



Physicochemical aspects of the tumour microenvironment as drivers of vasculogenic mimicry

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Abstract

Tumour vascularisation is vital for cancer sustainment representing not only the main source of nutrients and oxygen supply but also an escape route for single or clustered cancer cells that, once detached from the primary mass, enter the blood circulation and disseminate to distant organs. Among the mechanisms identified to contribute to tumour vascularisation, vasculogenic mimicry (VM) is gaining increasing interest in the scientific community representing an intriguing target for cancer treatment. VM indeed associates with highly aggressive tumour phenotypes and strongly impairs patient outcomes. Differently from vessels of healthy tissues, tumour vasculature is extremely heterogeneous and tortuous, impeding efficient chemotherapy delivery, and at the meantime hyperpermeable and thus extremely accessible to metastasising cancer cells. Moreover, tumour vessel disorganisation creates a self-reinforcing vicious circle fuelling cancer malignancy and progression. Because of the inefficient oxygen delivery and metabolic waste removal from tumour vessels, many cells within the tumour mass indeed experience hypoxia and acidosis, now considered hallmarks of cancer. Being strong inducers of vascularisation, therapy resistance, inflammation and metastasis, hypoxia and acidosis create a permissive microenvironment for cancer progression and dissemination. Along with these considerations, we decided to focus our attention on the relationship between hypoxia/acidosis and VM. Indeed, besides tumour angiogenesis, VM is strongly influenced by both hypoxia and acidosis, which could potentiate each other and fuel this vicious circle. Thus, targeting hypoxia and acidosis may represent a potential target to treat VM to impair tumour perfusion and cancer cell sustainment.

Keywords Vasculogenic mimicry · Tumour microenvironment · Hypoxia · Extracellular acidosis · Tumour progression

1 Introduction

In 1889, Stephen Paget reported the “*seed and soil theory*”: “*When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil*” [1]. Many years after Paget’s observation, and after important revisions, it became clear that cancer cells get through their progression towards malignancy not only due to the expression of relevant ‘driver’ genes but also as a consequence of epigenetic, transcriptional and posttranslational modifications elicited by the multiple interactions, which cancer cells establish in the so-called tumour microenvironment (TME). TME contains cancer cells and host

cells co-evolving dynamically through indirect and direct interactions. Indeed, cancer cells adapt themselves to the various conditions offered by TME and TME is modified according to the secreted products generated by cancer cells themselves. This is a real evolving, well-organised tissue with only one critical project: to assist the survival and proliferation of cancer cells. Indeed, the progression of cancer cells occurs in tissue and is not the result of changing of one individual cell; for this reason, TME represents an essential part of cancer. Thus, we need to consider TME and cancer cells as a single functional entity for elaborating new measures of prevention and therapy [2]. In 2011, Hanahan and Weinberg found it necessary to extend the initial hallmarks of cancer to new two characteristics identified as the genomic instability of cancer cells and inflammation of TME [3]. TME may exert multiple epigenetic effects on cancer cells, and this may happen following the several interactions among cancer cells and host inflammatory and immune cells or host-cell derived factors secreted into TME. These

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changes in cancer cell phenotype may be transient or associated with a more stable character. Along with this reprogramming, cancer cells under the TME influence might gain the ability to disseminate in distant organs and/or lymph nodes. The multifaceted cascade of metastatic spread of cancer cells consists of several steps including primary tumour cell expansion and invasion into the surrounding host tissues, the angiogenic drift that promotes intravasation into the blood vessels, the survival in the circulation and adhesion on the endothelial cells of a distant organ, extravasation and the final colonisation in the target site. In each step of the metastatic cascade, cancer cells need to adapt their abilities, such as detaching/re-attaching, invading using a mesenchymal or amoeboid style of migration, and adapting their metabolic attitude to survive in a quiescent state or proliferate. All these adaptive characteristics are influenced by host cells, such as endothelial cells, cancer-associated fibroblasts (CAF), immune cells including the various types of tumour-infiltrating lymphocytes, and tumour-associated macrophages (TAM). However, to deeply understand the complex interactions between TME and cancer cells, not only in primary tumours but also in metastatic sites, we also need to consider the local physicochemical gradient of O_2 tension (pO_2) and pH. Solid tumours are indeed usually hypoxic and acidic, and such physicochemical aspects of the TME are even sustained by the chaotic tumour perfusion. Tumour vasculature consists indeed of irregular and tortuous branching with a consequent establishment of a heterogeneous blood flow favouring a gradient of hypoxia and acidosis. This microenvironment resembles the characteristic of the stem cell niche.

In such hypoxic and acidic TME, cancer cells change their phenotype accordingly to survive and disseminate. They adapt themselves to the reduced pO_2 modifying their metabolism but also a series of biological properties which have a profound impact on cancer progression. Further, the Warburg effect (i.e. the aerobic glycolytic metabolism of proliferating tumour subpopulations), as well as the anaerobic metabolism acquired by hypoxic cancer cells, together with CO_2 hydration by the carbonic anhydrase IX promote the acidification of extracellular pH of solid tumours [4]. The dramatic reduction of pH is also sustained by the reduced lymphatic vessels of tumours [5]. Thus, in solid tumours, we observe a reverse gradient of pH, characterising, in contrast to normal tissue, by a higher intracellular pH and a much lower extracellular pH, ranging from 6.5 to 7.1 [6]. As for hypoxia, changes in intracellular as extracellular pH may influence the behaviour of cancer cells contributing to heterogeneity and plasticity re-adaptation of cancer cells towards malignant progression.

