Neutrophil extracellular traps: key drivers of severe COVID-19

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Les filets du neutrophile: acteurs clés des formes sévères de la Covid-19

Covid-19, neutrophil, respiratory failure, inflammation, thrombosis
Covid-19, neutrophile, défaillance respiratoire, inflammation, thrombose

Abstract

In severe COVID-19, hyperinflammatory tissue-damaging, thromboembolic or immunothrombotic responses triggered by SARS-CoV-2 are major causes of respiratory failure and death. Neutrophil extracellular traps (NETs), released by activated neutrophils during a process known as "NETosis", can be formed in the lungs upon infection with respiratory viruses. They have the ability to promote lung damage, thrombosis and fibrosis, three cardinal features encountered in severe COVID-19. In a recent study, we sought to understand how NETosis could be related to lung immunopathological changes associated with fatal cases of the disease. We assessed whether NET structures could be identified in post-mortem lung biopsies from COVID-19 patients, and whether they were located in particular lesions and microanatomical lung compartments. We performed immunofluorescence staining of myeloperoxidase, citrullinated histone H3 and nuclear acid (DAPI) on sections of paraffin-embedded lung biopsies from four COVID-19 patients who succumbed to COVID-19 and from four patients who died from a cause unrelated to COVID-19. The former four patients represented prototypical severe and fatal cases of COVID-19, characterised by pneumonia and fatal respiratory distress associated with signs of systemic inflammation, neutrophilia and coagulopathy. NETs were uniquely detected in the lungs of all the

Résumé

Dans les formes sévères de la Covid-19, les réponses inflammatoires exacerbées, les dommages tissulaires subséquents, ainsi que les évènements thromboemboliques ou immunothrombotiques déclenchés par le Sars-Cov-2 sont les principales causes d'insuffisance respiratoire et de décès. Il est connu que les filets du neutrophile (NET), libérés lors d'un processus appelé NETose, peuvent se former dans les poumons suite à une infection par des virus respiratoires. Ces structures extracellulaires ont la capacité de provoquer des lésions pulmonaires et de favoriser la formation de thrombi et la fibrose, complications majeures de la Covid-19 sévère. S Dans une étude récente, nous avons cherché à comprendre comment la NETose pouvait être liée aux changements immunopathologiques pulmonaires associés à des cas mortels de la maladie. Nous avons recherché ces structures NET dans les biopsies pulmonaires post-mortem de patients Covid-19. Nous avons ensuite analysé leur localisation dans les lésions et dans les différents compartiments micro-anatomiques des poumons. À cette fin, nous avons effectué une coloration par immunofluorescence de la myéloperoxydase, de l'histone citrullinée H3 et de l'acide nucléique (DAPI) sur des coupes de biopsies pulmonaires incluses dans la paraffine de quatre patients Covid-19 et de quatre patients décédés d'une cause non liée à la Covid-19. Les premiers représentaient des cas

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COVID-19 patients. Detailed histopathological analysis revealed widely distributed NET-infiltrating areas encompassing several lung compartments, including arteriolar microthrombi, neutrophil-rich inflammatory areas of the lung interstitium, as well as the alveoli or bronchioles, where they often co-localised with occluding fibrin-rich deposits. Another study published simultaneously to ours provides the first experimental evidence of causality between NETosis and lung injury in severe COVID-19. However, the possible involvement of NETs in thrombogenesis remains to be addressed. Nevertheless, these data support the hypothesis that NETs may represent drivers of COVID-19-associated severe pulmonary complications and suggests that NET-targeting approaches could represent potential future avenues for the treatment of uncontrolled tissue-damaging, thrombotic or fibrotic responses to SARS-CoV-2.

prototypiques graves et mortels de la Covid-19, caractérisés par une pneumonie et une insuffisance respiratoire fatale associées à une inflammation systémique, une neutrophilie et une coagulopathie. Nous avons détecté des NET dans les poumons de chaque patient Covid-19. Une analyse histopathologique détaillée a révélé de larges zones d'infiltration de NET dans plusieurs compartiments pulmonaires correspondant aux microthrombi artériolaires, aux zones inflammatoires riches en neutrophiles de l'interstitium pulmonaire ainsi qu'aux alvéoles ou bronchioles où elles étaient souvent colocalisées avec des dépôts occlusifs de fibrine. Une autre étude, publiée simultanément à la nôtre, a fourni les premières preuves expérimentales de l'existence d'un lien causal possible entre la NETose et les lésions pulmonaires. Môme si l'implication des NET dans les évènements thrombotiques associés à la Covid-19 n'a pas encore été démontrée, ces données soutiennent l'hypothèse selon laquelle les NET représenteraient des acteurs clé des complications pulmonaires associées à la maladie. Elles suggèrent également que le ciblage des NET pourrait représenter des voies potentielles pour le traitement des réponses inflammatoires et thrombotiques à l'infection par le Sars-Cov -2.

