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Metastatic pathways in patients with cutaneous melanoma

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Summary

Metastasis represents the end-product of an elaborate biological process, which is determined by a complex interplay between metastatic tumour cells, host factors and homeostatic mechanisms. Cutaneous melanoma can metastasise haematogenously or lymphogenously. The three predominant models that endeavour to explain the patterns of melanoma progression are the stepwise spread model, the simultaneous spread model and the model of differential spread. The time course to the development of metastases differs between the different metastatic routes. There are several clinical and histopathological risk factors for the different metastatic pathways. In particular, patient sex and the anatomical location of the primary tumour influences patterns of disease progression. There is limited existing evidence regarding the relationship between tumour mutation status, other diagnostic and prognostic biomarkers and the metastatic pathways of primary cutaneous melanoma. This knowledge gap needs to be addressed to better identify patients at high risk of disease recurrence and personalise surveillance strategies.

Key Words: Cutaneous melanoma, metastasis, metastatic pathways, biomarker.

Running Title: Metastatic pathways in patients with cutaneous melanoma

Introduction

Recent advances in melanoma treatment have led to more intensive surveillance of high risk patients as there is evidence that treatments are more effective in patients with low volume metastatic disease (Hodi et al., 2010; Sosman et al., 2012). An improved understanding of the pathways of metastatic disease and the biology that influences these pathways is important in order to improve surveillance strategies and to personalise follow-up of high risk patients. The former part of this review will explore the pathogenesis of metastasis and the patterns of progression of cutaneous melanoma. The existing models that endeavour to explain these patterns of progression will be described in the context of our current understanding of the biology underlying each mechanism of spread. Additionally, the time course to the development of metastases in patients with cutaneous melanoma and the recognised risk factors for the different metastatic pathways will be discussed. The effect of mutation status and other biomarkers on tumour behaviour and clinical outcomes in patients with cutaneous melanoma will subsequently be outlined. Finally, the limited existing body of evidence regarding the relationship between tumour mutation status and metastatic pathways of primary cutaneous melanoma will be examined and future directions proposed.

Pathogenesis of metastasis in primary cutaneous melanoma

Metastasis represents the end-product of an intricate biological process, which necessarily involves dissemination of neoplastic cells to different anatomic sites and adaptation of neoplastic cells to foreign tissue microenvironments (Gupta and Massague, 2006; Valastyan and Weinberg, 2011). The

process of metastasis is determined by the interplay between metastatic tumour cells, various host factors and homeostatic mechanisms (Fidler, 1988; Leiter et al., 2004). Moreover, metastasis is a multistep process, which includes proliferation, neovascularization, immune system evasion, lymphangiogenesis, invasion, circulation, embolism, extravasation and colonisation (Fidler, 1988; Leiter et al., 2004; Nguyen and Massague, 2007). The interactions between the neoplastic cells and the non-neoplastic stromal cells are important in the progression of the invasion-metastasis cascade (Gupta and Massague, 2006; Valastyan and Weinberg, 2011).

Furthermore, epithelial-mesenchymal transition (EMT) is a complex biological process, which plays an important role in the biology underlying carcinoma metastasis. During the process of EMT, a differentiated polarized epithelial cell undergoes multiple biochemical transitory changes to enable it to obtain a mesenchymal cell phenotype (Alonso et al., 2007; Kalluri and Weinberg, 2009; Rowe and Khosrotehrani, 2015). Mesenchymal cells have enhanced migratory capacity, invasiveness, production of extracellular matrix components and resistance to apoptosis (Kalluri and Weinberg, 2009). Consequently, tumour cells detach from the epithelial layer, interact with the extracellular matrix, become motile and acquire the capacity for metastasis (Alonso et al., 2007; Kalluri and Weinberg, 2009). There are multiple molecular processes involved in EMT, which include activation of transcription factors, expression of certain cell-surface proteins and cytoskeletal proteins, production of extracellular matrix degrading enzymes and changes in the expression of specific microRNAs (Kalluri and Weinberg, 2009). Epithelial tumour cells may undergo EMT to different extents; some tumour cells may retain several epithelial features, while others may adopt a complete mesenchymal phenotype (Kalluri and Weinberg, 2009). It is likely that EMT is a plastic phenomenon, whereby cells may become mesenchymal in order to migrate and invade, before switching back to an epithelial phenotype as mesenchymal tumour cells may be unable to proliferate in the organ in which they have seeded (Alix-Panabieres and Pantel, 2014). Furthermore, a recent study has demonstrated that melanocytes directly influence the formation of the dermal tumour niche by microRNA trafficking prior to invasion (Dror et al., 2016). Melanocytes were shown to release melanosomes, which carry mircoRNA into primary fibroblasts and serve to increase proliferation, migration and pro-inflammatory gene expression (Dror et al., 2016).

As melanoma originates from neural crest-derived melanocytes and not from epithelial cells it does not progress through classical EMT; rather, melanoma progresses through a distinct EMT-like process (Caramel et al., 2013; Li et al., 2015; Vandamme and Berx, 2014). In this EMT-like process, certain EMT transcription factors appear to be tumour suppressive in nature, while others promote invasion and progression. In particular, melanoma cells cycle between a differentiated state, which is characterised by high levels of ZEB2 and Slug, and an oncogenic invasive phenotype, which is characterised by high levels of ZEB1 and TWIST (Li et al., 2015; Vandamme and Berx, 2014). Importantly, the reversible phenotypic switch between differentiated and invasive phenotypes, which

is a mechanism that may account for melanoma heterogeneity, is coupled with the EMT transcription factor signalling switch. Oncogenic signalling and changes in the micro-environment drive phenotype-switching (Vandamme and Berx, 2014). Indeed, both proliferative and invasive cells are present within heterogeneous metastatic melanomas (Hoek et al., 2008; Li et al., 2015).

Lymphatic flow, chemotaxis and chemokine/chemokine receptors are responsible for homing of melanoma cells to different anatomic sites (Zbytek et al., 2008). Extravasation of tumour cells into the surrounding tissue requires expression of adhesion molecules and degradation of components of the extracellular matrix (Leiter et al., 2004; Zbytek et al., 2008). Adhesion molecules of the integrin, cadherin and immunoglobulin families are involved in the metastasis of cutaneous melanoma (Leiter et al., 2004). Furthermore, formation of new bloods vessels by vasculogenic mimicry, in which tumour cells acquire endothelial-like features, is an important mechanism in the pathogenesis of melanoma metastasis (Zbytek et al., 2008). Notably, vascular endothelial growth factor (VEGF) is an key factor in angiogenesis in metastatic melanoma (Zbytek et al., 2008).