Due to the metabolic reprogramming of cancer cells along the gradient of pO_2 , in several regions of solid tumours, different levels of hypoxia and acidosis come together, even

though in some circumstances acidosis was detected in well-oxygenated areas as well. In fact, at a determinate level, hypoxia and acidosis have the ability to induce in cancer cells similar programs, such as genomic instability [7, 8], increased resistance to apoptosis [9, 10] and an epithelial-to-mesenchymal transition (EMT) [11, 12] as stem-like features [13, 14]. In comparison, the metabolic adaptation of cancer cells to hypoxia and acidosis is different, as hypoxic cancer cells undergo anaerobic glycolysis [15] while acidic cancer cells prefer a more respiratory trait, using substrates such as glutamine and free fatty acids [16].

What is particularly intriguing is the role of hypoxia and acidosis in tumour vascularisation. Tumour vasculature can arise from different mechanisms: (1) angiogenesis, consisting of the formation by endothelial cells (ECs) of new blood vessels from existing tissue-resident ones; (2) vasculogenesis, which instead involves EC precursors; (3) intussusception, defined as the vessels splitting through the insertion of tissue pillars; (4) vessel co-option, consisting of the tumour cell migration along the existing vasculature; (5) cancer stem cell (CSC) trans-differentiation to ECs, which in turn generate blood vessels; and (6) vasculogenic mimicry (VM), the phenomenon by which tumour cells mimic ECs and form vascular channels themselves [17–19]. Among them, VM is gaining increasing interest in the scientific community. Being a capacity of the tumour to provide by itself sufficient blood supply for its sustainment [20], VM refers to the ability of aggressive cancer cells to produce fluid-conducting vessel-like structures in an EC-independent way [21]. First discovered in uveal melanoma in 1999 [20], during the following 20 years, VM has been reported in several malignant tumours, including melanoma [22, 23], glioblastoma [24, 25], osteosarcoma [26, 27], hepatocellular carcinoma [28, 29], and breast [30, 31], lung [32, 33], gastric [19, 34], colorectal [35, 36] and prostate [37, 38] cancers. VM is associated with a high tumour grade, invasion, metastasis and poor prognosis in patients with malignant tumours [39–43], including breast [44], colorectal [36], prostate [45], hepatocellular [46], lung [45], ovarian [47], gastric [48] and bladder [49] cancers. To date, the precise mechanisms underlying VM formation are still under investigation; what is known is its correlation with ECM remodelling, certain tumour environmental conditions, and a dedifferentiated state proper of stem-like cells or cancer stem cells (CSCs). Indeed, the ability of VM tumour cells to alter their markers and behave like ECs allows considering them as aggressive tumour cells with high plasticity [50]. Thus, although VM and CSCs-to-ECs trans-differentiation have been proposed as separate ways to form tumour vessels, they could be seen as intertwined pathways towards the same goal. Considering the impact of hypoxia and acidosis on tumour perfusion, we know that hypoxia promotes tumour angiogenesis via HIF-1 α signalling, while acidosis induces a reversible

mechanism of EC-driven angiogenesis inhibition [51]. However, more importantly, both hypoxia and acidosis stimulate in cancer cells a VM programme [20]. To date, the relationship between VM and angiogenesis remains unclear; indeed, it was found that antiangiogenic therapy does not modify VM [52–54] and VM may sustain tumour growth also during anti-angiogenic therapy [55–57].

Overall, we believe that a reviewing work on the role of hypoxia/acidosis on VM is crucial to outline the dynamic structures of TME useful to establish new adjuvant therapy for tumours.

2 Hypoxia and acidosis in TME

Before reviewing the role of acidity and hypoxia on VM, it is useful to summarise the effects of these two biochemical aspects of TME on tumour initiation, development, invasion, metastasis and therapeutic response. Gillies and Gatenby suggest that cell adaptation to this acidic/hypoxic TME represents a key step in the transition from a benign tumour to a malignant carcinoma [58, 59]. The multi-step process of metastatic cascade encompasses local infiltration of cancer cells into the neighbouring tissues, intravasation, survival in the circulatory system, extravasation and subsequent colonisation in distant sites from the primary tumour. Neovascularisation is a fundamental step for tumour development, invasion and spread. Indeed, tumour growth requires the contemporary growth and new formation of a supporting vasculature network to maintain oxygen and nutrient supply to the tumour; moreover, invasive cells need to reside near blood or lymphatic vessels to access the blood circulation or the lymphatic system [60]. Hypoxia, through the induction of HIF-1 α , is involved in all steps of blood vessel formation [61]: it contributes to the recruitment of endothelial progenitor cells from the bone marrow and their differentiation into endothelial cells (ECs) by the regulation of the vascular endothelial growth factor (VEGF) and the stimulation of other pro-angiogenic agents such as the VEGF-R2, the members of the FGF family and the platelet-derived growth factor (PDGF) [61]. HIF-1 α is also involved in the angiogenic process by the induction of matrix metalloproteinase (MMP) release favouring EC activation, sprouting from pre-existing vessels and proliferation towards a hypoxic area [62, 63]. Finally, hypoxia and HIF-1 α support the creation of mature and stable blood vessels by inducing Ang-1, PDGF and TGF- β to recruit supporting cells such as pericytes and smooth muscle cells [64]. Recent evidence indicates that the HIF-1 α -mediated hypoxia response pathway plays a crucial role also in lymphangiogenesis, upregulating the VEGFR-3 ligands VEGF-C and VEGF-D [65].

