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Translation of single-cell transcriptomic analysis of uveal melanomas to clinical oncology.

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Short title: Intratumor heterogeneity in uveal melanomas

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Abstract

Uveal melanoma (UM) is an aggressive and deadly neoplasm. In recent decades, great efforts have been made to obtain a more comprehensive understanding of genetics, genomics and molecular changes in UM, enabling the identification of key cellular processes and signalling pathways. Still, there is no effective treatment for the metastatic disease. Intratumoural heterogeneity (ITH) is thought to be one of the leading determinants of metastasis, therapeutic resistance and recurrence. Crucially, tumours are complex ecosystems, where cancer cells, and diverse cell types from their microenvironment engage in dynamic spatiotemporal crosstalk that allows cancer progression, adaptation and evolution. This highlights the urgent need to gain insight into ITH in UM and its intersection with the microenvironment to overcome treatment failure. Here we provide an overview of the studies and technologies to study ITH in human UMs and tumour micro-environmental composition. We discuss how to incorporate ITH into clinical consideration for the purpose of advocating for new clinical management. We focus on the application of single-cell transcriptomic analysis and propose that understanding the driving forces and functional consequences of the observed tumour heterogeneity holds promise for changing the treatment paradigm of metastatic UMs, surmounting resistance and improving patient prognosis.

1. Introduction

Uveal melanoma (UM) is the most common primary intraocular malignancy in the adult population. UM mainly arises from choroidal melanocytes (90%) and less frequently from melanocytes in the ciliary body (6%) or iris (4%) (Shields et al., 2009). UM is an aggressive and deadly neoplasm and approximately 50% of patients will die within 10 years of the diagnosis because of the tumour’s high propensity to metastasize (Damato, 2018; Damato et al., 2011; Gamel et al., 1993; Mathis et al., 2019). The fact that nearly 50% of patients develop metastases while their primary lesions have successfully been eradicated implies that UM cells disseminate early during primary tumour progression (Eide et al., 2013; Eskelin et al., 2001). This hypothesis has been corroborated by calculating UM metastasis doubling times,
indicating that primary UM cells metastasize several years before diagnosis and initial treatment (Eskelin et al., 2001).

Because of this highly threatening behaviour of UMs, an improved classification and prognostication, using clinical, histological and cellular parameters, and innovative molecular tools are of paramount importance, as it will allow a better treatment to minimize metastatic development and facilitate the design of rational therapeutic options for metastases.

2. Overview of the understanding of uveal melanomas and consequences for patient prognosis

The first attempts at histological and cellular classification date back to 1931 and described for the first-time that UMs are composed of epithelioid cells, spindle shaped cells or a mixture of both cell types (Callender, 1931). In 1962, a study established a link between these cell morphologies and patient survival (Paul et al., 1962). The survival rate at 15 years is almost three times lower in patients with epithelioid cell tumours than in those with spindle cell tumours.

Currently, this histological classification is still used in addition to the American Joint Committee on Cancer staging (AJCC), which is based on the tumour size (diameter and thickness) and anatomical extent (ciliary body involvement and extra-scleral extension) (Amin et al., 2017; Kujala et al., 2013). It is worth mentioning that the Collaborative Ocular Melanoma Study (COMS), the largest study in ocular oncology based on UM size, have had tremendous impact on the diagnosis and individualized patient care (Margo, 2004; Straatsma et al., 1988). Several other clinical and histological features are used to complement the conventional AJCC UM staging and patient prognosis (Kaliki et al., 2015; Mäkitie et al., 1999) (Figure 1). However, a
major twist came from cytogenetic studies identifying that the recurrent loss of chromosome 3 and gain of chromosome 8q in tumours (Horsman et al., 1990; Prescher et al., 1992; Sisley et al., 1990) were associated with poor prognosis tumours (Damato et al., 2009, 2007; Jager et al., 2020; Pandiani et al., 2017; Smit et al., 2020; Trolet et al., 2009). Adding chromosome status together with the AJCC resulted in an even better system (Bagger et al., 2015). Finally, transcriptomic and genomic studies also contributed to a better characterisation and understanding of UM pathogenesis, with clear applications in their classification and prognostication. The comparison of the transcriptomic profile of primary UMs with either disomy or monosomy of chromosome 3, identified a set of genes associated with patient survival (Tschentscher et al., 2003). Similarly, unsupervised clustering in primary UM transcriptomic analysis disclosed two molecular classes, with class 2 being associated with poor patient survival (Onken et al., 2004). Both signatures partially overlapped and identified \textit{HTR2B} as a marker of poor prognosis (Onken et al., 2004; Tschentscher et al., 2003). High preferentially expressed antigen in melanoma (\textit{PRAME}) expression was proposed as an indicator of a further sub-classification of tumours at increased risk for metastasis (Field et al., 2016). Globally, the signature of class 2 UM largely overlaps with that of chromosome 3 monosomy, an observation best shown in the TCGA research network report (Robertson et al., 2017). Later, a signature based on gene expression and the DNA methylation profile was reported to predict metastatic risk (Li et al., 2018).

Then, genetic analyses added an additional layer to the molecular characterization of UMs. Activating mutations have been found in \textit{GNAQ} and its parologue \textit{GNA11} in approximately 80% of UM (Van Raamsdonk et al., 2010, 2009). These driver
mutations are present in choroidal naevi, and do not necessarily give rise to melanoma (Vader et al., 2017). Although these mutations have no prognostic value, it cannot be concluded that GNAQ and GNA11 activating mutations do not influence the metastatic behaviour or patient survival. Additional rare mutations in CYSLTR2 and PLCB4 have also been identified (Johansson et al., 2016; Karlsson et al., 2020; Moore et al., 2016). These four driver mutations are maintained in metastatic UMs (Karlsson et al., 2020). CYSLTR2 and PLCB4 function upstream and downstream of GNAQ/GNA11, respectively. Therefore, the GNAQ/GNA11 signalling pathway is activated in virtually all UM cells and it is difficult to obtain consistent data on the metastasis and survival of the few patients without mutations in the GNAQ/GNA11 module. Nevertheless, it has been observed that GNA11 mutations confer a slightly increased risk of metastasis compared to GNAQ mutations, which might be attributable to variations in their effector spectrum (Griewank et al., 2014; Van Raamsdonk et al., 2010).

