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MCAM and its Isoforms as Novel Targets in Angiogenesis Research and Therapy

Jimmy Stalin, Lucie Vivancos, Nathalie Bardin,
Françoise Dignat-George and Marcel Blot-Chabaud

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Abstract

Melanoma cell adhesion molecule (MCAM) (CD146) is a membrane glycoprotein of the mucin family. It is one of the numerous proteins composing the junction of the vascular endothelium, and it is expressed in other cell types such as cancer cells, smooth muscle cells, and pericytes. Some recent works were designed to highlight its structural features, its location in the endothelium, and its role in angiogenesis, vascular permeability, and monocyte transmigration, but also in the maintenance of endothelial junctions and tumor development. MCAM exists in different splice variants and is shedded from the vascular membrane by metalloproteases. Studies about MCAM spliced and cleaved variant on human angiogenic physiological and pathological models permit a better understanding on the roles initially described for this protein. Furthermore, this knowledge will help in the future to develop therapeutic and diagnostic tools targeting specifically the different MCAM variant. Recent advances in research on angiogenesis and in the implication of MCAM in this process are discussed in this chapter.

Keywords: angiogenesis, MCAM (CD146), melanoma, physiology, pathology

1. Introduction

Angiogenesis is the process of new blood vessel formation from preexisting vessels. It contributes to physiological processes such as development and wound healing, but also to pathological processes, such as tumor angiogenesis. The identification of new targets involved in angiogenesis remains an important challenge to fully understand the involved mechanisms and to generate new therapeutic tools. Recent studies have highlighted CD146, an endothelial junctional molecule, as a key factor in angiogenesis. This molecule that displays different

isoforms and that is present on different cell types could hence constitute a novel target for therapy. Different reviews have underlined its structural features, localization, and functions in the endothelium. This chapter thus mainly addresses the differences in CD146 isoforms with a special focus on their role in angiogenesis and the therapeutic tools targeting the molecule.

Historically, CD146 was discovered in 1987 by Professor J.P. Jonhson for the first time. It was identified as a marker of melanoma progression. These data were obtained by using an antibody generated by mouse immunization with a cell lysate of metastasizing melanoma. This antibody (MUC18) allowed the identification of a 113 kDa transmembrane protein. MCAM (melanoma cell adhesion molecule) described as a marker of metastasizing melanoma [1].

In 1991, the team of Professor. F. Dignat-George identified Sendo-1 antigen as a marker of circulating endothelial cells in the blood by flow cytometry. This was made possible through the generation of a mouse monoclonal antibody named Sendo -1 [2] obtained by mice immunization with a HUVEC cell lysate. Sendo-1 was able to stain the human endothelium whatever the vessel size and its anatomical location within the vascular tree [3, 4]. Gicerin and HEMCAM refer both to the avian homologues of the molecule [5].

As reported in Kobé in 1997, CD146 (cluster of differentiation 146) is now the official name grouping Sendo-1/MUC18/MCAM/gicerin/HEMCAM (Sendo-MUC18 preCD, Workshop Report).

2. Structure and characteristics of CD146

2.1. Genomic description

The specific location of the CD146 gene is on the arm q23.3 of the chromosome 11 in humans and on the chromosome 9 in mice (www.ensembl.org). The gene encoding the CD146 protein extends over 14 kb. It consists of five immunoglobulin-like domains, two variable domains, and three constant domains of C2 type, as well as a transmembrane domain and an intracytoplasmic portion [6]. The extracellular part of the molecule, including the five immunoglobulin domains, is encoded by 13 exons; the transmembrane domain and the intracellular domain are encoded by three exons.

The promoter of CD146 presents different putative binding sites and motifs including AP1, AP-2, CRE, SP1, CArG, and c-myb. Analysis of this DNA segments suggests that the four SP-1 sites, the two AP-2 domains, and one response element to AMPc-(CRE) form the minimal promoter of CD146 [7]. Specific sites play a role in CD146 expression. The AP-2 sites, which are located at -131 and -302 by relative to the initial ATG, inhibit the expression of CD146 by 70 and 44%, respectively. Moreover, when mutated, the CRE site inhibits by 70% the transcription of the genes. Therefore, AP-2 [8] and CRE sites [9] have been described to modulate CD146 expression in melanoma cells, leading to an increase in tumor growth and metastatic potential in these cancers. In fact, the AP-2 binding site located in the promoter (located at -23 bp) is an inhibitor of the transcription of CD146 while the other AP-2 sites (located at -131 and -302, respectively) are transcription activators [8].

The size of CD146 mRNA is around 3.3 kb and has been first identified in human melanoma cancer cells [10]. Its encoding region is about 1940 bp. A large homology in the mRNA sequences exists between human and mouse, but differences can be noted. Thus, in humans, there is a lengthening of the 3' and 5' UTR region as well as a loss of 6 pb in exon 2. The encoding regions and 5'UTR have a homology of about 80 and 72%, respectively, between the murine and human genes and there is only 31% of homology for the 3'UTR fragment. Finally, the protein sequence shares about 76% of homology between these two species [1, 10, 11].

2.2. Proteic structure and isoforms

The proteic structure of CD146 is composed of a signal peptide of 28 amino acids (AA), five immunoglobulin domains (including two variable domains and three constant domains), a hydrophobic transmembrane region (AA 561–585), and an intracellular region. The protein sequence derived from the coding region of CD146 has a theoretical molecular weight of about 72 kDa. However, CD146 has a molecular weight of about 113 kDa. This difference is due to the glycosylation sites present on the protein sequence. Indeed, glycosylations represent about 35% of the total molecular weight of CD146 with mainly N-glycosylations. The presence of sialylation has also been shown [12].

CD146 has many similarities with other immunoglobulin family members such as BCAM (B-cell adhesion molecule) and ALCAM (activated leukocyte cell adhesion molecule), including the same number of immunoglobulin-like domains, similarity of functions and expression on tumor and endothelial cells. Thus, the ALCAM protein plays a role in CD4⁺ T lymphocytes and in tumor invasion [13, 14].

A short and a long isoform generated by alternative splicing have been identified as the two isoforms of membrane CD146. They have not been identified simultaneously. The long isoform was the first discovered in human melanocytes in 1987 and the short isoform was identified as a complementary DNA from chicken more recently [15]. In addition, a soluble form of CD146 was also identified in endothelial cell culture supernatant (HUVEC) and in bloodstream in patient [16].