While the effect of hypoxia on tumour angiogenesis is well known, the interplay between acidosis and angiogenesis

is controversial and the effect of low pH on pro-angiogenic factors, such as VEGF-A expression, remains unclear. Shi et al. reported that the incubation of tumour cells at different pH for different times reveals a correlation between low pH and stimulation of VEGF levels [66], while, on the contrary, Scott et al. observed an inverse regulation between acidity and VEGF mRNA expression in breast cancer cells [67]. We contributed to the understanding of the role of extracellular acidosis on lymphangiogenesis, demonstrating that exposure of melanoma cells to low pH (6.7 ± 0.1) promoted the NF- κ B-mediated induction of lymphangiogenic growth factor C (VEGF-C) [68], known to be expressed by several aggressive human melanoma cell lines *in vitro* [69]. These data suggest that an acidic microenvironment can influence lymph node metastasis probably by inducing functional changes in lymphatic endothelial cells. In line with this hypothesis, Nakanishi et al. [70] found that extracellular acidosis induces the expression of lymphangiogenic factors, especially IL-8, together with the proliferation of lymphatic endothelial cells. Concerning the effects of extracellular acidosis directly on endothelial cells, we have recently demonstrated that acidosis per se and lactic acidosis are sufficient conditions to dramatically impair *in vitro* EC capillary network/lumen formation and invasion without altering cell viability [51]. In line with our observations, Faes and colleagues observed a reduced EC proliferation and migration in response to VEGF upon acidic exposure [71]. The EC functions' impairment that we observed under extracellular acidosis was, however, restored following the re-establishment of standard pH conditions, in accordance with Mena and colleagues showing a potentiated angiogenic activity of human endothelial colony-forming cells that have undergone acidic preconditioning and then were re-exposed to standard pH [72, 73].

After the formation of new vessels, tumour cells will get in contact with venules, capillaries or lymphatics and can enter the circulation. To intravasate, tumour cells slow down proliferation, activate anti-apoptotic mechanisms, alter cellular phenotype from epithelial to mesenchymal, reduce cell–cell adhesion and degrade extracellular matrix components, all changes that tumour cells undergo during the EMT process [74, 75]. Several studies have shown the effect of hypoxia and acidic pH in inducing EMT in tumour cells, selecting cells with high plasticity, enhanced ability to invade [11, 12] and greater survival, by suppressing apoptosis [9, 10], promoting autophagy [76, 77] and avoiding anoikis cell death when cells are detached from extracellular matrix [78, 79].

There is a strong linkage between hypoxia, acidity and stemness. Low O₂ concentration has been closely associated with the maintenance of a stemness trait in tumour cells residing in specific stem cell niches [13]. Several studies have demonstrated that acute hypoxia selects for

cancer cells with stem cell characteristics, enhancing stem-like cell marker expression, such as OCT4, SOX2, NANOG and MYC, through upregulation of the HIF-1 α pathway [80, 81]. Moreover, increased expression of HIF-1 α and HIF-2 α has been found in stem cell-like populations of neuroblastomas [82] and gliomas [83]. Recently, we contributed to the understanding of the role of an acidic TME in cancer cell stemness demonstrating that exposure to low extracellular pH induces the expression of pluripotency markers such as Nanog, KLF4, OCT4 and Sox2 and the over-expression of stemness markers such as CD133, CD243 and ALDH1A1 in melanoma cells [14]. The correlation between acidity and stemness was also verified in prostate cancer [84], malignant glioma [85, 86] and osteosarcoma [87], and it is sustained by NF- κ B activation [14, 87, 88].

Tumour hypoxia and extracellular acidosis exert on cancer cells a selective pressure able to lead to the propagation of subpopulations characterised not only by an aggressive phenotype, but also by the ability to resist apoptotic stimuli, for example, after pharmacological treatment. In 1953, Gray et al. [89] demonstrated that hypoxia can confer resistance to radiotherapy. Moreover, several studies show the effect of low oxygen in increasing resistance to chemotherapy leading to tumour recurrence and ultimately limiting patients' prognosis [90–92]. Also, acidity is a contributing factor in the resistance of tumours to therapy. Thews et al. found an enhancement of pGP activity in acidic prostate carcinoma cells caused by an increase of intracellular Ca²⁺ levels and related to ERK and p38 pathway [93]. Several other mechanisms of low pH-induced drug resistance have been proposed; one of these has a chemical nature and it is called 'ion trapping', based on the ion protonation of drugs [94]. It has been widely demonstrated that low pH reduces uptake and efficacy of weak base chemotherapeutics such as anthracyclines, anthraquinones and vinca alkaloids [95, 96], thus alkalinisation could be useful to restore cell drug sensitivity. Autophagy, a self-digestive process that cells perform in response to nutrient stress, is now considered a mechanism of drug resistance in cancer; indeed, it has been found to maintain cancer stem cell phenotype and correlates with chemo- and radio-resistance [89, 97–100]. It was demonstrated that hypoxia can induce autophagy in different cellular settings [76, 101], acting as a survival mechanism for hypoxic cells through the recycling of cellular components. Wojtkowiak et al. proved that autophagy is induced also in response to acute and chronic acidity as a cellular adaptation to survival and that an autophagy inhibitor, 3-methyladenine, could be used to affect acidic cells [77], suggesting that autophagy could represent a valid therapeutic target. Also, other studies demonstrated that proton pump inhibitors which inhibit extracellular acidification, such as pantoprazole, lansoprazole and omeprazole, or buffering experiments

using sodium bicarbonate, can suppress the later stages of autophagy [102, 103].

