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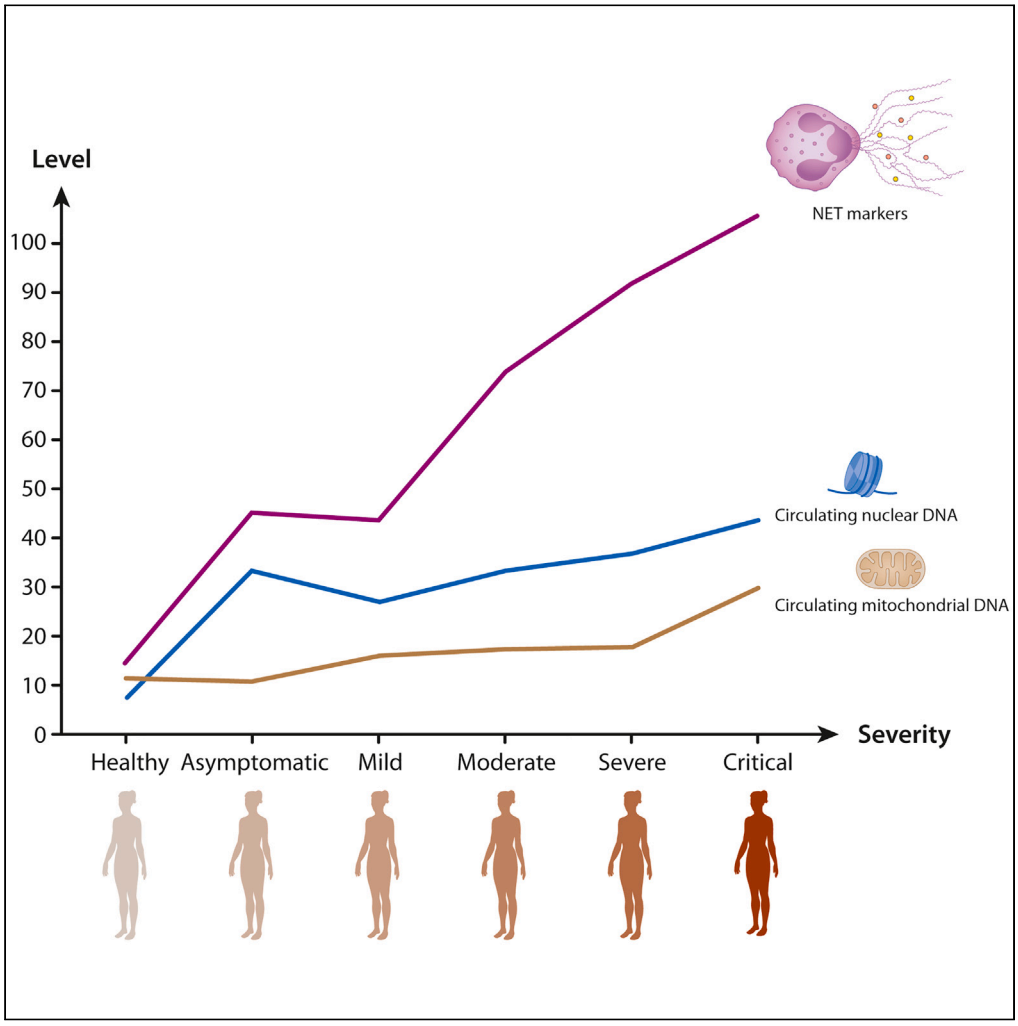

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Highlights

Covid-19 severity is associated with the elevation of NETs markers and cir-nDNA

Asymptomatic individuals unexpectedly showed also significant netosis formation

Similar trends were observed in Delta- and Omicron-infected patients

Circulating mitochondrial DNA level does not associate with disease severity

Kapoor et al., iScience *27*, 109573
May 17, 2024
https://doi.org/10.1016/j.isci.2024.109573

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Association of vascular netosis with COVID-19 severity in asymptomatic and symptomatic patients

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SUMMARY

We examined from a large exploratory study cohort of COVID-19 patients (*N* = 549) a validated panel of neutrophil extracellular traps (NETs) markers in different categories of disease severity. Neutrophil elastase (NE), myeloperoxidase (MPO), and circulating nuclear DNA (cir-nDNA) levels in plasma were seen to gradually and significantly ($p < 0.0001$) increase with the disease severity: mild (3.7, 48.9, and 15.8 ng/mL, respectively); moderate (9.8, 77.5, and 27.7 ng/mL, respectively); severe (11.7, 99.5, and 29.0 ng/mL, respectively); and critical (13.1, 110.2, and 46.0 ng/mL, respectively); and are also statistically different with healthy individuals (N = 140; $p < 0.0001$). All observations made in relation to the Delta variant-infected patients are in line with Omicron-infected patients. We unexpectedly observed significantly higher levels of NETs in asymptomatic individuals as compared to healthy subjects ($p < 0.0001$). Moreover, the balance of cir-nDNA and circulating mitochondrial DNA level was affected in COVID-19 infected patients attesting to mitochondrial dysfunction.

INTRODUCTION

Despite intense basic and clinical investigation, a comprehensive account of all pathophysiological contributors to COVID-19 has not yet emerged.[1] Although this infectious disease is respiratory, for instance, the symptoms and severe complications observed thus far have been multiple, systemic, and multi-organ in nature.[2] Indeed, while a large majority of deaths are due to respiratory complications, severe myocardial and vascular symptoms account for a significant proportion of lethal events.[3] The multiplicity of symptoms makes the prognosis and follow-up of patients more difficult. The development of new diagnostic tools which are adequate for that task is therefore necessary.