Mechanistically, downstream of GNAQ, ARF6 orchestrates the activation of a broad range of events, including the activation of the PKC, PI3K/AKT/mTOR, ERK/MAPK and HIPPO/YAP signalling pathways (Yoo et al., 2016). However, compelling recent data suggest that the ERK module is the pivotal signalling axis in UM cell proliferation (Ma et al., 2020; Truong et al., 2020), bringing attention back to MEK and ERK inhibitors as rational UM treatments.

Additional recurrent mutations in EIF1AX, SF3B1 (Martin et al., 2013) and BAP1 have been identified (Harbour et al., 2010; Van De Nes et al., 2016). They are generally mutually exclusive of each other (Robertson et al., 2017), yet co-occurrence of BAP1 and SF3B1 mutations can be observed in a few metastatic UMs (Moore et al., 2016). EIF1AX and SF3B1 mutations are prognostically associated
with a low to intermediate metastatic risk (Yavuzyigitoglu et al., 2016), and mutations in \( BAP1 \), which is located on chromosome 3, correlate with a high metastatic risk and a poor survival (Field et al., 2018; Shain et al., 2019; Smit et al., 2020). However, most \( BAP1 \) mutations are in tumours with chromosome 3 monosomy, which by itself confers a poor prognosis. Analysis of patients with chromosome 3 monosomy has failed to demonstrate clearly an association of \( BAP1 \) mutations with poor prognosis (Kalirai et al., 2014; Koopmans et al., 2014; Van De Nes et al., 2016). Furthermore, analysis of the whole exome sequencing (WES) TCGA dataset shows that \( BAP1 \) mutations do not predict a worse outcome within patients with chromosome 3 monosomy (Figure 2A). However, WES can miss intronic mutations leading to aberrant splicing (Karlsson et al., 2020) and overlook the alterations in \( BAP1 \). Interestingly, using whole genome sequencing datasets (Johansson et al., 2020), we showed that \( BAP1 \) mutations conferred a high metastatic risk in patients with chromosome 3 monosomy (Figure 2B). In line with this, immunohistochemical staining for nuclear BAP1 protein is now used routinely as a surrogate marker for BAP1 wild-type genetic status. The absence of nuclear BAP1 is a marker of poor prognosis within the patients with chromosome 3 monosomy (Farquhar et al., 2018). Furthermore, the key role of \( BAP1 \) mutations in metastasis and patients’ survival is strengthened by the huge amount of mutations (91% of the tumours analysed) observed in UM metastases (Karlsson et al., 2020).

Finally, the Cancer Genome Atlas (TCGA) recently proposed, based on a combination of molecular parameters (copy number, gene alterations, DNA methylation, and gene expression), a classification of the UMs into four distinct profiles (Robertson et al., 2017) that seems to provide a superior accuracy in predicting metastasis compared to chromosome, gene expression profile, or AJCC
staging alone (Mazloumi et al., 2020). In line with this, multi-parameter prognostic algorithms have already been developed that combine clinical, genetic and histological parameters, and are used regularly for several years in clinical care (Damato et al., 2011; Rola et al., 2020).

This large amount of data has provided invaluable information on the biology of UMs and has enabled accurate staging and therefore better patient management and care. However, none of the molecular markers identified so far have been shown to be an effective therapeutic target for preventing or eliminating UM metastases. New technical approaches allowing transcriptomic, epigenetic and genomic analyses at the single-cell level has emerged. These approaches provide key insights into the molecular characterization of intratumour heterogeneity (ITH) and may lead to the discovery of new markers and therapeutic targets after their validation by classical biological and preclinical methods. In this review, we mainly focus on the recent studies using single-cell analysis in UMs (Figure 3).

3. Dissection of tumour heterogeneity and molecular determinants in the single-cell era

Solid tumours are heterogeneous neoplasms composed of a complex architecture of malignant cells, that dynamically interact with different types of non-malignant cells within their microenvironment. Within the same tumour, malignant cells can vary in molecular and phenotypic profiles over time and space, which is also known as ITH (Chakrabarty et al., 1985; Meacham and Morrison, 2013). ITH poses a huge challenge for precision medicine, since a single sample may not accurately capture the entire genomic and phenotypic complexity of a tumour, especially because ITH may evolve during disease progression and treatment.
Moreover, tumours are not composed exclusively of cancer cells. Precise knowledge of the nature and function of the cells that make up a tumour is therefore critical, as this tumour microenvironment (TME) plays an essential role in the development of the tumour, the immune response and the effectiveness of the treatment (Binnewies et al., 2018). Conventional bulk approaches (sequencing and arrays) provide only an average expression signal or mutation profile for a group of cells. Even though elaborate bioinformatic methods have been developed to infer the cellular composition from bulk analyses, these algorithms are not flawless and may miss minority cell sub-types (Jimenez-Sanchez et al., 2019). In the case of UM with a good prognosis, as defined by classical cytogenetics, whole exome sequencing or gene expression analysis, the presence of a small percentage of cells with, for instance, a chromosome 3 monosomy, will be overlooked, but these cells might metastasize and confer a poor prognosis.

Recently, high-resolution single-cell RNA sequencing (scRNA-seq) has revolutionized the field by allowing unique transcriptomes to be obtained from individual cells. In addition to uncovering that gene expression may be heterogeneous among cancer cells in the same tumour, it has also opened-up information on the cellular composition of the TME and on rare tumour cells. It is expected that these data will be essential for elucidating the interactions between cancer cells and their ecosystem and for furthering our understanding of drug resistance and relapse (Emert et al., 2017; Kemper et al., 2015, 2014; Rambow et al., 2018; Tirosh et al., 2016).