Since its emergence in the Wuhan region of China in late December 2019, the new coronavirus SARS-CoV-2 (for severe acute respiratory syndrome coronavirus 2) has spread rapidly around the world [1]. The World Health Organization declared it a pandemic on 11th March 2020. As of 28th July 2021, the number of confirmed cases of COVID-19, the disease caused by SARS-CoV-2, stood at 195,368,552 and the virus had caused the deaths of 4,178,287 patients (John Hopkins Center for Systems Science and Engineering, https://coronavirus.jhu.edu/map.html). After mid-April 2020, which saw the first wave and first peak of the pandemic, the world experienced two other successive waves which varied in severity depending on the continent. At the time of writing, Europe is facing the beginning of a fourth wave, probably due to the emergence and high contagiousness of the delta variant of the virus.

Clinical characteristics of COVID-19

Patients with COVID-19 most often present with fever, dry cough, fatigue and/or myalgia. Less common symptoms may include gastrointestinal disorders, conjunctivitis, headaches, loss of smell or taste, and skin manifestations. Although most infected patients develop a mild form of the disease that does not require hospitalisation, in others, COVID-19 can rapidly progress to acute respiratory distress syndrome (ARDS) and respiratory failure due to severe lung injury in the form of interstitial pneumonia and diffuse alveolar damage, accompanied by arterial thrombi and numerous microthrombi in the microvessels [2, 3]. Furthermore, the progression of COVID-19 to a critical state is accompanied by a significant increase in systemic cytokines, such as interleukin (IL)-1β, IL-4, IL-6, IL-10, tumour necrosis factor (TNF)-α and interferon (IFN)-γs? This is similar to a cytokine storm, as described in sepsis. According to initial studies, approximately 70% of patients who died following COVID-19 developed disseminated intravascular coagulation (DIC), as evidenced by systemic activation of coagulation with clot formation in small and medium-sized blood vessels, responsible for the development of systemic multivisceral failure [4].

In addition, a number of the most severely affected patients have concomitant cardiac damage, such as acute myocarditis, acute coronary syndrome, rhythm disturbances or heart failure, which may progress to cardiogenic shock and death. Overall, about 25% of patients hospitalised with COVID-19 require admission to an intensive care unit (ICU) and 10% require respiratory support.

In addition to increased markers of inflammation, the majority of hospitalised patients have elevated D-dimer levels, which reflect both a thrombotic process followed by activation of fibrinolysis and systemic inflammation. Several studies indicate that high D-dimer levels are associated with increased mortality [5]. Patients with COVID-19 are at an increased risk of venous thromboembolism (VTE) and arterial thromboembolism (myocardial infarction and stroke). Clot formation in extracorporeal circuits or vascular access routes is also frequently observed. For example, VTE, pulmonary embolism or deep vein thrombosis were initially reported in 25–69% of patients admitted to ICUs and were responsible for high morbidity and mortality. According to a recent meta-analysis, the incidence rate of VTE is as high as 28% in the ICU, while the rate is about 7% for patients hospitalised outside the ICU. The risk factors for VTE are the development of ARDS and the advanced age of the patients. People with pre-existing health problems are more likely to become seriously ill as a result of the infection and are, therefore, more likely to develop thrombotic complications associated with COVID-19. Nevertheless, the rate of pulmonary embolism in ICUs is higher than that usually seen in non-COVID-19 patients. In addition to pulmonary embolism, several ante- and post-mortem pathological studies reveal a generalised thrombotic phenomenon in the lungs. This is characterised by the presence of non-occlusive arterial thrombi and numerous microthrombi in the alveolar capillaries [3]. It is estimated that there are up to nine times more microthrombi in COVID-19 cases than in cases of ARDS caused by the influenza virus. Interestingly, not only are the microthrombi observed in the lungs of COVID-19 patients rich in platelets and fibrin, but they also contain neutrophils, indicating an underlying thrombo-inflammation process (or immunothrombosis) [6]. Extravascular fibrin deposits are also observed, due to breaches and unrepaired vascular damage.

