditioning, one out of five treated melanoma patients has obtained an ongoing partial response (+13 months). We are currently screening the TIL cultures for the occurrence of tumor associated antigen (TAA) specificities by measuring INFγ in Elispot. This enables us to identify the specific combination of TAA specificities in each patient, which potentially can be identified during immune monitoring of the patient samples. In addition, we are establishing and validating a flow cytometry-based method of identifying TAA-specific T-cell populations and obtain information on the kinetics of T-cell memory and effector stages before and after treatment. Information providing more insight into the prognostic values of adoptively transferred TIL.

Conflict of Interests

The authors state no potential conflict of interests.

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Research Article

Nonsteroidal Anti-Inflammatory Drugs and Risk of Melanoma

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Because nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit tumor growth *in vitro*, we investigated the association between NSAIDs and melanoma to determine if there was epidemiologic evidence of a chemopreventive effect from these medications. Three hundred twenty-seven subjects with incident melanoma and 119 melanoma-free controls completed a structured interview assessing melanoma risk factors. The unadjusted odds ratio (OR) for use of nonaspirin NSAIDs was 0.58 (95% CI 0.31–1.11), in a comparison of subjects with melanoma to controls. After adjustment for melanoma risk factors, the OR was 0.71 (95% CI 0.23–2.02). Aspirin users had an unadjusted OR of 0.85 (95% CI 0.45–1.69) and an adjusted OR of 1.45 (95% CI 0.44–4.74). In this pilot study, we found no evidence of a significant association between analgesic use and melanoma risk when potential confounders are assessed. Based on conflicting reports in the literature, meta-analysis may be appropriate.

1. Introduction

Melanoma is a potentially fatal tumor that continues to increase in incidence despite public education measures to limit sun exposure [1]. Chemoprevention has been identified as an important strategy for reducing melanoma incidence [2]. Among the candidate agents, nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to affect the mechanisms of cellular damage caused by ultraviolet radiation [3]. Expression of cyclooxygenase-2 (COX-2), the primary pharmacologic target of NSAIDs, has been reported in up to 93% of melanomas [4]. In addition, *in vitro* and animal studies have demonstrated a protective effect of NSAIDs against melanoma; [4–6] however, studies in humans have had conflicting results [7–9].

Using a case-control design, we tested the hypothesis that NSAIDs convey a chemoprotective effect against melanoma.

2. Methods

The Genes, Environment, and Melanoma (GEM) study is a multicenter case-control study investigating heredity and environment in melanoma. A full description of the GEM study design has been published separately [10–12]. In this report, only the University of Michigan subjects were used, since supplemental data regarding medication usage was not collected from other centers.

Individuals with a first primary invasive melanoma diagnosed in the year 2000 and individuals with a second or higher order invasive primary melanoma diagnosed between January 1, 2000, and August 31, 2003, were eligible for participation. At the University of Michigan site, subjects with a first primary melanoma *in situ* diagnosed in 2000 were also enrolled. This site also enrolled spouses of the melanoma subjects to act as controls. The inclusion criteria for controls were a current spousal relationship to a melanoma subject and willingness to provide informed consent to participate. Spouses were excluded from participation as controls if they had a personal history of melanoma. The current analysis is restricted to cases diagnosed with first primary invasive melanoma or melanoma *in situ*.

Participants provided written informed consent at the time of enrollment. The protocol was approved by the Institutional Review Board at the University of Michigan.

Subjects completed an interview assessing personal and family history of cancer, skin phenotype, medication use, and

Table 1: Demographic characteristics.