Concerning the extracellular sequence, it is common to both isoforms. The difference is located in the intracytoplasmic portion [15]. The two isoforms are the result of an alternative splicing on exon 15 causing a reading frame shift. The short isoform displays a shorter intracytoplasmic domain containing a phosphorylation site for protein kinase C (PKC) and an interaction site for the protein with PDZ domain while the long isoform displays two domains for phosphorylation by PKC and an endocytosis signal sequence [15].

The intracytoplasmic domain sequence is similar to mice and human at 95 and 93% for the short isoform and long isoform, respectively. This conservation across species is in accordance with the important functions carried by the intracytoplasmic domain of CD146.

Finally, a soluble CD146 isoform with a molecular mass around 100 kDa, was identified for the first time in 1998 [16]. This isoform is detectable in human plasma and serum [17] and is generated by a metalloprotease-dependant shedding of the extracellular domain of CD146. The use of nonspecific inhibitors of metalloproteinase (GM6001) inhibits the formation of soluble CD146 [18].

3. CD146 localization

All the data concerning the expression of the different isoforms of CD146 and their functions are summarized in **Figures 1** and **2**.

3.1. Localization in cancer cells

CD146 has been identified for the first time in melanoma where it plays an important role in disease progression. Thereafter, CD146 has been shown to be expressed in various cancers, such as pancreatic/breast/prostate/ovarian/lung/kidney cancers, osteosarcoma, Kaposi sarcoma, angiosarcoma, Schwann cell tumors, or leiomyosarcoma (**Figure 1**). The mechanism of this neo-expression is still largely unknown but, in prostate cancer, it was reported that high expression of CD146 resulted from hypermethylation at the promoter of the CD146 gene [19].

However, almost nothing is known on the differential expressions and localizations of the different isoforms of CD146 in these cells. A recent study has shown that many cancer cells expressing CD146 were able to secrete soluble CD146 through a metalloprotease-dependant shedding [20].

Tissue/Organs/Cell lines	CD146 isoform expression	Effects/roles	Pathology	References
Human endothelial cell and cell lines HUVEC/EPC	CD146	Interaction endothelial-stromal cells / angiogenesis	Tumor angiogenesis	22, 33, 37, 59, 64
	short CD146	Angiogenesis	nd	23, 39
	long CD146	Stabilization of new vascular structure	nd	23
	soluble CD146	Pro-angiogenic factor-chemotactic properties	nd	16,17, 33, 39, 41
Zebrafish endothelial cells	CD146	Involved in VEGF-a signalization / regulate blood flow rate and vessel lumen size	nd	33, 34
Central Nervous System	CD146	Increases neurite extension / involved in optic tectum process extension	nd	30, 31, 32, 55
Bone marrow stroma	CD146	Able to generate bone tissue and hematopoietic environment	nd	29
Mesenchymal cells	CD146	Increases differentiation capacity	nd	28
Intermediate trophoblast	CD146	Regulates embryon implantation	Obstetric complication	70, 71
	soluble CD146	Decreases EVT migration	Obstetric complication	72
Human B lymphocyte	CD146	Increases adhesion	Rheumatoid arthritis	25
Human T lymphocyte	CD146	Increases adhesion	Rheumatoid arthritis	25, 26, 34,36
	long CD146	Increases adhesion	nd	26
Human Natural Killer cells	CD146	Increases adhesion	Rheumatoid arthritis	25
Mesengial/tubular cells	CD146	Endocapillary proliferation/proteinuria/inflammatory syndrome	Nephropathy	73
	soluble CD146	Increased in blood patient with chronic renal failure	Type 2 diabetic nephropathy patient	74
Non Small Cell Lung Cancer	soluble CD146	Increases in plasma	Poor prognosis factor	51
Gastric cancer	CD146	Increases metastasis/EMT inducer	Cancer development	49
	soluble CD146	nd	nd	74
Breast cancer	CD146	Increased cell motility	Poor prognosis factor	46, 47, 48, 50
Osteosarcoma	CD146	Increases metastatic dissemination	Cancer development	44, 45
Ovarian cancer	CD146	Increases metastatic dissemination and cancer cell survival	Cancer development	43
Prostate cancer	CD146	Increases invasiveness and metastatic potential	Cancer development/poor prognosis marker	19, 42
	soluble CD146	nd	nd	74
Human melanoma cancer	CD146	Increases metastatic dissemination	Poor prognosis marker	8, 9, 10, 61, 62, 63
	soluble CD146	Increases tumor angiogenesis and cancer cell survival	nd	20
Murin melanoma cell line	CD146	Increases metastatic dissemination	nd	11
Suprabasal Keratinocyte	CD146	nd	Kaposi's sarcoma/acute chronic dermatitis	75

Figure 1. Summary table for the different isoforms of CD146 expressed in several organs and cells related to their functions, pathologies, and references associated.

