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Gonçalo Branco Madureira

Intussusceptive Angiogenic features of Covid-19: molecular mechanisms, stimuli and therapeutic implications

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Doutora Raquel Ângela Silva Soares Lino

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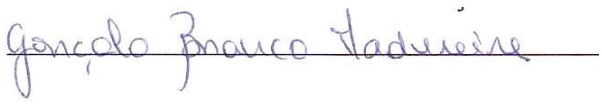

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NOME

Gonçalo Branco Madureira

NÚMERO DE ESTUDANTE	E-MAIL
201505288	gbmadureira.12@gmail.com

DESIGNAÇÃO DA ÁREA DO PROJECTO

Bioquímica

TÍTULO ~~DISSERTAÇÃO~~/MONOGRAFIA (riscar o que não interessa)

Intussusceptive Angiogenic features of Covid-19: molecular mechanisms, stimuli and therapeutic implications

ORIENTADOR

Raquel Ângela Silva Soares Lino

COORIENTADOR (se aplicável)

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Abstract

Novel Coronavirus Disease 2019 (Covid-19) is associated with multi-systemic derangement, including circulatory dysfunction with features of endothelial dysfunction, microangiopathic thrombosis and angiocentric inflammation. Recently, intussusceptive angiogenesis has been implicated in the pathogenesis of the disease.

In this article, we have broadly reviewed and discussed data regarding splitting angiogenesis including mechanisms, drivers, regulators and putative roles. We have reviewed other relevant angiogenic features in Covid-19, including their potential role in inflammation, endothelial dysfunction and permeability as well as their use as prognostic markers and therapeutical roles.

We conclude that splitting angiogenesis in Covid-19 is likely the result of a combination of factors. We hypothesize that hypoxia, hypoxia-inducible factors as well as other classic angiogenic mediators, such as the Vascular Endothelial Growth Factor (VEGF) and Angiopoietins pathway, hyperinflammation and cytokine storm and dysregulation of the Renin-Angiotensin-Aldosterone System interact to promote intussusception. However, splitting angiogenesis remains poorly understood and thus further studies are needed to better characterize this phenomenon.

We have also summarized the main data regarding the use of angiogenic mediators as prognostic tools. Data suggests that angiopoietins and VEGF are elevated in Covid-19 patients and are predictors of adverse outcomes. However, this is the first time that an attempt to relate these findings to intussusception was made.

Finally, we reviewed the scarce data regarding angiogenic mediators as therapeutic targets in Covid-19. These preliminary findings suggest a potential benefit of bevacizumab as an add-on therapy. Whether this relates to intussusception or not requires further studies.

Keywords: Intussusception; cytokine storm; SARS-CoV-2 infection; endothelialitis; microenvironment factors

Introduction

Novel Coronavirus Disease 2019 (Covid-19) is potentially life-threatening disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection. [1] Since its first identification in December 2019, the disease evolved into an international public health emergency, and in March 2020, the World Health Organization declared it a pandemic.

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is associated with a plethora of clinical manifestations ranging from pauci-symptomatic to an invasive, severe infection with acute respiratory distress, systemic hyperinflammation and multi-organ failure. [1] SARS-CoV-2 uses the same receptor as SARS-CoV, the angiotensin-converting enzyme 2 (ACE2). [2] As endothelial cells express abundant ACE2, they are a target to SARS-CoV-2 infection. In fact, SARS-CoV-2 mediated endothelial damage is an important aspect regarding to the effects of the virus. [2-4]

Covid-19 as a vascular disease

Studies suggest that vascular disease plays a major role in Covid-19 course influencing both the susceptibility to and the outcomes of the infection. Classical cardiovascular risk factors such as hypertension, cardiovascular disease, diabetes, and obesity are the most prevalent comorbid conditions among Covid-19 patients. [2,3]. These conditions are associated with worse outcomes and are independently associated with Covid-19 related deaths. [2,3] Furthermore, they also correlate with age as aging is associated with complex vascular changes that result in endothelial disfunction. Age is the strongest predictor of Covid-19 mortality and it is very likely that endothelial disfunction is responsible for part of the excess mortality in this age group. [2,3].

SARS-CoV-2 infection results in the disruption of endothelial homeostasis with loss of Renin-angiotensin-aldosterone system (RAAS) balance, anti-thrombotic and immune functions [5]. The hyperinflammatory state potentiated by SARS-CoV-2 infection is deleterious to the endothelium. High circulating levels of cytokines in the context of cytokine storm, such as IL-6, TNF-α act as endothelial activators shifting endothelial cell's phenotypes to a pro-inflammatory, chemotactic phenotype with high permeability and pro-thrombotic features [5,6]. A maladaptive innate immune response is also responsible for endothelial damage as DAMPs and PAMPs mediated TLR activation promotes oxidative stress [7]. Neutrophil extracellular traps are also associated with endothelial injury [8].

