Original Article

Circulating cell-free DNA, peripheral lymphocyte subsets alterations and neutrophil lymphocyte ratio in assessment of COVID-19 severity

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Abstract

Cell destruction results in plasma accumulation of cell-free DNA (cfDNA). Dynamic changes in circulating lymphocytes are features of COVID-19. We aimed to investigate if cfDNA level can serve in stratification of COVID-19 patients, and if cfDNA level is associated with alterations in lymphocyte subsets and neutrophil-to-lymphocyte ratio (NLR). This cross-sectional comparative study enrolled 64 SARS-CoV-2-positive patients. Patients were subdivided to severe and non-severe groups. Plasma cfDNA concentration was determined by real-time quantitative PCR. Lymphocyte subsets were assessed by flow cytometry. There was significant increase in cfDNA among severe cases when compared with non-severe cases. cfDNA showed positive correlation with NLR and inverse correlation with T cell percentage. cfDNA positively correlated with ferritin and C-reactive protein. The output data of performed ROC curves to differentiate severe from non-severe cases revealed that cfDNA at cut-off ≥ 17.31 ng/µl and AUC of 0.96 yielded (93%) sensitivity and (73%) specificity. In summary, excessive release of cfDNA can serve as sensitive COVID-19 severity predictor. There is an association between cfDNA up-regulation and NLR up-regulation and T cell percentage down-regulation. cfDNA level can be used in stratification and personalized monitoring strategies in COVID-19 patients.

Keywords

cfDNA, COVID-19 patients' stratification, COVID-19 monitoring, COVID-19 severity predictors, personalized biomarkers, NLR

Introduction

COVID-19 was declared as an outbreak in January 2020. Shortly, it became a pandemic, and by mid-March 2020, COVID-19 had generated 24 times more cases than the severe acute respiratory syndrome (SARS) outbreak,[1] which highlights the importance for personalized severity predictors biomarkers of COVID-19.

Circulating cell-free DNA (cfDNA) is extracellular DNA found in plasma or serum. Accumulation of cfDNA in plasma may be the result of excess release from massive cell destruction, insufficient elimination

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of dead cells, extracellular DNA traps formed during inflammation, or a combination of all these causes.[2]

The cfDNA is detected in plasma and other body fluids. Release of cfDNA into the circulation is likely due to cellular breakdown mechanisms, such as apoptosis and necrosis, as well as active DNA-release mechanisms.[3] In the context of many pathological conditions, cfDNA is an excellent biomarker candidate for clinical application, mainly circulating cell-free mitochondrial DNA.[4]

Leukocytes are a major source of cfDNA which is produced from apoptotic and dying cells in COVID-19 patients and can be brought up by dying lymphocytes. More than 60% of patients with COVID-19 had lymphopenia, which can produce abundant free DNA in these patients.[5]

Phagocytic cells usually remove apoptotic debris, decreasing the consequences of the presence of dead cell materials.[6] In a state of disease, when cell death exceeds the clearance capacity, this phagocytic system is overwhelmed.[7] Previous data suggested a link between the level of inflammation and the amount of cfDNA released from damaged cells in the circulation, which gives a potentiality of cfDNA in determination of COVID-19 severity.

The level of lymphocytes is thought to be a means of early identification of risk factors for severe COVID-19. Neutrophil-to-lymphocyte ratio (NLR) was claimed to serve as an indicator for the systematic inflammatory response with COVID-19.[8] NLR is calculated as the absolute count of neutrophils divided by the absolute count of lymphocytes. High NLR points to a predominance of inflammatory factor.[9] Dynamic changes in peripheral blood lymphocyte subsets were also reported in COVID-19 patients.[1] Still, the link between the peripheral lymphocyte subsets alterations and cfDNA needs to be explored.

Hence, our aim was to find out if levels of plasma cfDNA and NLR may serve as monitoring biomarkers and predictors of severity in COVID-19 disease, and to assess their role as strategies for stratification of patients with COVID-19. Also, we aimed to investigate the presence of a link between cfDNA level, NLR, lymphocyte subsets alterations and COVID-19 severity.

