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Neutrophil Extracellular Traps in Coronavirus Infection: Interaction Network Analysis

Amal Bouzid[1], Asmaa T Uthman [2], Noor N Al-Rawi[3], Natheer H Al-Rawi[4*]

[1] Laboratory of Molecular and Cellular Screening Processes, Center of Biotechnology of Sfax, Sfax, Tunisia.
[2] Diagnostic and Surgical Dental Sciences Dept., College of Dentistry, Gulf Medical University, Ajman, UAE
[3] Sharjah Institute of Medical Research, University of Sharjah, UAE
[4] Dept. Oral & Craniofacial Health Sciences, College of Dental Medicine, University of Sharjah, UAE

Corresponding author: Natheer Hashim Al-Rawi
E mail: nhabdulla@sharjah.ac.ae

ABSTRACT

Background: This study objective was to investigate, through interaction network analysis, the target genes involved in the pathogenesis of neutrophil extracellular traps (NETs) in coronavirus infection.
Method: Genes participating in the pathogenesis of NETs were recognized in GeneCards database. Gene list was extended, and the gene interactions network was mapped using the STRING software. Weighted number of links (WNL) were calculated to identify "leader genes". Total interactions score (TIS) was calculated using all interaction data generated by the STRING database. The ontological analyses were also performed using BinGO plugin and Cytoscape software.
Results: Seven sets of genes (IL6, TNF, CRP, CXCL8, IL-1ß, IL17A and IL-1a) were identified in the GeneCards database. The suggested leader genes from the results of interaction scores were IL-6, TNF, IL-1ß and CXCL-8 with the highest adjusted WNL values. However, the most influential genes in the network were IL-17 and CRP, with WNL/TIS ratios of 0.988 and 0.986, respectively.
Conclusion: The above results might suggest the participation of potential genes to facilitate the understanding of complex pathogenesis mechanisms of coronavirus infection. The clinical course of SARS-CoV-2 infection can be modulated by evaluating the activity of NETosis which represents a promising therapeutic target for the COVID-19.

Keywords: COVID-19, SARS-CoV-2, Coronavirus, Neutrophil extracellular traps, Target genes.

Correspondence:
Natheer H Al-Rawi
Dept. Oral & Craniofacial Health Sciences, College of Dental Medicine, University of Sharjah, United Arab Emirates
E mail: nhabdulla@sharjah.ac.ae

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has recently erupted as a respiratory disease caused by SARS-CoV-2 (severe acute respiratory syndrome). COVID-19 was declared a pandemic by the World Health Organization (WHO) on March 11, 2020 after the first declaration in Wuhan, China in December 2019. It has progressed to spread aggressively worldwide, infecting more than 64.5 million cases to date (1). The SARS-CoV-2 seems to activate innate and adaptive immune responses. Moreover, unregulated inherent inflammation and reduced adaptive immune function can lead to harmful tissue injury (2). While the currently available therapy directly affects the virus or interferes with viral access (3), therapies that aimed at the immunopathology of COVID-19 infection have become a promising motivation.

The key cells of innate immunity are neutrophils. The development of neutrophil extracellular traps (NETs) is one of the neutrophil action mechanisms (4). The process of NETs generation is called NETosis, which is a different type of cell death from necrosis and apoptosis. It is a cell death program of multi-steps where nuclear chromatin, associated with nuclear histones and granular antimicrobial neutrophil-dead proteins, forms trap-retaining scaffolds and kills pathogens such as bacteria, fungi and viruses (5). NETosis was described to be involved in several diseases, other than infections, like cancer, autoimmune diseases, atherosclerosis, venous thromboembolism, diabetes, etc (6–8).

Virus-induced NETs will circulate uncontrollably, contributing to the body's intense systemic response by generating immune complexes, cytokines, chemokines, and ultimately promoting inflammation. To date, limited data on the role of NETs in coronavirus infection is available in the medical literature/research. For instance, NETosis has been described to tend to be closely related to the pulmonary diseases' inflammatory response. In fact, it has been found that NETs were increased in patients having acute respiratory distress syndrome (ARDS) (9,10), also, in patients with acute respiratory failure midst chronic obstructive pulmonary disease (COPD) exacerbation, as seen in studies on bronchoalveolar lavage fluid (11). Likewise, advanced cases of COVID-19 are frequently characterized by a hyper-inflammation associated to an ARDS-like syndrome 'cytokine storm' (3). So far, several publications have reported the existence of multiple thrombotic complications (both arterial and venous thrombotic complications) in COVID-19 infections (12,13). In addition, micro and macro thrombotic phenomena like pulmonary embolism and microangiopathy have been commonly reported (14), leading to a detailed assessment regimen of anti-thrombotic prophylaxis/ coagulation in patients with COVID-19 (12–14). In fact, all conditions with arterial and venous thrombosis, NETosis seems to play a significant role, as numerous evidence has been considered (15–17). NETosis, as in many vasculitis and thrombotic microangiopathies like Moschowitz syndrome, has been reported (18).

