Melanoma is one of the deadliest and most aggressive forms of skin cancer, with the occurrence of metastasis often being incurable and predictive of poor survival.\(^1\) Local invasion and metastatic spread are responsible for the morbidity and mortality in melanoma. Patients with localized or regional disease have a relatively good prognosis with the 5-year relative survival rate of 98% and 64%, respectively. In contrast, the 5-year survival rate is reduced to 23% in patients with metastatic melanoma (stage IV).\(^2\) Clinically, the Breslow index or the absolute depth of local invasion, measured directly by histopathologic analysis, is the principal prognostic factor and primary criterion in melanoma staging.\(^3\) Over the past years, several therapies have been approved for melanoma.\(^4\) Depending on the features of the tumor (location, stage, and genetic profile), the therapeutic options may be surgical resection, chemotherapy, radiotherapy, photodynamic therapy (PDT), targeted therapy or immunotherapy. Despite the fact that surgery is the primary treatment in thin melanomas (up to 2 mm), adjuvant therapies are recommended.\(^5\) Melanoma is highly metastatic and heterogeneous at early stages of disease, posing a great challenge in the clinic.

Melanocytes are derived from the embryonic neural crest which has undergone morphological changes accompanied by altered adhesion and migration abilities to colonize the epidermis.\(^6\) Therefore,
transformed melanocytes suffer a de-differentiation process that brings them closer to their former neural crest cell precursors.7 Due to its plastic nature, melanoma cells can migrate individually or collectively as multicellular groups.8 Melanoma cell migration requires both actin polymerization and Myosin II driven forces. Rho A/C- are crucial regulators of the cytoskeleton and activate Rho-associated protein kinases (ROCK1 and ROCK2).9 ROCK1/2 promote actomyosin contractile force generation directly phosphorylating Myosin Light Chain 2 (MLC2) or indirectly by decreasing Myosin Phosphatase (MYPT) activity, therefore activating Myosin II complex.10-12 Amoeboid cancer cell migration is propelled by very high levels of Rho-ROCK driven Myosin II activity and membrane blebs as functional protrusions.11,13

We have found an enrichment of amoeboid invasive cells at the edge of human and mouse melanoma tumors 10,14-17 (Figure 1A, B). Such cells are therefore uniquely positioned to receive physical-chemical signals from the surrounding tissue; while they are capable of modifying that tissue more vigorously than cells at the tumor core. Since these cells are at the edge of the tumor, they are well located to leave the tumor and access the vasculature (Figure 1B). Acquiring invasive properties involves remodelling of the cytoskeleton leading to re-programming of the transcriptional landscape of the invading cancer cells. Indeed, Rho-ROCK-Myosin II have been implicated in all stages of the metastatic cascade18 via crosstalk with several key pro-tumorigenic transcriptional programs.

2 | MOLECULAR PATHOLOGY: DO WE NEED AMOEBOID MELANOMA CELL MARKERS?

Oncogenic activation of the MAPK pathway can occur via multiple mechanisms, the most common of which in melanoma is constitutive activation of the BRAF kinase via mutation, which occurs in ~40%-60% of cases. The second most common MAPK pathway aberration in melanoma is mutated NRAS, occurring in ~15%-30% of cases.19 MAPK-directed therapies have significantly improved melanoma treatment in the last decade.4,5 Molecular pathology is currently evolving and includes the use of biomarkers such mutant BRAFV600E, proliferation markers and markers of distant metastasis20-24 -which include markers of differentiated melanomas such as Tyrosinase, Melan A, S100b and may not always detect undifferentiated melanoma.25 Moreover, morphology-based classification methods do not provide relevant information for selecting treatments for patients whose tumors have metastasized.26 It is therefore critical to improve clinical and histopathological criteria to predict metastasis risk. Apart from targeted therapies, immunotherapies have also significantly improved melanoma patient outcome27 but partial responses or development of therapy resistance are a major challenge in the clinic.28,29 therefore biomarkers of therapy response are also needed.

We propose that detecting amoeboid melanoma cells (AMCs) could improve clinical practice since these cells are highly metastatic and involved in therapy resistance. Signalling pathways supporting amoeboid behaviour and possible markers for detecting AMCs are detailed below.