Patients and methods

Type of study

This was a comparative cross-sectional study.

Study participants

A total of 64 acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-positive patients were enrolled in this study. Sample size was calculated using PASS 11 program, setting power 80% and alpha error at 0.05. Patients were subdivided into severe group ($n = 34$) and non-severe group ($n = 30$). Patients were recruited from Ain-Shams University Specialized Hospital.

Candidates were informed about the aim of the study and gave their informed consent before enrolment in the study. The study was done after approval from the Research Ethical Committee of Faculty of Medicine Ain-Shams University.

Inclusion criteria: adult patients >18 yr with positive result of real-time reverse transcriptase-PCR assay (RT-PCR) for nasal swab specimens for SARS-CoV-2 RNA. Cases were diagnosed based on the interim guidance of the World Health Organization.[11] Non-severe patients met all following conditions: (a) history of exposure to a confirmed SARS-CoV-2 patient, (b) fever or other respiratory symptoms, and (c) typical chest computed tomography image abnormities compatible with viral pneumonia. Severe patients additionally met at least one of the following conditions: (a) Shortness of breath, respiration rate $\geq$ 30 times/min, (b) oxygen saturation (resting state) $\leq$ 93%.[12]

Assessment of participants

Personal history: name, age, sex, occupation, contact with a known positive case of severe acute SARS-CoV-2 infection. History of present illness: onset (date of diagnosis), disease severity (non-severe, severe with critical needing intensive care unit (ICU) admission or mechanical ventilation), duration of the disease symptoms (namely, fever, cough, shortness of breath and fatigue) (in d). Drug history and past history: history of drug intake especially immunomodulators, chronic diseases especially respiratory disease (e.g. bronchial asthma).

Sample collection

Approximately 10 ml of venous blood was drawn from each COVID-19 patient and divided into five aliquots; the first aliquot was 2 ml blood transferred to a plain tube for serum ferritin, C-reactive protein (CRP), liver enzymes and creatinine. The second aliquot was 2 ml transferred to a heparin tube for flow cytometry to be analysed within 24 h. The third aliquot was 2 ml blood transferred into an EDTA tube for complete blood count. The fourth aliquot was transferred into citrate tube for D-dimer measurement. The fifth aliquot was transferred into EDTA tube, centrifuged at 1370 g for 10 min, then plasma was removed carefully and re-centrifuged at 18,894 g for 10 min. The supernatant was then transferred to microcentrifuge tubes and

stored at –80°C for extraction and measurement of cfDNA by real-time quantitative PCR (qPCR)

Study tools

Complete blood cell count was done using automated haematology cell counters (Siemens, Advia 560, Germany). Biochemical study measured alanine transaminase (ALT), aspartate transaminase (AST), creatinine and ferritin (Biolis-24i, Tokyo Boeki Medisys Inc. Japan). Analysis of D-dimer was performed on (Immunoassay, VIDAS PC, Biomerieux, France. Serial number; IVD3002806).

Flow cytometry assay

Flow cytometry assay was conducted at Clinical Pathology Department, Al-Zahraa Hospital, Al-Azhar University, using four-colour FACS Calibur (BD Biosciences, San Jose, CA). Cell Quest Pro software (BD Biosciences) was used for data analysis. The compensation setting was established before acquiring the samples using colour-calibrated beads. Acquisition count was raised to 100,000 events, to consider analysis of lymphopenic samples in COVID-19 cases, isotype control was acquired to detect positive cut-off.

Three tubes were used with 50 μl of fresh blood sample each. The first tube was incubated with 5 μl cocktail of mouse-stained anti-human controls IgG1 FITC/IgG2a PE (catalogue no. 34240, lot no.90642). The second tube was incubated with 5 μl of FITC-conjugated anti-human CD3/PE-conjugated anti-human CD16 + CD56 cocktail (catalogue no. 95131, lot no. 6012680, BD Biosciences, USA). The third tube was incubated with 5 μl of FITC-conjugated anti-human CD3/PE-conjugated anti-human CD19 cocktail (catalogue no. 349217, lot no. 79439, BD Biosciences, USA). All tubes were incubated for 20 min. Then, lysis reagent (BD Biosciences) was added for destruction of red blood corpuscles for 8 min before using FACS buffer for washing, and the sample was then centrifuged at 500 g.