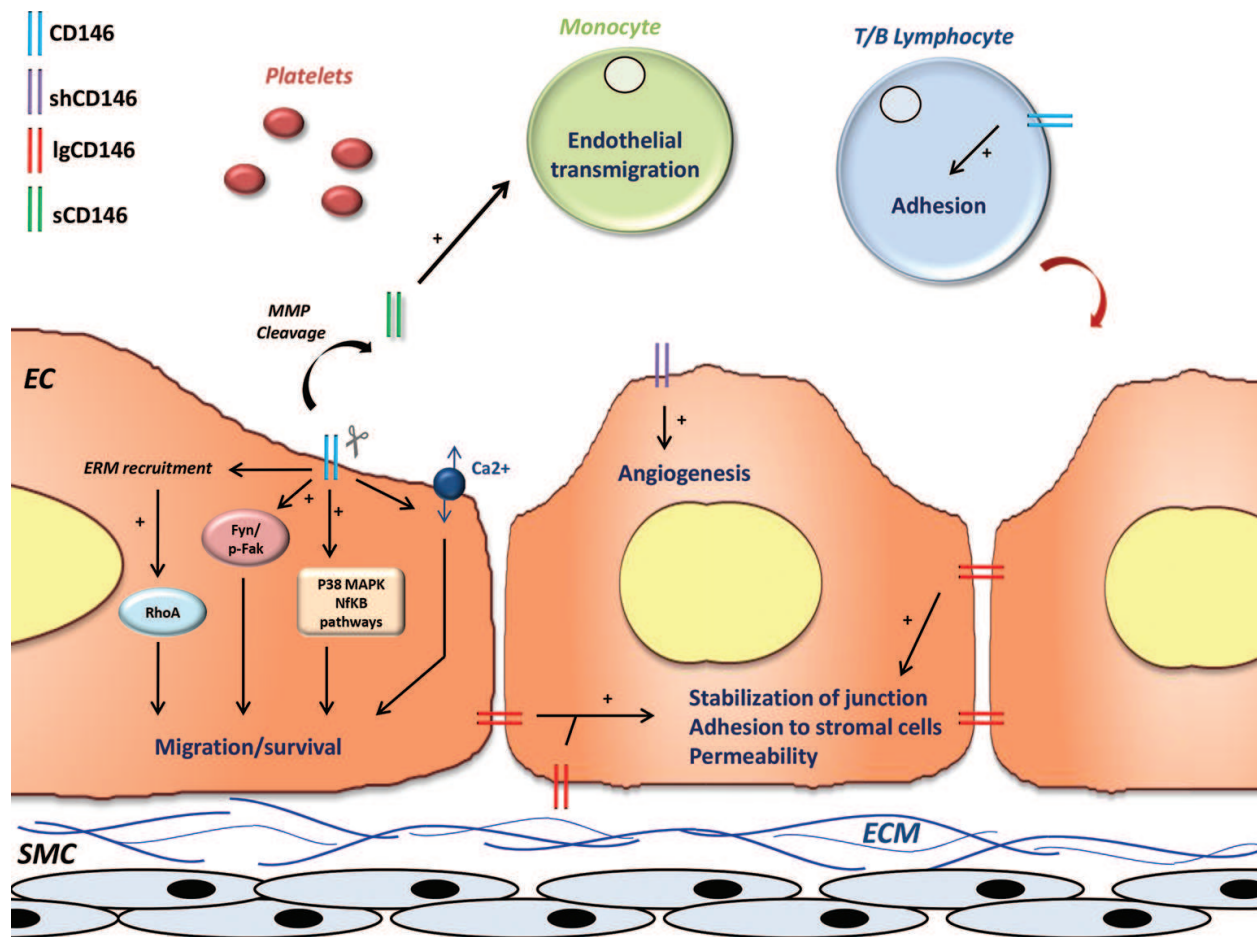


Figure 2. Expression, cell localization, and functions of the different isoforms of CD146 by endothelial cells (EC) and blood circulating cells.

3.2. Vascular localization

CD146 is expressed on the whole vascular tree whatever the vessel anatomical location and caliber. The localization of the long and short isoforms of CD146 is different. The induction of long CD146 expression in the CHO cell line (which does not constitutively express CD146) results in the expression of the protein at the intercellular junctions. Costaining of CD146 with VE-cadherin, focal adhesion kinase (FAK), PECAM, and the complex catenin/cadherin shows no colocalization, suggesting that CD146 is not located in the adherent junctions, tight junctions, or focal adhesions sites [21, 22].

Overexpression of the long form of CD146 in the MDCK cell line (Madin-Darby canine kidney) leads to a basolateral localization of the protein. A dileucine motif on its intracytoplasmic peptide sequence is necessary for this localization [21]. An immunohistochemical staining of long CD146 in endothelial colony-forming cells (ECFC) confirmed this junctional localization of the protein. In addition, the presence of a cytoplasmic pool of long CD146 that can be redistributed to the cell membrane was also described in Ref. [23].

The short isoform of CD146 does not share the same cellular localization. Transfection shows an apical localization of the protein in MDCK cells [21] that was confirmed in ECFC in a culture with a specific antibody generated against this isoform [23].

The confluence state of endothelial cells appears to regulate the spatial distribution of the two isoforms. Indeed, the long CD146 isoform was not detected at the junction in nonconfluent endothelial cells. Under this condition, the long CD146 isoform was intracytoplasmic and the short CD146 isoform was essentially nuclear and at the migration front [23]. In other experiments performed in chickens, it was shown a preferential localization of the long isoform of CD146 in the microvilli where the protein plays a role in their formation. Overexpression of CD146 increased the size of these microvilli [24].

3.3. Localization on immune cells

On peripheral blood of healthy patients approximately 1% of blood mononuclear cells express CD146. An analysis by flow cytometry of different lymphocyte populations showed an expression of CD146 on B and T lymphocytes in humans [25].

Research has shown that about 1% of B lymphocytes cells express CD146 and its expression is upregulated by a factor 5 following stimulation with IL-4 and CD40. Moreover, CD146 can be neo-expressed on some cell populations after stimulation [25]. The generation of two antibodies by rat immunization using cells from the T lymphocytic cell line HUT102 deepened these studies and shown that 2% of CD3+, CD3+/CD4+, and CD3+/CD8+ lymphocytes express CD146.

Moreover, stimulations with IL-2 [25] and PHA (phytohemagglutinin) [26] increases the amount of CD146+ T lymphocytes. The cells are also found *in vivo* in the synovial fluid of patients with rheumatoid arthritis [26].

In mice, a leucocytes screening was carried out which demonstrated that CD146 is not detectable on T/B lymphocyte populations, monocytes, and dendritic cells while 30% of neutrophils and 60% of NK cells express CD146. CD146 expression was correlated with an increased expression of CD11b and CD27 reflecting the maturity of NK. These CD146+ NK cells have a decreased cytotoxicity and produce gamma interferon in smaller quantities [27].

3.4. Bone marrow environment

In adults, hematopoiesis takes place in the bone marrow located in long bones of the human body. It is composed of a dense network of discontinuous capillaries allowing easy passage of cells produced in the bone marrow into the blood. A vascular sinus network which is mainly composed of stromal cells (reticular, endothelial, adipocyte, and osteoblast) serves to support the hematopoiesis process.

In one particular study, a subpopulation of bone marrow stroma cells was shown to express CD146 and to display characteristics of mural cells. They were characterized as a subpopulation of adventitial reticular cells which are abundant in the bone marrow and are able to generate bone tissue and a hematopoietic environment after isolation and implantation into an immunodeficient mouse [28].

Furthermore, angiopoietin -1 is regulated by CD146+ stromal cells. A decrease in the expression of CD146 by siRNA or FGF-2 (CD146 and Ang-1 regulator) reduces the capacity of these cells to participate in the remodeling and the assembly of pseudovascular structures *in vitro*

and to form hematopoietic microenvironment *in vivo*. From data on the spatial location of adventitial reticular cells and the expression of Tie-2 (the angiopoietin-1 receptor), it was suggested that CD146 and angiopoietin-1 are involved in the interaction between endothelial and stromal cells [29].