Both hypoxia and acidity-driven adaptive mechanisms allow tumour cells to continue to survive in the hypoxic/acidic TME while also creating an inhospitable environment for immune cells and damaging key regulatory pathways [104, 105], inducing immune suppression and contributing to a reduced anti-tumour response. Immune surveillance represents the first line of defence against cancer, but often cancer cells manage to escape it. Indeed, upon exposure to acidosis or hypoxia, tumour cells release a large variety of immunosuppressive molecules, such as IL10, TGF β and VEGF, that act on the immune system in three main ways: inhibiting immune cell proliferation and survival [106, 107], affecting immune cell function [106, 108] and regulating the signalling of downstream processes, such as PDL-1 overexpression [109, 110]. An important reason for immune dysfunction in the acidic condition is that the elevated glycolytic activity of tumour cells reduces glucose availability and activated T cells do not survive without glucose, differently from tumour cells that enter quiescence [111]; especially CD8⁺ T cytolytic activity results significantly reduced by acidification [112]. Also, hypoxia was shown to decrease T-cell survival [113] and proliferation [114]; Lukashev et al. [115] demonstrated that CD4⁺ and CD8⁺ T cells derived from HIF-1 α -deficient mice show increased proliferation, can secrete higher levels of interferon- γ and exhibit enhanced antitumour responses. Moreover, hypoxia has been shown to promote regulatory T cell (Treg) induction [116]. Acidosis and hypoxia not only decrease T-cell functionality, but they also impair dendritic cell maturation [117, 118], lymphokine-activated killer cells [119, 120] and natural killer (NK) cells [121, 122]. Colegio et al. [123] and Ke et al. [124] found respectively that low pH and hypoxia are responsible for TAM polarisation to M2 and the induction of VEGF production. Similarly, other authors found a correlation between the acidic/hypoxic microenvironment and the release of pro-angiogenic mediators by macrophages [125, 126]. Recent studies demonstrated *in vivo* that neutralisation of tumour acidity using oral buffers [127] or treatment with proton pump inhibitors [128] respectively ameliorates T-cell activity and TAM activation in the tumour microenvironment.

Accumulating evidence indicates that metastasising cells undergo dynamic metabolic changes, a necessary step contributing to their plasticity, useful to successfully survive the microenvironmental changes during the metastatic cascade [129]. Under hypoxic conditions, HIF-1 α triggers the expression of genes involved in the metabolic shift towards anaerobic glycolysis, such as pyruvate dehydrogenase kinase 1 (PDK1) to decrease mitochondrial oxygen consumption [130], hexokinase 2 (HK2), lactate dehydrogenase A (LDHA), pyruvate kinase isoform M2 (PKM2) and enolase 1

(ENO1), and promotes the expression of glucose transporters (GLUT1 and GLUT3) [15, 131]. In addition, hypoxic cancer cells activate the glycogen synthesis pathway to reserve glucose. Another enzyme involved in glycolysis, PFKFB4 (6-phosphofructo2-kinase/fructose-2,6-biphosphatase 4), was demonstrated to be upregulated following hypoxia exposure, and its high expression correlates with poor prognosis [132]. Thus, the HIF-1 α pathway increases glucose uptake and secretion of lactate, which accumulates in hypoxic cells together with H⁺. These products are exported via monocarboxylate transporter 4 (MCT-4), carbonic anhydrase (CA9) and Na⁺/H⁺ exchanger (NHE-1) which are all under HIF-1 α control [133]. Hypoxia, through HIF-1 α , regulates also glutamine metabolism; it causes reduced glucose-derived citrate by decreasing PDH activity and an increase in aKG levels induced by the reduction of aKGDH activity [134]. In contrast to hypoxia, which induces glucose uptake and metabolism, tumour acidosis leads to a dramatic reduction in the use of glucose by tumour cells [16], shifting the metabolism to oxidative phosphorylation (Oxphos) with the decrease of glycolic markers, such as GLUT1, GLUT3, HK2, G6PD, PKM2 and LDHA, and the increase of CytC and PGC1 α . We demonstrated that this metabolism can be reverted using metformin, a biguanide commonly used for type 2 diabetes with a mitochondrial antagonist activity [135], and that Oxphos is sustained by the expression of SOX-2 increased by acidic conditioning [136]. The same metabolic behaviour in acidic conditions was found in breast carcinoma cells [16] and malignant neuroblastoma cells [137]. Oxidative metabolism allows cells to get more bioenergetic molecules instead of biomass for cell division [16, 135], and the main energetic source of acidic pH-adapted cancer cells derives from fatty acid oxidation [138]. Corbet et al. [138] demonstrated

that under chronic acidosis, acetyl-CoA generation derives from fatty acids and glutamine, instead of glucose. Moreover, several studies have identified HIF2 α as a key driver of metabolic adaptation to tumour acidosis [85, 139], while the synthesis and activity of HIF-1 α were generally found reduced [139, 140].