Regarding the gating strategy for identification of lymphocyte subsets, initial gating was taken from the lymphocyte area on the forward scatter/side scatter (FS/SS) [R1]. Then T lymphocytes were identified as being CD3 positive, NK as CD16+CD56 positive and CD3 negative while T-natural killer cells (TNK) were identified as CD16+CD56 positive and CD3 positive. Finally, B cells were CD19-positive cells (Figure 1).

Real-time PCR for cfDNA quantitation

Real-time PCR for cfDNA quantitation was conducted at the Clinical Pathology Department, Al-Zahraa Hospital, Al-Azhar University. Extraction of cfDNA

was done manually from 200 μl of plasma using QIAamp DNA Blood Mini kit (Qiagen, Germany) according to the protocol provided by the manufacturer; it was eluted in 30 μl of elution buffer and concentrations were measured using QIAXpert (Qiagen, Germany).

Plasma cfDNA was determined by real-time quantitative PCR for β-actin gene found in all nucleated cells. The sequence of primers was forward (5′–3′): GCGCCGTTCCGAAAGTT; reverse (5′–3′): CGGC GGATCGGCAAA. The PCR was performed using Quanti Tect SYBR Green Master Mix (Qiagen, Germany) on a Rotor-Gene Q detection system (Qiagen, Hiden Germany). The real-time PCR was carried out in 25 μl of total reaction volume containing 12.5 μl SYBR green master mix (Qiagen, Hiden, Germany), 1 μl of each primer, and 10.5 μl of the extracted plasma supernatant. Fluorescence measurements were made in every cycle. The cycling conditions used were as follows: PCR initial active step at 95°C for 15 min, then 40 cycles which includes denaturation at 94°C for 15 s, annealing at 55°C for 30 s then extension at 70°C for 30 s. Melting curve analysis was performed to confirm specificity of the PCR product. The absolute DNA concentration was calculated according to the standard curve generated by serial dilutions of genomic DNA ranging from 0.00001 to 100 ng/μl.

Statistical analysis

Data were coded and entered using the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Data were summarized using mean $\pm$ SD for quantitative parametric data, median (interquartile range) for non-parametric data. In addition frequency (count) and relative frequency (percentage) were used for categorical data. The comparisons between quantitative variables were done using student t-test for parametric and Mann–Whitney test for the non-parametric measures. For comparing categorical data, Chi square (χ^2) test was performed. The correlations between quantitative variables were done using Spearman correlation coefficient. P Values less than 0.05 were considered statistically significant. Receiver operating characteristic (ROC curve) was constructed, with area under curve (AUC) analysis performed to detect best cut-off value of different parameters for differentiating severe and non-severe COVID-19 infections. P Values less than 0.05 were considered as statistically significant.

