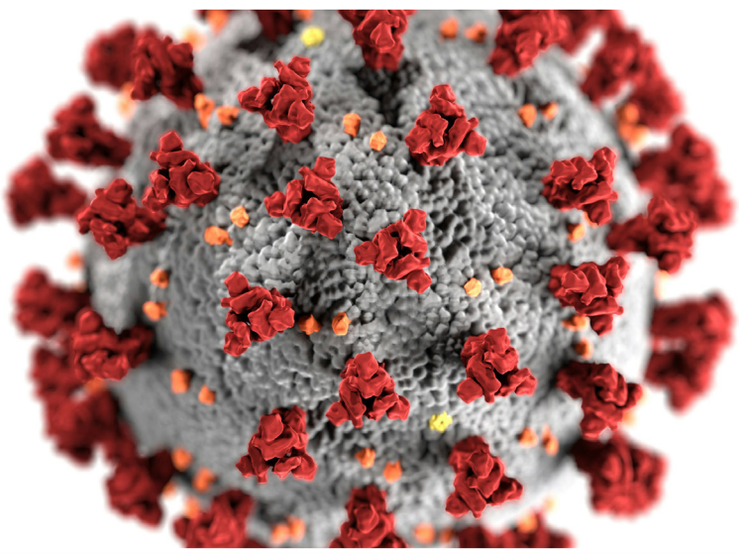
Review

The impact of COVID-19 on neutrophil NETosis and NET-induced immuno-thrombosis

K. K. Duvindi Nehansa Kularatne
Year 2, BMBS, University of Exeter
Email: dk462@exeter.ac.uk



Abstract

Coronavirus disease 2019 (COVID-19), a global health concern, is a severe virus-induced respiratory disease that could progress onto acute respiratory distress syndrome and isolated thrombosis. Excessive neutrophils in the blood of COVID-19 patients have drawn significant attention. This review aims to explore the relationship between elevated neutrophil NETosis and COVID-19 disease severity. Additionally, there is a focus on how NET-induced immuno-thrombosis in COVID-19 leads to thrombotic complications. The literature required to accomplish this was acquired using online databases such as PubMed. Research found elevated serum markers associated with neutrophil NETosis capable of inducing lung tissue damage by apoptosis, including citrullinated histone H3, neutrophil elastase (NE) and myeloperoxidase (MPO) in COVID-19 patients. Studies have shown the virus inducing neutrophil, platelet and complement activation, leading to the production of NETs that release MPO, NE and inhibit anti-thrombin 3, which ultimately progresses to thrombosis. Furthermore, COVID-19 plasma was able to trigger NET formation. Importantly, research shows increased cell-free DNA and MPO-DNA in hospitalised COVID-19 patients on mechanical ventilation compared to patients breathing room air. Therapies targeting dysregulated NETosis via its inhibition and promotion of NET degradation could be beneficial in preventing disease exacerbation.

Abbreviations

ACE – angiotensin-converting enzyme
ARDS – acute respiratory distress syndrome
Cit-H3 – citrullinated-H3
COVID-19 – Coronavirus disease 2019
DNA – deoxyribonucleic acid
MPO – myeloperoxidase
NE – neutrophil elastase
NET – neutrophil extracellular traps
ROS – reactive oxygen species
SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2
vWF – von Willebrand Factor

Introduction

Coronavirus disease 2019 (COVID-19) is a contagious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a single-stranded RNA enveloped virus.[1] The virus breaches alveolar epithelial membrane by binding to human angiotensin-converting enzyme (ACE) 2 receptor found on lung pneumocytes, epithelial and endothelial cells.[2] To complete fusion, the viral membrane spike protein needs to be cleaved by serine proteases such as TMPRSS2.[3] After spreading rapidly across the globe, COVID-19 was declared a pandemic on 11 March 2020 by the World Health Organization.[4,5] Some typical symptoms of the disease include fever, cough, difficulty breathing and loss of taste, whereas acute respiratory distress syndrome (ARDS) and multiple organ failure are seen more commonly in individuals with severe COVID-19.[6,7] Global deaths attributable to the COVID-19 pandemic are currently exceeding 3.4 million.[8]