In UM, ITH is supported by the co-existence, in some tumours, of cells with epithelioid and spindle shapes. Cells with these different morphological
presentations might each exhibit distinct gene expression patterns as a result of different transcriptional states (Onken et al., 2006; Seftor et al., 2002). The molecular characterization of ITH and the identification of transcriptional states and TME cell composition in UMs should lead to the identification of subpopulation(s) prone to metastatic dissemination and might reveal new biomarkers. More importantly, as until now classical approaches have failed to identify valuable therapeutic targets, single-cell analysis approaches are expected to provide crucial information for the design of rational therapeutic options.

Hereafter, we discuss recent studies describing the analysis of UMs at the single cell level. These analyses provide not only insight into ITH and the different transcriptional cell states that co-exist in UMs but also into the complexity of their microenvironment. We next discuss how to improve our understanding of ITH functionality and the potential impact of these studies on future directions of the management of UM patients.

4. Single-cell analysis of uveal melanomas

An important study reported the RNA-seq analysis of 59,915 single cells from eight primary and three metastatic tumours (Durante et al., 2020). These tumours had a considerable number of infiltrating immune cells: 7-10% in primary class 1 UM (n= 1), 7 to 45% in primary class 2 UM (n= 3) and 45 to 85% in metastases (n= 3). These large numbers of infiltrating immune cells allowed a dissection of the nature of these cells and provided important information for developing rational immunotherapies. More recently, another RNA-seq analysis of 7,890 single cells from six primary UM lesions allowed the characterisation of molecular heterogeneity and the identification of a new gene signature that is associated with a poor clinical
outcome and shorter overall survival (Pandiani et al., 2021). Both studies, by inferring copy number variation (CNV) from scRNA-seq datasets, also highlighted genomic heterogeneity, the origin of which remains to be uncovered.

5. Deciphering the tumour ecosystem with single-cell RNA-seq analysis.

As mentioned above, immunotherapies that have had successful results in several neoplasms, including cutaneous melanomas, have failed to improve the outcome in patients with UM (Niederkorn, 2009). The improvement of the response to immunotherapies in UM requires a better understanding of the TME and especially of the immune infiltrate. Using scRNA-seq, Durante et al. studied the complexity of the UM immune infiltrate (Durante et al., 2020). As previously reported (Robertson et al., 2017), class 2 tumours exhibited more immune infiltrate (Durante et al., 2020). The infiltrates were mainly composed of macrophages with an imbalance towards M2 polarization, and the pro-tumoural function of these cells has been well documented. Interestingly, tumours with monosomy 3, which are associated with a poor outcome, displayed significantly more M2-type macrophages than the group with chromosome 3 disomy (Bronkhorst et al., 2012; Herwig and Grossniklaus, 2011). In accordance with this finding, the 10-year UM-specific mortality rate increased with higher numbers of macrophages (Mäkitie et al., 2001). Notably, other studies showed that the influx of macrophages was related to extra copies of chromosome 8q (Gezgin et al., 2017). Likewise, the detection of proteins at the single cell level by CyTOF mass cytometry, in five primary UMs, revealed a predominant cluster of macrophages (Figueiredo et al., 2020; Krishna et al., 2017).

The immune infiltrate comprised few natural killer (NK) cells (Durante et al., 2020; Figueiredo et al., 2020). Given that the disruption of NK function in vivo has
been shown to enhance the spontaneous hepatic metastasis of intraocular melanomas in mice, this is consistent with a role of NK activity in suppressing the metastatic process, as previously proposed (Dithmar et al., 1999; Ma et al., 1995). It is worth noting that a clonal expansion of plasma cells was found in one indolent metastasis containing class 1 PRAME+ cells (Durante et al., 2020). This observation prompted the authors to propose a role for antibody-dependent immunity in metastatic surveillance.

T cells are also frequently found in infiltrates and are mostly CD8+, including naïve, cytotoxic and effector memory cells (De Cruz et al., 1990; De Waard-Siebinga et al., 1996; Durante et al., 2020). More interestingly, in three class 2 primary UMIs, the T cell subset included clonally expanded exhausted T cells, with strong expression of the immune checkpoint Lymphocyte antigen gene-3 (LAG3) and, to a lesser extent, T cell immunoreceptor with Ig and ITIM domains (TIGIT) (Durante et al., 2020). These observations confirm those of Triozzi et al. who reported a high LAG3 level in tumour-infiltrating lymphocytes (TILs) in UMIs (Triozzi et al., 2014). Another significant study described the immune composition of the largest metastatic UM cohort and revealed the expression of the immune checkpoint receptors TIM-3, TIGIT and LAG3 on UM TILs, which likely represent a means of immune evasion (Karlsson et al., 2020). BAP1 has potentially important consequences for tumour-immune interactions. Indeed, BAP1 loss in UM cells has been associated with higher numbers of infiltrating T cells, likely a consequence of the secretion of chemokines known to mediate the homing of immune cells to the tumour (Gezgin et al., 2017). Furthermore, an increase in genes with immunosuppressive functions, including PD1, TIGIT, and LAG3, has been reported following BAP1 loss in UM (Figueiredo et al., 2020; Krishna et al., 2020). Consistent with this, the downregulation of TIM-3 and
TIGIT ligand-related genes was observed after restoring BAP1 function in the metastatic cells (Karlsson et al., 2020). Together, these data might explain the resistance to anti-CTLA4 and anti-PD1 antibodies in metastatic UM and provide new therapeutic opportunities. Another explanation of immunotherapy failure may be linked to the immune suppressive environment of liver (Yu et al., 2021).