Lugassy and Barnhill have described an alternate model for melanoma metastasis, whereby angiotropic melanoma cells migrate in a pericyte-like manner (pericytic mimicry) along the abluminal vascular surface, without intravasation (Lugassy and Barnhill, 2007). This model is termed ‘extravascular migratory metastasis’ and is distinct from intravascular dissemination (Lugassy and Barnhill, 2007; Lugassy et al., 2014). There is accumulating evidence to support angiotropism, pericytic mimicry and extravascular migratory metastasis as important alternative means of melanoma metastasis (Bald et al., 2014; Lugassy et al., 2014; Van Es et al., 2008).

Metastatic pathways in patients with primary cutaneous melanoma

Cutaneous melanoma is considered to have a high metastatic potential (Meier et al., 2002; Mervic, 2012; Tejera-Vaquerizo et al., 2007). Cutaneous melanoma can metastasise haematogenously or by the lymphatic system (Mervic, 2012). There are three predominant metastatic pathways in the progression of primary cutaneous melanoma (Meier et al., 2002; Mervic, 2012). Specifically, cutaneous melanoma can metastasise as satellite or in-transit metastases, as lymph node metastases or as distant metastases (Leiter et al., 2004; Meier et al., 2002). Satellite metastasis represents the development of metastatic nodules within two centimetres of the primary tumour, while in-transit metastasis is defined as the development of metastasis within the dermal and subdermal lymphatics in the drainage area before the first regional lymph node basin (Leiter et al., 2004; Meier et al., 2002). Intralymphatic metastasis includes both satellite and in-transit metastasis (Grotz et al., 2011). Satellite metastasis, in-transit metastasis and lymph node metastasis represent loco-regional metastasis. Occasionally, loco-regional disease can occur distal to the primary tumour in the limbs. Distant metastasis represents metastasis beyond regional lymph nodes and frequently involves visceral sites.

There has been limited research investigating the pattern of metastatic pathways in patients with primary cutaneous melanoma. A landmark study on the patterns of progression in patients with primary cutaneous melanoma, which was conducted by Meier and colleagues at the Department of Dermatology at Tuebingen University in Germany, analysed data from the German Central Malignant Melanoma Registry (Meier et al., 2002). This study traced the metastatic pathways of 3,001 patients with primary cutaneous melanoma from 1976-1996 (Meier et al., 2002). Of the patients who had disease confined to the primary tumour at diagnosis, 466 developed metastases during the study period (Meier et al., 2002). Of the patients who developed metastases, 50% developed regional lymph node metastases, 28% developed distant metastases and 22% developed satellite or in-transit metastases as the site of first tumour recurrence (Meier et al., 2002). The results from this study are consistent with other studies, which have confirmed that approximately two-thirds of patients who develop metastases initially present with loco-regional metastases and one-third present with distant metastases (Cohn-Cedermark et al., 1999; Reintgen et al., 1992; Soong et al., 1998; Tejera-Vaquerizo et al., 2007).

Existing models to explain the progression of cutaneous melanoma

There are three predominant models that endeavour to explain the progression of primary cutaneous melanoma (Figure 1) (Pizarro, 2015). The stepwise spread model posits that melanoma metastasises initially via the lymphatic system towards regional lymph nodes and subsequently, systemic dissemination occurs (Mervic, 2012; Tejera-Vaquerizo et al., 2007). Proponents of the stepwise spread model initially used this model to argue in favour of routine sentinel lymph node biopsy, maintaining that melanoma spreads to regional lymph nodes prior to systemic metastases (Morton et al., 2006). Nevertheless, defenders of the stepwise spread model acknowledge that direct haematogenous spread may occur in exceptional cases (Leong and Tseng, 2014).

The second predominant model is the simultaneous spread model, which maintains that primary cutaneous melanoma metastasises simultaneously by haematogenous and lymphatic pathways (Pizarro, 2015). Proponents of this model, such as Medalie and Ackerman, contend that lymph node involvement is therefore a marker of systemic disease (Medalie and Ackerman, 2004; Mervic, 2012; Pizarro, 2015). Conversely, opponents maintain that this model is not able to account for the fact that in patients who undergo regional lymph node dissection, approximately 30% of patients do not develop further disease progression (Meier et al., 2002; Mervic, 2012; Pizarro, 2015; Tejera-Vaquerizo et al., 2007).

The third model, which attempts to explain the patterns of progression of cutaneous melanoma, has been coined the model of differential spread (Tejera-Vaquerizo et al., 2007). This model proposes that there are multiple independent dissemination pathways (Tejera-Vaquerizo et al., 2007). That is, some

cutaneous melanomas do not have the biological potential to metastasise at all, others are able to metastasise only to regional lymph nodes, others are able to metastasise only haematogenously, while others still are able to metastasise both haematogenously and via the lymphatic system (Clark, 1991; Mervic, 2012).

The role of sentinel lymph node biopsy can be evaluated within the context of the abovementioned models of melanoma progression. The Multicentre Selective Lymphadenectomy Trial-I (MSLT-I) evaluated outcomes of patients randomised to either sentinel lymph node biopsy followed by immediate completion lymphadenectomy if nodal disease was identified, or observation with lymphadenectomy for clinical evidence of nodal disease (Morton et al., 2014). Whilst there was no benefit to overall survival, sentinel lymph node biopsy was shown to provide prognostic information and regional disease control (Morton et al., 2014). While the hypothesis of this randomised interventional trial was premised on the stepwise spread model, Pizarro argues that in fact, the model of differential spread most accurately accounts for the results (Pizarro, 2015). The fact that a negative sentinel lymph node biopsy does not guarantee survival can be accounted for by the model of differential spread as it purports that some cutaneous melanomas metastasise exclusively via the bloodstream (Tejera-Vaquerizo et al., 2007).

Time course to the development of metastases in patients with cutaneous melanoma

The time course to the development of metastases in patients with primary cutaneous melanoma ought to be considered within the context of the abovementioned models of disease progression. In Meier and colleagues' study, the time to development of primary tumour recurrence differed significantly between the different routes of metastasis (Meier et al., 2002). The median time course to the development of distant metastases as first tumour recurrence was twenty-five months (Meier et al., 2002). The median time course for regional lymph node metastases and satellite/in-transit metastases as primary tumour recurrence was sixteen months and seventeen months, respectively (Meier et al., 2002). It is important to consider lead time bias in the time course to the development of metastases. That is, small in-transit and lymph node metastases are more likely to be detected by patients, leading to earlier detection, compared to distant visceral metastases of the same size. Similarly, very small visceral metastases may not be detected on radiological surveillance with Computed Tomography (CT) or Positron Emission Tomography (PET) imaging (Friedman and Wahl, 2004). In addition, routine radiological surveillance is not a universal practice.