Neutrophils account for roughly 70% of circulating human leukocytes.[1] One of their effector mechanisms, NETosis, which utilises NETs (neutrophil extracellular traps) has been suggested as a significant cause of the immunopathological manifestations of the SARS-CoV-2 virus.[6] NETosis is a special form of programmed cell death which is regulated in normal conditions. There are three types of NETosis: suicidal, where the neutrophil ruptures releasing its DNA; vital, where NETs are exocytosed in vesicles; and mitochondrial, where mitochondrial DNA is released as extracellular traps in a

reactive oxygen species (ROS) dependent manner.[9] For a detailed overview of the mechanism of NETosis, refer to **Figure 1**. There are numerous ways in which SARS-CoV-2 virus can induce NETosis which is detailed in **Figure 2**.[6,10-12] Excessive activation of NETs in circulation can also cause hypercoagulability and immuno-thrombosis.[6] Platelet coagulation can occur via two main pathways, namely the intrinsic and extrinsic coagulation pathways (**Figure 3**).[13] Incidence of immuno-thrombosis has been found in 22.7% of critically ill patients with COVID-19 in ICU and 7.9% of non-ICU patients.[14]

There has been an exponential rise in the number of COVID-19 cases worldwide, with no effective treatment. This paper aims to explore the relationship between COVID-19 and NETosis, and to subsequently focus on NET induced immuno-thrombosis.

Methodology

The literature for this review was obtained from PubMed and Google Scholar databases online. Two additional papers were found whilst reviewing reference lists of highly relevant papers which did not appear during initial database searches. Keywords used for theme one were: "neutrophils", "NETosis", "COVID-19" in conjunction with AND to refine the search. Keywords used for theme two were: "neutrophils", "NETosis", "immuno-thrombosis", "COVID-19" in conjunction with "AND" to obtain relevant articles. For both themes synonyms were used with each word using "OR" alongside it to ensure no relevant papers were missed out due to different wording. For example, alternatives for "neutrophils" such as "granulocyte", "polymorphonuclear leukocyte" were used, and "SARS-Cov-2" were used as an alternative to "COVID-19". Furthermore, the truncation symbol "*" was used alongside each word, e.g. "neutrophils*" to ensure no alternatives of each word were missed.

Papers from any country within the last six years were used. This ensured relevance to the rapidly evolving field of COVID-19 and NETosis, reflecting the most current understanding and methodologies. Additionally, this incorporated significant technological advancements in biomedical research, such as improved imaging and advanced data analysis tools. Further resources were found through citations on other papers and clinical reviews. Twenty-four papers and one website were obtained for this review. Refer to **Figure 4** for a full method.

Discussion

Influence of COVID-19 on NET formation

Serum markers of NETs are elevated in COVID-19 patient sera. These include myeloperoxidase (MPO)-DNA, cell-free DNA and citrullinated-H3 (Cit-H3), which are NETosis-related enzymes capable of inducing lung tissue damage.[4] Thereby we can also infer a rise in neutrophil elastase (NE), which if in a complex with DNA and Cit-H3, could be another serum marker of NETs. This suggests the potential of excessive NETosis in contributing to exacerbation of the disease, perhaps by disrupting alveolar endothelium, promoting lung epithelial apoptosis and degrading alveolar basement membrane.[2,15]

Various studies have demonstrated elevated levels of neutrophil NETosis in individuals with COVID-19.[3,5,16] In one study, blood samples of 16 hospitalised patients with COVID-19 were obtained and their neutrophils were isolated.[5] NET production was then investigated ex vivo by relative fluorescence, which depicted that without stimulation more NETs were produced in the infected cohort compared to their control ($p < 0.0001$). Furthermore, increased levels of NETs were identified in the airways and alveoli of 40% of patients infected with SARS-CoV-2. A limitation of the study is that confounding factors such as diabetes mellitus present within the patient cohort were not mirrored in the control group. This could make the results an overestimation as increased NETosis is observed in individuals with diabetes, hence this may challenge the integrity of results.[17] Similar results were shown in a study on 32 patients with severe COVID-19, demonstrating their plasma NET levels to be elevated using confocal microscopy analysis. In addition, more than 80% of their neutrophils were positive for NETs compared to their control.[3] The study also looked at NET concentration in tracheal aspirate of patients with severe COVID-19 under mechanical ventilation and found a 10-fold increase in plasma concentration. The results may also be influenced by being on mechanical ventilation as it has been found to contribute to an increased level of NET markers in alveoli of critically ill patients.[18] Trauma from mechanical ventilation in lungs may result in the release of damage-associated molecular patterns (DAMPs), leading to neutrophil activation and increased NETosis. Hence, caution is advised in clinical practice when starting ventilation in patients with COVID-19. Perhaps measuring neutrophil count beforehand may allow the risk level of NETosis to be monitored. The study's methodical drawback lies in using qualitative confocal immunofluorescence, hindering quantification without additional techniques such as enzyme-linked immunosorbent assays (ELISA) or flow cytometry. Another prospective cohort study on 28 hospitalised patients with COVID-19 and 5 convalescent patients measured plasma MPO-DNA complexes. They found that survivors of COVID-19 had significantly lower NET levels than non-survivors ($p=0.0004$), suggesting that upon viral clearance NET levels fall.[19]