Our team was among the first to reveal that the vast majority of COVID-19 comorbidities consist of diseases in which neutrophil extracellular traps (NETs) formation is deregulated (NETopathies).[4–8] Based on these observations, several publications have since shown that NETs markers are elevated in both severe and non-severe phases of the disease.[9,10] NETs are produced by activated neutrophils and consist of long fibers of partially decondensed DNA containing granular proteins, in particular powerful enzymes such as neutrophil elastase (NE) and myeloperoxidase (MPO).[11–14] The formation of NETs (or "netosis") plays a role in the innate immune response which occurs during the first hours of infection and whose function is to neutralize microbes, physically and enzymatically.[15–17] However, the accumulation, incomplete elimination, or abnormal localization of NETs can promote inflammation and cell damage during microbial infection or in sterile inflammatory conditions.[18–20] In this way, the action of NETs is double-edged: besides its rapidity and relative effectiveness in the first hours of infection, it can also be deleterious and can participate in or lead to autoimmune diseases or serious physiological disorders.[20–22] It is by such mechanisms that the uncontrolled formation of NETs appears to play a key role in the pathophysiology of COVID-19.[5,23,24]

We previously validated the use of a set of markers, consisting of two protein markers (NE and MPO) and cir-nDNA concentrations, to indirectly quantify NETs formation in metastatic colorectal cancer[25,26] and in COVID-19[26,27] patients. First, we demonstrated that the quantitative analysis of cir-nDNA empowers the assessment of NETs formation when combined with the quantification of the granular enzymes (NE and MPO) that are essential in NETs production and in the digestion of trapped microbes.[25,26] Second, we have recently demonstrated that the degradation of NETs' chromatin fibers are degraded by DNases in blood and could be autocatalytic, leading to DNA fragmentation, thus producing mainly mono-nucleosomes as well as a very small proportion of di-nucleosomes.[26] Several studies showed that plasma-extracted

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https://doi.org/10.1016/j.isci.2024.109573

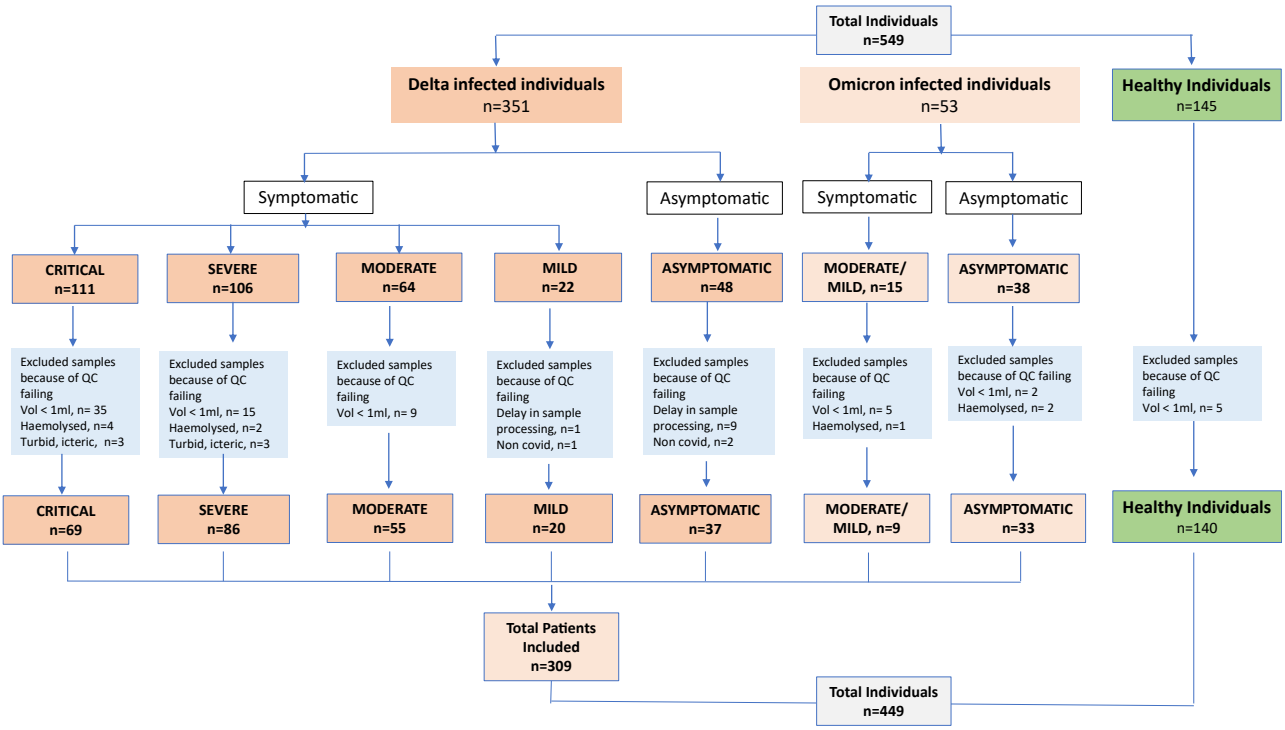

Figure 1. Study flowchart

DNA is mainly associated with mono-nucleosomes and to a lesser extent di-nucleosomes (>90% in healthy individuals).[28–31] Consequently, cir-nDNA can therefore be considered as a potential NETs marker.

While the dysregulation of netosis has been described in the literature in general terms, principally either in patients with non-severe or severe clinical conditions, the objectives of our exploratory study are (1) to confirm in a large cohort of infected individuals the formation of NETs according to specific categories of patient, notably in view of their clinical condition; (2) to evaluate NETs markers' predictive potential as to disease severity; (3) to observe NETs formation in Omicron-infected individuals and (4) to examine whether NETs formation exists in asymptomatic individuals.

RESULTS

Cohort study, design, and patient characteristics

Following the inclusion of 549 individuals, plasma examination was performed in critical ($N = 69$), severe ($N = 86$), moderate ($N = 55$), mild ($N = 20$), and asymptomatic ($N = 37$) conditions concerning Delta-infected individuals, and moderate/mild ($N = 9$), and asymptomatic ($N = 33$) conditions concerning Omicron-infected individuals and in 140 healthy individuals (Figure 1). This categorization followed national guidelines, which are based on radiological findings, oxygen requirements, and the need for intensive therapy and treatment (see STAR Methods). Briefly, the asymptomatic and mild patients were mostly outpatients, with a few mild patients being admitted to the hospital solely for the purpose of isolation. Patient management mostly included vitamin supplementation for high-risk mild patients, with a small number of these being administered through an early monoclonal antibody infusion. The moderate, severe, and critical categorizations were largely based on oxygen requirements. Those patients underwent a variety of treatment and modalities and were all treated as inpatients (see STAR Methods). Patient characteristics and comorbidities are listed in Table 1.