3.5. Localization in the central nervous system (CNS)

CD146 is found in the central nervous system (CNS). It is expressed during fetal development of the embryo but decreases after birth. Studies performed in chickens and rats have shown an expression of CD146 in the cerebellum, hippocampus, Purkinje cells, and sensorimotor cells of the spinal cord [30]. In chickens, CD146 binds NOF (neurite outgrowth factor), causing neurite extension [31], and increases the extension of the optic tectum process [32].

4. The different functions of CD146

CD146 was reported to be involved in many physiological processes. It has been described to play a role during the vascular development but also during the angiogenic process. As others junction molecules, it was described to be an actor during inflammation by modulating the migration of leucocytes through vascular endothelium.

4.1. CD146 during the vascular development

The role of CD146 was studied during vascular development. To this end, a model of CD146 inactivation by antisense morpholino-oligonucleotides was developed in zebrafish. Authors observed a decrease in intersomitic vessels followed by a decrease in blood flow and a reduction in vessel lumen observed by microangiography after CD146 inactivation [33]. It was also shown an inhibition of the VEGF-dependent angiogenesis [34].

4.2. Permeability and leucocytes migration

CD146 has been shown to be involved in endothelial permeability [33]. Using both a monocyte cell line, THP-1, and freshly isolated monocytes, it was also showed that it modulates monocytes transmigration. Junctional CD146 was shown to bind monocytes through a heterophilic interaction to increase their transmigration. In addition, an increased transmigration was observed following the binding of soluble CD146 on monocytes [33]. Another study showed that neo-expression of CD146 on lymphocytes induced new cellular properties. Indeed, an increase in the adhesion of CD146+ T lymphocytes effectors was observed after stimulation with IL-1 beta. This effect was blocked by the addition of anti-CD146 blocking antibodies [34]. An increase in the adhesion of CD4+/CD146+ T lymphocytes on endothelium was also observed after an inflammatory stimulus. In this study, *in vitro* transfection of the long isoform of CD146 in NKL.1 cell line induced a reduction of rolling cells and an increased adhesion to the endothelial monolayer. Moreover, these phenomena were accompanied by an increase in microvilli in T lymphocyte cell membrane. Another study showed an increased permeability of HMVEC (human microvascular endothelial cells) following incubation with

an anti-CD146 antibody (P1H12) [35]. Finally, CD146 is coexpressed with CCR6 on a population of TH17 lymphocytic cells [36].

4.3. Angiogenesis

Angiogenesis is an important mechanism, both in fetal life and at adulthood. Endothelial cells with angiogenic capacities are able to proliferate, migrate, adhere, and generate new capillaries from a preexisting one.

The injection of an anti-CD146 antibody (AA98) led to a decrease of 70% in the number of vessels in a membrane model, chorioallantoic membrane model, in chicken. Furthermore, in mice, this antibody reduced the number of vessels in different models of xenografted tumors (hepatocellular carcinoma, pancreatic, and leiomyosarcoma) [37], demonstrating a role of CD146 in tumor angiogenesis.

The recent discovery of the existence of two isoforms of CD146 and the description of a soluble form of CD146 led to study their implications in the angiogenic process. Specific siRNA directed against these two isoforms has shown that the absence of the short CD146 decreased the proliferation, migration, and adhesion of endothelial cells, whereas its overexpression led to the reverse phenomena. These experiments showed that the long CD146 was also necessary to generate pseudocapillaries in Matrigel *in vitro* by stabilizing the junctions of neovessels. It thus appears that both the short and long isoforms of CD146 display complementary effects to generate neovessels. The effects of the short CD146 were confirmed *in vivo* by the transplantation of endothelial colony-forming cells (ECFC) overexpressing this isoform in a mouse model of hind limb ischemia. Indeed, it increased the incorporation of ECFC in ischemic muscle and favored the generation of neovessels [23]. A study of the mechanism showed that the short CD146 is associated with VEGF-R2 [38], but also angiomin and VEGF-R1 at the endothelial cell surface [39, 40]. This association is essential for these different pathways. Indeed, the absence of the short CD146 isoform decreases the phosphorylation of VEGF-R2 in endothelial cells and prevents the proangiogenic effect of vascular endothelial growth factor (VEGF).

Soluble CD146 is also able to increase the formation of pseudocapillaries *in vitro* and to induce neovascularization in a rat model of hindlimb ischemia. In addition, subcutaneous injection of Matrigel containing soluble CD146 in mice increased the recruitment of both mature and immature endothelial cells, as well as smooth muscle cells, resulting in the formation of capillary-like structures [41]. Of interest, it was reported that soluble CD146 stimulates the short CD146 isoform through its binding on angiomin [39] and that the angiogenic properties of soluble CD146 are additive to those of VEGF [41]. The roles of the different forms of CD146 are summarized in **Figure 2**.

4.4. Cancer cell growth and dissemination

CD146, which is neo-expressed on cancer cells, modulates their growth and dissemination. In prostate cancer, CD146 expression was observed in different cell lines. CD146 overexpression increased their invasiveness and metastatic potential [42]. CD146 overexpression was also

observed in biopsies of patients. Its expression was correlated with a poor prognosis. In ovarian carcinomas, CD146 expression was also correlated with the increase of the metastatic potential. In addition, inhibition of CD146 protein expression in ovarian cancer cell lines led to inhibition of invasiveness, tumor spread and induced cancer cell apoptosis. This was explained by the fact that a lack of CD146 induced a decreased activity of Rho GTPase [43] involved in the invasion, proliferation, and metastatic spread of cancer cells.

It was also demonstrated that CD146 expression is increased in osteosarcomas as compared to nonpathological osteoblasts [44]. Injection of antibodies against CD146 decreased the amount of lung metastases in an immunodeficient mouse model injected with cells derived from human osteosarcoma [45].

In breast cancer, it was reported that CD146 would act as a tumor suppressor [46] while other studies have described CD146 as a poor prognosis marker [47]. Indeed, CD146 overexpression in a breast cancer cell line induced an increased motility and tumorigenicity [48]. Recent studies have also shown that CD146 induces the epithelial-mesenchymal transition (EMT) in so far as its expression is correlated with markers of EMT in gastric cancer [49]. Moreover, in triple negative breast cancers, an increase of CD146 expression in epithelial cells correlates with a loss of epithelial markers in favor of mesenchymal markers, increasing their invasiveness, migration, and the number of mammospheres. In addition CD44 expression increases and CD24 expression decreases on the cell surface suggesting that cells acquire phenotypic characteristics of cancer stem cells [50].