In summary, these studies confirmed the first level of ITH generated by the TME and showed that LAG3, TIGIT and PD1 are immune checkpoint proteins expressed by UM TILs. Of note, PD1 appears to be expressed together with LAG3 (Durante et al., 2020) and given that UM cells express PD-L1 (Zoroquiain et al., 2018), these observations suggest the possible synergistic action of using anti-PD1 and anti-LAG3. Such combination therapy is indeed being evaluated in the treatment of other solid tumours (NCT01968109).

6. Evidence of the heterogeneity of genomic alterations by single-cells RNA-seq.

The studies by both Durante and Pandiani inferred CNVs from scRNA-seq data (Durante et al., 2020; Pandiani et al., 2021). These analyses confirmed the existence of genomic ITH, discovered by earlier bulk studies (Dopierala et al., 2010; Lake et al., 2011; Schoenfield et al., 2009). Genomic ITH appears to be generated during tumour development and allows for the acquisition of metastatic behaviours (Shain et al., 2019). In addition to canonical CNVs, these approaches revealed new cryptic genomic alterations that cannot be detected at the bulk level (Durante et al., 2020; Pandiani et al., 2021). Globally, inferred CNVs parallel CNVs detected by CGH array analysis (Pandiani et al., 2021) or scDNA-seq performed in two tumours (Durante et al., 2020). However, some differences exist. For instance, the
amplification of chromosome 19 inferred from scRNA-seq in a subset of cells was not detected by scDNA-seq (Durante et al., 2020). Likewise, in Pandiani’s study, inferred-CNVs identified a chromosome 6q loss in a subset (5%) of cells in tumour #A, which was not found by CGH array. More importantly, in tumour #F that is classified as having a good prognosis due to chromosome 3 disomy, a subset (5%) of cells exhibits chromosome 3 loss, and could give rise to metastasis (Pandiani et al., 2021). We can also hypothesize that these cryptic alterations identified by inferred CNV, are caused by a broad regional transcriptional regulation at the level of topology-associating domains or chromatin nanodomains. These types of regulation involve interactions between distant DNA segments, that are mediated by insulators such as CTCF, or SATB1 that belongs to the GEP signature (Onken et al., 2004). High expression of these two genes is associated with the long-term survival of patients with UM (Figure 4), supporting the role of topology-associating domains in UM pathogenesis.

7. Single-cell RNA-seq established intratumour transcriptional heterogeneity and identified new prognostic signatures.

Although previous studies have suggested the existence of different transcriptional programmes associated with different UM cell morphologies (Onken et al., 2006), the scRNA-seq approach has provided invaluable knowledge on the transcriptional states that govern the behaviour of UM cells. Using a supervised clustering based on a 12-gene expression profiling (GEP) signature classifying UM into classes 1 (low metastatic risk) and 2 (high metastatic risk) (Onken et al., 2004), and an additional level of stratification according to PRAME expression, which was identified as a biomarker of metastasis in both classes (Field et al., 2016), Durante et
al. showed that the 11 tumours contained a mix of class 1 and class 2 cells, that did or did not express PRAME (Durante et al., 2020).

Our group performed the single-cell analysis of 6 primary UMs. Principal component analysis based the 1,000 most variable genes indicated that PC1 constituted the majority of the variance within the dataset. Kaplan-Meier analysis of UM patients from the TCGA cohort showed that the expression of the top 10 genes with the highest PC1 value correlated with metastasis development and low overall survival, while the expression of the top 10 genes with the lowest PC1 value correlated with a favourable prognosis (Pandiani et al., 2021). This unsupervised approach established a new prognostic signature (hereafter the PC1 signature) comprising HTR2B, which was already known to confer a poor prognosis and disclosed a list of new valuable prognostic markers.

For instance, the common MHC class I components β2-microglobulin (β2M), and HLA-A, components of the major histocompatibility complex class I (MHC-I) molecules, were among the top genes associated with a poor prognosis within the PC1 signature (Pandiani et al., 2021). In contrast with many other tumours, UMs with high HLA class I or HLA class II antigen expression in the primary lesions have a significantly decreased survival (Blom et al., 1997; Ericsson et al., 2001; Van Essen et al., 2016). In agreement with this finding, elevated serum concentrations of IFNγ, a factor known to increase HLA class I and class II expression, correlated with the metastatic spread and represented a poor prognostic marker (De Waard-Siebinga et al., 1995; Likhvantseva et al., 1999). Likewise, IFNγ-treated UM cells were less efficiently lysed by CD8+ MHC class I-restricted cytotoxic T lymphocytes, due to resistance to granule-mediated cytolysis than untreated parental cells or TNFα-treated counterparts (Hallermalm et al., 2008). Mechanistically, while IFNγ treatment
did not affect the UM cell capacity to trigger T cell activation or their intrinsic ability to undergo apoptosis (Hallermalm et al., 2008), it impaired both granzyme B binding to UM cells and perforin-mediated cell permeabilization (Hallermalm et al., 2008). The expression of β2M by cancer cells directly protected them from the phagocytic function of macrophages (Barkal et al., 2018). This finding agrees with previous reports showing an inverse correlation between B2M expression, and survival in UM (Jager et al., 2002; Souri et al., 2019). Notably, the sensitivity of UM cells to NK cell-mediated lysis was shown to be inversely correlated with HLA protein expression (Jager et al., 2002; Ma et al., 1995). The data above were generated in primary tumor cell lines and tumor samples. Less is known about metastatic UMs. One can imagine an advantage of inducing HLA to avoid innate immunity killing during the hematogenous spread but a disadvantage of expressing HLA in the metastatic niche where cells may be sensitive to tissue-resident or primed T cells. Future studies will be required to test this hypothesis.