Evidence exists to support the hypothesis that NETs could be involved in the response to COVID-19 infection, so it is valuable to explore protein interactions, signaling pathways and biological processes to understand the pathological mechanisms of the disease by exploring *in-silico* approaches. Bioinformatics research has so far become a valuable screening method to classify biological

targets (19,20). Leaders and associated genes can be defined on the basis of the Weighted Number of Links (WNL) and Total Interactions Score (TIS), and linked to a specific biological phenomenon, according to the already available data, (21,22) which could be useful for prognostic factors screening (20) .The current research was intended to examine the interaction of NETs and their immunological targets using interaction network analysis in coronavirus infection.

METHODS

Bioinformatics and Interaction Network Analysis

First, keywords involved in NETosis for coronavirus infection were determined by searching large-scale databases. On the GeneCards website, a search based on human genes was conducted to find the leading set of target genes (23). The gene naming was described by the Human Genome Organization.

The study of the interaction network between the established genes was then carried out, mapping these protein-coding genes with the STRING software (version 11.0) (24). Indirect and direct interactions were assessed at a high confidence level (0.7). The resulting network was expanded only once, so new genes linked to the studied pathological mechanism could be found. Each interaction was scored among the studied networks. For each gene, cumulative association scores have been summed and modified by multiplying by 1000 to determine the Weighted Number of links (WNL) (25). In addition, to classify the overall connectivity of each gene, the Total Interactions Score (TIS) was evaluated by calculating interaction data resulting from the STRING database. The WNL/TIS ratio was also calculated to classify the most influential genes in the mapping network (25). Genes with the major WNL values were considered as leader genes. The genes with higher WNL and TIS values were known to be the genes with more interactions. While gene without interactions was considered as an orphan gene. Based on WNL and TIS scores, genes were clustered using K-means classification method. To assess the differences among clusters, Kruskal-Wallis test was evaluated at a p-value ≤ 0.05 (26). Statistics were performed using IBM SPSS Statistics software (version 23). Statistical significance was set at a p-value of 0.05. In addition, a biological system analysis based on ontological and topological analysis was carried out using Cytoscape software with the BinGO application (27).

RESULTS

Identification of NETs targets in Coronavirus infection by interaction network analyses

To establish the primary targets of NETs, a bioinformatic approach was carried out. A search on the GeneCards database returned a leader set of 7 genes with the keywords "Neutrophil extracellular traps", "Inflammation", "Thrombosis", "Cytokine storm" and "Coronavirus" as follows: *IL6*, *TNF*, *CRP*, *CXCL8*, *IL1B*, *IL17A* and *IL1A* (Figure 1). Gene characteristics were described in Table (1).

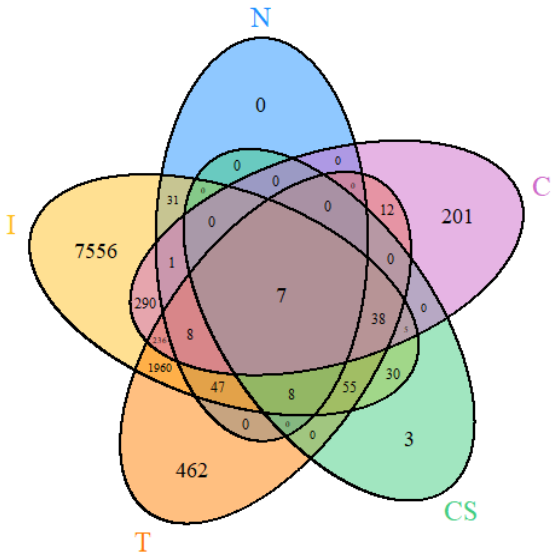

Figure 1: Venn diagram showing data mining using the following keywords: "Neutrophil extracellular traps"(N) AND "Inflammation"(I) AND "Thrombosis" (T) AND "Cytokine storm"(CS) AND "Coronavirus"

Table 1: Genes associated with “Neutrophil extracellular traps”, “Inflammation”, “Thrombosis”, “Cytokine storm”, and” Coronavirus” keywords.

Symbol	Description	Category	GIFtS	Score
IL 6	Interleukin 6	Protein coding	51	39.16
TNF	Tumor Necrosis Factor	Protein coding	53	29.68
CRP	C-Reactive Protein	Protein coding	48	20.01
CXCL8	C-X-C Motif Chemokine Ligand 8	Protein coding	43	18.24
IL 1B	Interleukin 1 Beta	Protein coding	50	14.00

IL 17 A	Interleukin 17A	Protein coding	44	10.03
IL-1 A	Interleukin 1 Alpha	Protein coding	45	8.82

GeneCards Inferred Functionality Scores (GIFtS)

In the second step, the interaction network analysis between the identified genes using the web-available software STRING mapped these protein-coding genes, as shown in Figure (2).