In terms of melanoma progression, the German Central Malignant Melanoma Registry characterised four distinct metastatic routes, which included; *1-Development of satellite or in-transit metastases followed by regional lymph node metastases and distant metastases, 2-Development of satellite or in-transit metastases followed by distant metastases, 3-Development of regional lymph node metastases*

followed by distant metastases and 4-Development of distant metastases as first tumour recurrence (Meier et al., 2002). Notably, in patients who developed distant metastases, irrespective of the site of primary recurrence, the time course to distant metastases was between twenty-four and thirty months following detection of the primary melanoma (Meier et al., 2002). Thus, the time course to the development of distant metastases was established to be independent of the metastatic pathway (Meier et al., 2002).

Several other studies have similarly demonstrated that the time course to distant metastases is comparable across the various metastatic routes (Dong et al., 2000; Tejera-Vaquerizo et al., 2007). In particular, Tejera-Vaquerizo and colleagues' performed a retrospective study of patients with primary melanoma in Spain from 1990-2004; of the 575 patients with primary melanoma, sixty-seven developed metastases (Tejera-Vaquerizo et al., 2007). In their study, the most common pattern of progression was metastases to lymph nodes followed by distant metastases, and the least common pattern was satellite/in-transit metastases followed by distant metastases (Tejera-Vaquerizo et al., 2007). Importantly, this study demonstrated that prior recurrence, either in the form of satellite/in-transit metastases or lymph node metastases, did not affect the time to the development of distant metastases (Tejera-Vaquerizo et al., 2007). Thus, similar to previous research, this study reported that the time course to distant metastases was independent of the metastatic pathway (Tejera-Vaquerizo et al., 2007).

Clinical and histological risk factors for the different metastatic pathways

While there are well-established clinical and histopathological risk factors for the development of tumour recurrence, there is limited data on the factors that influence the different metastatic pathways for patients with primary cutaneous melanoma. Data from the German Central Malignant Melanoma Registry was analysed to determine possible clinical and histological risk factors for the different metastatic pathways (Meier et al., 2002). Patient sex, anatomical location of the primary tumour, and tumour thickness were demonstrated to be significant risk factors for the development of metastases by the aforementioned metastatic pathways (Figure 1) (Meier et al., 2002). On the contrary, age, level of invasion and histological subtype did not impact the distribution of the metastatic pathways (Meier et al., 2002).

The most important factor to influence the different metastatic pathways was anatomical location of the primary tumour (Meier et al., 2002). Melanomas on the trunk and upper extremities were noted to display distinctly different patterns of progression compared to those on the lower extremities and the head and neck region. In particular, of the patients who developed metastases, greater than 30% of patients with melanoma on the lower extremity or head and neck region developed primary satellite or in-transit metastases, whereas greater than 30% of patients with melanoma on the trunk or upper

extremity developed direct distant metastases (Meier et al., 2002). Further, Cohn-Cedermark and colleagues' population-based study in Sweden, from 1976-1987, demonstrated that patients with head and neck primary melanomas had a higher frequency of developing direct distant metastases compared to those with primaries on all other anatomical locations (Cohn-Cedermark et al., 1999). In addition, in Tejera-Vaquerizo and colleagues' retrospective study, anatomical location of the primary melanoma significantly influenced which metastatic pathway was followed (Tejera-Vaquerizo et al., 2007). Specifically, patients who had a primary cutaneous melanoma on the lower extremity demonstrated a statistically significant lower risk of direct distant metastases compared to all other primary sites (Tejera-Vaquerizo et al., 2007). The abovementioned studies occurred prior to the routine testing of mutation status in patients with advanced disease and consequently, these studies did not control for this potential confounding factor. More specifically, *BRAF*-mutant tumours are associated with sites of intermittent sun exposure (Maldonado et al., 2003; Menzies et al., 2012; Poynter et al., 2006), whereas sites of chronic, cumulative sun exposure have a higher frequency of tumours with *NRAS* mutations (Platz et al., 2008). Therefore, the extent to which the anatomical location of the primary tumour influences the different metastatic pathways independent of the somatic mutational profile of the tumour is unclear.

Some authors suggest that the lower risk of developing distant metastases in patients with cutaneous melanoma of the lower extremities is a result of the longer lymphatic vessels and the greater number of lymph nodes that are required to be passed until the systemic circulation is reached (Garbe et al., 1995; Leiter et al., 2004; Meier et al., 2002). Therefore, the lymphatic drainage system associated with different anatomical locations may be responsible, at least in part, for the patterns of progression and consequent clinical course in patients with cutaneous melanoma (Meier et al., 2002). In fact, primary melanomas on the lower extremities have been reported to have a favourable prognosis compared to melanomas on other body sites (Leiter et al., 2004; Meier et al., 2002). In contrast, melanomas located on the head and neck region have been reported to have a poorer prognosis, with higher predilection for metastasis and poorer overall survival (de Giorgi et al., 2012; Lachiewicz et al., 2008; Pollack et al., 2011).

It has been well-established that primary melanomas on the lower extremities are more common in females, whereas those on the trunk are more common in males (Buettner and MacLennan, 2008; Erdei and Torres, 2010; Garbe and Leiter, 2009). In a large Australian study of 34,021 patients with invasive melanoma and 1,710 patients with in situ melanoma that were diagnosed between 1982-2002, incidence rates were the highest for the trunk in males and the lower extremities in females (Buettner and MacLennan, 2008). Some authors propose that differences in clothing, hairstyle and occupation are possible reasons to account for the sex differences in anatomical location of the primary tumour (Bulliard et al., 1997). However, the aforementioned socio-cultural factors are likely

to only partially explain these sex differences and there are likely to be sex-specific tumour-host interactions that play an important role (Joosse et al., 2011).