NETs marker levels increase with COVID-19 severity in Delta-infected patients

Figure 2 shows the median NE, MPO, cir-nDNA, and cir-mtDNA plasma concentration in Delta-infected patients with asymptomatic ($N = 37$), mild ($N = 20$), moderate ($N = 55$), severe ($N = 86$), and critical ($N = 69$) clinical conditions. As compared to healthy individuals (12.1 ng/mL), the MPO plasma level gradually increased by 4.1, 6.4, 8.2, and 9.1-fold in patients with mild, moderate, severe, and critical conditions (49.0, 77.5, 99.5, and 110.2 ng/mL; and $p < 0.0001$, $p < 0.0036$, $p < 0.0001$, and $p < 0.0001$, respectively) (Figures 2 and S1; Table S1). As compared to healthy individuals (2.6 ng/mL), the NE concentration gradually increased by 1.4, 3.8, 4.6, and 5.1-fold in patients with mild, moderate, severe, and critical conditions (3.7, 9.8, 11.7, and 13.1 ng/mL; and $p < 0.0001$, $p = 0.44$, $p < 0.0001$, and $p < 0.0001$, respectively) (Figures 2 and S1; Table S1). As compared to healthy individuals (4.3 ng/mL), the cir-nDNA plasma level gradually increased by 3.7, 6.4, 6.7, and 10.6-fold in

Table 1. Patient characteristics

	Number	Age: median and range	% female	% male	% with >1 comorbidities	% with one comorbidity
Delta infected						
Critical (expired)	20	67 (39–82)	25.0	75.0	70.0	10.0
Critical	69	53 (33–82)	28.3	71.7	36.2	18.8
Severe	86	47 (27–69)	25.0	75.0	16.3	33.7
Moderate	55	47 (19–79)	29.1	70.9	29.1	23.6
Mild	20	47 (29–67)	52.9	47.1	20.0	15.0
Asymptomatic	37	37 (14–64)	36.0	64.0	0	2.7
Omicron infected						
Moderate/mild	9	41 (26–78)	22.2	77.8		
Asymptomatic	33	41 (19–78)	60.6	39.4		

Plasma of 267 Delta and 42 Omicron infected patients were examined in the study out of 409 patients.

patients with mild, moderate, severe, and critical conditions (15.8, 27.7, 29.0, and 46.0 ng/mL; and $p < 0.0001$, $p < 0.0014$, $p < 0.0001$, and $p < 0.0001$, respectively) (Figures 2 and S3; Tables S2–S6). When dichotomizing the critical cohort and distinguishing patients who died from critical conditions and those who did not, we observed a slightly higher level of the NE, PO, and cir-nDNA, and a significant (~3-fold) increase of the cir-mt-DNA level (Figure S4).

The panel of NETs markers (MPO + NE + cir-nDNA) is termed "NETplex". It showed that the levels of NETs markers in asymptomatic patients are higher not only as compared to healthy subjects (2.0-, 3.3-, and 11.7-fold of NE, MPO, and cir-nDNA, respectively) but also as compared to COVID-19 patients with mild conditions (by 1.7-, 1.2-, and 2.4 fold-increase for NE, MPO, and cir-nDNA, respectively) (Figures 3 and S1–S3; Table S1). A comparison of the individual values shows 33 out of 37 (89%) of Delta-infected asymptomatic patients having significantly higher cir-nDNA value than healthy subjects. A comparison of individual values shows 32 out of 37 (86.5%) of Delta-infected asymptomatic patients having significantly higher MPO values than healthy subjects.

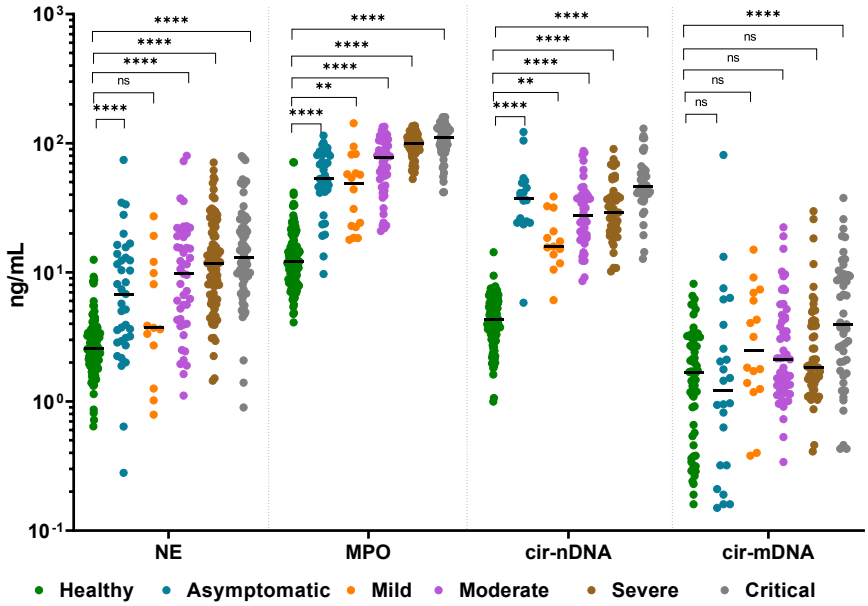

Figure 2. NETs marker levels increase with COVID-19 severity in Delta-infected patients

Each dot represents plasma concentration of NE, MPO, cir-nDNA, and cir-mtDNA of a patient with asymptomatic ($N = 37$), mild ($N = 20$), moderate ($N = 55$), severe ($N = 86$), or critical conditions ($N = 69$). (blue, orange, purple, brown, and gray color, respectively). Each green dot represents NETs marker value in a healthy individual ($N = 140$). For each of the markers, the median values of these subgroups are statistically different each other ($p < 0.0001$) using the Kruskall-Wallis statistical test, and they are also statistically different with healthy individuals ($N = 140$; $p < 0.0001$). A probability of ≤ 0.05 was considered to be statistically significant by Student's t test: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