At present, there is no data on the differential expression and roles of the two membrane isoforms of CD146 on cancer cells. However, recent studies have shown an important role of soluble CD146 in tumor development. First, an increase of soluble CD146 concentration was described in blood of cancer patients with nonsmall cell lung cancer as compared to patients with respiratory inflammatory disease and healthy subjects [51]. In this chapter, we showed that association between an increased soluble CD146 concentration and an increased number of circulating endothelial cells (CEC) constitute a poor prognostic factor [51].

Recently, a study showed that human cancer cells that express membrane CD146 on their surface have also the ability to secrete the soluble form of CD146 [20]. This was described in melanoma, colorectal and pancreatic cancer cell lines. The authors demonstrated that soluble CD146 secreted by cancer cells could display autocrine effects on cancer cells and paracrine effects on vascular endothelial cells. Indeed, *in vitro* stimulation of cancer cells with recombinant soluble CD146 increased their proliferation and the production of protumorigenic factor such as VEGF. They also demonstrated that this stimulation protected cancer cells from apoptosis induced by H₂O₂ and decreased cancer cell senescence. In particular, the c-myc signaling pathway appeared to be upregulated by soluble CD146. Soluble CD146 secreted by cancer cells also increased the proliferation of surrounding endothelial cells, stimulating tumor angiogenesis. These effects were confirmed *in vivo* in different models of xenografted mice and an antisoluble CD146 antibody was able to block these effects. Thus, soluble CD146 was described as a proangiogenic factor and seems to have a major role in tumor development.

5. Ligand and cell signalization

Historically, the first molecule interacting with the extracellular portion of CD146 is NOF (neurite outgrowth factor). A stable transfection of complementary DNA encoding for CD146 induces an adhesion of neuronal cells on a NOF matrix [32]. More recently, laminin-8 has been identified as a new vascular ligand of CD146 expressed by TH17 lymphocytes. In this study, it has been demonstrated that the laminin-411/CD146 interaction favors adhesion and tissue transmigration of these lymphocytes, leading to an increased inflammation [52]. Furthermore, one study showed that CD146 DNA transfection in the CD146-deficient melanoma cell line Mel-888 induced an increased aggregation between these cells and cells which do not express CD146 suggesting that there are other still unidentified partners [53].

The existence of a homophilic interaction for CD146 is controversial. One study showed that CD146 transfection in neuronal cells induced their aggregation, suggesting that CD146 could create homophilic bonds [32]. Another *in vitro* study demonstrated that the neurite growth of PC12 cells is increased when cells are in a chimeric CD146 protein substrate. In addition, under these conditions, the use of an anti-CD146 antibody blocks neurite growth. This inhibition would be associated with an inhibition of CD146-CD146 homophilic interaction [54]. CD146 dimerization at the cell membrane following stimulation with an activating CD146 antibody (clone AA98) was also demonstrated using fluorescence resonance energy transfer (FRET) and pull-down. The use of an NFkB signaling pathway inhibitor reduced this dimerization [55]. Finally, a recent study highlighted dimerization after stimulation with VEGF [56].

Conversely, other studies could not replicate the homophilic interaction, in particular, between soluble CD146 and CD146-Fc [33].

Recently, new ligands for CD146 were identified. A direct and strong interaction between CD146 and VEGFR-2 was demonstrated in endothelial cells and this association was important for VEGFR-2 phosphorylation by VEGF. These results were confirmed in a CD146 KO mouse model where the absence of CD146 inhibited vessel formation induced by VEGF. Experiments in mouse models of pancreas and melanoma cancer cell xenograft have shown that the combined use of anti-VEGF antibody (bevacizumab) and anti-CD146 antibody (AA98) displayed a synergistic effect on tumor development [57].

Another work identified galectin 1 as a new CD146 ligand on the endothelium [58]. This protein induced apoptosis of endothelial cells and specifically bound to CD146 via extracellular glycosylations. This interaction is specific for galectin 1 since it is not found with galectin 2. Using siRNA or antibodies able to block CD146 resulted in an increased cell apoptosis, suggesting a protective role of CD146 against apoptosis.

Different factors have been shown to modulate CD146 expression:

- A stimulation with TNF-alpha increases the amount of CD146 present at the endothelial cell surface [33].

- TGF-beta administered to hepatocyte cells pretreated with an inducer of acute hepatitis (tetrachloride carbonate) increases the amount of CD146 mRNA and the regenerative capacity of these cells [59].
- HSP27 (heat shock protein 27), a chaperone molecule involved mainly in tumor differentiation and tumorigenesis, inhibits the migration and invasion of melanoma cells and thus acts on the tumor phenotype [60]. Overexpression of HSP27 has been shown to decrease the expression of CD146 and increase the expression of E-cadherin in melanoma cell lines. These variations in protein expression determine, among other, malignant phenotype of melanoma cells [61].
- AKT activation by PD98059 and Wortmannin increases CD146 expression at the cell membrane in melanoma cell lines. Conversely in these cell lines, overexpression of CD146 increases AKT which inhibits BAD (Bcl-2-associated death promoter), increasing cell survival [62].

Membrane CD146 activates multiple signaling pathways, leading to the activation of the NFkB pathway. CD146 dimerization has been described in the membrane of endothelial cells following the addition of culture medium of tumor cells (A375 cell line). Inhibition of the NFkB pathway (by BAY11-7082 compound) causes a reduction of the nuclear translocation of NFkB but also inhibits the dimerization of CD146 [63, 64]. The junctional molecules involved in adhesion such as VE-cadherin or claudins are also involved in a phenomenon of actin cytoskeleton reorganization. CD146 is also connected to the actin cytoskeleton.

Indeed, CD146 targeting with the S-ENDO1 antibody led to FAK phosphorylation and an increase in the release of intracellular calcium and extracellular calcium entry. This mechanism of action of calcium flux was mediated by the recruitment and activation of Fyn leading to the phosphorylation of PLC gamma. Calcium entry also caused the recruitment of PYK2 and p130. On the other hand, FAK activation led to signaling pathways involved in the reorganization of the actin cytoskeleton and also modulated transcription factors involved in cells survival and migration. In these studies, there was no evidence of direct interaction between CD146, paxillin, and FAK. It seems therefore important to identify the intermediate partners [65, 66].