Patient sex, as well as influencing anatomical location, indepedently effects the different metastatic pathways for patients with primary cutaneous melanoma (Joosse et al., 2011; Meier et al., 2002). A German population-based cohort study, which was conducted by Joosse *et al.*, investigated gender differences in survival and disease progression at all progression phases in patients with cutaneous melanoma (Joosse et al., 2011). This study demonstrated a significant female advantage in melanoma-specific survival, a lower risk of progression in females, including a lower risk of lymph node metastases and visceral metastases, even after controlling for anatomical site of the primary tumour (Joosse et al., 2011). Gender independently affected melanoma in all phases of progression; thus, the results of this study lend further support to the notion that there are sex differences in biology and disease-host interactions (Joosse et al., 2011). Furthermore, Mervic analysed data from the German Central Malignant Melanoma Registry, which included 7,338 patients with primary cutaneous melanoma from 1976-2008 in order to identify sex differences in the patterns of melanoma progression (Mervic, 2012). In their analysis, the rates of primary lymph node metastasis and direct distant metastasis were similar among men and women (Mervic, 2012). However, females displayed a significantly greater predilection for primary satellite or in-transit metastasis; particularly, in 18.7% of men and 29.2% of women, the first metastasis was satellite or in-transit metastasis (Mervic, 2012). An important finding from this analysis was that the median time to distant metastasis was approximately forty months and thirty-three months in women and men, respectively (Mervic, 2012). Thus, the pattern of metastatic spread, with a higher frequency of primary satellite/in-transit metastases in females and the extended time course to distant metastasis in females, may contribute to the sex differences in melanoma prognosis (Mervic, 2012).

The sex differences in melanoma prognosis are well-recognised; females are reported to have better survival rates compared to their male counterparts (de Vries et al., 2008; Downing et al., 2006; Lasithiotakis et al., 2008). The enhanced survival rates cannot be ascribed solely to the differences in anatomical predilection of the primary tumour (Nikolaou and Stratigos, 2014). In fact, a Dutch study of 10,538 patients with cutaneous melanoma demonstrated that after adjusting for known phenotypic and histopathological prognostic factors, including age, anatomical location of the primary tumour, Breslow thickness, histologic subtype and metastatic involvement, males still had a significant excess mortality risk (de Vries et al., 2008). The results of this study and others highlight that a complex biological basis is likely to underlie females' survival advantage in cutaneous melanoma (de Vries et al., 2008; Lasithiotakis et al., 2008; Nikolaou and Stratigos, 2014).

Tumour thickness has also been reported to be a factor influencing the patterns of progression in patients with primary cutaneous melanoma (Meier et al., 2002). In Meier's discussed above, the majority of melanomas metastasised initially to lymph nodes. However, tumours less than 0.76mm

thick and those greater than 1.5mm thick preferentially developed satellite or in-transit metastases (Meier et al., 2002). Conversely, tumours between 0.75-1.5mm in thickness demonstrated the highest rate of direct distant metastases (Meier et al., 2002). In contrast to the above findings, Cohn-Cedermark and colleagues' study, which assessed the impact of multiple histological factors on melanoma metastatic pathways, demonstrated that metastatic pathways were similar with respect to primary tumour thickness, among other tumour factors, such as histologic subtype, Clark's level of invasion and ulceration (Cohn-Cedermark et al., 1999). Therefore, further research is required to elucidate the various patient- and tumour-related factors that may impact the patterns of progression in patients with cutaneous melanoma.

Effect of tumour mutation status on clinical outcomes of patients with cutaneous melanoma

Our understanding of the molecular basis underlying the pathogenesis of melanoma has improved considerably over recent years. Activation by mutation or amplification of various oncogenes, such as *BRAF*, *NRAS*, *KIT*, cyclin D and cyclin-dependent kinase 4, are significant events in the development of melanoma (Devitt et al., 2011). Activation of the mitogen-activated protein kinase (MAPK) pathway couples signals from cell surface receptors to transcription factors, thereby regulating gene expression and cell proliferation (McCubrey et al., 2007). Constitutive activation of MAPK signalling may be caused by mutations in the *BRAF* oncogene, while mutations in the *NRAS* oncogene may lead to upregulation of the MAPK pathway (Govindarajan et al., 2003; Mishra et al., 2010; Peyssonnaux and Eychene, 2001). Furthermore, inactivating mutations in NF1 (tumour suppressor gene) are present in 50% of wild-type tumours compared with 4% of *BRAF/NRAS*-mutant tumours (Mar et al., 2013). Inactivation of NF1 tumour suppression can result in constitutive MAPK pathway activation (Basu et al., 1992). Nonetheless, the precise role of *BRAF* and *NRAS* mutations in melanomagenesis and tumour progression is yet to be comprehensively established.

It is well-recognised that 40-50% and 15% of cutaneous melanomas harbour activating mutations of *BRAF* and *NRAS*, respectively (Devitt et al., 2011; Hocker and Tsao, 2007; Liu et al., 2007; Smalley, 2003). Mutations in *NRAS* and *BRAF* oncogenes are mutually exclusive of one another and are associated with distinct phenotypic and histopathological characteristics (Barbour et al., 2014; Colombino et al., 2012; Hodis et al., 2012). However, there is some evidence to suggest intra-tumour heterogeneity, whereby *BRAF* and *NRAS* activating mutations can co-exist in the same tumour specimen in different clonal sub-populations (Chiappetta et al., 2015; Sensi et al., 2006). *BRAF* mutant tumours are more common in patients who are younger, have multiple naevi and are more likely to arise in areas of intermittent sun exposure compared to areas of cumulative sun exposure (Barbour et al., 2014; Devitt et al., 2011; Krauthammer et al., 2012; Liu et al., 2007; Maldonado et al., 2003; Mar et al., 2013; Menzies et al., 2012; Poynter et al., 2006; Viros et al., 2008). *BRAF* mutations

are also more common in superficial spreading melanomas (Ekedahl et al., 2013; Liu et al., 2007; Long et al., 2011). Recent evidence suggests that *BRAF* and *NRAS* mutations, which confer distinct clinical and pathological characteristics, are also associated with poorer prognostic outcomes (Devitt et al., 2011; Long et al., 2011; Mann et al., 2013; Mar et al., 2015; Moreau et al., 2012; Si et al., 2012).

There are conflicting results in the literature regarding the clinical correlations and prognostic significance of *NRAS* mutant tumours. Devitt and colleagues' prospective study of 249 patients with cutaneous melanoma demonstrated that *NRAS* mutations were associated with a shorter melanoma-specific survival compared to wild-type and *BRAF* V600E mutations (Devitt et al., 2011). Similarly, Jakob *et al.'s* American study of 677 patients with cutaneous melanoma reported that *NRAS* mutation is independently associated with decreased overall survival after a diagnosis of stage IV disease (Jakob et al., 2012). In contrast, other studies have reported that *NRAS* mutation was not an independent prognostic factor in patients with metastatic melanoma (Barbour et al., 2014; Ekedahl et al., 2013).