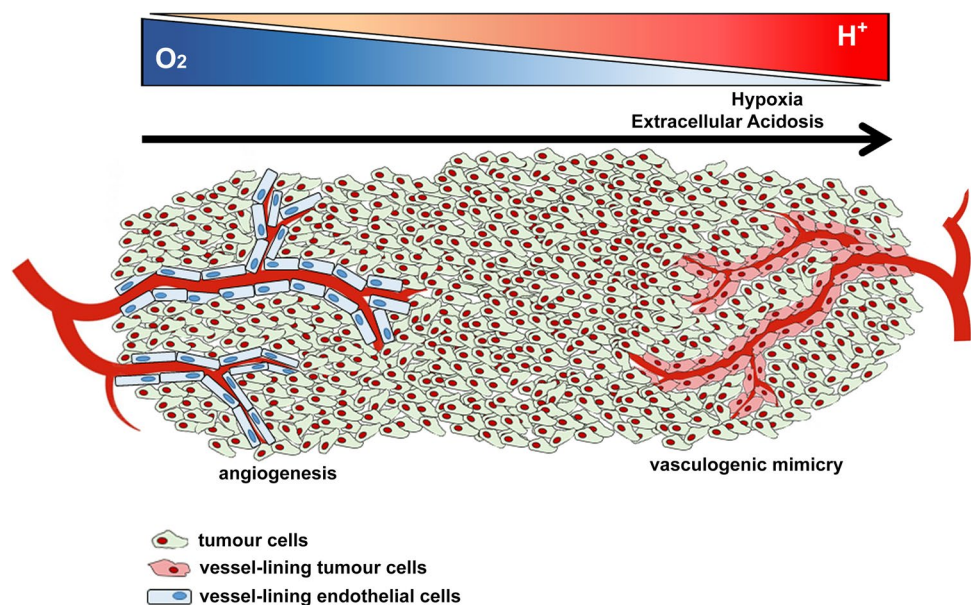
3 Hypoxia and acidosis in VM promotion

VM is finely regulated by hypoxia and acidosis of the TME (Fig. 1). Before going deeper into the mechanisms through which hypoxia and acidosis induce VM, let us briefly analyse the principal actors in this phenomenon.

3.1 VM regulating pathways

Two of the first proteins identified as central players in VM are VE-cadherin, a cell–cell adhesion molecule expressed by ECs allowing adherent junction formation [141], and EphA2, an epithelial cell receptor tyrosine kinase involved in EphA1-induced angiogenesis and found upregulated in a wide variety of cancers, whose expression has been linked to increased malignancy and a poor clinical prognosis [142]. Several studies appositely designed to disclose the contribution of these two proteins in VM allow understanding that they act in a coordinated way since VE-cadherin regulates the EphA2 localisation at the intercellular junctions between VM-forming tumour cells as well as its phosphorylation level [42, 143]. The signalling triggered by the kinase EphA2 leads to FAK-mediated PI3K and ERK1/2 pathway activation, which besides being associated with survival, proliferation and migration are also important in

Fig. 1 Hypoxia and extracellular acidosis of the tumour microenvironment induce vasculogenic mimicry. As the tumour mass grows, the support of oxygen provided by angiogenic vessels (left) decreases, and, at the meantime, H⁺ accumulate generating a hypoxic and acidic tumour microenvironment. Such a condition induces cancer cell vasculogenic mimicry (right) to support tumour survival and disease progression



the VM process; in fact, PI3K regulates extracellular matrix remodelling, crucial for VM-forming tumour cell migration, by inducing the activation of MMP-2 (mediated by MMP-14) and MMP-9. On the other hand, ERK1/2 signalling has been found to upregulate urokinase and MMP-2 activity and thus stimulates the invasion of VM-forming tumour cells [42, 144, 145]. VE-cadherin expression is also regulated by Twist-1, at least in hepatocellular carcinoma, astrocytoma and gastric cancer [146–148].

A discussed player of VM is the VEGF-A. Considered the main pro-angiogenic factor—for stimulating EC proliferation, survival and angiogenesis—there is evidence supporting its involvement in VM promotion, at least in melanoma and ovarian cancer [149, 150]. Among the signalling pathways triggered by VEGF-A binding to VEGF-R1, the activation of the tyrosine kinases Src and ERK1/2 leads to the promotion of cancer cell invasion and migration, known to be crucial steps for VM. Also, in melanoma, PI3K activation triggered by VEGF-A/VEGF-R1 binding contributes to VM by stimulating MMP-14-mediated MMP-2 activation and subsequent tumour cell migration and tube formation [42]. In ovarian cancer, VEGF-A/VEGF-R1 signalling increases the expression level of VM-specialised genes such as VE-cadherin, EphA2 and matrix-metalloproteinases MMP-2 and MMP-9 [151]. On the contrary, an opposite point of view has been proposed as well, based on the observation that VM increases in the absence of VEGF-A signalling: VEGF-A blocking strategies increase VM together with other alternative survival ways exploited by aggressive tumour cells [22]. Rather than VEGF-A blockage/absence itself, this could occur because upon VEGF-A inhibition and subsequent VEGF-A-driven angiogenesis impairment, the tumour microenvironment where cancer cells grow likely becomes increasingly hypoxic/acidic, conditions that stimulate the VM-driven tumour vascularisation. Focusing on the VEGF-R1, it has been demonstrated that the signalling it triggers is required for VM of melanoma cells to express VE-cadherin upon VEGF-A stimuli [149]. Even Cyclooxygenase-2 (COX-2) has been proven to induce VM by stimulating the VEGF-A expression [42, 152, 153]. COX-2, the enzyme that converts arachidonic acid into prostaglandin E2 (PGE2), upon binding prostanoid receptors (EP1–4), activates PKC signalling that in turn stimulates VEGF-A expression and subsequent tumour-derived vascular channel formation in glioblastoma, and lung and breast cancer [33]. Furthermore, the COX-2 product PGE2 signalling activates the EGF receptor (EGFR) with PKC-dependent ERK1/2 activation. Consequently to this signalling pathway, tumour cell proliferation, invasion, angiogenesis and VM are promoted [154]. The capacity of COX-2 to stimulate VM is in line with the observation that the inflamed tumour microenvironment and infiltration by the tumour-associated macrophages (TAMs) enhance VM-vessel network formation

[155, 156]. Keeping the focus on the VEGF family and VM, in melanoma, it has been observed that endothelin-1 (ET-1) increases the expression levels of VEGF-R3 together with its ligands VEGF-C and -D triggering VM signalling [157].