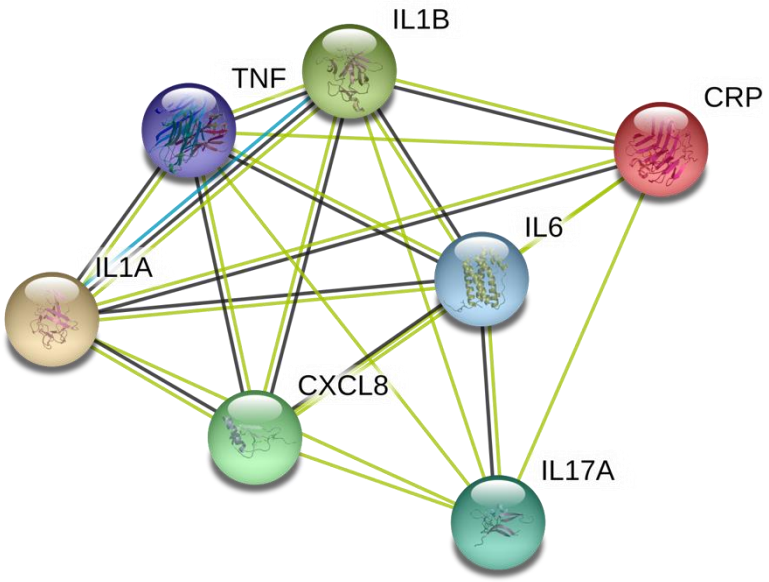

Figure 2: Protein interactions of the primary NETs targets.

Colored nodes revealed query proteins and first shell of interactors. Filled nodes means some 3D structure is predicted/known. Blue edge means known interactions from curated databases. Black edge presents co-expression. Green edge presents text mining, (level of confidence > 0.7).

Seven nodes (proteins), with 20 protein-protein interactions at a high degree of confidence (0.7) and an enrichment p-value <1.0 e^{-16}, were found in the gene interaction analysis. With an average local clustering coefficient of 0.952, the average node degree was 5.71. *IL-6*, *TNF*, *IL1b* and *CXCL8* with the highest adjusted WNL values were the suggested leader genes (Table 2). The adjusted TIS values were resulted in *IL-6* and *IL-1B* with the highest interaction scores of 7349 and 8812, respectively. Besides, *IL-17* and *CRP* were the most influential genes in the network, with WNL/TIS ratios of 0.988 and 0.986, respectively. The analysis did not recognize an orphan gene (a gene without a link).

Table 2: Total interaction scores of the primary NETs targets network.

Gene	No.	Adjusted WNL	Adjusted TIS	WNL/TIS	Notes
IL6	1	**5840ʊ**	**7349***	0.795	Leader gene with specific network interactions
TNF	2	**5701ʊ**	6683	0.854	Leader gene
IL-1B	3	**5665ʊ**	**8812***	0.643	Leader gene with specific network interactions
CXCL8	4	**5633ʊ**	7215	0.781	Leader gene
IL17A	5	5243	5302	**0.989μ**	Most influential gene
IL-1A	6	4525	6930	0.653	-
CRP	7	4397	4455	**0.987μ**	Most influential gene

ʊ: largest adjusted WNL values for leader genes

*: largest adjusted TIS values for genes with high interactions

μ: specificity scores

Data analysis of clustering and gene distribution was obtained from multiple clustering experiments with K-means. According to WNL and TIS parameters, genes were ranked in clusters. The results of initial clustering core of the seven genes listed in Table (3) display clusters as follows: 5 genes (*IL6, TNF, CXCL8, IL1B* and *IL1A*) for cluster 1 and 2 genes (*IL17A* and *CRP*) for cluster 2. The ANOVA test was used to show a statistically significant difference in the adjusted TIS ($p<0.05$). The TIS scores contribute the most to the broad F value of the cluster

solution, which provides the greatest distinction between clusters (Table 4).

Table 3: Initial clustering center of the NETs targets in Coronavirus Infection.

No.	Gene	Cluster	Distance
1	*IL-6*	1	370.429
2	*TNF*	1	750.343
3	*IL-1B*	1	1427.201
4	*CXCL8*	1	243.064
5	*IL-17A*	2	598.566
6	*IL-1A*	1	1056.959
7	*CRP*	2	598.566

Table 4: Differences between clusters using the ANOVA test.

	Cluster		Error		F	Significance
	Mean square	df	Mean square	df		
Adjusted WNL	608782.629	1	301139.760	5	2.022	0.214
Adjusted TIS	9066960.700	1	624847.860	5	14.511	0.013

To display NET target-related connectivity (WNL) versus global connectivity (TIS), a scatter diagram was created (Figure 3).