A study obtained similar results using serum samples from 50 hospitalised patients with COVID-19 comparing their results to 30 healthy controls.[16] They measured three NET markers: MPO-DNA ($p<0.001$), Cit-H3 ($p<0.0001$) and cell-free DNA ($p<0.0001$), with the latter not being specific to NETosis. All three of these markers were elevated in the serum of individuals with COVID-19 compared with controls. Surprisingly, Cit-H3 did not correlate as well as the other two markers and instead correlated strongly with platelet levels ($r=0.45$, $p<0.0001$). This could suggest the existence of multiple pathways to NETosis linked to COVID-19, as peptidyl arginine deiminase-4 (PAD4) is the primary catalyst for Cit-H3 synthesis. An above-mentioned study incubated neutrophils with Cl-Amidine, an inhibitor of PAD4, which showed a reduction in NET production from COVID-19 patient neutrophils.[3] This confirms patients with COVID-19 are more susceptible to PAD-4 dependent NETosis. The PAD4 pathway is only one of the many mechanisms of neutrophil NETosis, such as PAD4 independent, metabolism-mediated etc.[9] On the contrary, in vitro studies have demonstrated some pathways being somewhat non reliant on PAD4 activity.[16] For example, the stimulation of ROS production could be PAD4 independent. Furthermore, neutrophils may undergo cell death through various pathways such as apoptosis and necrosis. Hence, markers such as Cit-H3 may be produced by a type of cell death independent of NETosis as well.

All studies[3,5,16,19] unfortunately have not specified the type of NETosis that they were investigating. This makes finding a therapeutic target for NETosis-induced COVID-19 disease exacerbation challenging, as it is difficult to directly target a specific type of NETosis. However, one study[3] via the inhibition of PAD4 enzyme has shown that PAD4 dependent NETosis is involved in COVID-19, which allows for some targeted therapeutic approaches. Although this area requires more research as there is evidence disputing this claim.

Net induced immuno-thrombosis in COVID-19

Thrombotic complications, including venous, arterial and microvascular thrombosis, are observed in patients with COVID-19.[14] It is defined as immuno-thrombosis, where neutrophils interact directly with platelets and coagulation factors.[19] NETs have been identified as major elements of micro and macro vascular thrombi[20] and in patients with COVID-19, components of NETs, such as cell-free DNA and Cit-H3, can potentially act as coagulation inducers. When NETs are released into circulation, they bind to vessel walls, capturing platelets resulting in blood flow obstruction (**Figure 5**).

An above-mentioned prospective cohort study looked at 16 COVID-19 patient plasma samples for NET formation using confocal microscopy.[19] The study detected significantly higher circulating

platelet-neutrophil aggregates compared to healthy controls (p=0.006). However, they did not observe many thrombotic complications of SARS-Cov-2 in the cohort that other studies have reported on. Additionally, even though they found significantly elevated soluble markers of thrombosis (e.g. Plasma D-dimer and von Willebrand Factor (vWF) antigen levels) in patients with COVID-19 ($p<0.0001$), these did not correlate directly with plasma NET levels within the cohort. This may be due to markers such as vWF being released from activated endothelium early in COVID-19. In the study, the vWF levels measured later may reflect ongoing inflammation.[19] Another reason for the discrepancy may be because D-dimers result from active fibrinolysis, however, NET-rich clots are protected from fibrinolytic breakdown by tissue plasminogen activator. Therefore, this lack of correlation could be due to inhibited thrombus degradation in patients with COVID-19.

In contrast, a study on 36 patients with COVID-19 and 31 healthy controls found that there were significantly higher MPO-DNA complexes and Cit-H3 in COVID-19 patients with thrombotic events compared to those without.[21] In this study COVID-19 patients mainly had mild disease progression, however, they still found strong platelet and neutrophil activation, depicting that the virus itself triggers neutrophil modifications. Surprisingly, the study also found that NET biomarkers did not decrease to normal levels after COVID-19 recovery. This could suggest a degree of neutrophil activation persisting even after recovery.[22] Similar results were found in a study conducted on 44 patients with COVID-19 where cell-free DNA ($p<0.001$), MPO-DNA ($p<0.05$) and cit-H3 ($p<0.01$) were significantly raised in COVID-19 patients in their thrombosis group.[23] A limitation of this study was the use of discarded specimens at various sampling points rather than samples drawn up specifically for research purposes. This leads to the possibility of NETs being partially degraded over time, lowering their measurements, giving underestimates.