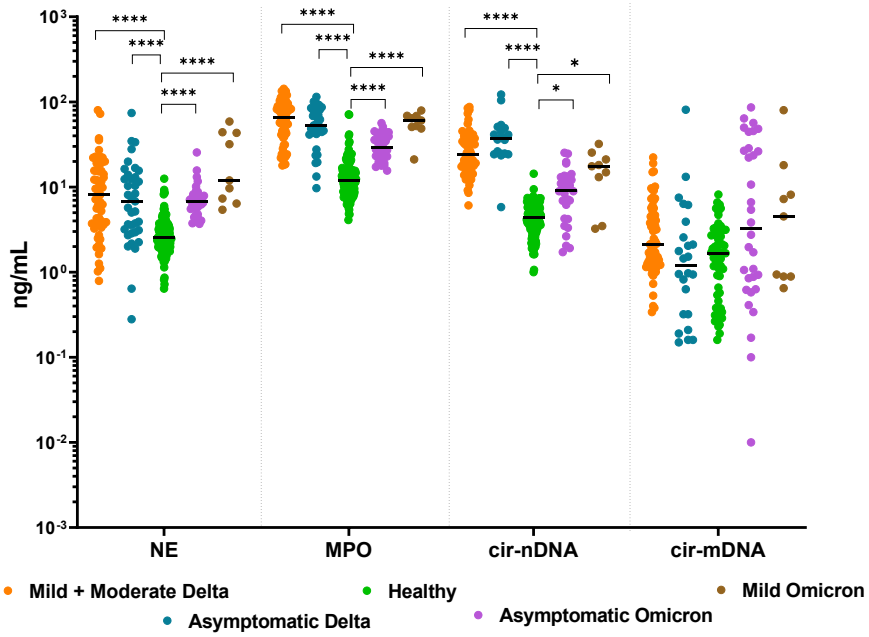

Figure 3. NETs marker levels increase with COVID-19 severity in Omicron-infected patients

Values of symptomatic (purple, *N* = 37) and mild/moderate (brown, *N* = 75) Omicron patients were compared with asymptomatic (blue, *N* = 33) and mild/moderate (orange, *N* = 9) Delta patients, as well as with healthy individuals (green, *N* = 140). A probability of ≤0.05 was considered to be statistically significant by Student's t test: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

In contrast to cir-nDNA, cir-mtDNA concentrations in asymptomatic and symptomatic patients in mild to severe conditions showed levels similar to those of healthy individuals (1.2–2.5 ng/mL, range). (Figures 2 and S6–S9; Table S1). Only critically ill patients showed a statistically significant difference in cir-mtDNA levels (3.9 vs. 1.7 ng/mL, $p < 0.05$) (Figure S4; Table S1).

NETs marker levels increase with COVID-19 severity in Omicron-infected patients

Plasma median concentrations of NE, MPO, and cir-nDNA are 6.8, 29.2, and 8.9 ng/mL in Omicron-infected asymptomatic patients being 2.6, 2.4, and 2.1-fold higher than in healthy individuals (Figure 3; Table S3). They are 11.9, 60.6, and 17.5 ng/mL in the Omicron-infected mild patients being also higher as compared to healthy individuals (4.6, 4.8, and 4.1-fold) (Figure 3; Table S3). A comparison of individual values shows 20 out of 33 (60.6%) Omicron-infected asymptomatic patients having significantly higher cir-nDNA values, as compared to healthy subjects. A comparison of individual values shows 27 out of 33 (81.8%) Omicron-infected asymptomatic patients having significantly higher MPO value, as compared to healthy subjects. The cir-mtDNA concentration in healthy individuals and Omicron-infected asymptomatic or symptomatic patients showed similar levels (Figures 3 and S6–S9).

Diagnostic potential of NETs markers

Using Kruskal-Wallis test, it is shown that the disease severity factor influences the three markers, whose values increase with increasing disease severity. But from Dunn's multiple comparisons test, it is seen that NE does not distinguish mild, moderate, severe, and critical conditions, MPO distinguishes critical exp. from healthy, moderate, mild, and asymptomatic; critical from moderate and asymptomatic; severe from asymptomatic and healthy; mild is not distinguished from severe, moderate, and critical; cir-DNA does not distinguish mild, moderate, severe, critical exp., and critical conditions (Tables S2–S5).The AUROC study showed AUC of 0.95, 0.94, 0.98, 1.00, and 1.00 for the MPO marker, and 0.99, 0.98, 1.00, 1.00, and 1.00 for the cir-nDNA marker in patients with asymptomatic, mild, moderate, severe, and critical conditions, respectively (Figure 4). Note, AUC using NE showed lower AUC values ranging from 0.75 to 0.98, respectively. Cir-mtDNA did not show diagnostic capacity except when comparing Delta-infected patients with critical illness to healthy subjects (AUC = 0.95). The MNR was found significantly lower in mild, moderate, severe, and critical conditions (2.1-, 5.3-, 6.4-, and 4.9-fold, respectively) (Figure S10). Note, since ROC analysis was used to compare the various disease severity groups with healthy individuals, it does not evaluate the diagnostic power to discriminate between different degrees of disease severity.

DISCUSSION

Using a cohort of patients which is larger and better categorized than many other studies[9,10] on this subject, this study showed that the median values of plasma concentrations of the NETs markers (NE, MPO, and cir-nDNA) significantly increase with disease severity from mild,