While the evidence regarding the prognostic significance of *NRAS* mutation has yielded somewhat inconsistent results, the emerging literature suggests that *BRAF* mutant tumours may confer a poorer prognosis (Long et al., 2011; Mar et al., 2015; Moreau et al., 2012). Long *et al.*'s prospective study of 197 patients with metastatic melanoma revealed that the presence of a *BRAF* mutation had no impact on the disease-free interval from primary melanoma diagnosis to first distant metastasis (Long et al., 2011). However, the median survival of patients with newly diagnosed metastatic melanoma was 5.7 months for patients with *BRAF*-mutant tumours and 8.5 months for patients with *BRAF* wild-type tumours. Therefore, these results demonstrate that the *BRAF* mutation had a significant impact on survival after the development of first distant metastasis (Long et al., 2011). Overall survival in patients with *BRAF* mutant melanoma may be improved by the use of BRAF inhibitors with or without MEK inhibitors (Chapman et al., 2011; Hauschild et al., 2012; Long et al., 2014; McArthur et al., 2014). In addition, Barbour and colleagues' study of patients with stage IIIB and IIIC cutaneous melanoma investigated the patterns of recurrence following therapeutic lymph node dissection associated with tumour mutation status (Barbour et al., 2014). Patients with tumours harbouring a *BRAF* mutation had a significantly poorer recurrence-free survival and disease-specific survival compared to patients with *BRAF* wild-type tumours (Barbour et al., 2014). This study established that the *BRAF* mutation is an independent prognostic factor for patients with resected stage IIIB and IIIC cutaneous melanoma (Barbour et al., 2014).

While the majority of studies investigating the relationship between tumour mutation status and clinical outcomes are focused on patients with metastatic disease (Long et al., 2011; Mann et al., 2013; Moreau et al., 2012; Si et al., 2012), a few studies have demonstrated that *BRAF* mutant melanomas are associated with a shorter disease-free and melanoma-specific survival in patients with

early-stage disease (Mar et al., 2015; Nagore et al.). While these studies were small cohorts (196 and 147 patients, respectively) the results suggest that mutation status may be an important consideration in assessing the risk of disease progression (Mar et al., 2015; Nagore et al.). Notably, a recent population-based study by Thomas *et al.* demonstrated that melanoma-specific survival was significantly decreased for higher risk tumours (T2b or higher stage), but not lower risk tumours (T2a or lower stage), harbouring *NRAS* or *BRAF* mutations compared to wild-type tumours (Thomas et al., 2015). The authors explained that the decreased melanoma-specific survival in *BRAF*- and *NRAS*-mutant melanomas, which was limited to higher risk tumours, may be a result of these tumours acquiring additional genetic alterations during their progression (Thomas et al., 2015). The results of this study provide support that mutational status may offer prognostic information for higher risk primary melanomas.

The relationship between tumour mutation status and metastatic pathways of primary cutaneous melanoma

Data on the relationship between tumour mutation status and the different metastatic pathways in patients with primary cutaneous melanoma is scarce. Notably, a large cohort study demonstrated that *BRAF* mutant tumours, but not *NRAS* mutant tumours, are associated with a greater risk of nodal metastasis at diagnosis (Mar et al., 2014). These results are consistent with Broekaert *et al.*'s previous study, which established that *BRAF* mutant tumours metastasise more frequently to regional lymph nodes, whereas *BRAF* wild-type tumours are more likely to metastasise to non-nodal sites (Figure 1) (Broekaert et al., 2010). The authors of the latter study conclude that *BRAF* mutant melanomas therefore represent a biologically distinct subtype of melanoma that differs in its pattern of metastasis (Broekaert et al., 2010). In contrast, Barbour and colleagues' study of patients with stage III disease revealed that, in patients with *BRAF* mutant melanomas, isolated regional lymph node metastases were rare and almost all primary recurrences represented distant metastases (Barbour et al., 2014). Moreover, Chang and co-worker's study demonstrated that melanomas harbouring *BRAF* mutations were more likely than BRAF wild-type tumours to metastasise to the liver (Chang et al., 2004). However, the results of this study are limited by its small sample size and retrospective study design.

Contrary to Chang *et al.* (Chang et al., 2004), Broekaert *et al.* (Broekaert et al., 2010), and Mar *et al.*'s (Mar et al., 2014) findings, Jakob and colleagues' study has demonstrated that BRAF mutant tumours were not associated with higher rates of either lymph node or liver metastases (Jakob et al., 2012). Rather, *BRAF* mutant tumours displayed significantly higher rates of central nervous system involvement and lower rates of pulmonary involvement at the time of diagnosis of distant metastatic disease (Jakob et al., 2012). The authors contend that if this finding is validated in other studies, a role

for heightened central nervous system surveillance in patients with tumours harbouring *BRAF* mutations may be warranted (Jakob et al., 2012).

Biomarkers in early stage disease

While there is limited research on tumour mutation status as a predictor of the metastatic pathways of disease progression, various other prognostic biomarkers have also been investigated. Biomarkers in early stage disease are of increasing importance in order to stratify the risk of progression in patients with primary cutaneous melanoma and to provide prognostic information (Gould Rothberg et al., 2009; Weinstein et al., 2014). However, there is currently a lack of reliable molecular biomarkers to predict the course of melanoma progression despite extensive efforts and an abundance of investigational studies (Gould Rothberg et al., 2009). While there is yet to be any novel diagnostic or prognostic biomarkers added to the current melanoma staging guidelines, recent research into this area has provided invaluable insight into the biology of melanomagenesis and tumour progression.

Recent studies have investigated the factors involved in EMT as potential biomarkers in cutaneous melanoma (Figure 1). The process of EMT involves the loss of the multifunctional transmembrane protein, E-cadherin (epithelial cadherin), and increased expression of N-cadherin (neural cadherin) (Miller and Mihm, 2006). In melanoma, the EMT-like process may be affected by the transcription factor, SNAI1 (snail 1), by modulating expression of E-cadherin and inducing N-cadherin (Bennett, 2008; Kuphal et al., 2005). In other malignant process, such as breast, endometrial, ovarian, cervical and oral squamous cell carcinoma, the upregulation of the transcriptional repressor Snail and the reduced expression of E-cadherin has been correlated with poor prognosis (Abouhashem et al., 2016; Blanco et al., 2002; Blechschmidt et al., 2008; Peng et al., 2016; Yokoyama et al., 2001). In fact, several studies have demonstrated that reduced E-cadherin expression may be have a role in promoting melanoma cell invasion and metastasis and thus, may be of prognostic significance in cutaneous melanoma (Kreizenbeck et al., 2008; Tucci et al., 2007). Alonso and colleagues analysed gene-expression profiles of vertical growth phase melanomas using cDNA microarrays and determined that expression of a set of proteins in the EMT group (N-cadherin, osteopontin and SPARPC/osteonectin) were significantly associated with the development of metastases (Alonso et al., 2007). The authors concluded that EMT-related genes contribute to the promotion of the metastatic phenotype by supporting specific adhesive, invasive and migratory properties (Alonso et al., 2007).