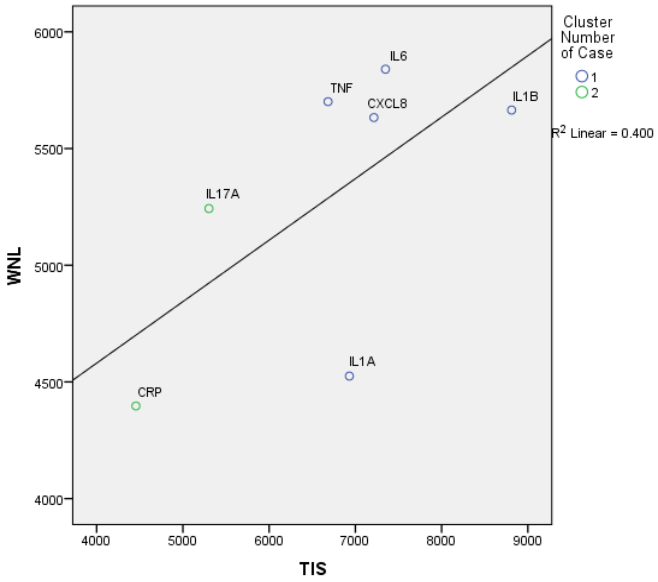

Figure 3: Scatter diagram showing the condition-related connectivities (WNL: weighted number of links) versus the global connectivities (TIS: total interactions score) of the identified NETs targets.

Moreover, the previous acquired network was extended once, discovering new potential genes associated with the pathological mechanisms sought. Thus, 12 nodes (proteins) with 46 protein-protein interactions at a high confidence level of 0.7 and an enrichment p-value < 4.23 e-12 were discovered by the extended network (Figure 4). With a mean local clustering coefficient of 0.844, the average node degree was 7.67. The study of the interaction scores revealed that the TNF gene showed the highest adjusted WNL and highest adjusted TIS values to be regarded as a "leader gene" (Table 5). Further, *Il-17* and *CRP*, deducted from the WNL/TIS ratio of 0.989 and 0.987, were the most influential genes in the network.

Table 5: Total interaction scores of the primary NETs targets network after one expansion.

No.	Gene	Adjusted WNL	Adjusted TIS	WNL/TIS
1	*CRP*	4397	4455	**0.987**µ
2	*CXCL8*	6357	7939	0.801
3	*IKBKB*	6397	13183	0.486
4	*IKBKG*	4957	12347	0.402

5	*IL-17A*	5243	5302	**0.989**µ
6	*IL-1A*	4.525	6930	0.653
7	*IL-1B*	5665	8812	0.643
8	*IL-6*	6.636	8191	0.811
9	*RIPK1*	4946	13552	0.365
10	*TNF*	10696Ů	19957*	0.536
11	*TRADD*	4911	11303	0.435
12	*TRAF2*	4968	12435	0.399

Ů: largest adjusted WNL values for leader genes
*: largest adjusted TIS values for genes with high interactions
µ: specificity scores

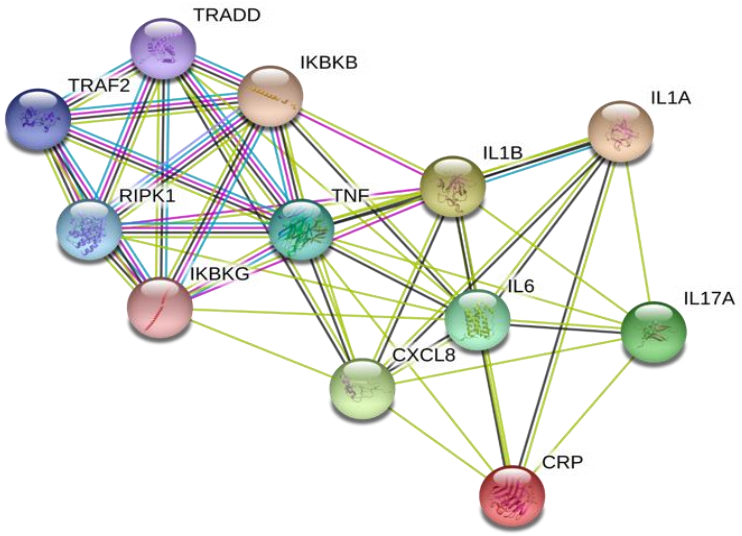

Figure 4: Demonstrated the genes involved in the pathological process of Coronavirus infection after NETs targets network expansion.

Figure was obtained from STRING software (level of confidence > 0.7). Colored nodes display query proteins and first shell of interactors. Filled nodes reveal some 3D structure is predicted/known. Blue edge presents known interactions from curated databases. Black edge means co-expression. Green edge reports textmining. Pink edge presents known interactions experimentally determined.
Clustering and gene distribution data analysis resulted in the following two clusters: 3 genes (*TNF, IKBKB* and *RIPK1*) in cluster 1 and 9 genes (*TRAF2*, *IKBKG*, *TRADD, IL1A, IL1B, CXCL8, IL6, IL17A* and *CRP*) in cluster 2 using the K-means classification process (Table 6). Genes were ranked in clusters according to the WNL and TIS parameters. A statistically significant difference in the adjusted TIS was revealed (p-value = 0.007) using the ANOVA test (Table 7). To optimize the differences between genes in different clusters, the F values were considered, and the clusters were chosen. A significant difference between the two clusters was deduced on the TIS scores (p-value = 0.009), using the Kruskal Wallis test.