These immune changes along with dysregulation of the RAAS are associated with hypercoagulability state [9]. SARS-CoV-2 infection leads to loss of ACE2 activity in ECs [10] which reduces angiotensin II metabolism and inactivation and consequently lower levels of $Angiotensin_{1-7}$ (the byproduct of angiotensin II degradation). Higher levels of AT II associated with lower levels of AT_{1-7} cause vasoconstriction, leucocyte and platelet adhesion, thus promoting thrombogenicity and suppressing fibrinolytic activity. [11] Furthermore AT II regulates NADPH oxidase 2 (Nox 2) and higher levels of AT II result in increased oxidative stress [12], which further amplifies vascular dysfunction.

This prothrombotic milieu has analytic repercussions. Patients with Covid-19 have increased levels of fibrinogen, D-dimer and fibrin degradation products, von Willebrand factor/factor VIII and lower levels of Plasminogen Activator Inhibitor 1 (PAI-1). [9] Data suggests that these mediators correlate with disease severity and thrombotic risk [9,11] This hypercoagulability state is an important part of the pathophysiological basis of cardiovascular complications in Covid-

19 patients, such as myocardial injury and dysfunction, deep venous thrombosis, pulmonary thromboembolism and some obstetric complications in pregnant women with SARS-CoV-2 infection such as pre-eclampsia and HELLP syndrome [5].

Pulmonary vascular bed alterations in Covid-19

The endothelial dysfunction and subsequent prothrombotic environment extends beyond the systemic circulation. An important feature of COVID-19 associated coagulopathy is microcirculatory endothelial damage in pulmonary vascular beds. Although less well characterized, peripheral pulmonary vascular repercussions are an important pathogenic factor that may result in increasing perfusion-ventilation mismatch, leading to worsening hypoxemia [13].

Covid-19 associated coagulopathy is associated with two main forms of thrombotic repercussions in the pulmonary vascular bed. As Covid-19 is associated with a pro-thrombotic phenotype, the risk of deep vein thrombosis or pulmonary thromboembolism is significantly higher and is a potential cause of acute exacerbation in patients. [9] However, the most common form of thrombosis is microangiopathic thrombosis in the pulmonary microcirculation. Studies report features of microthrombosis in pulmonary capillaries [14, 15]. In fact, microthrombi in the pulmonary vascular beds are 9 times more common in SARS-CoV-2 infection than in Influenza infection [15] and are associated with worsening hypoxia, shunting and increasing pulmonary vascular resistance. Changes in pulmonary microvascular resistance, more specifically in venules, is responsible for the discrepancy between the relatively preserved ventilatory mechanics and the severity of hypoxemia in Covid-19 patients, that differs from Acute Respiratory Distress Syndrome (ARDS) caused by other agents [17]. Microangiopathic thrombosis is also responsible for a up to 90% reduction in capillary density in lungs of patients with Covid-19 [18].

This microangiopathic phenomena has been demonstrated by histopathological and ultrastructural studies. Typically, pathological evidence shows thrombi in pulmonary arterioles associated with diffuse alveolar damage and hyaline membrane formation [14]. The latter are a direct repercussion of increased permeability in the microcirculation and disruption of intercellular junctions of the pulmonary endothelium. Hyaline membranes impair alveolar-capillary barriers and jeopardize gas exchange, contributing to hypoxemia and V/Q mismatch.

Besides the thrombotic manifestations in pulmonary vascular plexus, Covid-19 is associated with features of small vessel vasculitis [15]. This is not exclusive to the lung, and Kawasaki-like cases of coronary vasculitis have been described [19] and are outside the scope of this review. In the pulmonary vascular beds, SARS-CoV-2 infection promotes angiocentric inflammation. Patients with SARS-CoV-2 infection, specially those with ARDS, exhibit features of vascular acute inflammatory infiltrates with perivascular infiltration with T-lymphocytes [15]. These inflammatory infiltrates contribute to endothelial barrier disruption that leads to hyaline membrane formation and aggravate respiratory failure. Interestingly, Covid-19 patients display a feature similar to solid organ rejection characterized by a pattern of a T-lymphocyte crown surrounding a EC that displays features of being highly activated. To emphasize the interaction between the immune environment and the endothelium, this pattern has been termed endothelialitis [15,16].

Angiogenesis and Covid-19

Ackermann *et al* analyzed 7 lungs from people who died from Covid-19 and compared them to lungs from patients who died from Influenza and healthy controls [15]. They found significant distortion of the angioarchitecture of the lung

with prominent variations in caliber of small vessels. Surprisingly, capillaries in the lungs of Covid-19 subjects cylindrical microstructures in the capillary lumina, implicating, for the first time, intussusceptive angiogenesis (IA) in the pathogenesis of Covid-19. IA offers the advantage of being faster and more efficient than sprouting angiogenesis (SA) in expanding a vascular plexus [20], promoting arborization through intussusceptive arborization (IAR), optimizing the microangioarchitecture by pruning ineffective or occluded branches (IBR).

However, the findings of Ackermann *et al* must be read with caution. As Hariri *et al* point, sample size is too small to make any extrapolation, as ARDS and Covid-19 are too heterogenous entities [21]. Further studies are needed to determine the relative frequency of IA in SARS-CoV-2 infected patients, and possible associated risk factors. An interesting hypothesis is that these vasculocentric features represent a particular ARDS endotype. Angiogenesis has been implicated in ARDS pathology in subgroups of patients, in the pre-Covid era [22]. However, this is the first time that IA has been described in the context of ARDS.