Boyd and co-workers applied whole-genome expression analyses to reveal that oncogenic BRAF (V600E) regulates genes associated with the EMT-like process in normal cutaneous human melanocytes (Boyd et al., 2013). In particular, this study determined that *BRAF* V600E induces the transcriptional repressor, Tbx3, which represses E-cadherin expression in human melanocytes and melanoma cells (Boyd et al., 2013). These authors propose that the BRAF/Tbx3/E-cadherin pathway

has a significant role in metastasis of *BRAF*-mutant melanomas and consequently, inhibiting Tbx3 expression or activity may represent a potential downstream therapeutic target (Boyd et al., 2013). Moreover, Mitchell and colleagues' recent study sought to elucidate the relationship between *BRAF*, Snail, E-cadherin and other prognostic markers in primary cutaneous melanoma (Mitchell et al., 2016). These investigators demonstrated that the *BRAF* mutation is correlated with the loss of E-cadherin. Thus, *BRAF* may act to repress E-cadherin expression and consequently, it may have a catalytic role in EMT (Mitchell et al., 2016). While this study had a robust methodology, the results are somewhat limited by the small sample size (n = 68) and hence, further research is required to validate their findings. In a larger cohort of 814 patients, *BRAF* mutant primary melanomas were significantly more likely to present with involvement of the regional lymph nodes and were also more likely to have *RAC1* immunoreactivity (Mar et al., 2014). *RAC1* is a member of the Rho subfamily, which is important for cell motility and may also have a role in EMT.

In addition to Snail and E-cadherin, Twist1 and Twist2 are major regulatory proteins that induce EMT (Ansieau et al., 2008). Several small studies have determined that elevated Twist expression is associated with poor prognostic outcomes in patients with melanoma (Caramel et al., 2013; Hoek et al., 2004). In addition, there is some evidence to suggest that Twist1 and Twist2 have the ability to override oncogene-induced premature senescence (Ansieau et al., 2008).

Cell senescence denotes an irreversible arrest of cellular proliferation and is a process that must be overcome for the development of melanoma (Bennett, 2008; Campisi and d'Adda di Fagagna, 2007). Inducers of senescence include oncogenic stress, telomere shortening (replicative senescence) and sustained signalling by certain anti-proliferative cytokines (Bennett, 2003; Campisi and d'Adda di Fagagna, 2007). Some authors maintain that oncogene-induced senescence is not a distinct process from telomere-induced senescence (Bennett, 2008; Campisi and d'Adda di Fagagna, 2007). Indeed, both oncogenes and short telomeres may activate DNA damage signalling, representing a common mechanism of senescence induction (Bennett, 2008; Di Micco et al., 2006; Herbig and Sedivy, 2006). Cellular senescence is established by the p53 and p16-pRB tumour suppressor pathways (Campisi and d'Adda di Fagagna, 2007). Senescence-inducing signals engage these pathways independently; however, these pathways may interact with one another to halt cell-cycle progression (Campisi and d'Adda di Fagagna, 2007).

Immunohistochemical staining for p16, a cell cycle regulator and tumour suppressor, has been demonstrated to be a potential diagnostic marker in differentiating between malignant melanoma and Spitz naevi (Al Dhaybi et al., 2011; George et al., 2010; Hilliard et al., 2009). While p16 expression has been reported as a potential diagnostic biomarker (Al Dhaybi et al., 2011; George et al., 2010; Hilliard et al., 2009), several studies have also demonstrated that p16 may have value as a prognostic biomarker (Lade-Keller et al., 2014). A large cohort study determined that loss of p16 expression predicted overall- and distant metastasis-free survival independent of known histopathological

prognostic markers and tumour stage (Lade-Keller et al., 2014). Thus, p16 expression may represent an independent prognostic biomarker for patients with cutaneous melanoma (Lade-Keller et al., 2014).

Furthermore, Gould Rothberg and colleagues conducted a meta-analysis of immunohistochemistry-based protein biomarkers of melanoma outcome and determined that p16/INK4A, melanoma cell adhesion molecule (MCAM)/MUC18, matrix metalloproteinase-2, Ki-67 and proliferating cell nuclear antigen are promising prognostic biomarkers (Gould Rothberg et al., 2009). The results of this meta-analysis support the role of effectors of DNA replication and cell proliferation, cyclin-dependent kinase inhibitors, transcription factors and regulators of tissue invasion as potential biomarkers in melanoma prognosis (Gould Rothberg et al., 2009). None of the abovementioned biomarkers have been adopted as standard of care for patients with melanoma. Therefore, further studies are required to validate the prognostic value and clinical utility of these biomarkers.

Biomarkers in advanced disease

While none of the serological or immunohistochemical biomarkers discussed above are routinely used in clinical practice in patients with early stage disease, lactate dehydrogenase (LDH) is a serum biomarker that has been included in the American Joint Committee on Cancer (AJCC) staging and classification guidelines for patients with advanced stage disease. Other prognostic biomarkers included in the AJCC staging system include, Breslow thickness, ulceration, mitoses, nodal involvement and the site of distant metastases (Balch et al., 2009).

The cytoplasmic enzyme LDH is one of the earliest studied biomarkers in cutaneous melanoma and is a surrogate marker of disease burden (Karagiannis et al., 2014; Weinstein et al., 2014). Various inflammatory, ischaemic and infective processes may result in elevated LDH; thus, it is not a specific marker of malignancy (Karagiannis et al., 2014). While the specificity of LDH in sera increases with disease progression, the sensitivity of this biomarker decreases (Brochez and Naeyaert, 2000; Deichmann et al., 1999; Sirott et al., 1993; Stark et al., 2015; Weide et al., 2012). It is noteworthy that LDH has been demonstrated to be a predictor of progression in patients with stage IV disease and numerous studies have reported an association between elevated LDH and reduced patient survival (Balch et al., 2009; Sirott et al., 1993; Weide et al., 2012; Weinstein et al., 2014). In particular, Weide and colleagues' cohort study determined that both elevated LDH and S100 independently predicted disease outcome in melanoma patients with advanced stage disease (Weide et al., 2012).