Neutrophil extracellular traps and lung damage

The lungs are the main organ affected in COVID-19, in the form of both ARDS and pulmonary thrombotic events, both of which are associated with a poor prognosis. In a recent study, we therefore looked at the immunopathological changes that accompany these severe forms of the disease [7]. Neutrophils were the focus of our attention. These cells of the innate immune system are the most abundant leukocytes in the blood and their numbers increase in severe forms of COVID-19. Furthermore, this increase in the circulating neutrophil count was associated with the severity of respiratory symptoms and a poor clinical prognosis [8, 9]. The presence of these cells in patients' lung tissue had also been described. An interesting feature of these cells is their ability to produce NETs, made up of DNA, modified histones and granule proteins such as elastase and myeloperoxidase (MPO) [10]. The process of releasing these NETs, known as “NETosis”, is involved in the eradication of microbes, but has also been implicated in a number of conditions such as rheumatoid arthritis, diabetes, sepsis and cancer. Mechanistically, this process is initiated by neutrophil activation via receptors recognising microbial component motifs or chemokines, followed by the production of reactive oxygen species and the mobilisation of intracellular calcium, which activates the protein PAD-4 (peptidyl arginine deiminase-4), an intracellular enzyme involved in the deamination of arginine residues on histones [11]. Interestingly, our team has previously shown that NETs can be formed in the lungs in response to viral infections [12]. They can increase tissue damage and contribute to the formation of

thrombi and fibrosis, three characteristics of severe COVID-19 lung disease. As such, based on several reports, NETs are proposed to play a role in the development of severe forms of COVID-19 [9]. In addition, components of NET have been detected in patient sera and correlated with disease severity [13].

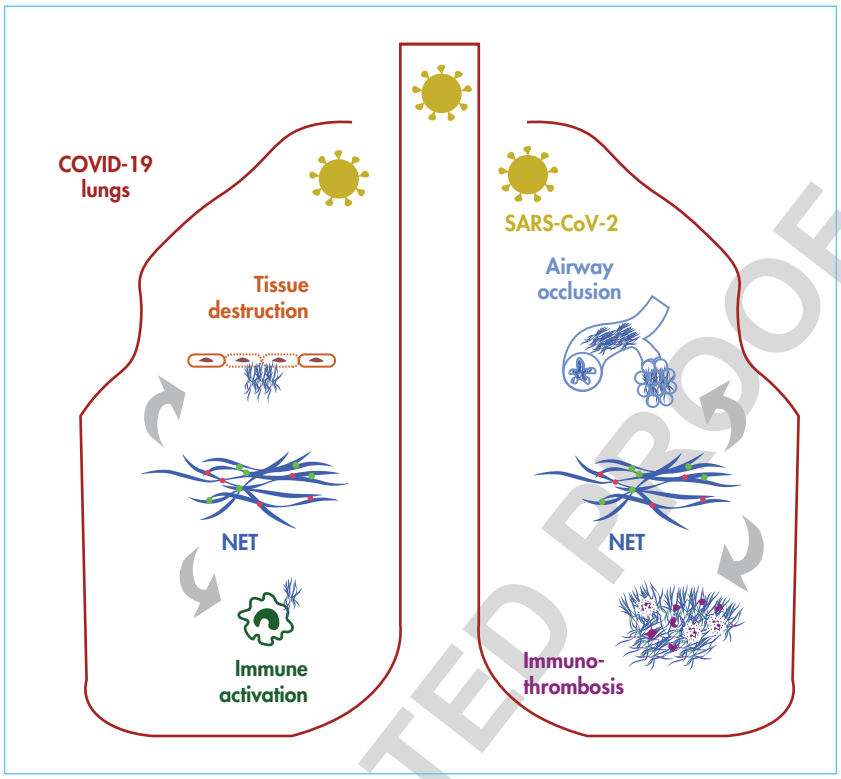
In our study, we performed immunofluorescence staining on post-mortem lung biopsies from COVID-19 patients to assess whether NETs could be identified in the lungs of these patients and, if so, whether they were localised in particular lesions and microanatomical compartments.