The populations compared in the study had major differences, as pointed out by Ackermann and his colleagues [15]. In the Covid-19 group, no single patient had been mechanically ventilated, whereas in the Influenza group, a significant proportion of the subjects had been intubated without protective lung measures. It is possible that mechanical ventilation has repercussions in microcirculatory dysfunction and angiogenesis. Comparisons between the two groups must take this factor into consideration.

The authors also suggest the possibility that differences noted between the two groups were attributable to the differences in the stages, as the Influenza subjects had more advanced and extensive diffuse alveolar damage. However, Ackermann and his colleagues report increasing levels of IA with increasing time of hospitalization and in Influenza it remained significantly lower and relatively constant, which renders this hypothesis unlikely.

We believe that IA has been overlooked in pathology reports of patients with ARDS and Covid-19. IP cannot be detected by light microscopy requiring corrosion casting or scanning electron microscopy to be identified. Most likely, the majority of pathological studies in Covid-19 patients did not use such techniques. Furthermore, IA remains largely unknown and poorly studied and a high degree of suspicion is necessary to identify IA. IP could be misidentified as artifacts or simply overlooked. Therefore, researchers should be aware of this phenomenon when conducting pathology studies in Covid-19 populations.

Overview of IA mechanisms and role

Definition of IA:

IA was first noticed in 1986 by Caduff *et al* while studying angiogenesis in the postnatal rat lung [23]. Caduff *et al* noticed small holes in sheet-like regions of the microvasculature which enlarge to constitute a microvascular network. This was later proved by his co-author Burri and his colleague Tarek in 1990 [24].

IA is a dynamic process of microvascular growth and development through the formation of IPs that span and divide the lumen of capillaries in order to form neovascular networks. This opposes the classical form of angiogenesis, the SA, whose mechanisms and regulations are outside the scope of this review and are better described elsewhere.

Mechanisms of IA:

Transluminal pillar morphogenesis is the hallmark of IA [25]. Pillar development starts with the protusion of diametrically opposed capillary walls into the lumen until direct contact between the endothelial cells in both sides is established. The result of this protrusion is the formation of a transluminal interendothelial cellular bridge. Further reorganization of the interendothelial junction results in a perforated core within the endothelial bilayer which is occupied in a centripetal fashion by cytoplasmatic processes of fibroblasts and pericytes. Pericytes and fibroblasts then secrete collagen fibrils further dividing the initial single lumen into two [26]. Alternative morphogenic mechanisms of pillar formation have been described [26] and include pillar formation by kissing or peg-like contacts, meso-like intravascular folds, merging of adjacent capillaries or splitting of intercapillary meshes.

From a mechanistic point of view, IA can be divided into three different processes: intussusceptive microvascular growth (IMG), IAR and intussusceptive branching remodeling (IBR). These processes have been well described in the embryogenesis of different organs where they occur in tandem. In post-natal organs however, the three processes mentioned have a temporospatial overlap and constitute a single process [25, 26].

IA starts with IMG, which initiates pillar morphogenesis and expansion, allowing a quick development of the capillary plexus. This constitutes a primordial vascular bed characterized by an increased surface area. IMG is a ubiquitous mechanism [27, 28] which allows rapid expansion of capillary plexus (which arise from SA or vasculogenesis) without jeopardizing vascular and hemodynamic efficiency [27]. This quick vascular expansion is particularly useful in tissues with high metabolic demands, ischemia or hypoxia allowing nutrients and oxygen to be readily delivered and metabolites to be swiftly removed [29].

However, this primordial vascular bed remains chaotic and hierarchical. IAR allows the establishment of the proper angioarchitecture by remodeling the capillary bed in an organized, branched, tree-like fashion with the differentiation of certain branches into pre-capillary arterioles and others as post-capillary venules [27-28].

The optimization of the organized vascular bed geometry is achieved with IBR. IBR increases the efficiency of nutrient and gas exchange by remodeling of some branches into areas that are poorly oxygenated and by pruning those branches that are superfluous or inefficient [27-28].

Role of IA:

IA is an important process of vascular remodeling under physiological and pathological conditions. Since its discovery, evidence of IA was found during the formation and remodeling of vascular beds in many organs including: the mammary gland [30, 31], the bone [32], the glomeruli [33, 34], the skeletal muscle [35], the ovaries [36], and others. This ubiquity of the process of IA in the human body was not exclusive of physiological mechanisms and soon, evidence of IA playing an important role in various pathological scenarios arose, including both non-neoplastic conditions [37-39] as well as neoplastic diseases [40-43].

This data proves that IA is a relevant form of angiogenesis, playing a pivotal role in a wide variety of settings, however its mechanisms remain poorly understood when compared to its counterpart, SA, namely which stimuli promote IA, which molecular mechanisms are implicated, whether it occurs in synergy with other vascularization processes, and whether it plays an advantageous role in pathological conditions.