B2M, HLA-A and HLA-B are expressed on UM cells and immune cells, but in a large proportion of the TCGA cohort, there were small amounts of immune infiltrate that were restricted to a small subgroup of tumours. Importantly, analysis of the TCGA cohort using a PC1 score based on the expression of the top up- and downregulated PC1 genes showed a very high predictive value for patient survival, demonstrating the potential usefulness of this new transcriptomic signature (Pandiani et al., 2021). Indeed, ROC analyses indicated that our PC1 signature performed as well, or even better than two previous signatures (Li et al., 2018; Onken et al., 2004) or chromosome 3 monosomy (Figure 5). Applying the PC1 score to our dataset allowed us to estimate the % of cells potentially at metastatic risk in the 6 primary UMs analysed (Pandiani et al., 2021) (Figure 6).
Using SCENIC (Single-Cell rEgulatory Network Inference and Clustering), a program that monitors the activity of the transcription factors and their predictive downstream target genes (Aibar et al., 2017), Durante et al., described several salient transcriptional states that are driven by MYC, ARNT, TAF1, TAF7 or JUN and that are associated with class 2 UM cells (Durante et al., 2020). Pandiani et al., using SCENIC also identified several transcriptional states, among which a differentiation-related state was driven by SOX10 and PAX3, two major regulators of microphthalmia-associated transcription factor (MITF), the master gene of melanocyte lineage development and function (Ballotti et al., 2020; Bertolotto et al., 2011; Cheli et al., 2010; Goding and Arnheiter, 2019). This state overlaps with cells presenting intermediate MITF activity and correlates with a low PC1 score and a good prognosis. Supporting this observation, a high level of SOX10 seems to confer a low metastatic risk (TCGA UM dataset). Pandiani et al. also identified transcriptional states driven by RELB, HES6 and MYC that are associated with a high PC1 score and therefore represent poor prognostic markers (Pandiani et al., 2021). These transcriptional states overlap with the class 1 PRAME+ and class 2 cells (Figure 7). Therefore, both studies identified a MYC-driven transcriptional state in cells conferring a poor prognosis (Durante et al., 2020; Pandiani et al., 2021). However, neither MYC itself nor the top 10 genes within its regulon displayed a predictive value for patient survival, although MYC has been described as a key metastatic driver in UM (Meir et al., 2007). Note however, that the role of MYC may be overshadowed by the influence of BAP1/chromosome 3 loss. To determine the influence of MYC, one should look at tumours retaining BAP1 expression/chromosome 3 normal status.
Additionally, neither TAF1 nor JUN expression is correlated with the metastatic risk or survival, while high ARNT or TAF7 expression is predictive of metastasis but is not correlated with patient survival (TCGA UM dataset). RELB is not correlated with patient outcome, but BAP1 loss, which is associated with a high metastatic risk, has been reported to influence RELB activation (Singh et al., 2019), and the overexpression of NF-κB p50/RELB has been reported to promote cutaneous melanoma cell migration (Gao et al., 2006).

It is worth noting that primary UMs analysed by Bertolotto and coworkers did not show much stroma or immune infiltrate (Pandiani et al., 2021). This was not due to bias from preparing single-cell suspension, because parallel flow cytometry analysis of bulk tumour cells prior to single-cell analysis showed less than 2% of CD45 positive cells (personal communication). In line with this finding, a comprehensive multiplatform analysis of 80 UMs by TCGA project reported that a large majority of primary UMs does not harbour large amounts of immune infiltrate (Robertson et al., 2017). In this respect, it is possible that primary UMs analysed by Bertolotto and coworkers are more representative of the majority of primary UMs (Pandiani et al., 2021), but that of Durante et al. reflects a minority with a large amount of immune infiltrate (De Waard-Siebinga et al., 1996; Durante et al., 2020). However, TILs are readily expanded from metastases (Karlsson et al., 2020) and have been used in adoptive cell therapy in trials with promising data (Chandran et al., 2017). Nevertheless, both studies detected ITH in UMs and demonstrated that almost all tumours contained a mix of cells expressing different transcriptional programmes. Accumulating evidence indicates that even an extremely small subset of cells that confer a poor prognosis within a UM with a good prognosis may be responsible for the development of metastases and patient death, weakening the accuracy of
prognosis. Whether these cells are genuine cancer stem cells remains to be determined.

Altogether, while these reports both studied UMs at the single-cell level, they focused on different aspects, i.e invasive properties and the immune system (Durante et al., 2020; Pandiani et al., 2021). Thus, they provide complementary information that serve a better understanding of two critical aspects of tumour biology, metastatic dissemination and immune evasion and disclose new actionable vulnerabilities.

8. HES6 and the NOTCH pathway are key drivers of the metastatic behaviour of UM cells.

Among the transcriptional states associated with poor prognosis, HES6 is of particular interest. Indeed, HES6 belongs to the top 10 upregulated genes of the PC1 signature and SCENIC identified an HES6-driven transcriptional state associated with the PC1 signature, that confers a poor prognosis. HES6 is a poorly studied member of the HES/HEY family of transcription factors in the NOTCH signalling pathway (Hojo et al., 2008). Its expression is enhanced in various tumours and it represents a poor prognostic marker in prostate and colorectal cancers (Ramos-Montoya et al., 2014; Swearingen et al., 2003; Xu et al., 2018). This latter is also true in UMs, as HES6 expression is associated with shorter progression-free survival and overall survival (TCGA cohort) (Pandiani et al., 2021). Additional evidence for the role of HES6 as a prognostic biomarker has been reported in primary cutaneous melanomas (Brunner et al., 2018). Also significant, HES6 overexpression in glioma cells resulted in the increased expression of nestin, a marker for cancer stem cells (Haapa-Paananen et al., 2012). Nestin has been
described as a possible biomarker for high-risk UMs (Djirackor et al., 2018). However, in UM cells, *in vitro* and *in vivo* experiments demonstrated a key role of HES6 in proliferation and metastatic dissemination, indicating that HES6 is more than a simple prognostic marker, but also emerged as a key driver of UM pathogenesis and a valuable therapeutic target.