Table 6: Clustering of the primary NETs targets network after one expansion.

No.	Gene	Cluster	Distance
1	*TNF*	1	5524.375
2	*IL6*	2	1412.135
3	*IKBKB*	1	2563.278
4	*CXCL8*	2	1269.315
5	*IL1B*	2	409.804
6	*IL17A*	2	3333.301
7	*TRAF2*	2	3814.192
8	*IKBKG*	2	3727.508
9	*RIPK1*	1	3132.051
10	*TRADD*	2	2695.666
11	*IL1A*	2	1870.890
12	*CRP*	2	4275.357

Table 7: Differences among clusters in the expanded network using the ANOVA test.

	Cluster		Error		F	Significance
	Mean square	df	Mean square	df		
WNL	9463826.778	1	2271676.089	10	4.166	0.069
TIS	108028306.8	1	9655411.689	10	11.188	0.007

Furthermore, ontology analysis resulted 702 significant biological processes for the primary NETs targets in coronavirus infection (*IL6*, *TNF*, *CRP*, *CXCL8*, *IL1B*, *IL17A* and *IL1A*), performed by BinGO software (p-value < 0.05, Benjamini-Hochberg correction, hypergeometric clustering) . The most important pathways that involved the 7 identified genes versus whole set annotation, are shown in Table (8).

Table 8: Biological processes of the NETs targets in Coronavirus infection carried out by BinGO software.

Network description	ID	p-value	Adjusted p-value
Biological regulation	6943	6.33E-03	1.12E-02
Regulation of biological process	6554	4.23E-03	8.22E-03
Positive regulation of biological process	2208	2.07E-06	2.55E-05
Response to stimulus	3633	6.78E-05	2.91E-04
Immune response	619	2.75E-10	3.59E-08
Immune system process	948	5.50E-09	5.25E-07
Response to stress	1773	4.44E-07	8.94E-06
Response to wounding	541	1.07E-10	3.59E-08
Defense response	620	2.78E-10	3.59E-08
Inflammatory response	315	2.35E-12	1.80E-09
Regulation of cellular process	6224	2.94E-03	5.95E-03

DISCUSSION

COVID-19 is a new viral respiratory disease, with around 10-15% of affected patients progressing to cytokine storm-induced ARDS (28). Combined with its pandemic spread, the severity of COVID-19 has put an immense pressure on the healthcare system, therefore, treatment tactics are desperately needed. Dysregulated NETosis is also encountered by COVID-19 patients who shown life-threatening complications including blood clots and inflammation (5). Neutrophils are early recruited to infection sites where, through oxidative burst and phagocytosis mechanisms, they kill the pathogen (29). Neutrophils, however, have another less understood way of destroying pathogens: the formation of NETs (30). Three NETs markers were identified: myeloperoxidase-DNA (MPO-DNA), citrullinated histone H3 (Cit-H3), and Cell-free DNA (31). Besides decreased neutrophil count, cell-free DNA is closely related with acute phase reactant (APR), like D-dimer, LDH, and C-reactive protein. On the other hand, Cit-H3 is associated with high platelet levels. In hospitalized patients who received mechanical ventilation, both cell-free DNA and MPO-DNA were detected at high levels.

Netosis and COVID-19 infection

In patients with extreme COVID-19 infection, aberrant activation of neutrophils triggers an amplified host reaction. Neutrophilia predicts bad outcomes in SARS-CoV-2 patients (32), and the neutrophil: lymphocyte ratio is considered as an independent risk factor for serious illnesses (33). NET production is a controlled process, though the involved signals are not fully understood. In fact, major enzymes are involved in the formation of NETs; neutrophil elastase (NE), that degrades intracellular proteins and induces nuclear disintegration; peptidyl arginine deiminase type 4 (PAD4), that citrullates histones to promote chromosomal DNA decondensation and release; and gasdermin D, which produces neutrophil membrane pores to facilitate cell membrane rupture (34–36). Netosis is also associated with increased levels of intracellular Reactive Oxygen Species (ROS) of neutrophils incubated in the presence of COVID–19. Reactive oxygen species can kill the virus directly by causing oxidative damage or indirectly in neutrophils by stimulating pathogen elimination via neutrophil extracellular trap formation(37,38) . ROS also have a detrimental role promoting venous thrombus formation through modulation of the enzymatic cascade of fibrinolysis systems, coagulation and the complement system (39). These findings undoubtedly point to a critical role for neutrophils in the pathology of infection. Some authors suggests that in the presence of some neutrophil stimuli, ROS may not be needed to form NETs (40,41) .