Post-mortem lung samples were taken from four patients who died of COVID-19 at the University Hospital of Liège (CHU Liège, Belgium) in March or April 2020. The age of the patients ranged from 51 to 73 years, and one patient was female. Each case had tested positive for SARS-CoV-2 by nasopharyngeal swab and PCR at the time of admission. The total duration of hospitalisation until death was between eight and 32 days. Respiratory support was in place at the time of admission for three patients, and at three days after admission for the remaining patient. All patients were treated in hospital with hydroxychloroquine and antibiotic therapy. In the 24 hours prior to death, each patient had blood neutrophilia and elevated serum C-reactive protein levels. Serum IL-6 levels were elevated in all three patients for whom they were assessed; D-dimer levels were also above normal in all four patients (>500 mg/L). Lymphopaenia and thrombocytopaenia were observed in three of the four patients. The causes of death identified were pneumonia, ARDS or multivisceral failure. Typical diffuse alveolar lesions were confirmed by histopathological examination. Thus, the four patients represented prototypical severe cases of COVID-19, characterised by fatal pneumonia and respiratory failure associated with signs of systemic inflammation, neutrophilia and coagulation disorders. We detected NETs–defined as extracellular triple-positive areas for DNA (by diamidino-phenylindole [DAPI]), MPO and citrullinated histone H3 (Cit-H3)–in lung sections from COVID-19 patients, but not in lung tissue sections from four patients who died due to a non-COVID-19 related cardiac cause, in the absence of other infections. In addition, in order to determine whether NET formation was specific to lung tissue in COVID-19 patients, we performed similar staining on liver, heart, kidney and pancreas sections from COVID-19 patients for whom such samples were available. We found no evidence of NET formation in these organs, suggesting that the systemic presence of NET components reported in the sera of COVID-19 patients is not associated with generalised multi-organ NETosis [13].

Detailed histopathological analysis revealed multiple areas of NET infiltration in the lungs of COVID-19 patients (*figure* 1). Firstly, NETs were detected in the alveolar or bronchial compartments in all four patients, where they were often associated with fibrin, resulting in almost complete occlusion of some alveoli or bronchioles. Therefore, in the airway compartment, NETs could represent major procoagulant triggers, leading to fibrin deposition and impaired lung ventilation [14]. In advanced stages, NETs may be replaced by collagen fibres, thus contributing to the lung fibrosis previously described. They may also facilitate secondary infections, such as those seen in cystic fibrosis. In our study, two of the four patients developed a secondary lung infection during hospitalisation. Although NET formation can be induced by bacterial mediators during secondary infection, we found a massive NET presence in every patient, regardless of the possible occurrence of secondary infection. It is therefore unlikely that secondary infection alone is responsible for the massive and multifocal infiltration of NETs in our study.

Secondly, the pulmonary interstitial compartment also contained NETs in two patients, particularly in areas infiltrated by neutrophils and macrophages; few lymphocytes were observed. The proximity of NET-releasing neutrophils to macrophages is consistent with the hypothesis that NETs may contribute to the

FIGURE 1



Neutrophil nets (NET), located in the airways, interstitium and pulmonary microvessels, are thought to play a key role in severe forms of COVID-19.

cytokine storm associated with COVID-19, by promoting IL-1β by macrophages, which in turn would increase NET formation and IL-6 secretion, as described in other contexts [15].

Thirdly, NET-rich areas were located in the vascular compartment in the lungs of three patients, mainly in arterioles containing microthrombi. Importantly, in the microthrombi we observed many Cit-H3 and MPO double-positive neutrophils rather than filamentous NETs, indicating an earlier stage of NET formation than in the other compartments. This observation is consistent with the study by Middleton *et al.* [6]. Whether NETs contribute to the formation of pulmonary microthrombi associated with COVID-19 remains an open question and deserves further study. Indeed, in the inflammatory context induced by infection, NETs represent a mechanism by which neutrophils could promote the formation of thrombi [14]. NETs are a structure that allows the adhesion of platelets, adhesion molecules such as Von Willebrand factor (VWF) and fibrinogen, and red blood cells. They induce platelet activation and aggregation via histones and NET components, such as DNA, elastase and cathepsin G, and can activate tissue factor or factor XII-dependent coagulation, resulting in the generation of fibrin and the formation of platelet-rich and fibrin-rich thrombi.