How HES6 operates, remains to be fully demonstrated. However, HES6 seems to play a key role in NOTCH signalling in UM cells as it is essential for the migration mediated by Delta-Like Ligand 4 (DLL4), one of the five NOTCH ligands (DLL1, DLL3, DLL4, Jagged (Jag) 1, and Jag2). Notably, DLL4 is the member whose expression is the most strongly associated with UM metastatic ability (Figure 7). Interestingly, NOTCH activation by DLL4 triggers the induction of the TRIO-RHO module to drive the invasion and metastasis of colorectal cancer (Sonoshita et al., 2015). In UM cells, the TRIO-RHO/RAC signaling axis lies downstream of oncogenic GNAQ/11 (Feng et al., 2014). Given that the NOTCH signalling pathway involves the interaction between two adjacent cells, one expressing a ligand (either Delta or Jagged) and the other expressing NOTCH receptor, this may facilitate a collective form of UM cell migration. In this circumstance, UM cells may not need to undergo a pseudo epithelial-to-mesenchymal transition. This could explain why the epithelioid shape represents a determinant of poor prognosis. Of note, tumors (TCGA UM cohort) with high percentage of epithelioid cells express higher HES6 level than tumor with low epithelioid cell content (Figure 9). It would be interesting to determine whether HES6 favours a stem-cell phenotype in UM cells.

To date, there are no available direct HES6 inhibitors. Thus, targeting the upstream or downstream events remains a logical goal. The *HES6* regulon provides a list of valid therapeutic targets, including the histone deacetylase 4 (*HDAC4*) and the ChaC
glutathione-specific γ-glutamylcyclotransferase 1 (CHAC1). The expression of both correlates with a poor prognosis in UM (TCGA dataset). It is worth noticing that CHAC1 is suppressed when BAP1 is reintroduced into a BAP1-deficient UM cell line (Karlsson et al., 2020). Hence, CHAC1 expression correlating to survival could also be a consequence of BAP1 status.

HDAC4 plays a critical role in UM progression mediated by BAP1 loss. Indeed, BAP1-loss, through the regulation of H3K27ac-mediated transcriptional activation, favours a dedifferentiated phenotype. Pharmacologic inhibition of histone deacetylase (HDAC) activity or specific knockdown of HDAC4 rescued the BAP1 loss-driven phenotype (Kuznetsov et al., 2019). Consistently, BAP1 expression is inversely correlated to that of HES6 (Figure 10).

CHAC1 knockdown inhibits the proliferation and motility of UM cells (Liu et al., 2019). CHAC1 has an important role in the oxidative balance and cell survival by regulating the degradation of glutathione through its γ-glutamylcyclotransferase activity (Kumar et al., 2012). This can affect ferroptosis, and the key regulator of ferroptosis occurrence is glutathione peroxidase 4 (GPX4) (Stockwell et al., 2017). In cutaneous melanoma, resistance to BRAFi treatment was reduced by combination treatment of vemurafenib and ferroptosis inducers (Tsoi et al., 2018). Furthermore, CHAC1 is one of the endoplasmic reticulum (ER) stress proteins, which are involved in UM cell survival (Bellini et al., 2020).

Regarding upstream regulators of HES6, in human T cell leukaemia cells and prostate cancer cells, HES6 expression is regulated by c-MYC (Margolin et al., 2012; Ramos-Montoya et al., 2014). MYC is located on chromosome 8, whose amplification is one of the most common genetic abnormalities in UM and is strongly associated with metastatic risk (Parrella et al., 2001). Interestingly, NOTCH1 directly
regulates MYC in leukaemic cells (Palomero et al., 2006). Furthermore, in prostate cancer, HES6 has been described as a target of hypoxia-inducible factor (HIF1α) (Qi et al., 2010), a global regulator of oxygen homeostasis. This is consistent with the fact that HIF-1α and c-MYC may bind directly to many of the same promoters (Dang, 2007). Given that ARNT has been identified as part of the class 2 transcriptional state, HES6 may also be regulated by the HIF pathway in UM. Altogether, these data highlight a relationship between expression of HIF1, NOTCH, MYC and HES6 that may fuel UM progression.

Finally, Notch signalling, through the production of an anti-inflammatory secretome, has been shown to promote an immunosuppressive TME (Colombo et al., 2018). Although an intersection between HES6 and the immune system remains to be demonstrated, high HES6 expression correlates with immune cell infiltration, as illustrated by increased PTPRC expression (TCGA dataset) (Figure 11A). Tumour-infiltrating immune cells displayed enhanced expression of the immune checkpoint proteins PD1, CD27, TIGIT and LAG3 (Figure 10A). A CIBERSORT analysis (https://cibersort.stanford.edu/) was carried out to compare the 25% tumours with the highest and lowest HES6 expression. Among the 22 phenotypes identified by CIBERSORT, only naïve B cells were found to be significantly increased in the low HES6 tumours (Figure 11B).

Thus, single-cell transcriptomic profiling can be translated into mechanistic insights for the regulation of the metastatic process and therapeutic resistance, thereby highlighting the potential clinical impact of this technology.

9. Future direction and conclusions
9.1. Methodological improvements for investigating ITH in UM cells

To uncover its role in cellular function and understand how gene expression can promote beneficial or harmful states, attempts have been made to obtain high-resolution views of single-cell heterogeneity on a global scale. Such analyses led to the discovery of transcriptomic ITH in UMs.

In UM, survival correlates with primary tumour size (Rietschel et al., 2005), thus samples with different sizes may reflect very different disease biology. Given that a subset of UM cells disseminates early, genuine metastatic and innate drug resistant subpopulations might have already left the primary site at the time of analysis, preventing their detection in large lesions compared to the smaller ones. To circumvent this limitation and capture the entire genetic, genomic and phenotypic heterogeneity, single-cell RNA sequencing of specimens from small lesions, that are growing at a comparable latency after diagnosis, should also be carried out. In the meantime, parallel sequencing of DNA and RNA from the same single cell will directly link genomic variation in a population of cells with transcriptional variation. In the same vein, epigenetics is thought to play a critical role in UM progression since BAP1, which acts as a deubiquitinase of histone H2A, is lost during UM progression. One conundrum is whether cell subpopulations with different epigenetic statuses have a tendency to acquire particular aberrations, which needs to be determined.