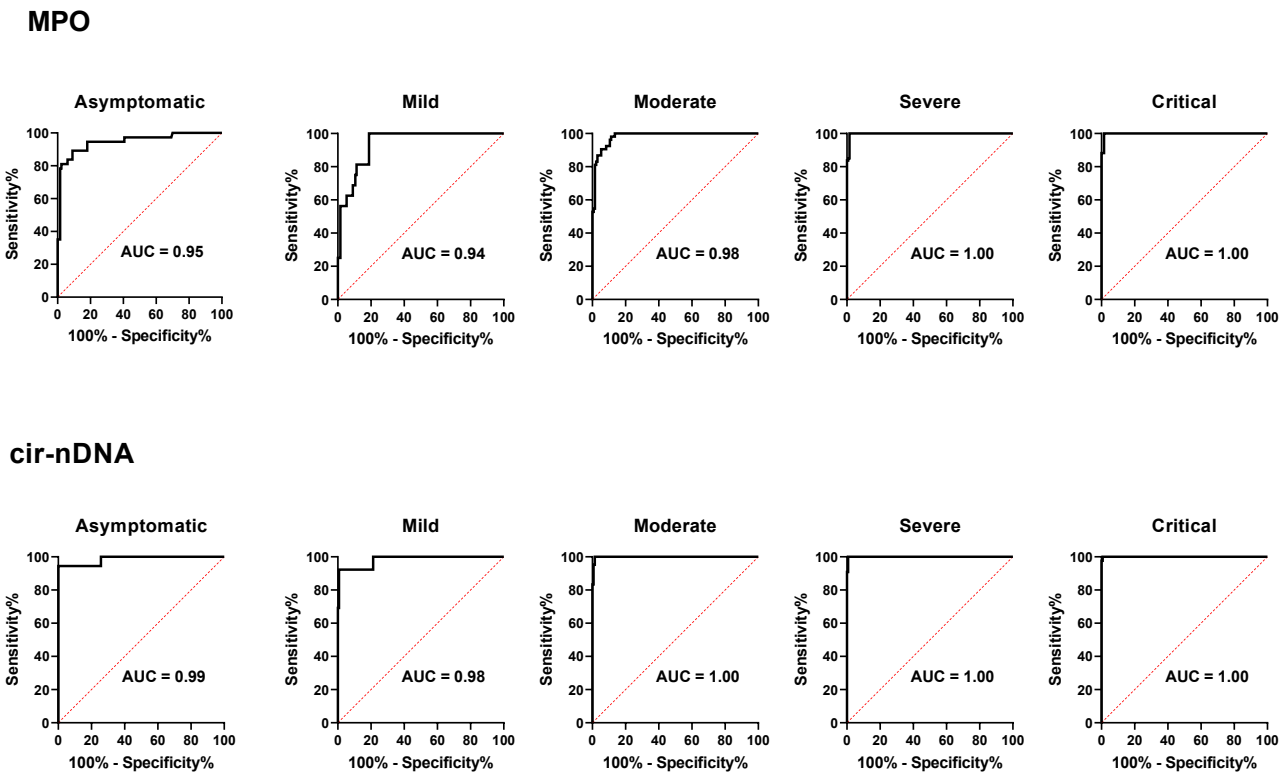

Figure 4. Diagnostic potential of NETs markers

Area under receiver operating curves (AUROC) of MPO and cir-nDNA in Delta-infected patients with asymptomatic, mild, moderate, severe, and critical conditions. AUC, Area under curve. Note, (i), AUROC using NE are not shown here but exhibited lower AUC values ranging from 0.75 to 0.98 from asymptomatic to critical conditions, respectively; (ii), Cir-mtDNA did not showed diagnostic capacity except when comparing Delta-infected patients with critical illness to healthy subjects (AUC = 0.95); and (iii), AUROC using Omicron-infected patients were not determined because of the low number of patients (42).

moderate, severe to critical conditions. On the other hand, the diagnostic value of these three markers is attested to by the fact that their concentrations can be more than 10 times greater in critical patients than in healthy individuals. The specificity appears high since the median value in mild cases is at least twice as high as that of healthy individuals. Ninety percent of the examined plasma samples, present NE, MPO, and cir-nDNA values that are higher than the highest values obtained in healthy individuals. As a whole, this shows the robustness of these markers' ability to categorize patients' clinical conditions. AUROC analysis showed that cir-nDNA and MPO and to a lesser extent NE exhibit high diagnostic power in all infected individuals. Although the Omicron-patient cohort is low (42 mild/moderate and asymptomatic patients), our data for that cohort are in line with all observations made in relation to the Delta variant-infected individuals.

Unbalanced NETs formation was found to contribute to various illnesses, in regard to inflammation, thrombosis, or auto-immunity.[18,32,33] The cardiovascular complications observed in the acute and post-acute phases of COVID-19,[34,35] along with the generalized presence of micro-clots detected during the autopsies of patients who have died of the disease,[36] point to the involvement of thrombotic events in its pathophysiology. Recently, Pretorius team showed that micro-clots are largely present in the blood of COVID-19 and long COVID patients.[37] Thus, in addition to the nature of the comorbidities associated with COVID-19,[5] and the high values of the NETs markers,[9,10,27,37–40] the aforementioned clinical observations lead us to believe that NETs favor the formation of micro-thromboses in a systemic way. Specifically, we postulate that NETs physically bind to the platelets that are activated by the infection and resulting inflammation, associate with fibrin, and thus favor the formation of thrombus. This would be amplified by NETs degradation products such as circulating DNA of nuclear or mitochondrial origin and histones, which cause inflammation and the production of autoantibodies, which in turn stimulate neutrophils to produce even more NETs, leading to a self-amplification loop.[41] It is possible that this self-amplification loop may persist after the acute phase of the infection, and therefore after the disappearance of the viral particles.[42] Further supporting this is our recent postulation that the persistence of NETs formation following acute SARS-CoV2 infection might be due in large part to three feedback loops involving (1) inflammation, (2) thrombosis, and (3) autoimmunity.[42] Thus, we speculate that this feedback loop maintenance may be the molecular and physiopathological explanation of post-acute sequelae of COVID.

Given our previous works,[26,27] along with that of other teams,[9,10] the combination of protein markers (for example, NE and MPO) and nucleic markers (cirDNA) for NETs analysis appears to be a potential tool for the monitoring of patients infected with SARS-CoV-2. Further

studies are needed to assess the clinical utility of these markers of netosis in the acute and post-acute phase of COVID-19 infection and particularly to predict the occurrence of sequelae in its post-acute phase (long COVID).