During mild SARS-CoV-2, physiological immuno-thrombosis is developed, which is controlled by homeostatic mechanisms as opposed to in severe infections where it is uncontrolled leading to pathological immuno-thrombosis.[24] A case-controlled study showed that both the activation of neutrophils and complement were associated with inducing thrombosis in patients with COVID-19.[25] Complement activation is a biochemical process that enhances immune responses against pathogens by producing proteins such as C3. Findings were confirmed by confocal immunofluorescence microscopy in neutrophils collected from four patients with severe COVID-19, where spontaneous NET formation expressing tissue factor (TF) was observed.[25] When C3 activation was blocked using Cp40, TF expression in control neutrophils was significantly decreased ($p < 0.05$). This indicates C3 inhibition disrupts TF release as a result of NETosis. A limitation of this study is the specificity regarding the use of confocal immunofluorescence. This technique cannot distinguish between NETs, other extracellular structures and debris present in samples, requiring additional validation of results. Furthermore, the study has not considered the likelihood of active fibrinolysis, which is the breakdown of clots, occurring simultaneously with thrombosis within the human body. This can obscure the true extent of clot formation caused by neutrophil and complement activation. Therefore, the study has found the true extent of clot formation whilst the body's response would be lower, making the results of this study an overestimation.

Conclusion

To conclude, studies show increased NETosis in COVID-19 patient plasma highlighting its influence on exacerbating the disease. Alarmingly, critically ill COVID-19 patients being on mechanical ventilation have been shown to increase NETosis, which can further accentuate disease progression. More studies specifically looking at the mechanism of how mechanical ventilation induced NETosis are required, perhaps by measuring DAMPs, such as MPO-DNA in patient serum, investigating its correlation to NET release.

Studies have shown components of NETs and NETosis itself to increase micro and macro vascular thrombi in blood vessels of COVID-19 patients. The virus, by activation of the complement system and platelet activation induced NETosis, leads to immuno-thrombosis. These studies have used confocal immunofluorescence for imaging which is qualitative and nonspecific; hence, future studies should use ELISA or flow cytometry to quantify results and facilitate statistical analysis. Additionally, some studies have not accounted for confounding factors such as the presence of active fibrinolysis. Therefore, in future studies researchers need to measure and control fibrinolytic activity by assessing levels of fibrinolytic markers such as plasminogen or D-dimer and factor these findings into their analysis. Finally, it is integral that studies specify the type of NETosis that they observe in COVID-19 patients along with which NET-related markers correlate more with each type of NETosis.

References

1. Gillot C, Favresse J, Mullier F, Lecompte T, Dogne J-M, Douxfils J. NETosis and the immune system in COVID-19: mechanisms and potential treatments. Frontiers in Pharmacology. 2021;12:708302.
2. Keane C, Coalter M, Martin-Loeches I. Immune system disequilibrium—Neutrophils, their extracellular traps, and COVID-19-induced sepsis. Frontiers in Medicine. 2021;8:711397.
3. Veras FP, Pontelli MC, Silva CM, Toller-Kawahisa JE, de Lima M, Nascimento DC, et al. SARS-CoV-2–triggered neutrophil extracellular traps mediate COVID-19 pathology. Journal of Experimental Medicine. 2020;217(12):e20201129.
4. Borges L, Pithon-Curi TC, Curi R, Hatanaka E. COVID-19 and neutrophils: the relationship between hyperinflammation and neutrophil extracellular traps. Mediators of inflammation. 2020;2020.
5. Masso-Silva JA, Moshensky A, Lam MT, Odish M, Patel A, Xu L, et al. Increased peripheral blood neutrophil activation phenotypes and NETosis in critically ill COVID-19 patients. medRxiv. 2021:2021.01. 14.21249831.
6. Zhu Y, Chen X, Liu X. NETosis and neutrophil extracellular traps in COVID-19: immunothrombosis and beyond. Frontiers in Immunology. 2022;13:838011.
7. Zhang R, Sun C, Han Y, Huang L, Sheng H, Wang J, et al. Neutrophil autophagy and NETosis in COVID-19: perspectives. Autophagy. 2023;19(3):758-67.
8. Organization WH. Global excess deaths associated with COVID-19 (modelled estimates) [Internet] 2023 [cited 2024 May 15] Available from: https://www.who.int/data/stories/the-true-death-toll-of-covid-19-estimating-global-excess-mortality.
9. Tan C, Aziz M, Wang P. The vitals of NETs. J Leukoc Biol. 2021;110(4):797-808.
10. Liao T-L, Liu H-J, Chen D-Y, Tang K-T, Chen Y-M, Liu P-Y. SARS-CoV-2 primed platelets–derived microRNAs enhance NETs formation by extracellular vesicle transmission and TLR7/8 activation. Cell Communication and Signaling. 2023;21(1):304.
11. Hsieh K-H, Chao C-H, Cheng Y-L, Lai Y-C, Chuang Y-C, Wang J-R, et al. Enhancement of NETosis by ACE2-cross-reactive anti-SARS-CoV-2 RBD antibodies in patients with COVID-19. Journal of Biomedical Science. 2024;31(1):39.
12. Allen KC, Ramos-Benitez MJ, Teague H, Chertow DS, Strich JR, Warner S. 249. SARS-CoV-2 spike immune complexes induce NETosis in COVID-19. Open Forum Infectious Diseases. 2023;10(Supplement_2).
13. Morris G, Bortolasci CC, Puri BK, Olive L, Marx W, O'Neil A, et al. The pathophysiology of SARS-CoV-2: a suggested model and therapeutic approach. Life Sciences. 2020;258:118166.
14. Shaw RJ, Bradbury C, Abrams ST, Wang G, Toh CH. COVID-19 and immunothrombosis: emerging understanding and clinical management. British Journal of Haematology. 2021;194(3):518-29.
15. Wang J, Li Q, Yin Y, Zhang Y, Cao Y, Lin X, et al. Excessive neutrophils and neutrophil extracellular traps in COVID-19. Frontiers in Immunology. 2020;11:2063.
16. Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, et al. Neutrophil extracellular traps in COVID-19. JCI insight. 2020;5(11).
17. Njeim R, Azar WS, Fares AH, Azar ST, Kfoury Kassouf H, Eid AA. NETosis contributes to the pathogenesis of diabetes and its complications. J Mol Endocrinol. 2020;65(4):R65-r76.