3 | AMOEBOID MELANOMA CELLS AND THEIR TRANSCRIPTIONAL REWIRING, MICROENVIRONMENT REMODELLING, AND CLINICAL RELEVANCE

Cancer cells can use different molecular mechanisms to migrate away from the primary tumor. Cell migration is a well-organized biological phenomenon which is modulated by multiple intrinsic (adhesion, actomyosin contractility, nuclear deformability) and extrinsic factors (matrix organization and composition).30,31 Cancer cells switch between different modes of migration depending on environmental signals. Indeed, cells turn on amoeboid migration when cell-Extracellular Matrix (ECM) adhesion is reduced in pliable matrices.32-34 However, under high confinement and low adhesive conditions, distinct amoeboid motility modes have been described (A1 and A2).31,35,36 Indeed, conditions of high contractility generated by myosin II favour the A2 mode. Interestingly, tumor cells, as well as leukocytes, prefer the A2 mode.31,35,36 We have also observed that AMCs present high levels of cortical Myosin II activity driven by Rho-ROCK signalling in pliable complex matrices10,14-17,37,38 suggesting that amoeboid cells retain a mechanical or a chemical memory (or both). In the past decade, we have reported several signalling pathways that re-inforce and sustain an amoeboid cancer cell state via secreted factors and transcriptional reprogramming. We described how- in melanoma cells - Myosin II activity is perpetuated by establishing a positive feedback loop with the cytokines leukaemia inhibitory factor (LIF)/IL6 and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to maintain Rho-ROCK activity10 (Figure 2A). As a result of high STAT3 activity, AMCs secrete different factors, including matrix metalloprotease 9 (MMP-9). MMP-9 non-catalytic activity promotes Myosin II activation via binding to CD44 and sustains amoeboid invasion and metastatic colonization38 (Figure 2A). Moreover, AMCs secrete high levels of transforming growth factor beta (TGFβ) which activate Sma- and Mad-related protein 2 (SMAD2)-CITED1 driven transcription17 (Figure 2A). TGFβ-SMAD2-CITED1 control a series of genes that sustain Myosin II levels and support metastatic dissemination.17 On the other hand, AMCs harbour lower Reactive Oxygen Species (ROS)39 (Figure 2B). This results in lower Myosin II activity being linked to higher ROS-dependent DNA damage and ATM-mediated p53-PIG3 protein stabilization39 (Figure 2B). Importantly, MAPK signalling supports Myosin II activity, while re-activation of ROCK-Myosin II plays a crucial role in the development of melanoma therapy resistance.15 Furthermore, ROCK1/2 play an indispensable role in cell cycle progression and tumorigenesis, possibly through the maintenance of cellular contractility.40 Moreover, a WNT11/5B-FZD7-DAAM1 signalling axis supports an amoeboid state via direct activation of Rho A-ROCK16 (Figure 2A). High levels
FIGURE 1 Regional distribution of amoeboid melanoma cells in human. (A) Invasive front of human melanoma from a Tissue Microarray cylinder. Immuno-histochemical staining of pMLC2 (top left), cocktail of two melanoma markers (HMB45-MelanA) (bottom left) and combination of both staining using a cyclic IHC and transformed in Fiji-ImageJ (Virtual IHC) (top right). Inset of pMLC2 (magenta; middle left), HMB45-MelanA (cyan, middle middle) and Nuclei (Blue; middle right). Scale bars: (TMA cylinders) 200 µm, (insets) 50 µm (B) Invasive area of thick melanoma tumor. Representative merge of virtual IHC image of pMLC2 (magenta), HMB45-MelanA (Cyan) and Nuclei (Blue). (Bottom) Insets of amoeboid cancer cells invading. (Left) Merge image, pMLC2 (magenta), HMB45-MelanA (cyan) and Nuclei (Blue). Scale bars: (top) 50 µm, (insets) 10 µm. (C) Representative cartoon showing the distribution of amoeboid melanoma cells. Cartoon also depicts tumor microenvironment components such as tumor-associated macrophages, Tregs and endothelial cells cross-talking with amoeboid cells at the invasive front. IF, Invasive front; TB, Tumour body. Panel (C) was created using Servier Medical Art templates licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com)
FIGURE 2  Transcriptional rewiring and impact of amoeboid melanoma cells. (A) Pathways that positively regulate Myosin II activity. (B) Pathways that negatively regulate Myosin II. (C) The amoeboid cancer cell state. Figure was created using Servier Medical Art templates licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com)
of non-canonical Wnt signalling in melanoma cells leads to a pro-invasive and pro-metastatic program that shares many genes with an EMT signature and confers cancer stem cell (CSC) properties.\(^{16}\) (Figure 2A). This indicates that amoeboid cancer cells are part of the EMT spectrum.\(^{41,42}\) During “Phenotype switching”\(^{41,42}\) melanoma cells switch from a proliferative to a migratory state, all orchestrated by MITF and AXL.\(^{43,44}\) However, a population of AXL\(^{\text{high}}\)-MITF\(^{\text{high}}\) melanoma cells was compatible with increased invasiveness and proliferation.\(^{45}\) In agreement with this, we observed that AMC at the edge of mouse and human tumors were both proliferative and invasive\(^{16}\) and in our transcriptome studies we observed that they expressed MITF mRNA.\(^{16}\) As a result of high transcriptional TGF\(\beta\) signalling activation, a subpopulation of melanoma cells was identified that simultaneously displayed proliferative and invasive properties.\(^{46}\) Since their gene ontology analysis showed an enrichment in amoeboid features,\(^{45}\) it is possible that this the same population of AMC. Since MITF plays a mechanosensitive role in melanoma\(^{46}\) how ROCK driven contractility and MITF functions are balanced in amoeboid cancer cells remains to be fully understood.