Possible hypothesis on the occurrence of IA in Covid-19 patients

IA molecular regulation and other factors involved remain largely unknown. However, we believe that IA occurrence in Covid-19 results from the dynamic interaction between different factors:

1. Hypoxia, VEGF and angiopoietins:

Hypoxia and Hypoxia Inducible Factors:

In ARDS, inflammatory markers (e.g. IL-1) and neutrophilic mediators (e.g. ROS, elastasis, LPS) cause extensive damage to the alveoli capillary plexus and endothelium. Such damage leads to transudation of fluid to the interstitium and air spaces (during the exudative phase) and, later, exudation of neutrophils and protein-rich fluid, culminating in the formation of hyaline membranes that impair gas exchange and ultimately cause hypoxemic respiratory failure with regional alveolar hypoxia [44].

Hypoxia is a known promoter of angiogenesis [45]. In fact, local hypoxia associated with ongoing inflammatory processes results in HIF-1α stabilization and induction of its downstream pathway. HIF-1α signaling is associated to metabolic changes (induces glycolytic enzymes and GLUT translocation to maximize ATP production in anaerobic conditions, a Warburg-like effect) and acts as a gene regulator [46]. HIF-1α regulates the expression of a wide range of genes involved in vasodilation (e.g. eNOS), extracellular matrix remodeling (e.g. metalloproteinases, uPAR), and pathways classically associated with angiogenesis such as the VEGF pathway (VEGF, flt-1) and Angiopoietin/Tie2 pathway (Ang-1/2, Tie2, thrombospondin 1).

Viral infections (e.g HCV, EBV, HPV) *per se* can induce HIF-1α activation [47]. This phenomenon seems to be dual as HIF-1α can contribute to the pathogenesis of the infection itself (e.g. HIF-1α promotes neoangiogenesis in HPV-related cervical cancer). Interestingly, HIF-1α overexpression reduces ACE2 membrane expression [48]. However, this effect is damped if AT II is inhibited, suggesting a close relationship between HIF-1alpha and AT II [48]. This might explain why populations living under chronic hypoxic conditions (e.g. communities living in high altitudes such as in the Himalaya and the Andes) display less severe features of the disease [49]. Thus, it is likely that HIF-1α is induced in Covid-19 associated ARDS, however its role in IA is unknown and whether HIF-1α promotes SA and IA to the same extent requires further studies.

VEGF family:

VEGF and its receptors are widely expressed in the lung (mainly in the alveolar epithelium) even in a non-pathological state which suggests a potential physiological role of VEGF in the lung [50]. In fact, VEGF acts as a stimulant and mitogen of the alveolar epithelium [51] and primary human type 2 alveolar epithelial cells express VEGF-R2 [51] which suggests a possible autocrine role of VEGF in the air space beside its paracrine actions in the vascular bed [51]. VEGF also plays an important role in pulmonary organogenesis as VEGF antagonism during the embryonic period arrests alveolarization besides leading to impaired lung vasculogenesis [51]. On the other hand, transgenic mice lacking HIF-2alfa (leading to down-regulation of VEGF) have diminished surfactant production and die from neonatal respiratory distress syndrome (RDS). However, *in utero* administration of $VEGF_{165}$ to these mice is protective against RDS, which further corroborates the role of VEGF during embryogenesis [51]. Post-natal, VEGF-R2 blockage results in emphysema and

alveolar apoptosis without inflammatory features [51]. Thus, it is very likely that the pneumotrophic role of VEGF is not only important during embryogenesis but also in the post-natal life.

VEGF has been implicated in the pathogenesis of ARDS before the pandemic. In fact, VEGF seems to be a key regulator of the interstitial edema that happens in ARDS. This edema occurs despite normal pulmonary capillary wedge pressure and normal oncotic pressure, which implicates endothelial permeability as the main culprit. The regulation of vascular permeability is complex and many factors modulate it directly or indirectly. VEGF is one of the prominent mediators in this process. VEGF/VEGF-R2 interaction results in an intracellular cascade that among other factors, results in the downstream activation of nitric oxide synthase (NOS) and Rho-Rac pathway with subsequent involvement of junctional signaling proteins leading to increased vascular permeability [52]. Gene therapy delivering $VEGF_{165}$ and $VEGF_{164}$ to mice resulted in non-cardiogenic lung edema and an increased capillary permeability, a phenotype very similar to that of ARDS [51]. Pro-inflammatory stimuli such as LPS and neutrophil-derived enzymes are able to induce and stimulate VEGF secretion by alveolar cells and acute lung injury (ALI)/ARDS models demonstrated increased levels of VEGF in the lung, early after insult (about 96h after), simultaneously to increased protein levels and neutrophils in bronchoalveolar lavage (BAL) [53]. This suggests a pivotal role of VEGF in exudate and hyaline membranes formation phases of ARDS. However, despite increased levels of VEGF there is a loss of compartmentalization. Typically, there is a concentration gradient through the alveolar-capillary membrane with the concentration of VEGF in the air space being 500 times higher than in the capillary. In ARDS, however, there is a shift of this ratio and even though plasmatic VEGF levels increase, there is a marked reduction of the levels in the air space [51] It is possible that this reduction of VEGF in the air space results in a loss of its pneumotrophic protection and aggravation of alveolar damage.