Recently, a study confirmed the role of CD146 in the migration and induction of signals related to the actin cytoskeleton. Indeed, CD146 displays direct physical interaction with the ezrin-radixin-moesin (ERM) proteins, allowing the recruitment of ERM at the protrusions of melanoma cells. This phenomenon induces the elongation and expansion of microvilli at these protrusions [67].

Recruitment by CD146 allows the sequestration of a RhoA inhibitor (Rho guanine nucleotide dissociation inhibitory factor 1) leading to RhoA activation and an increased cell motility. Another study showed that CD146 is redistributed in a polarized structure named W-RAMP (Wnt5a-mediated actin-myosin receptor-polarity) in subconfluent melanoma cells stimulated with Wnt5a. W-RAMP is involved in membrane retraction and the direction of cell migration with an intervention of Rho-A [68].

In another study that focused on the priming of ECFC with soluble CD146 in order to improve the therapeutic potential of these cells *in vivo*, the authors showed that a priming of these cells

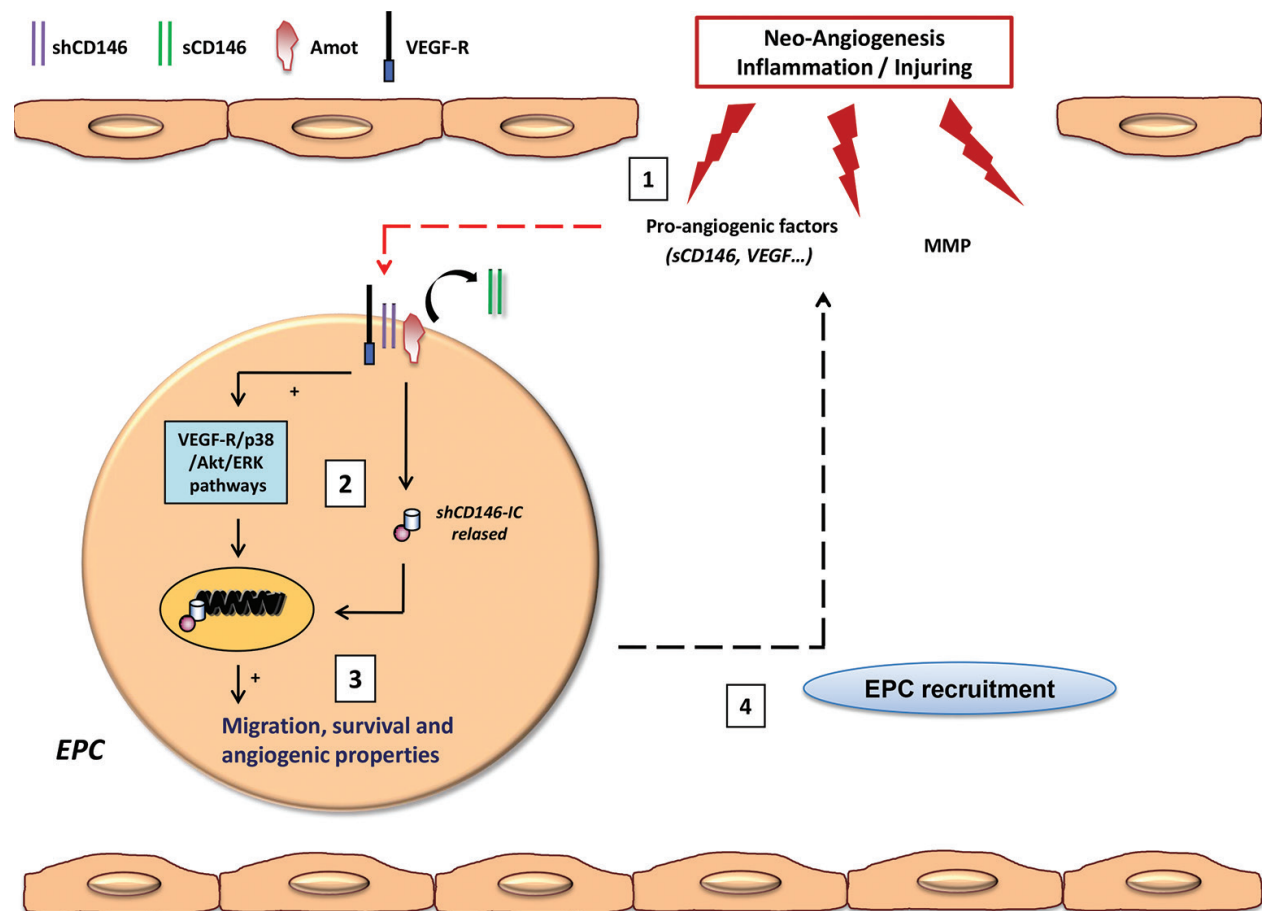


Figure 3. Mechanism of actions of endothelial progenitor cells (EPC) activation leads to their recruitment after inflammatory or angiogenic stimulation.

with soluble CD146 did not modify the number of engrafted ECFC in the ischemic muscle but improved their survival capacity leading to an enhanced revascularization [39]. They showed that in ECFC, it exists a signalosome that is located in a particular region of cell membrane called lipid rafts. This signalosome contains soluble CD146, the short isoform of CD146 (shCD146), presenilin-1 but also the two VEGF receptor called flt1 and flk1. The mechanism of action is characterized by a sequential proteolytic cleavage, induced by soluble CD146, with an extracellular shedding of the short CD146 followed by an intramembrane cleavage which is mediated by both the ADAM/matrix metalloproteases (MMP) and the gamma-secretase protein. The consequences of this shedding involved a nuclear translocation of the new intracellular peptide of shCD146 which binds to the transcription factor CSL and is associated with a modulation of gene transcription leading to angiogenesis (eNOS) and cell survival (FADD, Bcl-xl). The association between CD146 and VEGFR2 was described in a previous paper and based on these results the authors showed that the effect of soluble CD146 on EFCF is dependent on VEGFR2 but also VEGFR1 which are phosphorylated by soluble CD146. All these findings show that the stimulation of this cell by soluble CD146 and the proteolytic cleavage of shCD146 is a promising pathway to increase the regenerative properties of endothelial progenitor cells for the treatment of cardiovascular diseases (**Figure 3**).