18. Mikacenic C, Moore R, Dmyterko V, West TE, Altemeier WA, Liles WC, et al. Neutrophil extracellular traps (NETs) are increased in the alveolar spaces of patients with ventilator-associated pneumonia. Crit Care. 2018;22(1):358.
19. Middleton EA, He XY, Denorme F, Campbell RA, Ng D, Salvatore SP, et al. Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. Blood. 2020;136(10):1169-79.
20. Dolhnikoff M, Duarte-Neto AN, de Almeida Monteiro RA, da Silva LFF, de Oliveira EP, Saldiva PHN, et al. Pathological evidence of pulmonary thrombotic phenomena in severe COVID-19. J Thromb Haemost. 2020;18(6):1517-9.
21. Petito E, Falcinelli E, Paliani U, Cesari E, Vaudo G, Sebastiano M, et al. Association of Neutrophil Activation, More Than Platelet Activation, With Thrombotic Complications in Coronavirus Disease 2019. J Infect Dis. 2021;223(6):933-44.
22. Carfì A, Bernabei R, Landi F. Persistent Symptoms in Patients After Acute COVID-19. Jama. 2020;324(6):603-5.
23. Zuo Y, Zuo M, Yalavarthi S, Gockman K, Madison JA, Shi H, et al. Neutrophil extracellular traps and thrombosis in COVID-19. J Thromb Thrombolysis. 2021;51(2):446-53.
24. Al-Kuraishy HM, Al-Gareeb AI, Al-Hussaniy HA, Al-Harcan NAH, Alexiou A, Batiha GE-S. Neutrophil Extracellular Traps (NETs) and Covid-19: A new frontiers for therapeutic modality. International Immunopharmacology. 2022;104:108516.
25. Skendros P, Mitsios A, Chrysanthopoulou A, Mastellos DC, Metallidis S, Rafailidis P, et al. Complement and tissue factor-enriched neutrophil extracellular traps are key drivers in COVID-19 immunothrombosis. J Clin Invest. 2020;130(11):6151-7.

K. K. Duvindi Nehansa Kularatne

Duvindi Kularatne is a second-year medical student at the University of Exeter. She has a strong interest in public health and infectious diseases, with a particular focus on HIV and COVID-19. Duvindi has published an article on current HIV management and combating stigma in the Medic Mentor National Magazine. She is passionate about bridging gaps in healthcare access and fostering awareness around stigmatised health conditions.