S100 proteins are involved in a variety of cellular functions, including cell growth, cell cycle regulation and cell motility (Weinstein et al., 2014). S100 is frequently used as a diagnostic biomarker when the histopathological diagnosis of melanoma is uncertain (Weinstein et al., 2014). Numerous studies have demonstrated that S100 may be a useful serum prognostic biomarker for patients with stage III and IV disease (Egberts et al., 2008; Kaskel et al., 1999; Kruijff et al., 2009; Mohammed et

al., 2001; Smit et al., 2005; Tarhini et al., 2009; Weide et al., 2012) and may be useful for the stratification of stage III patients for adjuvant treatment (Kruijff et al., 2009). However, there is no current consensus on its routine use in the clinical setting. Indeed, both the sensitivity of serum S100 and LDH varies with disease progression and consequently, these are inadequate biomarkers for detecting early progression at all stages of disease (Stark et al., 2015). More reliable, sensitive and specific novel biomarkers are required in order to detect early disease progression and provide prognostication for all stages of disease.

There is evidence to suggest that in patients with advanced disease, treatment is more effective in those with a lower disease burden (M1a/M1b) compared to those with distal metastases (M1c) (Hodi et al., 2010; Sosman et al., 2012; Stark et al., 2015). This underscores the importance of early identification of disease progression in order to detect low volume metastatic disease, particularly in the context of the rapidly progressing landscape of melanoma therapeutics. Indeed, recent advances in melanoma treatment with new targeted therapies and checkpoint inhibitors has led to more intensive surveillance of high risk patients. Blood tests which are able to reliably detect circulating biomarkers may assist with providing improved and individualised surveillance strategies for these patients. Therefore, circulating tumour products, which include circulating tumour cells (CTCs), circulating tumour DNA (ctDNA) and microRNA (miRNA), represent areas of immense interest in melanoma research (Xu et al., 2016).

CTCs represent cells that have shed from primary tumours or metastatic deposits and are thus circulating in the vasculature (Haber and Velculescu, 2014). Examining CTCs may enhance our understanding of the metastatic cascade and may allow for the serial monitoring of tumour genotypes (Pantel and Speicher, 2016). CTCs are difficult to isolate from patients with melanoma as they do not express common CTC markers, such as epithelial cell adhesion molecule (EpCAM) or epithelial cytokeratins (Gkountela et al., 2016). Morphological and immunophenotypical profiling to detect CTCs remains challenging and therefore, sophisticated cellular isolation platforms are required (Haber and Velculescu, 2014; Hong and Zu, 2013). The multistep preparation process of CTC assays, including blood sample preparation, tumour cell separation, cell staining by antibodies or gene probing and CTC detection, may lead to challenges in interpreting the results (Hong and Zu, 2013). Just as primary melanomas are made up of a heterogeneous population of cells, CTC subpopulations in melanoma have also been shown to be heterogeneous (Gray et al., 2015a). It is unknown at this stage whether CTCs have specific protein expression signatures that enable metastasis and growth in specific organ sites, such as the brain.

Several studies have demonstrated that CTCs may represent potential prognostic biomarkers in patients with metastatic melanoma (Bidard et al., 2014; Khoja et al., 2013; Koyanagi et al., 2010; Reid et al., 2013). Khoja *et al.*'s prospective study used the method of CTC enumeration per 7.5 ml of blood as previously described by Rao *et al.* (Rao et al., 2011) and determined that 26% of patients had

≥ 2 CTCs at baseline and that a baseline CTC of ≥ 2 was an independent prognostic marker for overall survival in patients with metastatic melanoma (Khoja et al., 2013). These authors also demonstrated that CTC measured sequentially throughout treatment provided additional prognostic information and information regarding treatment response (Khoja et al., 2013). Moreover, in patients with uveal melanoma, a recent prospective study demonstrated that CTC count was strongly associated with progression-free survival and overall survival (Bidard et al., 2014). However, CTCs were only detected in 12 of the 40 (30%) patients with metastatic uveal melanoma in this study (Bidard et al., 2014). Notwithstanding the low sensitivity, the investigators maintain that detection of CTCs merits further investigation as it may be coupled to downstream molecular analysis to improve our understanding of the biology of metastasis (Bidard et al., 2014).

An additional circulating non-invasive biomarker, which may assist in improving our understanding of the biology of metastasis, is ctDNA. The isolation of ctDNA in the blood, which is derived from primary tumours, metastatic deposits or lysed CTC, is simpler than the isolation of CTCs (Haber and Velculescu, 2014). There is emerging evidence to demonstrate that ctDNA may be detected in patients with advanced stage melanoma and that ctDNA levels are increased in patients with a higher tumour burden (Bettegowda et al., 2014; Chang et al., 2016; Tsao et al., 2015). A recent American study of patients with unresectable stage IIIC/IV melanoma has demonstrated that, prior to initiation of treatment, in patients with low volume metastatic disease, ctDNA levels were elevated in five of seven (71%) patients and among patients with disease progression, ctDNA had a sensitivity of 82% (Chang et al., 2016). In fact, their study revealed that ctDNA was more sensitive than LDH at detecting metastatic disease (Chang et al., 2016).

Among patients receiving treatment for melanoma, ctDNA may serve as an early indicator of changes in tumour burden and as a monitoring tool for response to therapy (Gray et al., 2015b; Lipson et al., 2014; Schreuer et al., 2016; Tsao et al., 2015). Schreuer *et al.* quantitatively analysed *BRAF* V600 mutant ctDNA from plasma in patients treated with *BRAF*/MEK inhibitors (Schreuer et al., 2016). *BRAF* V600 mutant ctDNA decreased rapidly upon initiation of targeted therapy and was undetectable after six weeks of treatment in seven of 12 (60%) of patients. In patients whose disease progressed, an increase in the *BRAF* V600 mutant ctDNA fraction was detected prior to clinical evidence of progression in 12 of 27 (44%) patients and simultaneously in seven of 27 (26%) patients (Schreuer et al., 2016). Their findings suggest that plasma *BRAF* V600 mutant ctDNA may serve as a potential monitoring tool during treatment with *BRAF*/MEK inhibitors (Schreuer et al., 2016). Lipson *et al.*'s pilot study monitored serial plasma ctDNA levels in patients with metastatic melanoma undergoing treatment with immune checkpoint blocking drugs (Lipson et al., 2014). Of the ten patients who completed treatment, increasing levels of ctDNA were observed in conjunction with radiological evidence of progressive disease in three patients (Lipson et al., 2014). These findings support the notion that changes in ctDNA may predict anti-tumour activity of immune checkpoint

blockade; however, these findings require validation with larger patient numbers in future prospective studies (Lipson et al., 2014).