Besides its role on vascular permeability, it is likely that VEGF signaling plays an important role in Covid-19 associated IA. VEGF family members and their pathways are the best studied angiogenic stimulatory factors promoting mitogenesis, differentiation and migration of Endothelial cells (ECs). VEGF plays a critical role in both SA and IA [28] and its actions are therapeutically modulated in settings where angiogenesis is contributing to the pathogenesis of the underlying condition (e.g. retina neovascularization and tumoral neoangiogenesis) [54]. Its role in IA was demonstrated in models of the chorioallantoic membrane (CAM) [26] as well as in human skeletal muscle [55, 56]. VEGF seems to have a very heterogenous effect and its signaling in SA and IA are dependent of a multitude of factors such as experimental model, dose, location, process [55]. While high levels of VEGF promote sprouting, lower levels of VEGF may be crucial in promoting intussusception. Such dose-dependent effect explains the transient increase in intussusception in tumor neovascular beds after treatment with anti-VEGF therapies. However, in skeletal muscle, high levels of VEGF are associated with IA mainly because there is a disruption of the concentration gradient, which is important in tip and stalk cell signaling, therefore promoting a switch from SA to IA [55, 56]. Reduced levels of VEGF are also associated with vascular pruning and may play a role in IBR [28].

In Covid-19 patients, high plasmatic levels of VEGF have been reported, from the early stages of the disease [57]. This is consistent to previous findings in ARDS patients as stated above. It is therefore likely that there is a disruption of the alveolar-plasmatic VEGF gradient in Covid-19 patients, a factor that aggravates diffuse alveolar damage. It is also likely that this event occurs in the early stages of disease. As previously emphasized, disruption of the concentration gradient of VEGF may promote shifting from SA to IA. However, as IA seems to be a late feature, associated with prolonged hospitalization, it is unlikely that VEGF *per se* can cause angiogenesis by splitting. Other factors may as well contribute to IA in Covid-19 patients.

Angiopoietin family:

VEGF's action depends on the synergism with many factors. It is likely that VEGF and angiopoietins act synergistically in IA as VEGF overexpression associated with Ang-1/2 overexpression results in a higher number of small holes in primordial capillary plexuses, a sign of increased intussusception [28]

Angiopoietins are important signaling molecules that play an important role in EC-pericyte crosstalk. Angiopoietin 2 is stored in Weibel-Palade bodies of ECs. Secretion of Ang-2 is stimulated by hypoxia (the hallmark of ARDS), TNF-alpha (typically elevated in Covid-19), turbulent flow and thrombin (the latter two occur in CAC). Therefore angiopoetin-2 has been used as a marker of endothelial activation and is associated with endothelial disfunction [58]. In Covid-19 patients, Ang-2 levels rise several days after VEGF levels, probably reflecting extensive endothelial disfunction and activation [58].

High levels of VEGF in conjunction with high levels of Ang-2 in the latter stages of Covid-19 could be the molecular drive to promote IA. There are four Ang ligands (Ang-1 to 4) that bind to two receptors (Tie-1/2). Even though Ang-1 itself cannot initiate angiogenesis, it acts on the receptor Tie2 to promote vessel remodeling, maturation and stabilization. This is crucial and knockout rats lacking Ang1/Tie2 have impaired angiogenesis. Embryological evidence from knockout rats also supports this synergism as VEGF lacking mice die early during embryogenesis from absence of vascularization (elucidating its role in both SA and IA) and Ang-1 lacking mice die later in life with problems related to vessel manutention, remodeling and immaturity [28]. Thus, it is likely that while VEGF may act as a promoter of IA, Angiopoietins may play a crucial role in subsequent phases of this process [28]. Ang-2 acts as an Ang-1 antagonist and promotes vascular regression, disrupts vessel integrity and promotes endothelial cell death. Ang-2 also acts with PDGF to promote pericyte division, migration and recruitment, which is important in pillar morphogenesis [28]. This is corroborated by the fact that Tie2-knockout mice have impaired pillar morphogenesis [28]. Angiopoietin expression is modulated by shear stress. In fact, laminar shear stress down-regulates Ang-2 expression while turbulent shear stress promotes the opposite [28]. As discussed below, microthrombi in Covid-19 disrupt local hemodynamic conditions and promote turbulent blood flow. Oscillatory shear stress acts on the endothelium and promotes Ang-2 expression [28] as well as cell migration, proliferation and tubular formation. Increased shear stress also acts on the vascular endothelial growth factor (VEGF) pathway as it increases *in vitro* VEGFR-2 expression and enhances VEGF mitogenic activity via NO [28].

2. Microangiopathic thrombosis, hemodynamic and platelet derived factors:

Covid-19 is associated with extensive microangiopathic thrombosis. Obstruction of the pulmonary microcirculation likely result in significant changes in hemodynamic conditions. The MYSTIC study [16] reported significant reductions of V_{RBC} associated to capillary density loss in Covid-19 patients. This loss is almost exclusive to small capillaries between 4–6 μm and results in a lower area of gas exchange, contributing to worsening hypoxia. D-dimers correlate strongly with vascular density which suggests a causality between microthrombi and vascular regression.