Unexpectedly, the plasma concentrations of the NETs markers in asymptomatic patients were higher than those observed in patients with more severe disease. This observation is most striking for the cir-nDNA marker, which shows values more than twice those of patients with mild condition, and nearly 10 times higher than those of healthy individuals. The difference between the median concentrations in asymptomatic individuals and in patients with mild clinical conditions is statistically significant. This observation does not appear to be due to any technological bias since (1) although the analysis is performed on the same blood sample, the ELISA test for NE and MPO is performed on plasma, while the qPCR test for cirDNA is performed on a plasmatic DNA extract, and (2) cirDNA of mitochondrial origin derived from the same DNA extract as the cirDNA of nuclear origin, exhibits a lower median concentration in asymptomatic individuals than in individuals with normal conditions. Thus, the accuracy of our unanticipated observations regarding asymptomatic patients is largely supported by combining NETs proteic markers with cir-nDNA concentration values. This highlights the necessity of combining NETs proteic markers with cir-nDNA in order to obtain a robust interpretation of data. This phenomenon might result from the treatment offered to patients, especially anti-inflammatory and anti-coagulation drugs which are given to those with mild clinical conditions. An alternative if counterintuitive possibility to consider is that the higher inflammation state of a mild, symptomatic patient may result in greater inhibition of netosis. The slightly but significantly higher degree of netosis, such as observed in asymptomatic cases as compared to healthy individuals, as well as in mild cases (consequently a low grade of inflammation), needs to be confirmed and further investigated. As a result, the "asymptomatic" clinical condition of those patients could be questioned. This might have medical consequences, given the implication that an uncontrolled NETs production in part of these patients remains a distinct possibility, and offers an explanation of long-term post-infection sequelae, or long COVID. An examination of NETs markers in a large cohort of subjects experiencing long-term sequelae, but who were previously considered as asymptomatic individuals at the time of infection, is needed to evaluate this hypothesis.

The concentration levels of circulating DNA of mitochondrial origin (cir-mtDNA) follow an opposite trend to those of MPO, NE, or cir-nDNA markers. There is no statistical difference between healthy, mild, moderate, and severe patients individuals. Since cir-mtDNA and cir-nDNA assays are performed from the same plasma DNA extract, this suggests that the observation of these opposite trends might not be due to analytical bias. Moreover, this suggests a clear difference in the mechanism of release of cir-nDNA and cir-mtDNA, and, in addition, that cir-mtDNA levels do not associate with COVID-19 pathogenesis. One possible explanation could be the fact that suicidal netosis mostly release cir-nDNA whereas cir-mtDNA, are mainly released by vital netosis. In addition, cir-mtDNA level in patients in critical condition, especially those who died from the disease, is higher than that observed in the other patient categories. Cir-mtDNA analysis has been studied to identify biomarkers to predict mortality and disease severity in critical care patients.[43] This might be explained by the induction of the various cell death processes (necrosis, apoptosis, pyroptosis, or netosis) when patients are critically ill. Alternatively, it is likely that in response to critical illness, there is damage to the mitochondria leading to the release of mitochondrial DNA into the circulation. Some studies[44,45] have suggested an association between cir-mtDNA levels with increased mortality and disease severity in sepsis critical care patients. In particular, Hepokoski et al.[46] showed that mitochondrial DNA concentrations could be a real-time biomarker in critical care subjects with Covid-19 to guide clinical decision making.

In addition to other circulating NETs byproducts (histones, granules proteins such as NE or MPO, and structural proteins), cir-mtDNA may trigger inflammatory process by acting as damage-associated molecular patterns.[47–50] Thus, despite their presence in lower amounts than cir-nDNA in healthy individuals, cir-mtDNA might be a marker of interest for inflammatory diseases.[47,51] In this study, we confirmed in a higher cohort number that cir-mtDNA did not increase with disease severity, by contrast to cir-nDNA. In COVID-19 critically ill patients as compared to healthy individuals, the only statistically significant difference was seen in cir-mtDNA levels, as has been previously reported.[27] This might be due to general increased cell death in the bodies of these patients. That said, it should be noted that we observed in each group of COVID-19 infected patients an imbalance between the cir-nDNA and cir-mtDNA amounts. Since cir-mtDNA amount mainly corresponds to the number of circulating cell-free mitochondria, our observation[52,53] attests to a lower mitochondria production arising from impaired mitochondrial protein synthesis or some other mitochondrial dysfunction in COVID-19 and long COVID patients.[27] This postulate should be placed in the context of previous reports associating mitochondria dysfunction with COVID-19.[54–56] Further support for such a perspective is offered by the very recent study by Guarnieri et al.,[57] which describes the genetic mechanisms that damage mitochondria in organs and which, in doing so, may contribute to COVID-19 and perhaps to long COVID. It has been suggested that ROS overproduction is involved in the pathophysiological mechanisms of both pathologies,[57] and that in a similar manner, the formation of NETs is promoted by the mitochondrial ROS in neutrophils in patients with various NETopathies.[5,20] Genomic and transcriptomic research is needed to decipher whether NETs are linked to mitochondria dysfunction.

While NETs and consequently neutrophils clearly appear as a significant biological source of cir-nDNA,[26,58] cir-nDNA in COVID-19 patients may also derive from other origins, such as damaged organ tissue. For instance, Y. Dor's team recently reported elevated levels of lung-derived cir-nDNA among COVID-19 patients, with such levels being significantly associated with disease severity and mortality.[59,60] Endothelial, cardiac, or kidney cells undergoing a process of cell death (apoptosis, necrosis, …) or phagocytosis, may release their DNA into the bloodstream. A determination of the tissue-of-origin of COVID-19 cir-nDNA is necessary to better circumscribe the impact of NETs in the high elevation of cir-nDNA, and to infer possible tissue damage, thus improving patient management care, especially patient follow-up.

As determined using ELISA and qPCR assay, NE and MPO on the one hand, and cir-nDNA on the other, appear to provide a reliable estimation of COVID-19 severity, as observed in the gradual increase in their concentrations, the correlation study, and the AUROC assessment. This "NetPlex" panel could therefore be a strong surrogate biomarker in the quantitative follow-up of patients.