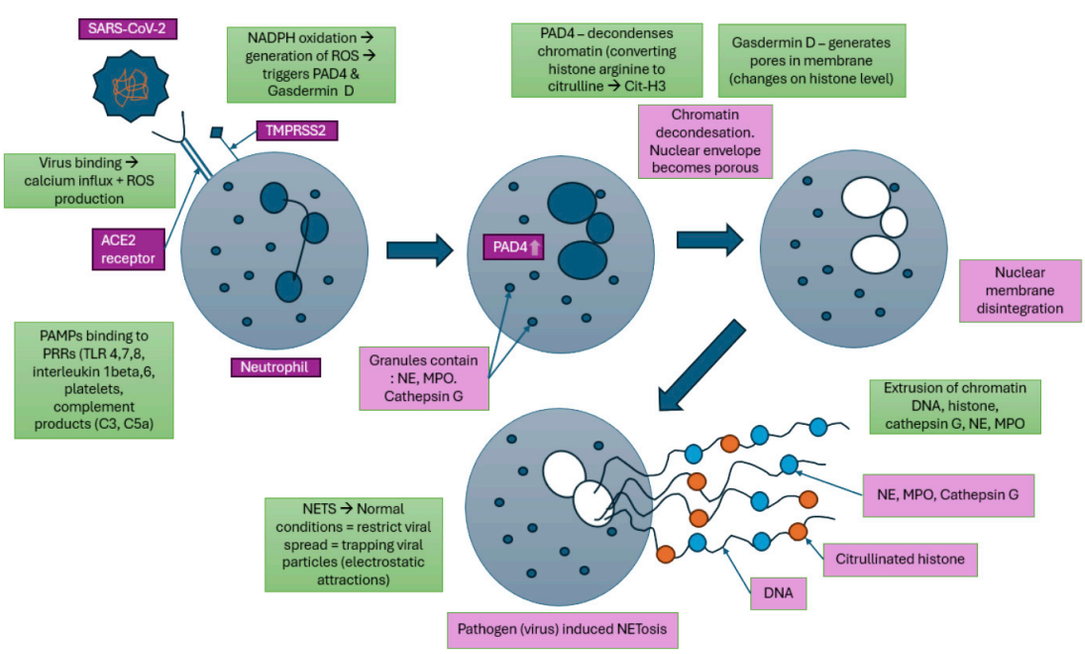


Figure 1. Mechanism of neutrophil NETosis in COVID-19

NETosis is the formation of Neutrophil Extracellular traps during cell apoptosis. It involves the extrusion of chromatin DNA, histones, and antimicrobial proteins into the extracellular space to restrict viral spread.

Created from information in Gillot et al[1], Keane et al[2], Borges et al[4], Zhu et al[6], Al-Kuraishy et al[24]. (No graphical elements from the above papers were used.)

Abbreviations - SARS-COV-2: Severe acute respiratory syndrome coronavirus 2, ACE 2: Angiotensin-converting enzyme 2, TMPSS2: Transmembrane serine protease 2, NADPH: Nicotinamide adenine dinucleotide phosphate, ROS: Reactive Oxygen species, PAD4: Peptidyl arginine deiminase 4, Cit-H3: Citrullinated histone H3, NE: Neutrophil elastase, MPO: Myeloperoxidase, PAMPs: Pathogen associated molecular patterns, PRR: Pattern recognition receptors, TLR: Toll-like receptors, DNA: Deoxyribonucleic acid.