Potentially, this could be explained by a mechanism of intussusceptive branching remodeling and pruning. Microthrombi result in vasooclusion and acute reductions in blood flow, shear stress together with high transmural pressures caused by the thrombi could promote IBR. As stated above, this would optimize the disrupted angioarchitecture, reconducting blood flow to spared areas. Local changes in hemodynamic forces have a strong repercussion in IA mechanisms. In fact, it has been demonstrated that IMG and IAR occur predominantly in regions with high blood flow [59,

60]. Blood flow redistribution to patent branches results in areas of high blood flow with high distending forces promoting IP morphogenesis and high shear stress promoting arborization of the newly formed branches by IAR.

Platelet derived factors may also play a role in IA apart from hemodynamic factors. PDGF-BB markedly accelerates the splitting process [56]. PDGF-BB modulates VEGFR-2, promotes pericyte survival and controls vascular circular expansion [56]. This is protective against aberrant angiogenesis promoted by high levels of VEGF [55,56].

3. Inflammation, endothelialitis and cytokine storm:

The immune system also plays an important role in IA. In fact, mononuclear cells stabilize IP by protruding uropod-like projections and producing collagen [61]. The role of mononuclear cells such as lymphocytes or monocytes was first described in a Notch knockout model [61]. Notch inhibition has been associated with mononuclear cell recruitment and migration as well as with increased pillar morphogenesis.

While the precise mechanism is unclear, it has been shown that this process depends on chemokines such as CXCL12 or SDF-1 [61,62]. SDF-1 is responsible for the Notch-dependent mobilization of mononuclear cells acting in MMP-9 [63]. Furthermore SDF-1 also acts in CXCR4 receptor in mononuclear cells to promote the formation of the uropod-like that stabilize the IP's [63].

VEGF and bFGF promote IA by acting on the SDF-1/CXCR4 pathway [62]. In fact, a study using a mouse model of diabetic retinopathy reported increased retinal neovascularization after vitreal injection of SDF-1 [64]. Hypoxia, a known angiogenic trigger, promotes SDF-1 up-regulation and SDF-1/CXCR4 pathway seems to play an important role in re-establishing blood flow to ischemic zones through IA [61-63].

SDF-1/CXCR4 are associated with T-lymphocyte infiltrates in Covid-19 [65]. It is therefore likely that a positive feedback between shear stress alterations induced by inflammation (particularly T-cell), SDF-1, hypoxia and eNOS promote angiogenesis. This represents a vascular adaptation in order to maintain high blood flow to areas with prominent inflammation. T-cell infiltrates have a dynamic interaction with endothelial cells and a phenomena of endotheliatis has been described in Covid-19. This further contributes to prolonged local inflammation and therefore, promotes angiogenesis. In endothelialitis, T-cell infiltrates activate ECs which in turn express a wide array of genes. Activated ECs express high levels of TLRs and MyD88. These molecules recognize SARS-CoV-2 and promote cytokine, chemokine and adhesion molecule expression as well as angiogenic factors such has VEGF, NOS and CD14 monocytes recruitment [65].

One of the most prominent features of COVID-19 is an inappropriate and excessive inflammatory response with concomitant release of large amounts of pro-inflammatory cytokines, a process termed "cytokine storm" (CS) [66]. Cytokine storm relates to the severity of Covid-19 and is directly correlated with lung injury, multi-organ disfunction and adverse outcomes in Covid-19 patients [66, 67].

The main contributors of CS are IL-6 and TNF-alfa. In fact, IL-6 is the most frequently reported cytokine to be elevated in Covid-19 and IL-6 levels are independently associated with higher mortality [67]. IL-6 has been implicated in pathogenic angiogenesis in different scenarios (rheumatoid arthritis, stroke and different cancers). Studies in tumors have shown that IL-6 levels are directly correlated with VEGF levels and vessel density [69]. The published literature suggests that IL-6 can drive aberrant angiogenesis [68]. IL-6 promotes the formation of defective vascular beds with abnormal pericyte coverage. Loss of pericytes has been described in Covid-19 [70], however it was postulated that this was a direct effect of viral infection as pericytes express ACE2. We suggest that IL-6 might be a contributor to this alteration. As stated above, pericytes are directly implicated in IA and pillar morphogenesis. Loss of pericytes has been previously implicated

as an angiogenic driver, promoting both SA and IA [71] Thus, the aberrant effects of IL-6 on pericytes could be related to IA in Covid-19 [68]. Furthermore, studies showed that IL-6-trans-signaling inhibits proliferation and tube formation of ECs that could also explain the shift from SA to IA in Covid-19 [71].

Prognostic and therapeutical implications of IA in Covid-19

Ackermann et al reported increasing density of angiogenic features with increasing duration of hospitalization, suggesting IA as a potential prognostic tool in Covid-19 patients. In fact, angiogenic mediators have an established prognostic role in ARDS. Positive correlations between VEGF, soluble form of VEGFR2 (sVEGFR2), Ang-2 and Ang-2/Ang-1 ratio and ARDS development in critical illness have been described [53]. Moreover, Ang-2 and sVEGFR2 are also associated with adverse outcomes and higher risk of mortality in ARDS patients [53, 72, 73].