Simultaneous profiling of gene expression and chromatin accessibility (ATAC-seq) in single cells (Reyes et al., 2019), will enable high-resolution cell state classification and more accurate mechanistic conclusions. Interestingly, single-cell triple OMICS sequencing that reveals the genetic, DNA methylation, and transcriptomic heterogeneity of an individual cell has been developed (Hou et al., 2016). Likewise, the integration of RNA-seq with T-cell receptor (TCR)-seq, or DNA-seq and RNA-seq
with TCR-seq was performed in UM (Durante et al., 2020; Karlsson et al., 2020), and allowed to suggest a role of heterogeneity in immune evasion (Figure 3).

Cell phenotypic diversity can also occur due to complex gene rearrangement and alternative RNA splicing events (Wang et al., 2008). To date, there are only two reports using short-read RNA-sequencing technologies to measure differential mRNA expression in single cells in UM (Durante et al., 2020; Pandiani et al., 2021). This approach generates short reads from one end of a cDNA template at high sequencing depth in a large number of UM cells (>1,000 cells), but prevents the reconstruction of highly diverse sequences (Ziegenhain et al., 2017), thereby impacting the detection of full cell phenotypic diversity. To decipher the critical alternative splicing of mRNA in UM the above approach can be coupled to a full-length single-cell RNA sequencing (~100 cells) method that, though at lower sequencing depths, can evaluate the entire sequence of gene transcripts and circumvent the limitation of short-reads scRNA-seq (Byrne et al., 2017).

Single-cell sequencing studies open up a large number of cell markers for testing. The clinical use of anti-LAG3 and anti-TIGIT warrants investigation. In support of this idea, a phase 2 study of relatlimab, an antibody that inhibits LAG-3, for the treatment of patients with solid tumours, including locally advanced UM of stage IIIB, IIIC, and metastatic IV, is ongoing (NCT02519322). An anti-TIGIT antibody (MTIG7192A) is also part of a clinical trial in locally advanced or metastatic tumours in combination with atezolizumab (an anti-PD-L1). However, these therapeutic strategies need to be considered with caution given that in the case of UM, the immune infiltrate of primary lesions is associated with a poor prognosis (De Cruz et al., 1990).
Crucially, it remains to be determined whether the cell states identified in primary lesions are maintained in the subsequent metastases. Indeed, UMs continue to genetically evolve as they progress from primary to metastatic disease, as illustrated by metastases having additional oncogenic mutations and distinct genomic alterations compared to primary tumours (Shain et al., 2019).

To date, various animal models have been developed, yet none fully mimics human UM disease (Richards et al., 2020). Patient-derived xenografts (PDX) models in which fresh tumours are implanted can allow the tracking of tumour heterogeneity, evolutionary dynamics and treatment-resistance (Amirouchene-Angelozzi et al., 2016). For instance, the CellTagging strategy can be used to longitudinally trace UM cell history from the primary lesions to the hepatic metastasis. This will help discriminate which cell subgroups and cognate markers, under the influence of the TME, including drug cytotoxic effects or immunotherapies, are dominant at each time point and provide insight into valuable therapeutic targets. However, PDX models suffer from the lack of a human microenvironment and immune system. Efforts to create new human-relevant, immune-competent pre-clinical models, that recapitulate human UM disease are thus vitally important for elucidating the cross-talk between ITH and the TME and gaining insight into how this dialogue influences cell state dynamics and tumour progression, dissemination and therapeutic resistance. Today it is indeed possible to immune-humanize PDX models (Jespersen et al., 2017; Somasundaram et al., 2021).

Collectively, these strategies may allow a better understanding of the dynamic evolution of the tumour and its ecosystem which in turn, will better reflect its functional heterogeneity, allowing us to identify the most harmful cells and relevant therapeutic opportunities.
9.2. ITH from the perspective of clinical relevance

No cure is available for metastatic UM, despite new insights into the molecular mechanisms of disease development and progression (Seth et al., 2020). In various tumours, several lines of evidence have shown that ITH fosters tumour evolution and therapeutic resistance. Taking ITH into account can allow the tracking of the evolutionary trajectory of cancers and the construction of comprehensive systems for the development of rational therapeutic options to improve patient outcomes. The dissection of ITH and the driving molecular forces represent a challenge for realizing the potential of precision medicine in cancers, including in UMs. Although the single-cell technology is expensive, large-collection-based studies and standardized methods of analysis are needed to generate accurate molecular data and to value the interest of investigating ITH in routine clinical practice. Only a few UM cases of primaries (n=14) and hepatic metastases (n=3) were analysed with this approach so far (Durante et al., 2020; Pandiani et al., 2021).

Analysis of circulating tumour cells (CTCs) and cell-free DNA (cfDNA) has also emerged as an interesting approach to gain insight into ITH and its contribution to therapeutic resistance and cancer recurrence. Genome-wide single-cell RNA-seq and DNA-seq performed in circulating tumour cells (CTCs) offered information on tumour heterogeneity (Keller and Pantel, 2019). Furthermore, in hepatocellular carcinoma, cell-free DNA-seq can reflect ITH (Cai et al., 2017; Huang et al., 2016). Thus, there is a rationale for assessing UM ITH with cell-free DNA-seq in liquid biopsies, which in addition is a minimally invasive approach (Martel et al., 2020). However, the results from blood need to be interpreted with caution because CTCs
or cfDNA can come from different and heterogeneous tumour sites and may not reflect ITH.

While our manuscript was under review, a manuscript has been published about the use of single cell technology in UMs, which also addresses future perspectives (Wang et al., 2021).