While the aforementioned studies by Lipson *et al*. (Lipson et al., 2014) and Schreuer *et al*. (Schreuer et al., 2016) assessed ctDNA levels in patients treated with immune checkpoint inhibitors or *BRAF*/MEK inhibitors, respectively, a recent Australian study monitored plasma ctDNA levels in both patients with advanced metastatic melanoma receiving targeted therapies (vemurafenib, dabrafenib or dabrafenib/trametinib combination) or immunotherapies (ipilimumab, nivolumab or pembrolizumab) (Gray et al., 2015b). This study revealed that plasma ctDNA levels decreased significantly in patients receiving MAPK inhibitors in accordance with response to therapy, whereas ctDNA levels did not decrease in patients treated with immunotherapies (Gray et al., 2015b). Thus, the findings of this study indicate that ctDNA may be a valuable non-invasive biomarker of response to kinase inhibitor therapy (Gray et al., 2015b). The utility of ctDNA to predict progression in early stage disease has yet to be evaluated.

Recent evidence is emerging that circulating miRNAs may be used as biomarkers in multiple malignant processes, including cutaneous melanoma (Allegra et al., 2012; De Guire et al., 2013; Fleming et al., 2015; Friedman et al., 2012; Stark et al., 2015; Weiland et al., 2012). miRNAs are small non-coding ribonucleic acids that are involved in the regulation of gene expression at the post-transcriptional level (Bartel, 2004). Tumour cells have been demonstrated to release miRNAs into the circulation. Thus, extracellular miRNAs are detected in serum, whereas intracellular miRNAs are profiled from tumour tissue (Margue et al., 2015). It is noteworthy that miRNAs in serum are highly stable as they are resistant to endogenous RNase activity, prolonged room temperature incubation and multiple freeze-thaw cycles (Mitchell et al., 2008). In contrast, the limited stability of ctDNA is due to the presence of DNAse activity in the bloodstream (Haber and Velculescu, 2014).

There is evolving evidence that various miRNAs and miRNA expression signatures may be used as biomarkers in patients with cutaneous melanoma (Mione and Bosserhoff, 2015). Stark and colleagues' multicentre study has recently identified a panel of seven melanoma-related miRNAs (MELmiR-7) that is able to detect the presence of melanoma in serum with 93% sensitivity and $\geq$ 82% specificity when at least 4 of the miRNAs are expressed (Stark et al., 2015). This melanoma-related miRNA panel was found to be superior to LDH and S100B at predicting overall survival, disease progression and recurrence in patients with stage IV melanoma (Stark et al., 2015). The panel was also able to discriminate stage I/II and stage III melanoma from controls, showing that tumour products are evident in the blood from an early stage, prior to any evidence of metastasis. Further, Armand-Labit and colleagues' recent prospective study identified a profile of two plasma miRNAs (miR-1246 and mirR-185) that was significantly related to metastatic melanoma compared to healthy controls with a sensitivity of 90.5% and a specificity of 89.1% (Armand-Labit et al., 2016). Therefore, this plasma miRNA profile may be a non-invasive biomarker for the timely detection of disease recurrence (Armand-Labit et al., 2016).

Furthermore, Friedman *et al.*'s study revealed that a signature of five miRNAs categorised patients into high and low recurrence risk groups with a significant separation of recurrence-free survival (Friedman et al., 2012). Hence, serum miRNAs may assist with identifying melanoma patients who are at high risk of recurrence (Friedman et al., 2012). Of note, a group of Australian investigators utilised an extensive validation approach among multiple independent melanoma cohorts and revealed that five miRNAs (miR-142-5p, miR-150-5p, miR-342-3p, miR-155-5p, and miR-146b-5p) were reproducibly correlated with patient outcome and thus, may have the potential for future clinical application (Jayawardana et al., 2016).

Moreover, Fleming and colleagues' recent study determined that four miRNAs (miR-150, miR-30d, miR-15b and miR-425) in combination with AJCC stage predicted recurrence-free survival and overall survival better than that predicted by AJCC stage alone (Fleming et al., 2015). Pathway analysis of the miRNAs that predicted disease recurrence showed that they may have a role in regulating immune signalling pathways and the cell cycle (Fleming et al., 2015). This study also reported that, in particular, miR-15b levels increased with time in patients with disease recurrence, while it did not significantly increase in non-recurrent patients (Fleming et al., 2015). These findings provide support for the clinical utility of serum-based miRNAs in improving stratification and surveillance practices.

The relationship between serum miRNA expression and tumour mutation status has been only scarcely described in the literature. Tembe *et al*. recently investigated the relationship between miRNA expression, *BRAF* mutation status and clinical outcomes of melanoma patients (Tembe et al., 2015). Their study revealed that there is an inverse relationship between miR-150-5p and *BRAF* mutation status with prognosis and disease stage. That is, elevated miR-150-5p and the absence of *BRAF* mutation were demonstrated to be positive prognostic markers in metastatic melanoma (Tembe et al., 2015). The relationship between mutational status and serum miRNA expression merits further investigation in order to understand its potential clinical application.

Conclusion

Melanoma metastasis is a complex, multi-step process. In view of the evidence, the model of differential spread, which posits that there are multiple independent dissemination pathways, most accurately accounts for our current understanding of melanoma progression. There is evidence to suggest that melanomas arising in different anatomical locations and melanomas associated with different degrees of sun exposure behave differently. Furthermore, it is well-established that some somatic mutations are more commonly associated with intermittent sun exposure and that tumours with a high mutation burden are associated with chronic ultraviolet damage. While there is some evidence to suggest that *BRAF* and *NRAS* mutant tumours may behave more aggressively than wild-

type tumours, the extent to which mutation status and other molecular characteristics of the tumour determine the pathway of metastasis is unknown.

With multiple new targeted therapies and checkpoint inhibitors now available and with results from adjuvant trials for patients with stage III disease on the horizon, accurate staging and close surveillance of high risk patients is of upmost importance. An understanding of the biology of metastasis, the clinicopathological factors that influence the pathways of progression and the utility of the various circulating biomarkers is required to better identify patients at high risk of recurrence, individualise surveillance strategies and improve our understanding of which pathway of progression any particular primary tumour is most likely to follow.

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Figure Legends:

Figure 1. Patterns of metastasis, time course to the development of metastasis and the clinicopathological and other biomarkers to predict the pathways of progression in patients with primary cutaneous melanoma. Primary cutaneous melanoma can metastasise as satellite, in transit, lymph node or distant metastases. Haematogenous and lymphogenous routes of melanoma metastasis are depicted. The stepwise spread model, the simultaneous spread model and the model of differential spread are three discrete models that endeavour to explain the patterns of melanoma progression. The simultaneous spread model maintains that melanoma metastasises simultaneously by haematogenous and lymphogenous routes, whereas the stepwise spread model upholds that lymphogenous spread necessarily precedes haematogenous spread. The model of differential spread proposes that some melanomas are able to metastasise only lymphogenously, others are able to metastasise only haematogenously, while others still are able to metastasise by both routes.