Characteristic	Melanoma cases	Controls	*P*-value
All subjects—no	327	119	
Sex—no (%)			
Male	162 (49.5%)	41 (34.5%)	.0047
Female	165 (50.5%)	78 (65.6%)	
Age—yr (median (interquartile range))	53 (41–66)	56 (46–66)	
Family history melanoma—no (%)	51 (15.8%)	11 (9.5%)	.0926
Skin Color—no (%)			
Light	287 (88.9%)	49 (69.0%)	<.0001
Dark	36 (11.1%)	22 (31.0%)	
Number of moles on back (median (interquartile range))	10 (4–29.5)	5 (1–12)	
Current medication use—no (%)	217 (66.4%)	91 (76.5%)	.0413
Past medication use—no (%)	106 (32.9%)	46 (40.0%)	.1918
Comorbidities—no (%)	168 (50.9%)	70 (58.3%)	.1634
Cardiovascular disease—no (%)	37 (8.2%)	9 (7.5%)	.7965
Musculoskeletal pain—no (%)	22 (4.9%)	18 (15.0%)	.0001

SD Standard deviation. *P*-values for categorical variables are from Mantel-Haenszel $\chi 2$ test.

comorbidities. To assess past and current use of analgesics, they were asked to recall medications used on a daily basis for at least three months.

Statistical analyses were performed using SAS software (version 9.1). Contingency tables were used to assess the crude associations between medication use and melanoma risk. Fisher's exact test was used to determine *P*-values when cells contained counts of less than 10. Unconditional logistic regression was used to assess the association between medication use and melanoma risk, to adjust for confounding, and to identify any potential effect modification. All reported *P* values are two-sided.

Potential variables evaluated for the multivariate model included age, gender, family history of melanoma, skin color, hair color, eye color, history of severe sunburn, history of cardiovascular disease, history of a musculoskeletal pain condition, tendency of skin to burn, tendency of skin to tan, age at first sunburn, number of moles on the back, smoking history, history of other medical diagnoses (yes/no), and statin use. Covariates were included in the initial multivariate model if the addition of that covariate to the univariate model of analgesic use and melanoma risk resulted in a 10% or greater change in the odds ratio. These covariates were then removed from the saturated multivariate model in a stepwise fashion to optimize the fit to the data as determined by the AIC.

3. Results

Seven hundred twenty-eight individuals were approached for study participation at the University of Michigan. Of these, 509 (69.8%) completed the telephone interview. Melanoma patients accounted for 390 of these subjects; the remaining 119 were melanoma-free spouses of the patients. Among the melanoma subjects, 104 had *in situ* melanoma, 223 had a first (single primary) melanoma, and 63 had a second or higher-order (multiple primary) melanoma. In this analysis, individuals with multiple primary melanomas were excluded, as we could not adjust for selection bias based on survival from the first primary melanoma. Demographic characteristics are described in Table 1, and results are summarized in Table 2. Due to the differences in gender and age between the melanoma and control groups, these variables were empirically included in the multivariate models. Covariates in the final models were age, gender, skin color, family history of melanoma, and number of moles on the back.

3.1. Aspirin. Regular use of aspirin was reported by 10.9% of controls. Current or past use of aspirin was associated with an unadjusted odds ratio of 0.85 (95% CI 0.43–1.69). The odds ratio was 1.45 (95% CI 0.44–4.74) in a multivariate model adjusted for age, gender, skin color, family history of melanoma, and number of moles. The number of moles had the most significant effect on the adjusted odds ratio. Restricting the analysis to current users or those with invasive melanoma yielded similar results. Due to small numbers of subjects, past use could not be evaluated. Subjects with *in situ* melanoma were more likely to take aspirin than those with invasive disease, but this difference was not statistically significant (OR 0.615; 95% CI 0.29–1.31).

3.2. Nonaspirin NSAIDs. Approximately 14% of control subjects reported regular use of nonaspirin NSAIDs in our study. The unadjusted odds ratio for melanoma risk with any use of nonaspirin NSAIDs was 0.58 (95% CI 0.31–1.11), and the adjusted odds ratio was 0.71 (95% CI 0.23–2.02). Covariates in the multivariate model were the same as in the aspirin model. The addition of aspirin use to the model did not affect the odds ratio, and results were similar when restricted to invasive melanoma. Control subjects were more

Table 2: Analgesic use and risk of first primary invasive or *In situ* melanoma.