10. Conclusion

ITH appears to be an unavoidable problem in solid cancer. Clearly, this research field is still in its infancy, especially in UM and faces immense challenges including the translation to clinical practice and the impact on rational therapeutic design. Single-cell analyses allow us to identify the different cell types that make up a tumour and to examine how individual tumour cells interact with each other and with cells composing the TME. These analyses can also depict the dynamic changes that occur during the natural history of tumour development or in response to external stimuli such as therapeutic challenges.

Understanding the biology of the cell states that co-exist within a tumour and their cognate markers will be critical in the future to predict the evolutionary trajectory of UM. We anticipate that the information gained will change the treatment paradigm of UMs, either by identifying new druggable frailties or by disclosing means to shift innate resistant cell states towards drug-sensitive states with the ultimate goal of curing patients with UM.

Declaration of competing interest

None.

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Figure Legends

Figure 1: Scheme of UM classification. The American Joint Committee on Cancer (AJCC) 8th edition defined by the basal diameter and tumour thickness and refined by the anatomical extent of the tumour based on ciliary body and/or extrascleral involvement is used for UM classification. AJCC staging serves as a universal standard for classification, therapeutic decision making, and the prediction of prognosis. Other criteria indicated in the figure can be used to complement the AJCC staging for prognostic prediction in UM. Existing (blue) and new (red) prognostic criteria identified from recent single-cells RNA-seq analyses are indicated.

Figure 2: Prognostic value of BAP1 mutations in UM patients. (A) Kaplan-Meier curves showing disease-specific survival (DSS) stratified by BAP1 mutations and chromosome 3 status (TCGA dataset). (B) Kaplan-Meier curves showing relapse-free survival (RFS) stratified by BAP1 mutations and chromosome 3 status obtained...
from whole genome sequencing datasets (Johansson et al., 2020). The numbers below the figures denote the number of patients "at risk" in each group.

**Figure 3: Schematic overview of single-cell transcriptomic analysis.**

Single-cell analyses and methods applied to compare OMICs data of thousands of individual cells and highlighting potential markers and therapeutic targets which are next used for functional validation. Principal component analysis is a dimensionality-reduction technique for graphical description of the information present in large datasets. Seurat is used to identify sources of heterogeneity (cancer, stroma and immune cells) within single-cell RNA-seq dataset and to plot cell clusters. InferCNV is used to explore copy number variation (CNV) from single cell RNA-seq datasets. SCENIC enables to infer gene regulatory networks and cell types from single-cell RNA-seq data. Monocle enables single-cell trajectory inference of individual cells according to progress through a biological process. ATAC-seq is used to study chromatin dynamics. TCR-seq allows to dissect the complexity of the T cell repertoire that directly reflects the diversity of immune responses. Immune profiling is a measure of the immune contexture of the tumors. Comprehensive integration of single cell data might allow to identify new biomarkers and targets for clinical applications.

**Figure 4: SATB1 and CTCF are associated with patient survival.** (A) Disease-specific survival (DSS) curves for mRNA expression levels of (A) SATB1 or (B) CTCF (median) were plotted using the Kaplan-Meier method. The numbers below the figures denote the number of patients "at risk" in each group.
Figure 5: Validation of UM prognostic signatures. Receiver operating characteristic (ROC) curves show the sensitivity and specificity of three-different mRNA signatures, GEP (Onken et al., 2004), PGS (Li et al., 2018) and PC1 (Pandiani et al., 2021), for predicting the patient overall survival. Chromosome 3 is shown as a reference.

Figure 6: Single-cell RNA-seq uncovers poor prognosis cell subpopulations. The histograms show the percentage of cells with a high (red) and low (blue) PC1 score, which are potentially at high and low metastatic risk respectively. Above the histograms are indicated the percentage of “at risk” cells per tumors.

Figure 7: HES6 expression overlaps with class 1 PRAME +, class 2 cells and PC1-positive cells. (A) Seurat analysis showing t-SNE plots of 7,890 UM cells coloured by the expression of HES6 (Pandiani et al., 2021). (B) Seurat analysis showing t-SNE plots of 7,890 UM cells coloured by class 1 PRAME -, class 1 PRAME + and class 2 based on the 12-gene expression signature (GEP) with or without PRAME expression (Field et al., 2016; Onken et al., 2004). (C) t-SNE shows cells coloured by PC1 scores.

Figure 8: Expression of HES6 according to UM cell morphology. HES6 expression has been compared in tumours (TCGA UM cohort) with more than 70% (n=20) versus less than 30% (n=52) of epithelioid cells.

Figure 9: Role of the NOTCH signalling pathway in UM cell progression. Expression of NOTCH pathway-related genes and their association with metastasis
(TCGA dataset). The y-axis shows the log(2)-expression in patients who did not
develop metastasis after a 2-year follow-up versus those who developed metastasis
before 18 months since UM diagnosis (Mets: No/Yes). Values indicated at the top of
the figure correspond to log(2)-fold changes and p-values of non-metastatic versus
metastatic patients. DLL4 expression is the most strongly associated with metastasis
formation.

**Figure 10: Expression of BAP1 and HES6 inversely correlates.** Pearson
correlation between *BAP1* and *HES6* (TCGA UM dataset).

**Figure 11: Correlation of HES6 with immune checkpoint proteins.** (A) The y-axis
indicates the log(2)-expression of immune checkpoint proteins in HES6-low (L) and -
high (H) UM tumours (TCGA UM). PTPRC is used as a marker of immune cell
infiltrate. Values indicated at the top of the figure correspond to log(2)-fold changes
and p-values in the two groups. (B) CIBERSORT analysis, which estimates cell
types using gene expression data, on the 25% tumours with the highest and lowest
HES6 expression (TCGA UM dataset). Shown are naïve B cells.
(Shields et al., 2017; Trolet et al., 2009)