In ARDS patients, the bronchoalveolar lavage fluid and plasma of extracellular histones, perhaps partially derived from NETs, are elevated (42). Bare histones are toxic to cells, though, reliable scientific evidence supports the function of histones in sepsis and ARDS (43). As an answer to a number of ARDS-inducing stimuli, NETs develop, and dissolving or preventing NETs decreases lung damage and increases survival (33), (12). Patients having a serious SARS-CoV-2 infection, acute cardiac and kidney injuries are commonly seen, leading to disease mortality (44). Furthermore, intravascular NETs were shown to play a key role in the initiation and accretion of arterial and vein thrombosis (45). For instance, NET complexes are elevated in serious coronary artery disease, and NET levels are positively correlated with thrombin levels that predict

adverse cardiac events (46). Besides, histones may further promote the activation of platelets by acting on platelets as ligands for the Toll-like receptors (47). At the same time, by digesting tissue factor pathway inhibitor, and major coagulation inhibitors antithrombin III, NE that is bound in its active form to NETs, also possibly plays an essential role (48). Equally important, there is almost certainly a feedback loop whereby the pro-coagulant activity contributes to platelet activation (e.g., that of thrombin), and then activated platelets further increase NETs formation (48),(49). Dissolving NETs with DNase I retrieves the normal perfusion of the kidney and heart microvasculature in animal models (50–52). Based on these findings, we argue that targeting intravascular NETs in patients with extreme COVID-19 infection can similarly reduce thrombosis.

Netosis and Cytokine storm

Increased serum levels of IFN-a, γ, IL-1b, IL-6, Il-12, Il-18, Il-33, TNF a,b, CCL-2&3 and CXCL 8,9,10 are characterized by uncontrolled systemic inflammatory response, leading to multiple organ failure. Seven target genes were found with the present *in-silico* study, of which four were leading genes (*IL-6, TNF*, *IL1b* and *CXCL8*) and two were of the most influential genes (*Il-17* and *CRP*) in the interaction network of the primary NETs targets of coronavirus infection. Elevated plasma concentrations of IL1β, IL2, IL6, IL7, IL8, IL10, IL17, IFNγ, IFNγ-inducible protein 10, G-CSF, monocyte chemoattractant protein 1 (MCP1), TNF alpha and macrophage inflammatory protein 1af are associated with extreme COVID-19 infection with cytokine storms (53–55). The activity of neutrophils is regulated by these inflammatory mediators, as well as the expression of chemoattractant, molecules that increase neutrophil trafficking at inflammation sites, is induced. In another hand, cytokine storms were reported to contribute to acute lung damage, ARDS, and death (56,57). It is particularly remarkable that NETs are able to induce IL1β secretion by macrophages and that IL1β increases the development of NETs in different diseases, including atherosclerosis, and aortic aneurysms (13,58). Together these data indicate that midst a cytokine storm, a signaling loop between neutrophils and macrophages can result in a progressive uncontrollable inflammation under conditions in which the usual signals to dampen inflammation are lost.

Netosis and Thrombosis

Recently, two Dutch studies reported cumulative occurrences of thrombotic events between 48% and 49%, respectively, in patients with COVID-19 pneumonia in their Intensive Care Units (ICUs) (59,60).

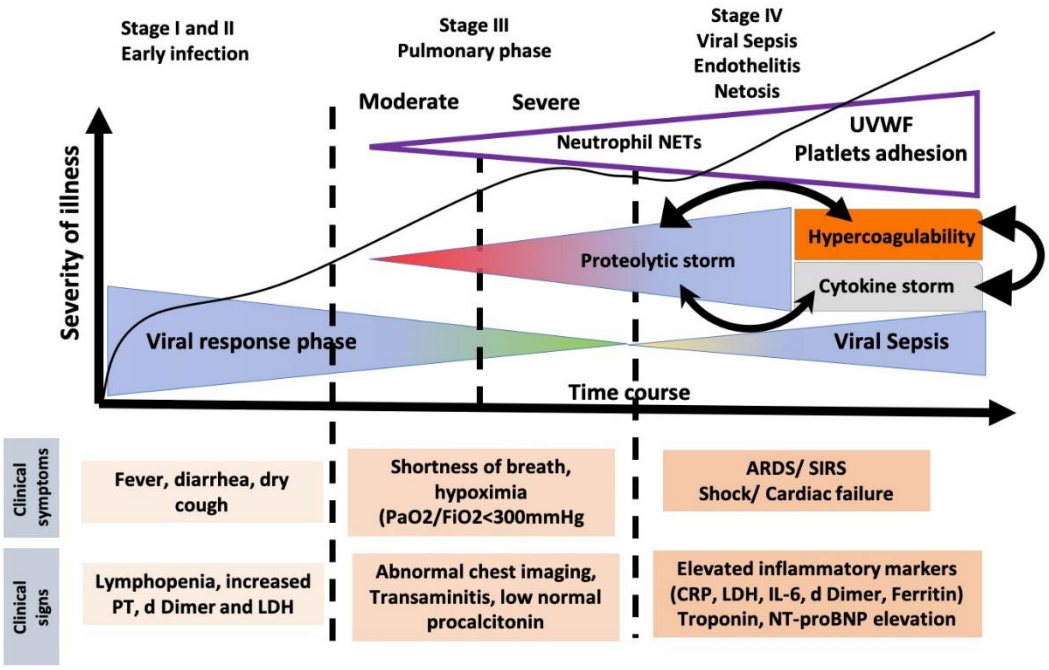

Figure 5: COVID-19 clinical evolution (Siddiqi *et al*, 2020).