This is the largest prospective clinical study on the quantification of NETs formation and of cir-nDNA and cir-mtDNA in symptomatic and asymptomatic SARS-CoV2 infected individuals. It enabled the definitive confirmation of the association of NETs with COVID-19 patient severity, and did so in several categories in a large cohort, further supporting our postulate that NETs formation is a key player in the COVID-19 pathogenesis. Similar observations are made in Delta- and Omicron-infected individuals. The unexpected observation of significantly higher NETs levels in asymptomatic individuals as compared to healthy subjects raises the question of their categorization as healthy individuals or otherwise and warrants further investigation in other cohorts. In addition, this observation suggests that NETs are significantly produced in the early phase of the SARS-CoV2 infection and that uncontrolled NETs formation might contribute to the acute phase of the disease. Furthermore, this work revealed that cir-mtDNA and consequently circulating cell-free mitochondria do not increase with disease severity, or only do so very slightly, which would point to the involvement of mitochondria dysfunction in the COVID-19 pathogenesis, as has been recently demonstrated elsewhere.[56,57] The NETs markers panel (NetPlex) used in this study provides accurate and reliable information as to the presence of NETs and therefore be a strong surrogate biomarker in the quantitative follow-up of patients. It could also potentially predict disease progression. Furthermore, it might have potential in the prognosis of long-term sequelae or "long COVID", given that the persistence of NETs formation during the post-acute phase has been observed six months after discharge from critical care.[27] Given the persistence of NETs formation in patients with post-acute sequelae of COVID-19 ("long COVID"), this test's potential should be evaluated with respect to the follow-up of both acute and post-acute phase COVID-19 patients or in diagnosing individuals for long COVID.

Limitations of the study

Although high statistical differences were shown between the severity groups of patients, especially with healthy individuals, the low number of symptomatic Omicron-infected patients limits the statistical significance of the difference of the values of the NETs markers between this cohort and the healthy individuals and the mild/moderate patient cohorts. This work lacks an analysis of the variation of NETs markers which may occur as patients move from one category of severity to another. A follow-up of patients who moved from the asymptomatic or mild category to a more severe category would have helped to circumscribe the potential of the NETs biomarker panel used in this study. Lastly, we could not provide details of the variation of the biological/biochemistry parameters of the severe and non-severe Delta-infected patients, with respect to the NETs markers, as we did in our previous report.[27]

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109573.

ACKNOWLEDGMENTS

The authors thank the excellent technical assistance of C. Sanchez and F. Frayssinoux (IRCM, Institut de Recherche en Cancérologie de Montpellier, INSERM U1194, Université de Montpellier, Institut régional du Cancer de Montpellier, Montpellier, F-34298, France) and Cormac Mc Carthy (Mc Carthy Consultant, Montpellier) for English editing (financial compensation). We are grateful to M. Ychou, A. Adenis, E. Crapez, T. Mazard, (Institut Régional du Cancer de Montpellier, ICM), and J.P. Cristol and K. Klouche (Center Hospitalier Universitaire de Montpellier) for fruitful discussion.

The study was partially supported by SIRIC Montpellier Cancer Grant INCa_Inserm_DGOS_12553, and Lucia M. was supported by the National Scholarship Program of the Slovak Republic and scholarship of Ludmila Sedlárová-Rabanová. The study is partially supported and funded by Gulf Medical University and Thumbay University Hospital and Labs Ajman, UAE, and the Société Française des Acides Nucléiques Circulants (SFAC).

AUTHOR CONTRIBUTIONS

S.K.: Data curation, formal analysis, investigation, methodology, validation, writing, and visualization. L.M.: Methodology, validation, investigation, and writing. E.P., A.M., and B.P.: Data curation, formal analysis, methodology, and validation. B.R.: Investigation. A.P.P.: Data curation, formal analysis, investigation, methodology, and validation. J.B.: Investigation, review and editing. S.C.: Investigation, review and editing, project administration, supervision, and funding acquisition. A.R.T.: Conceptualization, funding acquisition, methodology, project administration, supervision, validation, writing – original draft, and writing – review and editing.

DECLARATION OF INTERESTS

A.R.T., E.P., and B.P. are author of a patent: Thierry A.R., Pisareva E., and B. Pastor NEW METHOD TO DIAGNOSE INFLAMMATORY DISEASES 11194720 PCT application number PCT/EP2022/072147, Date of receipt 05 August 2022.

Received: September 25, 2023
Revised: December 19, 2023
Accepted: March 24, 2024
Published: March 26, 2024

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Plasma of patients	Thumbay Hospital, Dubai	
Blood samples from healthy individuals	Etablissement Français du Sang, Montpellier	
Chemicals, peptides, and recombinant proteins		
Mix q-PCR	BioRad (Marne la Vallée, France)	
Critical commercial assays		
Qiagen Blood Mini Kit (Qiagen, CA)		
ELISA assay using Human Myeloperoxidase DuoSet ELISA DY3174 and	R&D Systems	
Human Neutrophil Elastase/ELA2 DuoSet ELISA DY9167-05	R&D Systems	
Deposited data		
All data are provided in Supplemental		
Oligonucleotides		
CCTTGGGTTTCAAGTTATATG	IDT	Forward KRAS
CCCTGACATACTCCCAAGGA	IDT	Reverse KRAS
GACCCACCAATCACATGC	IDT	Forward COX3
TGAGAGGGCCCCTGTTAG	IDT	Reverse COXE

RESOURCE AVAILABILITY

Lead contact

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Materials availability

The data that support these findings of the study are available upon request from the corresponding authors.

Data and code availability

Data

Data reported in this paper will be shared by the lead contact upon request.

Code

The paper does not report original code.

All additional information

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

83% and 17% of SARS-CoV2 infected patients are from Arabic and Indian ethnicities. Healthy individuals are 8% and 92% are from Arabic and Western European ethnicities (Table 1).

The study complied with the Institutional Review Board: IRB/COMTRIPM/FAC/03/Marc and UAE Ministry of Health and Prevention Research Ethics Committee (MOHAP): Approval Reference No: MOHAP/DXB-REC/JJJ/No.53/2021. Every patient approved an informed consent.

METHOD DETAILS

Patients

All the COVID-19 patients were categorized as critical, severe, moderate, mild, and asymptomatic based on a positive COVID test, radiological findings, oxygen requirements, and the need for intensive therapy and treatment, following national guidelines (National Guidelines for Clinical Management and treatment of COVID-19, 19th January 2022, Version 7.0). Briefly, the characteristics of these categories are.

Asymptomatic (or presymptomatic infection)

Individuals who test positive for SARS-Cov2 using a virologic test (i.e., a nucleic acid amplification test or an antigen test), but who have no symptoms that are consistent with COVID-19. Mostly no change in X-ray or chest CTscan. Treatment: Vitamin C, Vitamin D3 and Zinc.