Going further, the players of VM, Tissue Factor (TF) and TF pathway inhibitors 1 and 2 (TFPI-1/2) have been found to be crucial for tumour cell-lined tube formation [42]. These proteins are finely involved in the coagulation pathway. In particular, the TF represents the cell surface receptor that initiates the coagulative cascade: upon binding the VIIa cofactor, the TF activates factor X, triggering haemostasis through downstream thrombin generation and subsequent fibrin formation and platelet activation. The TF is inhibited by the TFPI-1, which, by simultaneously binding the VIIa and Xa factors, locks TF into an inactive TF-VIIa-Xa-TFPI-1 complex. On the contrary, the TFPI-2, originally identified by TFPI-1 homology as placental trypsin and serine protease inhibitor associated with tumour cell ECM [158–160], does not physiologically exert any inhibitory activity on the TF pathway [161]. TFPI-2 is instead a potent inhibitor of plasmin and MMPs that can regulate the adhesion and migration of endothelial and tumour cells in a context-dependent manner [161]. TF, TFPI-1 and TFPI-2 are overexpressed by tumour cells, and experimental evidence highlights their role in VM. TFPI-1 was observed to serve as the predominant regulator of TF activity in aggressive melanoma cells (behaving thus as endothelial cells), and its anticoagulant activity could account for the fluid-conducting capacity of VM tumour cell-lined vessels. On the other hand, studies conducted with anti-TFPI-2 antibodies or with recombinant TFPI-2 highlight the crucial role of the TFPI-2 in the VM-vessel formation, linked with its complex MMP-2 activating function [161].

The Notch family has been found important in the VM process. It comprises four isoforms of transmembrane receptors (Notch1–4) and five possible membrane-bound ligands, Delta-like 1/3/4 and Jagged 1/2. Notch signalling is crucial for embryonic pluripotency and development. Briefly, the Notch intracellular domain (NICD) is released into the cytoplasm and localised in the nucleus after the Notch receptor is sequentially degraded. The role of Notch signalling in VM has been reported in melanoma, gastric cancer and hepatocellular carcinoma [39, 162–164]. In particular, Notch4 was found to induce VM in melanoma cells in a Nodal-dependent way since the treatment with recombinant human Nodal was able to rescue VM ability impaired by Notch4 inhibition [165].

Also, the Wnt/ β -catenin signalling pathway is involved in VM formation. It has been reported that Wnt/ β -catenin signalling disruption decreases VM in colon cancer, triple-negative breast cancer, melanoma and osteosarcoma [166–170].

The mammalian target of rapamycin (mTOR) represents a crucial player in VM. As we recently reported, mTOR

targeting by everolimus inhibits EphA2 and VE-cadherin expression and strongly impairs the ability of melanoma cells to perform VM [145]. Also, in glioblastoma, mTOR-specific inhibitor rapamycin and mTOR silencing were found able to completely disrupt the VM formation under both hypoxic and normoxic conditions [171].

Another important inducer of VM is the EMT. EMT is characterised by the loss of epithelial traits and the concomitant gain of mesenchymal phenotypes. It is accompanied by the expression of the transcription factors Twist, Snail, ZEB1 and ZEB2 which, upon binding to the E-cadherin promoter, mediate its inhibition in favour of an increased tumour cell invasion ability. Much evidence highlights that EMT plays a crucial role in VM, and EMT-related transcription factors are involved in the VM process. Indeed, as reviewed by Liu and colleagues, along with controlling the E-cadherin/N-cadherin and vimentin levels, Twist, Zeb1, Snail and Slug also modulate VE-cadherin and VEGF-R2 expression, activate MMP-2 and MMP-9, as well as guarantee stemness maintenance in VM-positive cancer cells [50].

3.2 VM regulation by hypoxia and acidosis of the TME

3.2.1 Hypoxia

Strong experimental evidence has demonstrated the major role of hypoxia in VM promotion. Under low oxygen conditions, HIF- α protein is stabilised and translocates into the nucleus where it binds the gene regulatory regions containing the hypoxia response elements (HREs) and subsequently activates the transcription of hypoxia-target genes. Hypoxia is a master regulator of several HIF- α -mediated signalling pathways inducing VM development in solid tumours [17, 42]. As reviewed by Seftor and colleagues, several genes involved in VM are directly (through the HIF- α binding to the HREs) or indirectly (via the involvement of an intermediate regulating protein or post-translational modifications) modulated by hypoxia. Among them, hypoxia directly modulates VEGF-A, VEGF-R1, EphA2, Twist and COX-2, and indirectly, VE-cadherin, TF and Notch [143]. Following the knockdown of HIF-1 α , VE-cadherin, EphA2 and laminin-5 γ 2 are inhibited in oesophageal cancer [172]. Hypoxia can modulate the expression of Notch-responsive genes—such as Nodal—via HIF-1 α -mediated NICD stabilisation. This non-canonical crosstalk between HIF-1 α and Notch may serve to promote tumour cellular undifferentiation and plasticity characterising VM-forming cells. Hypoxia also promotes VM via reactive oxygen species (ROS) generation. Indeed, in melanoma, VM is induced upon redox-dependent stabilisation of HIF-1 α [173]. These studies demonstrating the hypoxia-induced VM and VM-associated genes also highlight the critical role exerted by