Neutrophils are actively recruited by cytokines and platelets and released high level of serine proteases imbalance mainly in coagulation/complement/fibrinolytic cascade, inducing severe "proteolytic storm" with hypercoagulability, endothelial lesions and inflammation, and NETs formation.

NETs are phenomenally thrombogenic. Thus, it would seem that there is a related predisposition to thrombosis if there is neutrophilia. NETs are capable of inducing macrophages to secrete one of the leading genes, the *IL1β.* The speedy development of NETs and IL1β could accelerate respiratory decompensation, microthrombi formation, and aberrant immune responses if a NET-IL1β loop is triggered in severe COVID-19 (45). Importantly, *IL1β* induces *IL6*, another leading gene, that has recently emerged as a potential therapeutic candidate for COVID-19 (26). Neutrophilia, as well as IL1β, IL6, and D dimer elevations tend to characterize an extreme COVID-19 infection. Mechanistically, by electrostatic interactions between the platelet phospholipids and NET histones, NETs activate the coagulation contact pathway (61). Histones may also promote the activation of platelets by acting on platelets as ligands for the Toll-like receptors (62). Assuming the existence of a feedback loop where pro-coagulant activity contributes to platelet activation and thereby activated platelets further support NET formation (49). The link between NETosis and coagulation was made because of the presence of neutrophil elastase

(NE) on NETs. NE inactivates tissue factor pathway inhibitor (TFPI) through cleavage, thus resulting in increased procoagulant activity (63) . Procoagulant activity leads to platelet activation and activated platelets can enhance NET formation, but platelet depletion does not necessarily prevent NETosis (64,65).

CRP and Netosis

CRP is a protein produced by the liver, which is elevated in response to inflammation (66). The complement can be triggered, and phagocytosis can be enhanced by CRP levels, thereby cleaning up the pathogenic microorganisms entering the body. For early diagnosis of pneumonia, CRP levels may be used (67), again, patients with serious pneumonia have elevated levels of CRP. Besides, it is a substantial indicator for the diagnosis and evaluation of serious infectious lung diseases (68). The CPR levels were elevated, and were observed up to 86% in severe COVID-19 patients (69–71). The higher levels of CRP in SARS-CoV-2 patients may be viewed as a serum marker of disease progression. Moreover, before the disease progressed, elevated CRP levels occurred. Therefore, to forecast the risk of disease progression in non-severe COVID-19 cases, we suggest that elevated CRP levels can be a helpful early biomarker that can assist health workers to recognize such patients at an early stage of primary treatment. In addition, COVID-19 patients with high CRP levels require close monitoring and care, although symptoms have not been established to meet the requirements for the course of the serious disease. A substantial increase in CRP was observed in patients with COVID-19 with levels of 20 to 50 mg/L on average (69,72,73). CPR levels were 10 folds higher in patients who died from COVID-19 than the recovered ones (74). A significant correlation was perceived between CRP concentrations and the aggravation of non-severe COVID-19 patients (79). Consequently, researchers proposed the CRP as an effective marker with an optimal threshold value of 26.9 mg/L to anticipate the aggravation probability of non-severe COVID-19 cases (75).

IL-6 and Netosis

IL-6 is a powerful acute phase response inducer that is involved in the differentiation of B cells and monocytes and is necessary for T(H) 17 cells production. The IL-6 gene, with the highest interaction score in the present interaction network study, is present in over half of COVID-19 patients (76). Levels of IL-6 seem to be connected to the inflammatory response, needing for mechanical ventilation/intubation, respiratory failure, and mortality in COVID-19 patients (77),(78). A recent meta-analysis of nine studies (total of 1426 patients) had reported IL-6 and COVID-19 outcome, showing that IL-6 levels were more than three folds higher in patients having complicated COVID-19 relative to patients with uncomplicated disease, and the risk of mortality was correlated with IL-6 (78). It must also be noted that IL-6 levels at hospital admission tend to be a good prognosticator for the progression of the combined endpoint to serious disease/ in-hospital mortality, and tend to be the best negative outcome prognosticator. Therefore, targeting the SARS-CoV-2-induced cytokine storm by using anti-IL-6 drugs may be a viable therapeutic choice to improve outcomes in COVID-19 patients along with supportive care strategies (79). Humanized monoclonal antibodies like Sarilumab and tocilizumab target soluble and membrane-bound IL-6 receptors, and inhibit downstream IL-6 effects (80,81). Data from an open-label of uncontrolled case series (n= 21) presented that administration of tocilizumab resulted in a quick resolution of symptoms and radiographic abnormalities in patients with serious COVID-19 (82). Sinha *et al*. (83) found that prompt IL6ri treatment before the onset of critical disease is linked with decreased mortality from serious coronavirus infection, which may be used to direct present clinical management, although more conclusive RCT findings are required from the medical community.