Mild

Individuals who have any of the various signs and symptoms of COVID-19 (i.e., fever, cough, sore throat, malaise, headache and muscle pain) without shortness of breathe dyspnea, or abnormal chest imaging). Typical/Atypical changes: mostly no change in X-ray or chest CTscan. Treated outpatient, very rarely hospitalized for isolation purposes only. Treatment: Vitamin C, Vitamin D3, Zinc, monoclonal antibody infusion (on a case-to-case basis, if high risk), antivirals (favipravir), and symptomatic treatment.

Moderate

Individuals who show signs of lower respiratory disease on clinical assessment or imaging, and a saturation of oxygen (SpO2) $\geq$ 94% on room air at sea level. Clinical signs of pneumonia. Imaging: ground glass opacities/consolidation. No evidence of hypoxia in low risk patients. High risk patients may require minimal oxygen support via face mask, less 5L id spO2<94%. Treatment: Vitamin C, Vitamin D3, Zinc, Clexane, antibiotics, steroids (gupisone/dexamethasone}, monoclonal antibody infusion (on a case-to-case basis, if high risk), antivirals {flavipravir/Remdesivir}, symptomatic treatment.

Severe

Individuals who have respiratory frequency >30 breaths per minute, SpO2 <94% on room air (or, for patients with chronic hypoxemia, a decrease from baseline of >3%), ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) <300 mm Hg, or lung infiltrates >50%. Clinical signs of pneumonia. Imaging: ground glass opacities/consolidation. spO2 < 92% with tachypnea, high-grade fever, requiring O2 >10L, NRBM. Treatment: Vitamin C, Vitamin D3, Zinc, Clexane, antibiotics, steroids (gupisone/dexamethasone), antivirals (favipravir/Remdesivir), Tocilizumab, symptomatic treatment.

Critical

Individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction. Clinical signs of pneumonia. Imaging: ground glass opacities/consolidation. spO2 < 92% with tachypnea, high-grade fever, requiring O2 >10L, NRBM. Mostly ARDS, requiring NIV/MV. Treatment: Vitamin C, Vitamin D3, Zinc, Clexane, antibiotics, steroids (gupisone/dexamethasone), antivirals (favipravir/Remdesivir), Tocilizumab, symptomatic treatment.

The study complied with the Institutional Review Board

IRB/COMTRIPM/FAC/03/Marc and UAE Ministry of Health and Prevention Research Ethics Committee (MOHAP): Approval Reference No: MOHAP/DXB-REC/JJJ/No.53/2021.

Sample collection, treatment, and cfDNA extraction

All the samples were collected in EDTA tubes (BD Vacutainer R Blood Collection Tubes). Plasma was prepared within 6 h of sample collection from the patient. Plasma was included in the study following quality control and guidelines as previously described.[61] Plasma was harvested in Eppendorf tubes, centrifuged to remove all cells (1200 × g; 10 min) and, if required, frozen until use. Samples were centrifuged at 16,000 × g for 10 min at 4 °C to remove cellular debris and organelles. The supernatants were then transferred to 1.5 mL Eppendorf tubes and extracted using the Qiagen Blood Mini Kit (Qiagen, CA), according to the manufacturer's protocol. DNA was eluted from the column with 80 μL of elution buffer.

Measurement of cfDNA concentration by qPCR and evaluation of MNR and DNA integrity

Primers specific to the nuclear KRAS gene and mitochondrial COX3 gene were used to amplify sequences of nuclear and mitochondrial DNA.[61] Quantification of human nuclear DNA and mitochondrial DNA was done by amplifying the short 67 bp long sequence of the KRAS gene, and the 67 bp long sequence of the COX3 gene. The mitochondrial to nuclear ratio (MNR) was calculated as the ratio of the concentration of mitochondrial to nuclear cirDNA calculated using the 67 bp long amplicon quantification.[62,63] The quantification of cir-nDNA and of cir-mtDNA in clinical samples was validated in previous studies.[62,64,65]

Quantitative PCR (qPCR) was performed in a final reaction volume of 10 μL, which was composed of 5 μL of PCR mixture (Bio-Rad SYBR Green Supermix), 1 μL of each amplification primer (3 pmol/mL), 1 μL of PCR-quality water and 2 μL of DNA sample. Real-time qPCR was performed as follows: hot polymerisation activation-denaturation, performed for 3 min at 95 °C, followed by 40 repeated cycles at 95 °C for 10 s and then at 60 °C for 30 s. After amplification, melting curves were generated by increasing the temperature from 60 to 90 °C in increments of 0.2 °C, to confirm the specificity of the PCR product. Human nuclear DNA concentrations were calculated in ng/ml, using the standard curve of serial dilutions of human genomic DNA. Concentrations of the standard curve were expressed as ng/mL, and were used to determine the concentration of nuclear DNA and mitochondrial DNA in the plasma. Mean values were calculated from triplicate reactions, and internal negative controls with PCR-quality water were routinely used. List and sequence of human primers used for qPCR assays. Primer sequences for the detection of cir-nDNA by targeting *KRAS* are CCTTGGGTTTCAAGTTATATG (forward) and CCCTGACATA CTCCCAAGGA (reverse).[64] Primer sequences for the detection of cir-mtDNA by targeting *COX3* are GACCCACCAATCACATGC (forward) and TGAGAGGGCCCCTGTTAG (reverse).[63]

ELISA

Quantification of MPO and NE in the plasma samples of COVID-19 patients and healthy individuals was performed by ELISA assay using Human Myeloperoxidase DuoSet ELISA DY3174 and Human Neutrophil Elastase/ELA2 DuoSet ELISA DY9167-05 (R&D Systems) according to manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as the median (±) standard deviation. A non-parametric statistical Kruskal-Wallis Test was used to compare different groups of samples. Receiver operating characteristics and area under the ROC curves were constructed to measure the accuracy of biomarkers (GraphPad Prism 9, GraphPad Sowftware Inc., USA). Statistical analyses were presented in all figures at the conventional significant p value: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

ADDITIONAL RESOURCES

No additional resources.