Angiopoietin-2, is not only an angiogenic mediator but it is also a marker of endothelial activation [58]. Angiopoetin-2 levels have been consistently reported elevated in critical Covid-19 patients. High levels of Ang-2 are also associated with poor lung compliance, higher CRP and D-dimers [58, 74, 75], thus reflecting CAC in Covid-19 patients. Ang-2 is associated with worse outcomes in Covid-19, predicting ICU admission, mechanical ventilation, and death [58,74]. Therefore, Ang-2 is a strong prognostic biomarker in critical Covid-19 patients. However, whether that reflects only endotheliopathy and EC activation or also IA is unknown. It is possible that EC activation triggers Ang-2 secretion which in turn promotes angiogenesis, therefore linking endothelial dysfunction and IA. It is also unknown if Ang-2 levels correlate with IA in critical Covid-19 patients, although that hypothesis seems likely.

Similar results are found regarding VEGF. Data suggests that VEGF-A and Flt-1 levels are elevated in both non- and in critical patients [76]. VEGF-D levels are lower in patients in mechanical ventilation than those who are not [77]. However, plasmatic VEGF levels must be analyzed with caution as plasma VEGF changes may not reflect local VEGF changes and VEGF may be sequestered by tissues as most VEGF isoforms are heparin binding.

Other endothelial dysfunction markers have been described in critical ICU patients such as follistatin, PAI-1, sE-selectin and vWF [74-76]. These markers are also more elevated in patients that died than survivors, suggesting a potential prognostic role.

Biomarkers of angiogenesis are also associated with other factors such as vascular permeability and endothelial dysfunction. Further studies should focus on confirming whether IA is an independent prognostic factor in Covid-19. Direct quantification of angiogenic features would be a better way of establishing that, than using indirect biomarkers such as Ang or VEGF because the latter are susceptible to many factors implicated in Covid-19's pathogenesis.

Therapeutic modulation of IA is a potential adjuvant therapy in Covid-19. Currently, the anti-angiogenic drug, bevacizumab is being studied in Covid-19 (NCT04344782, NCT04305106 and NCT04275414). Bevacizumab is a monoclonal antibody that targets VEGF-A and inhibits its actions. It has been used in the oncotherapy of different neoplasms with efficacy and safety [78]. A recent single-arm trial (NCT04275414) suggested beneficial results of using Bevacizumab as an add-on therapy [79]. They report improvements in oxygenation (reflected by PaO_2/FiO_2 ratio, oxygen support), radiologic improvement (significant reductions in lesions and ratios in CT scan or X ray), fever and lymphocyte count. Although anti-angiogenic, degree of IA was not taken into consideration in this study. In fact, the authors suggest that the beneficial effects of bevacizumab in oxygenation are related to modulation of vascular leakage and diminishing pulmonary edema (a rationale similar to the effect of bevacizumab in capillary-leakiness in age-related macular degeneration). Interestingly, they report positive outcomes of fever resolution and lymphocyte count. In fact, VEGF has had a speculative role in immunopathology. It is known that VEGF promotes inflammatory cell mobilization to

pathological tissues, therefore bevacizumab's anti-pyretic effect could be related to antagonism of inflammation caused by SARS-CoV-2 infection, as bevacizumab also resulted in lower CRP levels in Covid-19 patients. Another striking finding is that bevacizumab improves lymphopenia, associated with worse outcomes in Covid-19. Although the exact mechanism is unknown, it is hypothesized that bevacizumab might affect lymphocyte extravasation and redistribution. Further randomized control trials are needed to establish the use of bevacizumab in Covid-19 and to assert its effects on intussusception.

Conclusion

The pathogenesis of SARS-CoV-2 infection is complex and different pathogenic factors contribute to the severity of the infection. Among those, vascular repercussions of the infection are a major threat, severely worsening prognosis and significantly increasing the mortality. Notwithstanding the fact that a lot of effort was put into studying these vascular repercussions (including the endothelial dysfunction), intussusceptive angiogenesis, a prominent feature of Covid-19, remains poorly understood. There are significant gaps in knowledge regarding splitting angiogenesis including molecular control and regulation, microenvironmental factors at play and therapeutic and prognosis implications. In this article, we reviewed the main characteristics and mechanisms of IA. We formulated a theoretical model hoping to explain the occurrence of splitting angiogenesis in Covid-19. We conclude that although there's much to learn regarding this subject, IA is likely the product of hypoxia and hypoxia-inducible factors, classical angiogenic molecular factors (e.g. VEGF, Ang-2), hyperinflammation and cytokine storm, thrombosis and associated hemodynamic changes and RAAS dysregulation. To our knowledge this is the most comprehensive review regarding this intriguing feature of Covid-19.

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Scale for the Assessment of Narrative Review Articles – SANRA

Please rate the quality of the narrative review article in question, using categories 0–2 on the following scale. For each aspect of quality, please choose the option which best fits your evaluation, using categories 0 and 2 freely to imply general low and high quality. These are not intended to imply the worst or best imaginable quality.

Nota: Para a UC de Dissertação/Projecto, todos os seis items devem ser cumpridos em nível 2.