Exposure	Patients	Controls	Total	Crude OR (95% CI)	Adjusted OR (95% CI)
Ever use of aspirin:					
Yes	31	13	44	0.854 (0.431–1.693)	1.447 (0.442–4.736)
No	296	106	402		
Ever use of nonaspirin NSAIDs:					
Yes	29	17	46	0.584 (0.308–1.107)	0.710 (0.234–2.024)
No	298	102	400		
Ever use of acetaminophen:					
Yes	6	3	9	0.723 (0.178–2.937)	Not assessed
No	321	116	437		

OR Odds ratio; CI Confidence interval; NSAIDs Nonsteroidal anti-inflammatory drugs.

likely to be current users of NSAIDs (OR 0.45; 95% CI 0.23–0.88); however, this association was no longer significant when adjusted for covariates (OR 0.55; 95% CI 0.19–1.58). Past use was not associated with a decreased melanoma risk. NSAID use in subjects with *in situ* disease was similar to use in those with invasive melanoma.

3.3. COX-2 Specific Inhibitors. Approximately 9% of controls reported regular use of COX-2 inhibitors. In an exploratory analysis, the crude odds ratio for melanoma in users of COX-2-inhibitors was 0.61 (95% CI 0.28–1.31), and the adjusted odds ratio was 0.42 (95% CI 0.14–1.27).

3.4. Acetaminophen. In order to assess whether the effects of NSAIDs were due to the medical indication rather than the mechanism of action, we evaluated the effect of acetaminophen on melanoma risk. Due to small numbers of users (2.5% of controls), Fisher's exact test was performed and yielded a two-sided *P*-value of .72.

4. Discussion

Our results suggest that aspirin and nonaspirin NSAIDs are not associated with a durable large reduction in risk of melanoma once potential confounders are taken into account. A small beneficial effect of these medications may be seen in a larger study and cannot be excluded. We were unable to fully assess the effects of COX-2 specific inhibitors or acetaminophen due to small numbers of regular users in our study.

The effect of NSAIDS on melanoma has been assessed in four other publications. The first describes a case-control study of 110 women with melanoma and 609 matched controls [7]. The relative risk of melanoma in women taking NSAIDs was 0.45 (95% CI 0.22–0.92). Our study builds on this result with the inclusion of men, an increased number of melanoma cases, and assessment of additional covariates. The second study investigated the effects of NSAIDs in individuals who had previously had melanoma [13]. Individuals taking COX inhibitors were less likely to have new primary melanoma, melanoma recurrence, or melanoma metastasis. Our study takes this question from a secondary/tertiary prevention setting to a primary prevention setting. The third study linked NSAID use in the Vitamins and Lifestyle (VITAL) study to the NCI Surveillance, Epidemiology, and End Results registry [9]. These investigators found no association between NSAID use and melanoma risk. The number of cases in this study is similar to ours; however, many had *in situ* rather than invasive melanomas. Our study expands upon this result by using a larger number of invasive melanoma cases and adding data on number of moles, which was the most significant covariate in our analysis. The fourth study assessed this question in the Dutch PHARMO pharmacy database and the PALGA pathology database [8]. They found a 46% reduction in melanoma risk for women who had continuous use of aspirin (OR 0.54, 95% CI 0.30–0.99). Our work adds melanoma risk factors, which can act as potential confounders.

Our study has several limitations. Unfortunately, we could not validate reports of medication use with prescription records in our study, as many NSAIDs are dispensed over the counter, and use of these medications is not always accurately collected in the medical record. Sun exposure could not be assessed as a potential confounder due to a lack of a simple, validated measure of this risk factor. However, we did evaluate self-reported history of severe sunburns, age at first sunburn, and tendency to burn as potential confounders; these did not have a significant effect on the odds ratios. Selection bias may be present in the control group, as not all spouses of melanoma subjects participated. Data are not available on nonconsenting spouses, so we are unable to assess whether the participating spouses were representative of the potential control population as a whole. Additionally, the odds ratios may be attenuated by overmatching of spouse controls, who may resemble the cases more than individuals in the general population.

In summary, in our pilot study we found no significant association between analgesic use and melanoma risk when potential confounders are assessed. Based on the conflicting data for this question, evaluation in a larger cohort that also allows for assessment of potential confounders or meta-analysis may be appropriate.

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