Results

Our study included 64 COVID-19 patients divided into two groups: group 1 included medium and
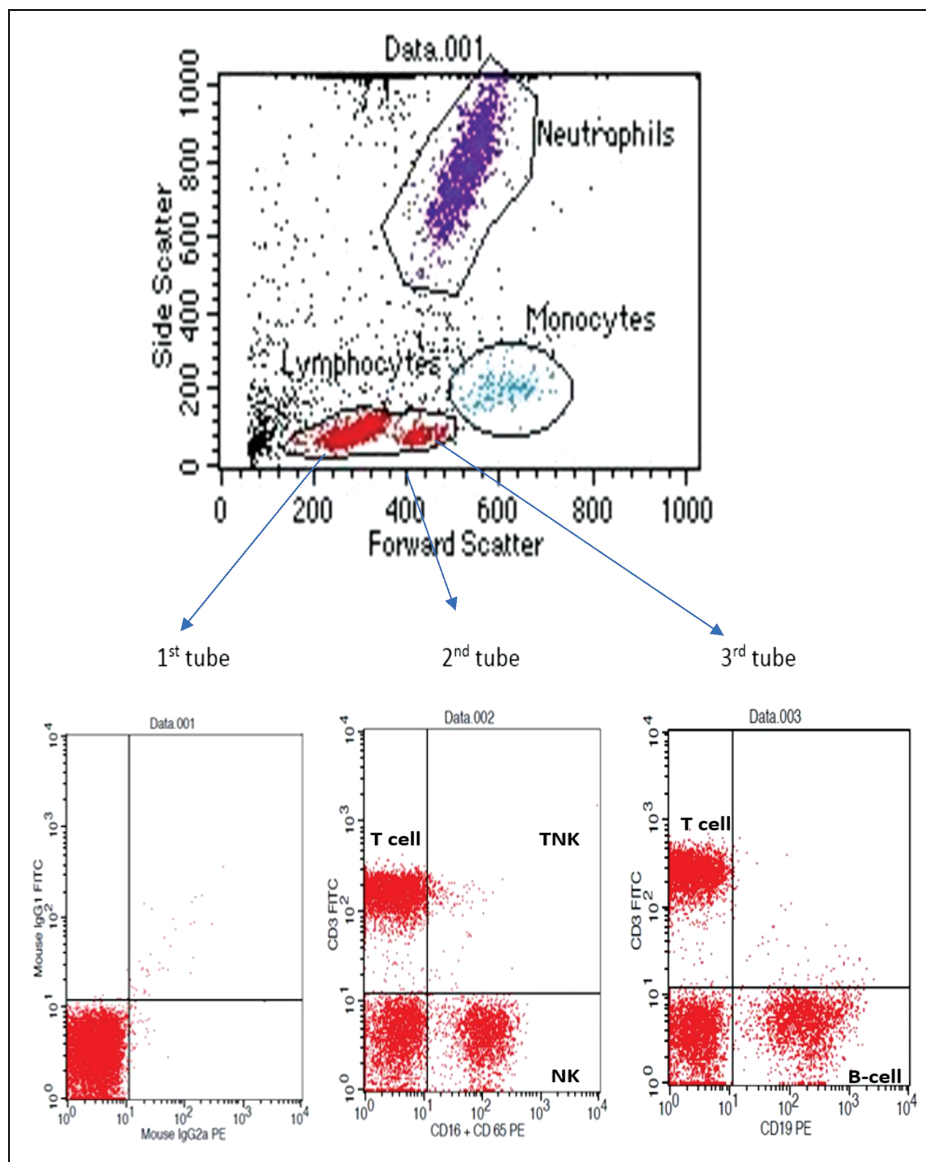
Figure 1. Gating strategy for identification of lymphocyte subsets, initial gating [R1] was taken from the lymphocyte area on FS/SS [R1]. Then T lymphocytes were identified as CD3 positive, NK as CD16 + CD56 positive and CD3 negative, TNK as CD16 + CD56 positive and CD3 positive. B cells as CD19-positive cells.

ICU-admitted severe cases of COVID-19 ($n = 34$) with median age of 60 yr, and group 2 included non-severe and non hospitalized cases [home isolated] ($n = 30$) with median age of 27 yr. The majority of patients were females (56.3%), 50% were asymptomatic, 15.6% had fever, 20.3% had cough, 4.7% had fever with cough, 6.3% had diarrhoea, and 3.1% had fever with diarrhoea. By the end of the study only two patients (3.1%) required mechanical ventilation and died. Ten patients (15.5%) were on continuous positive airway pressure therapy, while all other severe patients were on high flow nasal cannula or venturi. The median

duration between first symptoms and hospital admission in severe cases was 4.5 d. Hypertension, diabetes mellitus, and bronchial asthma were the most reported co-morbidities in 34.4%, 26.6%, and 14.1%, respectively. Comparative data between severe and non-severe cases are shown in Table 1. The correlation study of cfDNA with other parameters showed significant positive correlations with CRP, ferritin, total leukocyte count (TLC), absolute neutrophil count (ANC), B cells (%) and NLR. Significant negative correlations were also found with absolute lymphocyte count (ALC), platelets, T cells (%), NK (%) and TNK (%) (Table 2).