3.1 | Tumor microenvironment of AMCs

Within the tumor, a variety of normal cells interact with the cancer cells promoting tumorigenesis such as stromal, endothelial and immune cells.\(^{47}\) AMCs secrete a complex set of proteins that are crucially controlled by IL1\(\alpha\)-NF-\(\kappa\)B\(^{14}\) (Figure 2A). Such ROCK-Myosin II-NF-\(\kappa\)B driven secretion attracts monocytes and polarizes them into CD163+CD206+ pro-tumorigenic macrophages (Figure 2C). Therefore, amoeboid behaviour is sustained via a positive feedback loop between ROCK-Myosin-II-driven secretion and IL-1\(\alpha\)/NF-\(\kappa\)B, generating a strong circuit of signal amplification. While AMC-associated macrophages support melanoma cell growth,\(^{14}\) mural cells secrete factors that support MAPK-ROCK2-Myosin II-dependent growth.\(^{48}\) Moreover, AMCs disrupt endothelial junctions and increase endothelial cell permeability via secreted factors which aids during lung metastatic colonization\(^{14}\) (Figure 2C). These data suggest a complex set of feedback regulatory loops between the TME and melanoma cells to support contractility, cancer cell growth and dissemination. In line with this, therapy-resistant melanoma tumors with high Myosin II levels recruit/polarize pro-tumorigenic macrophages and immunosuppressive FoxP3+ T cells\(^{15}\) (Figure 2C). Using pre-clinical mouse models, ROCK inhibitor improved the efficacy of BRAF inhibitors or immunotherapy.\(^{15}\)

The ECM is a key component of the TME. Cancer cells switch migratory modes, depending on their environment.\(^{49}\) Interestingly, physical confinement can trigger amoeboid behaviours and Myosin II isoforms regulate actomyosin contractility levels under high levels of confinement in several tumor types.\(^{50}\) Increasing evidence suggests that the nucleus can act as cellular mechano-sensor while chromatin organization and gene expression can be affected by mechanical forces.\(^{51}\) The nucleus is capable of sensing physical confinement inducing actomyosin-dependent migratory behaviour.\(^{52,53}\)

4 | CLINICAL IMPACT OF AMCs

Using a large set of human melanoma tissues we have reported that the edge—or the invasive front (IF)—of such tumors is enriched in cells with a rounded morphology that harbour very high levels of Myosin II activity as measured by phospho-MLC2 (Figure 1A–C).\(^{10,14,16,17}\) We have measured an increase in the levels of several amoeboid markers (active STAT3, MMP-9, CITED1) at the edge of such melanomas\(^{10,17,38}\) and an association with pro-tumorigenic macrophages and vasculature.\(^{15}\) Furthermore, non-canonical Wnt markers and CSC markers were also enriched at the IF.\(^{16}\) Importantly, high levels of CITED1,\(^{17}\) high levels of ALDH1A1 - a CSC marker\(^{16}\) and high levels of ROCK-Myosin II pathway regulators\(^{15}\) conferred worse prognosis to patients. These data suggest that amoeboid cell content could predict worse outcomes for melanoma patients. On the other hand, therapy resistant melanoma cells were more sensitive to ROCK inhibitors and combination treatments improved targeted therapy and immunotherapy responses in tumors with high Myosin II levels.\(^{15}\) We speculate that the presence of AMCs could indicate a better response to ROCK inhibitors.

5 | CONCLUSIONS AND PERSPECTIVES

Our studies in hepatocellular carcinoma\(^{54,55}\) suggest that amoeboid cancer cells (ACCs) may be induced at the IF of other solid cancers. Identification of universal biomarkers of ACCs at the edge of tumors will be crucial for clinical pathologists to predict ROCK inhibitor sensitivity. Moreover whether all amoeboid cells are the same or there is heterogeneity within patient samples, also needs to be determined. Recently, the dual ROCK-akt inhibitor, AT13148 was used in a phase I clinical trial for solid tumors, mainly colorectal.\(^{56}\) Dose-limiting toxicity due to hypotension was reported and AT13148 pharmacodynamics was also a limitation for its efficacy, while lack of response was associated with no reduction of Myosin II activity in the biopsies analysed.\(^{56}\) After surgical removal, we suggest eradicating AMCs at the edge of melanomas. Since AMCs rely on ROCK for their aggressive behaviour, we suggest that AMC detection could improve clinical responses to ROCK inhibitors. Future research into this class of drugs will involve testing ROCK II isofrom specific inhibitors or soft ROCK inhibitors with less toxicities.\(^{57}\) For melanoma in particular, it will be important to consider the possibility of topical applications to reduce systemic side effects. Moreover, we have identified key signalling pathways that AMCs rely on and that are actionable in...
the clinic. JAK inhibitors, IKK inhibitors, TGFBR inhibitors or IL-1alpha blocking antibodies are all used for clinical applications and could be tested for abrogation of the amoeboid cell state.

ACKNOWLEDGEMENTS
This work was supported by Cancer Research UK (CRUK) C33043/A24478 and Barts Charity.

CONFLICT OF INTEREST
None.

AUTHOR CONTRIBUTIONS
OM and VSM wrote the manuscript. OM performed IHC and image analysis in Figure 1.

DATA AVAILABILITY STATEMENT
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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