IL-1 and Netosis

IL-1a is produced by activated macrophages. It is involved in inflammatory response and stimulate the release of prostaglandins. Another potent proinflammatory cytokine is IL-1b. It may induce prostaglandin synthesis, as well as neutrophil influx and activation, activation of T cells and production of cytokines, activation of B cells and formation of antibodies, and also promote the differentiation of T cells by Th17. An IL-1/IL-6 signature increases neutrophils and CRP levels. Anakinra, an interleukin-1 receptor antagonist, is a highly effective receptor antagonist due to the greater affinity for IL-1R1 than that for IL-1 itself (84). Anakinra is also used *in-vitro* to treat macrophage activation syndrome (MAS), associated with a severe cytokine storm (85). Anakinra resulted in a rapid reduction in fever and CRP with a total dose below 2,000 mg, decreased oxygen requirements and resulted in fewer days in the ICU without IMV compared with the control group. Early use of anakinra can be a significant clinical decision when respiratory conditions begin to deteriorate in the sense of increasing systemic inflammation.

IL-8 and Netosis

CXCL8 or IL-8 is a chemotactic factor that attracts neutrophils, basophils and T cells. It is involved in neutrophil activation (by 5-10 folds). The production of IL-8 is mainly regulated by NF-κB transcription factors (86). IL-8 is an important chemokine to promote tissue infiltration by polymorphonuclear leukocytes (87,88). Thus, these cytokines seem to play a fundamental role in systemic inflammatory response syndrome (SIRS) by augmenting NETs release that is mediated by enhanced formation of various reactive oxygen and nitrogen species. These species and NETs, in turn, might increase cytokine production, which points towards the idea of a positive loop to propagate and maintain the inflammatory condition (89). Besides, IL-8, a product of activated neutrophils and a cardinal neutrophil chemoattractant, can be blocked by neutralizing antibodies (HuMax-IL8). HuMax-IL8 effectively prevents IL-8 binding to neutrophils and inhibits the activation and recruitment of neutrophils towards inflammatory sites, which decreases inflammation. In a variety of cancer cell types and inflammatory diseases, many clinical trials were conducted using this neutralizing antibody to reduce neutrophils and subsequently NETosis among patients with severe COVID-19 infection (90).

IL-17a and Netosis

IL-17a is part of the IL-17 family, which causes stromal cells to develop proinflammatory and hematopoietic cytokines. Hou *et al*. found that the excessive amount of IL-6 promotes the generation of Th17 cells in murine viral models, and the resulting IL-6 and IL-17 synergistically promote viral survival by shielding virus-infected cells from apoptosis (89). A further important implication is in influenza virus infection, where acute pulmonary injury with high mortality is associated with a rise in airspace neutrophils promoted by IL-17 (91). Huang *et al*. reported that IL-17 is increased in intensive care SARS-CoV-2 patients compared to non-intensive care and controls. They hypothesized that blocking IL-17 may have the potential to improve COVID-19's aberrant immune

response and mortality associated with ARDS (92), (93). In spite of the effect of blocking IL-17 on Th2, the response should be more deeply investigated since SARS-CoV-2 also stimulates Th-2 cytokines production (IL-4 and IL-10) which suppress Th1/Th17 mediated inflammation (94). These findings suggest the remarkable need of investigating on IL-17 blocking role in COVID-19, which appears as a promising therapeutic target.

TNF-a and Netosis

Macrophages primarily secrete TNF and activate IL-1 secretion, which is responsible for fever and cachexia. In most types of inflammation, especially in the acute phase, TNF is known to be produced and is important in organizing and developing the inflammatory response. However, excess levels of TNF production becomes immune-suppressive for too long (95). In serious cases of autoimmune inflammatory disorders like inflammatory bowel disease, ankylosing spondylitis or rheumatoid arthritis, anti-tumor necrosis factor (TNF) antibodies were used for more than 20 years. As reported on September 29, 2019, US Food and Drug Administration approved anti-TNF therapy, indicating TNF as a valid target in many inflammatory diseases. Although, TNF is critical in almost all acute inflammatory reactions, acting as an inflammation amplifier, it is present in the blood and disease tissues of patients with SARS-CoV-2 infection. Recently, Feldman *et al*, indicated that in patients on hospital admission for COVID-19 infection, anti-TNF therapy should be tested to avoid progression to the need for intensive care support (95).

CONCLUSION

In this study, through interaction network analysis, seven target genes with a potential role in the pathogenesis of NETs in coronavirus were reported. Even with the limits of any *in-silico* study, our preliminary findings could highlight the implication of these genes for a further comprehensive data for the complex regulatory networks of COVID-19 infection. Targeted immunotherapy against the aforementioned cytokines (IL-1, 6, 8, 17 and TNF-a) may be promising therapeutic agents against NETs which may reduce the mortality rate of SARS-CoV-2 infection.

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