Although performed on a small number of patients, our study is the first to show the presence of NET in several lung compartments. Although no causal link has been demonstrated, our data support the hypothesis that neutrophils, NETs or the activation pathways involved in their formation could be potential therapeutic targets against the inflammatory, thrombotic or fibrotic manifestations of severe

forms of COVID-19, which are responsible for lung damage and subsequent respiratory failure.

There is currently no treatment for COVID-19, although several clinical trials are under way. Our study raises new hopes. On the one hand, since dysregulated NET formation could aggravate lung damage and impair gas exchange, inhalation of recombinant human DNAse I (pulmozyme/dornase alfa) could improve lung function and limit secondary infections. In this regard, the COVIDornase clinical trial is currently investigating the potential benefit of aerosolised dornase alfa in patients hospitalised for COVID-19 with ARDS [16].

On the other hand, since thrombotic events contribute to the morbidity and mortality of COVID-19, the guidelines issued by international medical societies recommend the use of heparin-based regimens for thromboprophylaxis and anticoagulation treatment of VTE [17]. Our study, therefore, reveals the possibility of considering a complementary antithrombotic approach that could be beneficial against both thrombo-inflammation (or immunothrombosis) and VTE. It is also interesting to note that the prothrombotic effect of NETs may be diminished by heparin [18]. This may be related to the results of early non-randomised studies showing lower mortality in patients undergoing heparin thromboprophylaxis than in untreated patients, particularly in patients with high D-dimer levels or who are on respiratory support [19]. Numerous randomised clinical trials are under way to define the optimal antithrombotic approach to be used depending on the level of severity of COVID-19 [20]. The complexity of choosing these treatments lies in the fine balance between protection and deleterious effects due to possible bleeding complications. The first results of two clinical trials have just been published. The INSPIRATION clinical trial indicated that increased doses of heparin (intermediate dose) in the ICU did not reduce arterial or venous thrombotic complications, or mortality compared with standard prophylactic doses [21]. Similarly, the ACTION randomised trial in hospitalised patients with elevated D-dimer levels showed that therapeutic anticoagulation did not improve clinical outcomes compared to standard heparin prophylaxis [22].

In parallel to our study, the study by Veras *et al.* [23] indicates a high concentration of NETs in lung autopsy samples from COVID-19 patients. This observation also shows an increase in MPO-DNA complexes in plasma and tracheal aspirate fluid from COVID-19 patients compared to the same samples from healthy donors. Given that the initial stage of SARS-CoV-2 infection involves the interaction between a viral glycoprotein, the spike protein and the ACE2 receptor (for angiotensin-converting enzyme 2) present on the surface of the host cells, such as pneumocytes, epithelial and endothelial cells, these authors reveal that neutrophils can also be infected by the virus, which induces the formation of NETs through a mechanism that is dependent on ACE2, viral replication and the enzyme PAD-4. The causal link between the presence of NETs and tissue damage is further supported by experiments conducted *in vitro* in which NETs produced by virus-infected neutrophils caused the apoptotic death of A549 lung epithelial cells. Neutrophils from COVID-19 patients produce more NETs than neutrophils from healthy donors and are also more cytotoxic towards these cells.

In conclusion, therapeutic targeting of NETs through the use of inhibitors of their formation or agents that degrade them has the potential to limit the severe complications of COVID-19. In addition, the beneficial effects of the anti-inflammatory or antithrombotic approaches being tested may be due, in part, to their indirect action of inhibiting NETosis. It would therefore be interesting to develop preclinical models that would allow a better understanding of the interrelationships between these processes during the host response to SARS-CoV-2 infection in order to define the best therapeutic approaches to adopt.

Conflict of interestQ1: The authors declare no conflict on interest concerning this article.]

References

[1] Wu F, Zhao S, Yu B, *et al.* A new coronavirus associated with human respiratory disease in China. *Nature* 2020 ; 579 : 265-9.

[2] Guan W, Ni Z, Hu Y, *et al.* Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med* 2020 ; 382 : 1708-20.

[3] Ackermann M, Verleden SE, Kuehnel M, *et al.* Pulmonary vascular endothelialitis, thrombosis and angiogenesis in Covid-19. *N Engl J Med* 2020 ; 383 (2): 120-8.

[4] Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost* 2020 ; 18 (4): 844-7.