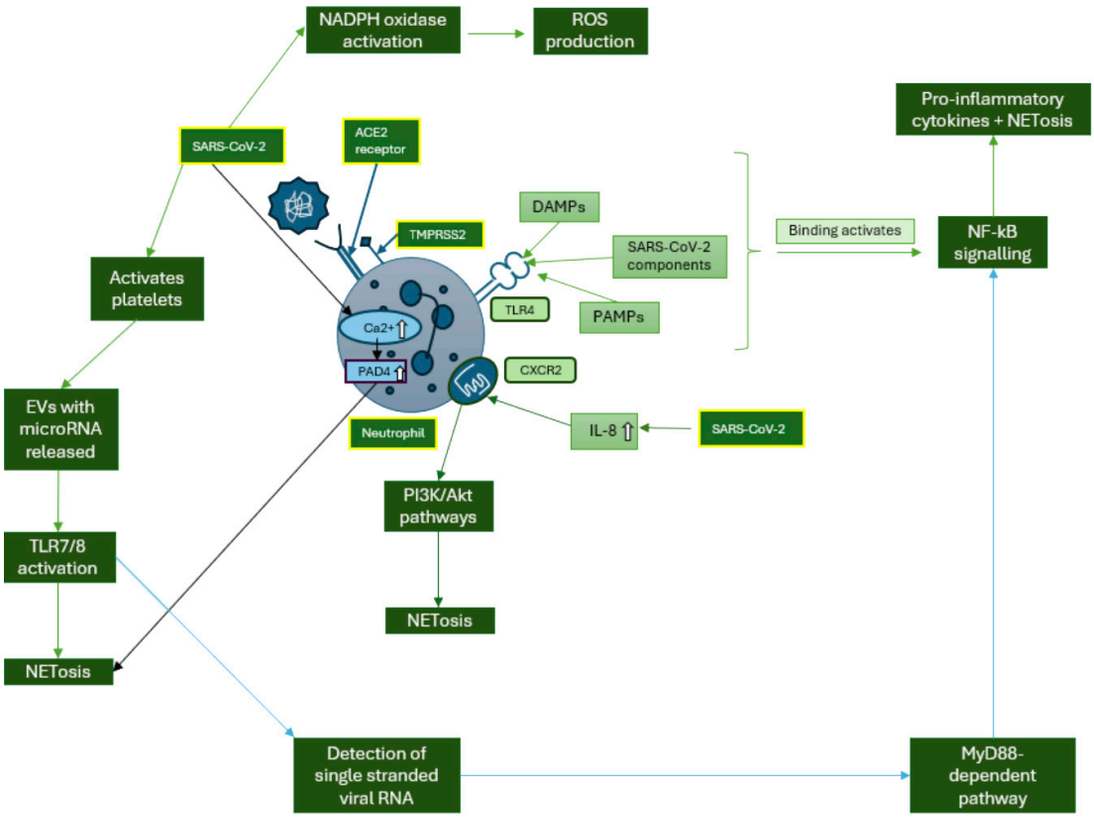


Figure 2. Mechanism of SARS-CoV-2 inducing neutrophil NETosis
Created based on text in Zhu et al[6], Liao et al[10], Hsieh et al[11] and Allen[12]. (No graphical elements from the above papers were used.)
Abbreviations - SARS-COV-2: Severe acute respiratory syndrome coronavirus 2, ACE 2: Angiotensin-converting enzyme 2, TMPSS2: Transmembrane serine protease 2, NADPH: Nicotinamide adenine dinucleotide phosphate, ROS: Reactive Oxygen species, PAD4: Peptidyl arginine deiminase 4, DAMPs: Damage associated molecular patterns, PAMPs: Pathogen associated molecular patterns, TLR: Toll-like receptors, EVs: Extracellular vesicles, microRNA: micro ribonucleic acid, MyD88: myeloid differentiation primary response 88, IL-8: Interleukin 8, CXCR2: C-X-C Motif chemokine receptor 2, PI3K: Phophatidylinositol-4,5-bisphosphate 3-kinase, Akt: protein kinase B, NF-kB: Nuclear factor kappa B.

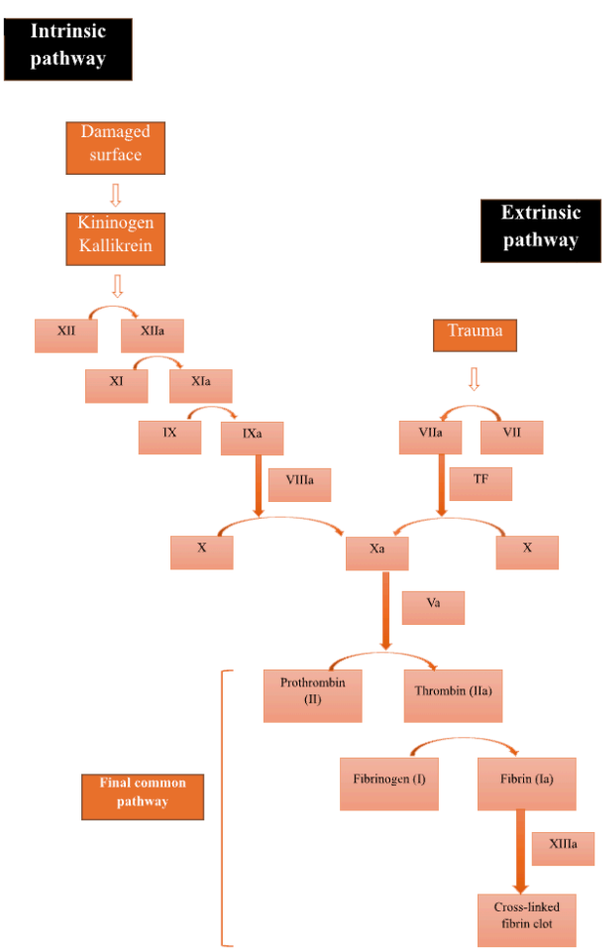


Figure 3. Intrinsic and extrinsic coagulation pathways
Roman numerals represent coagulation factors.
Figure created using data from Morris et al1[3]. (No graphical elements from the above paper was used.)