hypoxia in tumour migration since it also activates MMP-2 and MMP-9, thus promoting tumour cell invasion to adjacent tissue [174]. In melanoma, it was observed that hypoxia stimulates the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) which in turn increases VE-cadherin expression [175]. VE-cadherin expression, whose modulation under hypoxia can be dependent on both the isoforms 1 and 2 of HIF- α (HIF-1 α and HIF-2 α) [176], is also indirectly increased by hypoxia-induced BNIP3, a protein belonging to Bcl-2 family that contributes to VM by promoting cancer cell migration and cytoskeleton organisation during tube formation [175]. Bcl-2 and Bcl-XL proteins were also found to cooperate with hypoxia to induce MMP-2 expression in melanoma [177]. Moreover, HIF-1 α and HIF-2 α promoted *in vitro* VM tube formation by upregulating the VEGF-C and -D together with their receptor VEGF-R3 [172]. Hypoxia is also an inducer of the EMT and EMT-related transcription factors further enhancing VM as described above [178]. Upon hypoxic tumour microenvironment and subsequent HIF-1 α stabilisation, the urokinase-type plasminogen activator receptor (uPAR) was found stimulated as well [179]. It is especially known for its pro-angiogenic activity, but recent findings highlight its role even in VM [145, 180, 181], reinforcing uPAR's prognostic value in cancer progression and vascularisation [182].

3.2.2 Extracellular acidosis

Despite much evidence in the literature linking hypoxia to VM, very little has been reported to date about the contribution of the acidic tumour microenvironment in tumour-derived vessel network formation. We recently demonstrated that acid-adapted melanoma cells are endowed with a higher ability to organise themselves in a capillary-like network. The increased VM ability that we observed in acid-adapted melanoma cells is driven by uPAR and goes with an aggressive tumour phenotype endowed with drug resistance [145]. Interestingly, by studying the effects of the acidic tumour microenvironment on VM and angiogenesis, we observed that if, on one hand, extracellular acidosis severely impairs EC-derived vessel formation [51], on the other hand, it strongly promotes vascularisation from tumour cells themselves [145]. Considering the fast proliferative rate of cancer cells and their boosted glycolytic metabolism, we could imagine a rapidly growing tumour mass with an acidified extracellular milieu which confers several aggressive features including the ability to organise itself in tumour-derived vessels, accomplishing tumour mass vascularisation even in a context where angiogenesis is inhibited or just slowed down. VM likely represents an alternate/complementary route compared to angiogenesis exploited by aggressive tumours to guarantee perfusion and thus sufficient nutrients and oxygen supply needed for the tumour mass expansion.

VM can exist just for the period needed by ECs to perform angiogenesis and substitute VM-derived vessels. Indeed, as reported by Zhang and colleagues, VM seems to provide blood supply in the early stages of tumour expansion, while as the mass grows, tumour cells lining the VM vessel wall are little by little, but completely, replaced by ECs, creating a middle transitional phase where both tumour and ECs line the vessels in the so-called mosaic vessels [41]. Indirect evidence also supports the contribution of extracellular acidosis in VM. Indeed, acidosis in recent years has been defined as a new hallmark of cancer and demonstrated to endow tumour cells with aggressive tumour EMT phenotype characterised by a high plasticity, apoptosis resistance, immune surveillance evasion, increased resistance to radio-, chemo- and target therapies, enhanced migration/invasion and metastatic dissemination. So, VM becomes a part of this complex aggressive and plastic tumour phenotype selected by the acidic microenvironment and drastically able to pursue disease progression. Much evidence also links hypoxia and extracellular acidosis in VM promotion since crucial VM promoting pathways are shared by these environmental conditions: (1) EMT, initially, is indeed promoted by both hypoxic and acidic conditions [183]; (2) uPAR is over-expressed under hypoxia but also in the presence of low extracellular pH [145]; (3) mTOR is up-regulated under acidic conditions [184] and, in turn, mediates HIF-1 α expression [42]; (4) last but not least, both the hypoxic and acidic tumour microenvironments are important for selecting the CSCs and maintenance of stemness [14].

4 Final scenario and possible new strategies for treatment

The activation of a VM program is a paradigmatic example of how cancers deploy their devastating strategy. The emergence of VM is not only part of the natural history of cancer progression but also occurs wickedly as the consequence of anti-tumoural treatments. Accordingly, particular attention must be devoted to planning treatments that target VM.

As part of the natural history of cancer, VM is considered a marker of malignant progression; indeed, this discontinuous and leaky vessel network is strongly associated with aggressiveness and poor prognosis in different types of cancers. In this context, the discovery of novel VM biomarkers will facilitate the diagnosis and clinical evaluation of cancer patients during their treatments. The identification of novel markers will improve treatment monitoring for anticipating the emergence of drug resistance and, consequently, will help physicians in changing the therapeutic strategy when needed. Alongside classic biomarkers of VM such as the overexpression of VE-cadherin (CD144), the periodic acid-Schiff (PAS) positivity [185, 186] and the cell

surface glycoprotein CD133, novel biomarkers have been recently identified belonging to the PI3K/MMPs/Ln-5 γ 2 signalling pathway, to the EphA2/FAK/Paxillin signalling pathway and to the Sp1-dependent twist/VE-cadherin/AKT signalling pathway [56, 187, 188]. Importantly, the critical step in VM-marker future research should consider the discrepancy that exists between cancer patients and xenograft models. Indeed, VM, although undoubtedly detected in cancer patients, appears not so well developed as that developed and detected in xenograft tumours. Two interrelated factors contributed to this: the boosted tumour expansion in xenografts that still overwhelms murine angiogenesis, and, at the same time, a hypoxic and acidic microenvironment that strongly favours VM in xenografts [189].