1) Justification of the article's importance for the readership

The importance is not justified. — 0
The importance is alluded to, but not explicitly justified. — 1
The importance is explicitly justified. — 2

2

2) Statement of concrete aims or formulation of questions

No aims or questions are formulated. — 0
Aims are formulated generally but not concretely or in terms of clear questions. — 1
One or more concrete aims or questions are formulated. — 2

2

3) Description of the literature search

The search strategy is not presented. — 0
The literature search is described briefly. — 1
The literature search is described in detail, including search terms and inclusion criteria. — 2

2

4) Referencing

Key statements are not supported by references. — 0
The referencing of key statements is inconsistent. — 1
Key statements are supported by references. — 2

2

5) Scientific reasoning

(e.g., incorporation of appropriate evidence, such as RCTs in clinical medicine)

The article's point is not based on appropriate arguments. — 0
Appropriate evidence is introduced selectively. — 1
Appropriate evidence is generally present. — 2

2

6) Appropriate presentation of data

(e.g., absolute vs relative risk; effect sizes without confidence intervals)

Data are presented inadequately. — 0
Data are often not presented in the most appropriate way. — 1
Relevant outcome data are generally presented appropriately. — 2

2

Sumscore 12

Fig. 1 SANRA - Scale

: O ponto 5 diz respeito à contextualização da evidência científica em relação ao tipo de estudo(s) que a produziu (e, idealmente, à qualidade do(s))(s)). O ponto 6 diz respeito a sustentar as afirmações com os dados quantitativos mais apropriado. A título de exemplo, para cumprir o ponto 5 e c :s de afirmar "já foi evidenciado que os testes de alergia às penicilinas apresentam alta especificidade e moderada sensibilidade", dever-se-á indica) sistemática com meta-análise de acuidade diagnóstica evidenciou que os testes de alergia às penicilinas apresentam alta especificidade (valor me :o: 97%; IC95%=94-98%) e moderada sensibilidade (valor meta-analítico: 31%; IC95%=19-46%).

SANRA – explanations and instructions

This scale is intended to help editors assess the quality of a narrative review article based on formal criteria accessible to the reader. It cannot cover other elements of editorial decision making such as degree of originality, topicality, conflicts of interest or the plausibility, correctness or completeness of the content itself. SANRA is an instrument for editors, authors, and reviewers evaluating individual manuscripts. It may also help editors to document average manuscript quality within their journal and researchers to document the manuscript quality, for example in peer review research. Using only three scoring options, 0, 1 and 2, SANRA is intended to provide a swift and pragmatic sum score for quality, for everyday use with real manuscripts, in a field where established quality standards have previously been lacking. It is not designed as an exact measurement of the quality of all theoretically possible manuscripts. For this reason, the extreme values (0 and 2) should be used relatively freely and not reserved only for perfect or hopeless articles.

We recommend that users test-rate a few manuscripts to familiarize themselves with the scale, before using it on the intended group of manuscripts. Ratings should assess the totality of a manuscript, including the abstract. The following comments clarify how each question is designed to be used.

Item 1 – Justification of the article's importance for the readership

Justification of importance for the readership must be seen in the context of each journal's readership.
Consider how well the manuscript outlines the clinical problem and highlights unanswered questions or evidence gaps – thoroughly (2), superficially (1), or not at all (0).

Item 2 – Statement of concrete/specific aims or formulation of questions

A good paper will propose one or more specific aims or questions which will be dealt with or topics which will be reviewed.
Please rate whether this has been done thoroughly and clearly (2), vaguely or unclearly (1), or not at all (0).

Item 3 – Description of the literature search

A convincing narrative review will be transparent about the sources of information on which the text is based. Please rate the degree to which you think this has been achieved. To achieve a rating of 2, it is not necessary to describe the literature search in as much detail as for a systematic review (searching multiple databases, including exact descriptions of search history, flowcharts, etc.), but it is necessary to specify search terms, and the types of literature included. A manuscript which only refers briefly to its literature search would score 1, while one not mentioning its methods would score 0.

Item 4 – Referencing

No manuscript references all statements. However, those that are essential for the arguments of the manuscript – "key statements" – should be backed by references in all or almost all cases. Exceptions could reasonably be made for rating purposes where a key statement has uncontroversial face-validity, such as "Diabetes is among the commonest causes of chronic morbidity worldwide."
Please rate the completeness of referencing: for most or all relevant key statements (2), inconsistently (1), sporadically (0).

Item 5 – Scientific reasoning

The item describes the quality of the scientific point made. A convincing narrative review presents evidence for key arguments. It should mention study design (randomized controlled trial, qualitative study, etc), and where available, levels of evidence.
Please rate whether you feel this has been done thoroughly (2), superficially (1), or hardly at all (0). Unlike item 6, which is concerned with the selection and presentation of concrete outcome data, this item relates to the use of evidence and of types of evidence in the manuscript's arguments.

Item 6 – Appropriate presentation of data:

This item describes the correct presentation of data central to the article's argument. Which data are considered relevant varies from field to field. In some areas relevant data would be absolute rather than relative risks or clinical versus surrogate or intermediate endpoints. These outcomes must be presented correctly. For example, it is appropriate that effect sizes are accompanied by confidence intervals. Please rate how far the paper achieves this – thoroughgoingly (2), partially (1), or hardly at all (0). Unlike item 5, which relates to the use of evidence and of types of evidence in the manuscript's arguments, this item is concerned with the selection and presentation of concrete outcome data.