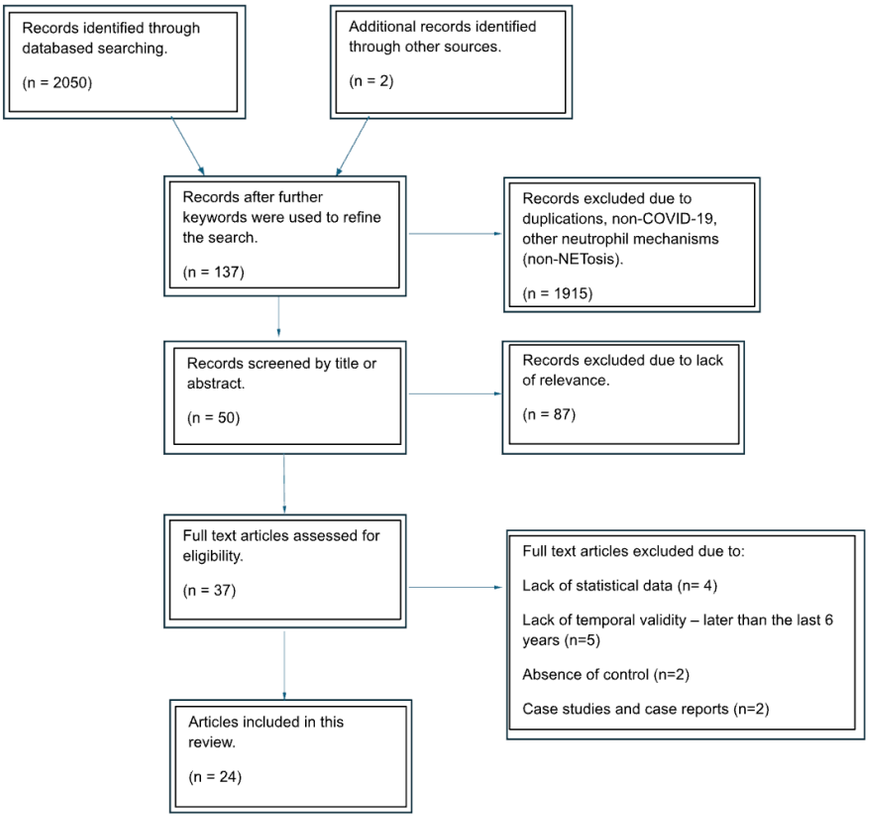


Figure 4. PRISMA diagram depicting the full method.

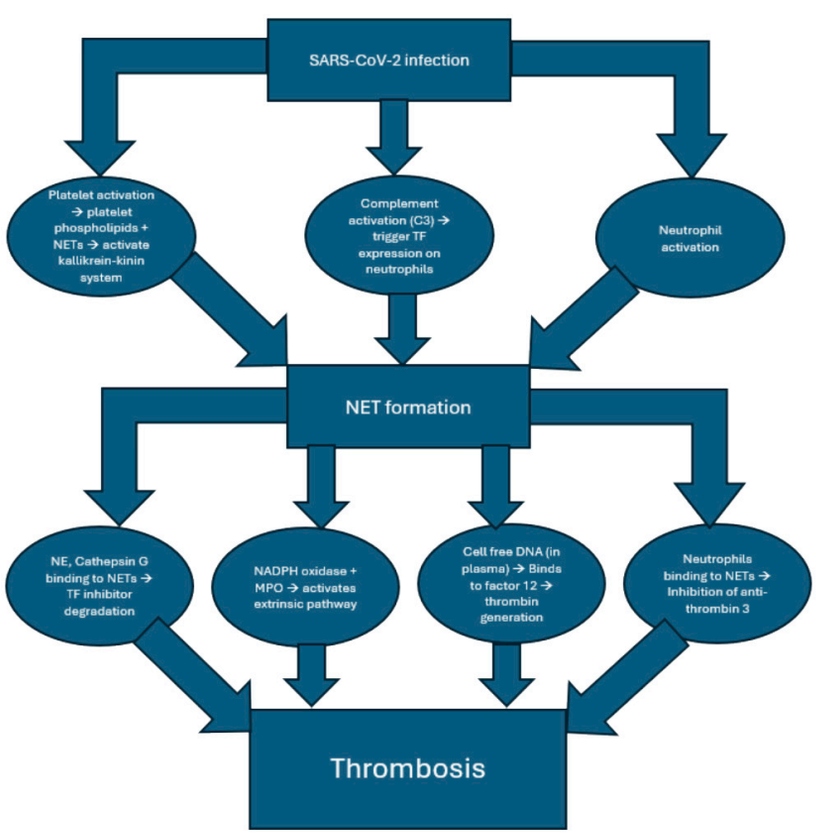


Figure 5. Summary of NETosis and coagulopathy in COVID-19
Created based on information in Zhu et al[6], Al-Kuraishy et al[24]. (No graphical elements from the above papers were used.)
Abbreviations - SARS-COV-2: Severe acute respiratory syndrome coronavirus 2, NETs: Neutrophil extracellular traps, NADPH: Nicotinamide adenine dinucleotide phosphate, NE: Neutrophil elastase, MPO: Myeloperoxidase, TF: Tissue factor, DNA: Deoxyribonucleic acid.