In addition to the spontaneous appearance of VM during tumour progression and its role as a marker for histopathology, VM occurs in response to anti-tumoural treatments, sustaining the acquisition of a therapy-resistant phenotype in cancer cells [22, 190]. Among the different anti-tumoural treatments, anti-angiogenic treatment reveals the most dangerous and tricky features of VM. In this context, two different aspects must be mentioned. The first is that anti-angiogenic treatments are reported to be the cause of VM development [52]; the second, conversely, is that anti-angiogenic treatments might be very useful against the development of VM and cancer progression. Recently, methionine aminopeptidase-2 (MetAP2), which is involved in the angiogenic process, but also has a pivotal role in VM, became an interesting dual target. Indeed, the use of angiogenic inhibitors, such as fumagillin and TNP-470, interfering with MetAP2, significantly suppressed VM in various human cancer cell lines [191, 192]. Sunitinib, a potent anti-angiogenic agent, represents another interesting example of how tricky the use of anti-angiogenic treatments is. Sunitinib is used in the treatment of renal cell carcinoma (RCC). In RCC cells, the treatment with sunitinib promotes *in vivo* tumour resistance, which is reduced with everolimus in second-line treatment [53]. Interestingly, during sunitinib treatment of RCC, the emergence of VM is used as a known target to be exploited, revealing the increasing potential efficacy of sunitinib [193]. Keeping in line with this approach, additional anti-angiogenic treatments that revealed promising efficacy against VM should be mentioned. Preclinical investigations demonstrated that Arg-Gly-Asp integrin antagonists such as cilengitide inhibited angiogenesis and VM. An interesting lesson should be learned from cilengitide. When used as a single agent, this cyclic pentapeptide has shown minimal clinical efficacy in patients with metastatic melanoma. Conversely, if used in combination therapies, it demonstrated antiproliferative effects against melanoma and endothelial cells, both *in vitro* and *in vivo* [194]. Moreover, RGD functionalised nanoparticles

targeting $\alpha v\beta 3$ integrin expressing cells efficiently and simultaneously inhibit angiogenesis and VM [195, 196]. In this scenario, RGD-conjugated drugs might exert a versatile anti-angiogenic/anti-VM effect [197–199].

We explored the role of TME in VM emergence, and we have extensively mentioned how extracellular acidity and hypoxia play a crucial role in this process. Accordingly, therapeutic strategy devoted to inhibiting extracellular acidity, hypoxia and/or EMT signalling pathways revealed also their inhibitory effect on VM [200]. The knockdown of ZEB1, one of the major inducers of EMT, or the re-expression of ZEB1-repressed microRNA (miRNA) clusters, inhibits VM [201]. The knockdown of Neuropilin-1, which is up-regulated by a HIF-1 α -dependent mechanism, inhibited VM formation [202]. Also, the inhibition of the Wnt/beta-catenin pathway, which correlates with EMT, promotes cancer drug resistance and is strongly associated with VM, is used for VM treatment [203]. In view of this, the development of a selective inhibitor directed towards VM reveals as an extremely challenging mission. Conversely, to fight the emergence of VM, a preclinical investigation should consider a drug repurposing strategy. Drugs that are already developed and approved might reveal a novel application in this field [204].

Finally, there is a growing interest in the use of phytochemicals and naturally derived compounds for the inhibition of VM, in view of their wide spectrum of pharmacological activity [205]. It is well known that natural compounds affect multiple signalling pathways, revealing to be interesting multi-target drugs in different types of diseases. This is the case of curcumin, derived from the rhizome of *Curcuma longa*, and recognised as a bioactive compound responsible for numerous pharmacological activities, one of which is the suppression of VM in hepatocellular carcinoma [206]; of ginsenoside, extracted from ginseng, with anticancer activity against several types of cancers which inhibit VM through the downregulation of VE-cadherin/EphA2/MMP9/MMP2 axis [207]; of the *Celastrus orbiculatus* extract (COE), a mixture of 26 compounds isolated from the Chinese herb *Celastrus orbiculatus* vine, which has shown to have anti-cancer activity and inhibit tumour growth and VM downregulating EphA2 [28]; of niclosamide, which derives from salicylic acid, and has been used worldwide as anti-helminthic drug (for approximately 50 years) and inhibit VM through downregulation of the expression of VEGFA, MMP2, ROCK1 and Cdc42 [208]; and of triptonide, used in a wide variety of inflammatory and autoimmune disorders, isolated from the herb *Tripterygium wilfordii* Hook F, which inhibit VM-related gene expression (VE-cadherin and CXCL2) [209]. It is recognised that the naturally derived compounds are characterised by a wide spectrum of pharmacological activity; therefore, they could be exploited to target the multiple and pleiotropic mechanisms of activation of VM.

5 Conclusion

In this review, we focused our efforts on the description of the tight connection between TME and the emergence of VM, which is a clear sign of malignancy, and thus could be used as a prognostic factor in clinical assessments. The overall picture suggests considering VM as the tip of an iceberg. The expression of specific biomarkers helps to identify the presence of VM in a tumour lesion and thus understanding how severe the prognosis is, but the emergence of these signs, above sea level, depends on the extremely complicated network of signalling pathway interactions that take place under sea level. Thus, the huge future challenge would be not only to characterise the distinct pathways driving VM—which may vary from cancer to cancer, and from patient to patient—but also the opportunity to plan a therapeutic strategy that answers to the continuous dynamic adaptation of cancer cells to the effect of the used drugs.

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Declarations

Conflict of interest The authors declare no competing interests.

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