[5] Bikdeli B, Madhavan MV, Jimenez D, *et al.* Covid-19 and thrombotic or thromboembolic disease: implications for prevention, antithrombotic therapy, and follow-up: JACC state-of-the-art review. *J Am Coll Cardiol* 2020 ; 75 : 2950-73.

[6] Middleton EA, He X-Y, Denorme F, *et al.* Neutrophil extracellular traps (NETs) contribute to immunothrombosis in Covid-19 acute respiratory distress syndrome. *Blood* 2020 ; 136 (10): 1169-79.

[7] Radermecker C, Detrembleur N, Guiot J, *et al.* Neutrophil extracellular traps infiltrate the lung airway, interstitial and vascular compartments in severe Covid-19. *J Exp Med* 2020 ; 217 (12): e20201012.

[8] Li S, Jiang L, Li X, *et al.* Clinical and pathological investigation of severe Covid-19 patients. *JCI Insight* 2020 ; 5 (12): e138070.

[9] Barnes BJ, Adrover JM, Baxter-Stoltzfus A, *et al.* Targeting potential drivers of Covid-19: neutrophil extracellular traps. *J Exp Med* 2020 ; 217 (6): e20200652.

[10] Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* 2018 ; 18 : 134-47.

[11] Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* 2010 ; 207 : 1853-62.

[12] Radermecker C, Sabatel C, Vanwinge C, *et al.* Locally instructed CXCR4hi neutrophils trigger environment-driven allergic asthma through the release of neutrophil extracellular traps. *Nat Immunol* 2019 ; 20 : 1444-55.

[13] Zuo Y, Yalavarthi S, Shi H, *et al.* Neutrophil extracellular traps in Covid-19. *JCI Insight* 2020 ; 5 (11): e138999.

[14] Laridan E, Denorme F, Desender L, *et al.* Neutrophil extracellular traps in ischemic stroke thrombi. *Ann Neurol* 2017 ; 82 : 223-32.

[15] Meher AK, Spinosa M, Davis JP, *et al.* Novel role of IL (Interleukin)-1β in neutrophil extracellular trap formation and abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol* 2018 ; 38 : 843-53.

[16] Desilles JP, Gregoire C, Le Cossec C, *et al.* Efficacy and safety of aerosolized intra-tracheal dornase alfa administration in patients with Sars-CoV-2-induced acute respiratory distress syndrome (ARDS): a structured summary of a study protocol for a randomised controlled trial. *Trials* 2020 ; 21 : 548.

[17] Spyropoulos AC, Levy JH, Ageno W, *et al.* Scientific and standardization committee communication: clinical guidance on the diagnosis, prevention and treatment of venous thromboembolism in hospitalized patients with Covid-19. *J Thromb Haemost* 2020 ; 18 (8): 1859-65.

[18] Sandra G, Volker H, Ppapelbaum KI, *et al.* von Willebrand factor directly interacts with DNA from neutrophil extracellular traps. *Arterioscler Thromb Vasc Biol* 2014 ; 34 : 1382-9.

[19] Bikdeli B, Madhavan MV, Gupta A, *et al.* Pharmacological agents targeting thromboinflammation in Covid-19: review and implications for future research. *Thromb Haemost* 2020 ; 120 (7): 1004-24.

[20] Talasaz AH, Sadeghipour P, Kakavand H, *et al.* Recent randomized trials of antithrombotic therapy for patients with Covid-19. *J Am Coll Cardiol* 2021 ; 77 : 1903-21.

[21] Mazloomzadeh S, Khaleghparast S, Ghadrdoost B, *et al.* Effect of intermediate-dose *versus* standard-dose prophylactic anticoagulation on thrombotic events, extracorporeal membrane oxygenation treatment, or mortality among patients with Covid-19 admitted to the intensive care unit. *JAMA* 2021 ; 325 : 1620.

[22] Lopes RD, de Barros e Silva PGM, Furtado RHM, *et al.* Therapeutic *versus* prophylactic anticoagulation for patients admitted to hospital with Covid-19 and elevated D-dimer concentration (ACTION): an open-label, multicentre, randomised, controlled trial. *Lancet* 2021 ; 397 : 2253-63.

[23] Veras FP, Pontelli MC, Silva CM, *et al.* Sars-CoV-2-triggered neutrophil extracellular traps mediate Covid-19 pathology. *J Exp Med* 2020 ; 217 (12): e20201129.

Question à l'auteur

Q1 Please check and confirm.