6. CD146 in pathology

6.1. Obstetrical pathologies

About 2% of fertile women are affected by spontaneous fetal loss. The mechanism of this fetal loss is not yet understood.

One study showed that CD146 is highly expressed during the implantation window. During the following steps, the level of CD146 decreased rapidly and CD146 blocking with an antibody caused abortion [69]. CD146 is expressed by the intermediate trophoblasts (or extravillous) in humans but is not detected on the syncytiotrophoblasts and cytotrophoblasts [70].

After this work, soluble CD146 was described as a novel physiological factor with angiogenic properties involved in the regulation of placenta vascular development by acting on extravillous trophoblast (EVT). Using placenta explants, soluble CD146 was demonstrated to inhibit the growth of extravillous trophoblasts and the ability of EVT to migrate and form pseudocapillary tubes on Matrigel. A clinical study on the role of soluble CD146 in 50 pregnant women was also conducted. A physiological decrease of plasmatic soluble CD146 was observed in pregnant women as compared to nongestational women. These results inspired the authors to study the effects of prolonged administration of soluble CD146 in a pregnant rat model. Repeated systemic injection of soluble CD146 after mating caused a significant decrease in the pregnancy rate and the number of embryos. Histological studies of placenta showed a decreased migration of glycogen cells (cells that are similar to the EVT in rat) in female rat treated with soluble CD146.

In mice the use of a specific antibody blocking CD146 (AA98) caused a decrease in the blastocysts adhesion on a uterine epithelium cell monolayer and a decrease in the growth of trophoblastic cells. In addition, injection of this antibody in the uterine horn of the mouse at 3.5 dpc (days post coitum) resulted in a decrease of embryo implantation at 7.5 dpc. Histological analysis showed that the embryos were present but smaller and in poor condition [69]. Two clinical studies were in line with these observations. A first clinical study showed that the rate of membrane CD146 expression was lower on intermediate trophoblasts in the placenta of preeclamptic patients when compared to patients with nonpathological pregnancies [70]. In a second study, two populations of women have been used to compare the blood level of soluble CD146. In this study, the authors used 100 blood samples which were taken 2 months after the last obstetrical events between women with no pregnancy lost which have at least one living child and 100 blood samples from women with at least two consecutive losses at/or before 21 weeks of gestation. They found an increase in the level of soluble CD146 in the second population compared to the first control population [71]. In this study, the two populations used are age matched.

Thus, in view of these results soluble CD146 may represent an attractive biomarker of vascular placental development as well as a therapeutic target in obstetric complications.

6.2. Inflammatory diseases

Endothelial functions are altered in inflammatory diseases.

In inflammatory kidney disease, biopsies of patients with nephropathy show an increased expression of membrane CD146 on endothelial cells, but also on the mesangial cells and a neo-expression of CD146 on tubular cells [72]. In addition, there is a correlation between CD146 expression and proteinuria, endocapillary proliferation and inflammatory syndrome. The serum level of soluble CD146 is also modulated. Thus, an increase in CD146 secretion was observed in chronic renal failure which was correlated with the severity of this disease in type 2 diabetic nephropathy patients [73].

In a second type of inflammatory disease, CD146 has also been identified in primary cultures of keratinocytes while its expression was not observed on healthy skin. An increase in the expression of CD146 has been observed in various skin diseases. For example, CD146 is expressed in suprabasals keratinocytes of psoriasis patients [74]. CD146 is also detected in Kaposi's sarcoma, lichen planus, on the skin overlying skin neoplasms or in chronic and acute chronic dermatitis [74]. On the other hand, the expression of CD146 is not increased in other skin diseases such as lupus erythematosus.

6.3. Tumor pathologies

CD146 is expressed in many cancers, such as melanoma, prostate cancer, breast cancer, pancreatic cancer, lung cancer, Kaposi sarcoma, angiosarcoma, Schwann cells tumors, or leiomyosarcoma.

The role of tumor CD146 was first studied in melanoma. A direct correlation has been demonstrated between the increase in metastasizing capacities and the increased expression of CD146 [75]. The level of expression of CD146 by human melanoma cell lines has been shown to correlate with their ability to form tumors and metastasis in a mouse xenograft model in immunodeficient nude or SCID mice [76]. In addition, CD146 increases the number of lung metastases following intravenous injection of melanoma cells in nude mice *in vivo* [77]. These observations were confirmed through the use of interfering RNA directed against CD146 leading to a decrease in migration, proliferation, and invasion *in vitro* [78].

CD146 immunohistochemistry staining was performed on human primary melanoma tissue, showing a CD146 expression on tumor-associated endothelium and on smooth muscle cells [79]. The role of CD146 in tumor angiogenesis has been described in particular thanks to the use of the AA98 antibody [37, 64] that is able to block tumor angiogenesis and decrease tumor growth of human melanoma xenograft model in immunodeficient mice.

Currently, mechanisms involved in melanoma progression are unclear. A study showed that a particular population of B lymphocyte cells, the B1 lymphocytes, has a prometastatic potential. Indeed, depletion of this population caused a decrease of tumor growth and metastasis dissemination in mice in an experimental metastasis model, following a B16 cell line injection. The decrease in metastases dissemination involved homophilic interactions between B1 and B16 cells thanks to CD146 [80]. In addition, coculture of B1 cells with melanoma cells

increased the expression of CD146 at the cell membrane of cancer cells, increasing the number of metastases *in vivo*.

A clinical study was conducted on a cohort of patients with skin cancer. Patients were divided into two groups: early and late stage melanoma, in order to analyze the presence of different commonly used cancer markers including CD146. Analysis in the blood of patients showed that CD146 was the only protein correlated with the advanced stages of the disease [81]. Another study confirmed this finding by demonstrating that CD146 is a marker of poor prognosis and survival in melanoma patients. CD146 constitutes a better marker than biopsies analysis of sentinel lymph node [82].

6.4. Angiogenesis-related diseases and therapeutic approaches

Recent studies revealed that both isoforms of CD146 are involved in angiogenesis with a promigratory and a proliferative role of the short CD146 and a vessel stabilization role of long CD146, which is also described in this chapter. Soluble CD146 secreted by both endothelial and cancer cells is also able to stimulate angiogenesis. These different forms are involved in physiological angiogenesis but also in pathological angiogenesis, in particular in tumor angiogenesis.

Therefore, different antibodies have been generated to block its functions. The first one was ABX-MA1, an antibody recognizing the human form of this molecule. This antibody was able to inhibit the formation of spheroids containing melanoma cells, reducing metastasis, tumorigenicity, and vascularization of the tumor *in vivo*. This reduction was related to the inhibition of MMP-2 expression which is heavily involved in metastasis formation [83].

Another team-generated monoclonal antibody specifically directed against the vascular endothelium of tumors. During the screening of these antibodies, the authors focused on the AA98 antibody. This antibody recognizes CD146 localized in the intratumoral vasculature but not recognizes CD146 expressed on blood vessels in healthy tissues [37]. This antibody inhibits both *in vitro* and *in vivo* angiogenic properties of CD146 in human tumors xenografted in immunodeficient mice. In addition, it was demonstrated that the AA98 antibody is a potential diagnostic and therapeutic agent in vascular and cancer diseases. Following this work, it was shown that AA98 antibody inhibits phosphorylation of p38/MAPK, suppresses NFκB activation, and inhibits MMP-9 and ICAM-1 expression. This suggests that deleting NFκB is a pivotal point of the inhibitory effects of the antibody on endothelial cell migration, angiogenesis, and development of tumor metastases [64].

Of interest, this antibody displays additive inhibitory effects when used in combination with the anti-VEGF antibody bevacizumab in xenografted models of human pancreatic tumors and melanoma [57]. In addition, it reduces significantly the chronic inflammation in the colon in a mouse model and prevents the development of cancer associated with this chronic inflammation [38].

Recently, a novel antibody was generated against the soluble form of CD146 [20]. The authors demonstrated that this antibody was able to decrease tumor angiogenesis and growth but to also induce apoptosis of human melanoma and pancreatic tumors xenografted in immunodeficient mice. Of interest, this antibody cannot bind membrane CD146, a property that should limit the side effects that could be observed with antibodies targeting the membrane form.

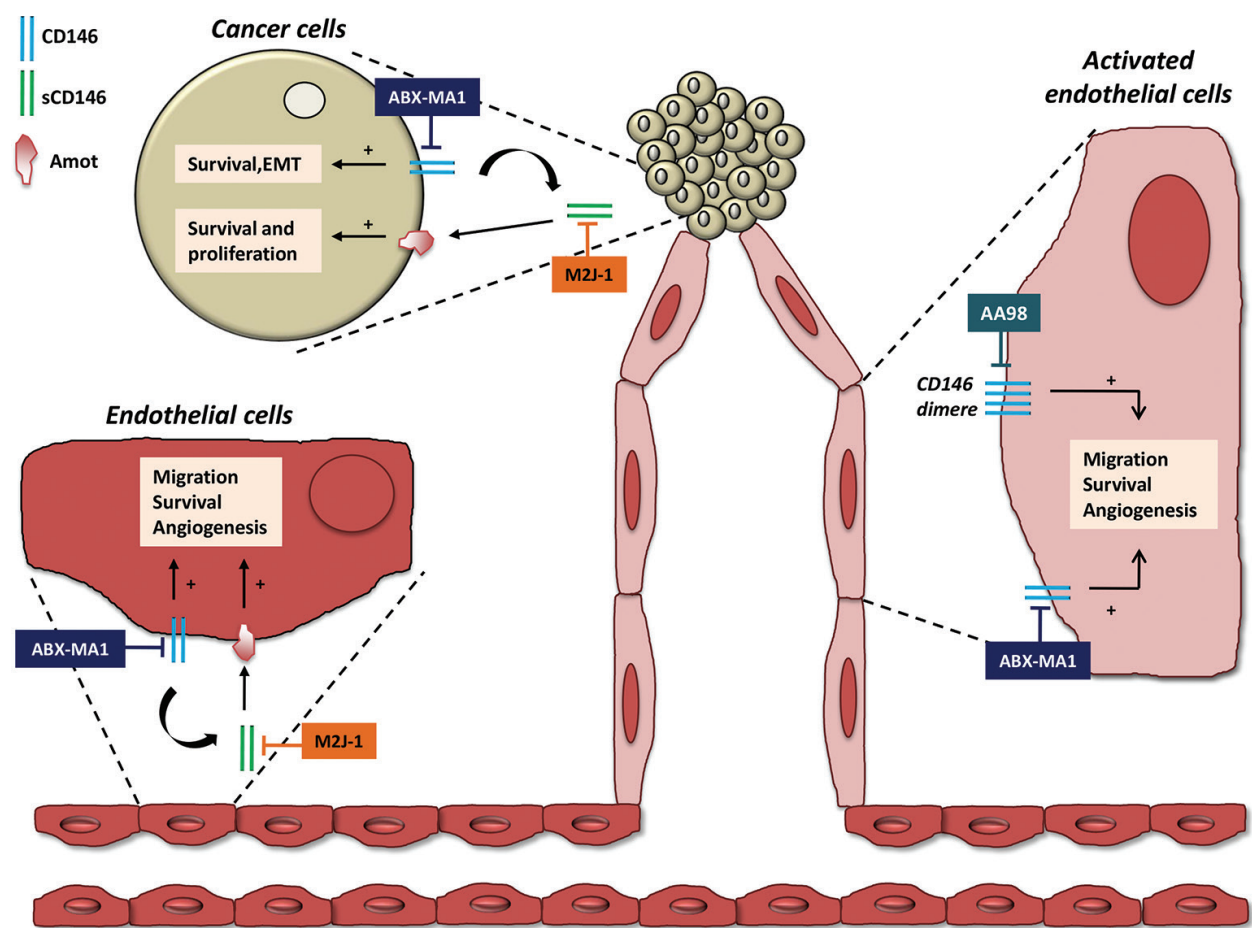


Figure 4. Functions of the different isoforms of CD146 and the inhibitory antibodies associated during tumor growth and angiogenesis.

Functions of the different CD146 isoforms and the inhibitory antibodies associated are summarized in **Figure 4**.

Author details

Jimmy Stalin^{1*}, Lucie Vivancos², Nathalie Bardin³, Françoise Dignat-George³ and Marcel Blot-Chabaud³

*Address all correspondence to: jimmy.stalin@unige.ch

1 Department of Pathology and Immunology, University Medical Center, Geneva University, Switzerland

2 Pediatric Hematology-Oncology Research Laboratory, Pediatric Division, University Hospital CHUV, Lausanne, Switzerland

3 INSERM UMR-S 1076, Aix-Marseille University, UFR Pharmacy, Marseille, France

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