Table 1. Comparative data between severe and non-severe cases.

Variables	Severe $n = 34$	Non-severe $n = 30$	Test	P Value
Age (yr)	60	27	Mann–Whitney	< 0.001
Interquartile range	(52–67)	(24–29.5)		
cfDNA (ng/µl)	24.562 ± 4.387	15.018 ± 4.068	*t*-test	< 0.001
CRP (mg/l)	29.5	1.5	Mann–Whitney	< 0.001
Interquartile range	(12.88–65.4)	(1.2 ± 4.03)		
Ferritin (ng/ml)	637	35.65	Mann–Whitney	< 0.001
Interquartile range	(427–1078.25)	(18.75–72)		
D-dimer ng/ml	1720	426	Mann–Whitney	< 0.001
Interquartile range	(1217–2898)	(301–450)		
Serum ALT(U/l)	26	17	Mann–Whitney	0.12
Interquartile range	(11–42)	(12.5–23)		
Serum AST (U/l)	28	20.5	Mann–Whitney	0.189
Interquartile range	(15–39.75)	(16.75–28.25)		
Creatinine (mg/dl)	0.8	0.7	Mann–Whitney	0.194
Interquartile range	(0.6–1.65)	(0.6–0.9)		
TLC ($\times 10^3/mm^3$)	8.25	6	Mann–Whitney	0.002
Interquartile range	(6.57–14.05)	(4.8–7.53)		
ANC ($\times 10^3/mm^3$)	6.85	3.5	Mann–Whitney	< 0.001
Interquartile range	(4.53–13.1)	(2.35–4.18)		
ALC ($\times 10^3/mm^3$)	0.7	2.3	Mann–Whitney	< 0.001
Interquartile range	(0.5–1.53)	(1.98–2.73)		
NLR	10	1.23	Mann–Whitney	< 0.001
Interquartile range	(6.47–16.75)	(0.95–1.75)		
T cells (%)	42.92	57.25	Mann–Whitney	< 0.001
Interquartile range	(20.84–56.55)	(55.37–62.07)		
NK (%)	4.96	10.14	Mann–Whitney	< 0.01
Interquartile range	(2–10.97)	(6.94–14.02)		
TNK (%9	2.23	4.95	Mann–Whitney	0.04
Interquartile range	(1.17–6.04)	(3.49–6.37)		
B cells (%)	46.87	23.75	Mann–Whitney	< 0.001
Interquartile range	(32.96–68)	(19–31.18)		
PLTs ($\times 10^3/mm^3$)	225	252	Mann–Whitney	0.07
Interquartile range	(95–280)	(198–295)		
Hb (gm/dl)	10.85 ± 2.23	12.67 ± 1.58	*t*-test	< 0.001

Data are expressed as mean ± SD (for parametric data) and expressed as median (Interquartile Range) in case of non-parametric data. $P > 0.05$ is not significant (NS). ANC, absolute neutrophil count; ALC, absolute lymphocyte count; ALT, Alanine transaminase; AST, Aspartate transaminase; cfDNA, cell-free DNA; Hb, haemoglobin; NK, natural killer; NLR, neutrophil-to-lymphocyte ratio; PLTs, platelets; TLC, total leukocytic count; TNK, T-natural killer.

The comparison of mean values of cfDNA in severe and non-severe groups showed significantly higher values in severe cases than in non-severe cases ($P < 0.001$) (Figure 2). On comparing alteration of lymphocytes in severe and non-severe groups, we found that the frequency of circulating T lymphocytes was significantly decreased in severe cases compared with non-severe cases ($P < 0.001$) and the frequency of B cells was significantly higher in severe cases than in non-severe cases ($P < 0.001$) (Figure 3).

The output data of performed ROC curves revealed that the cfDNA at a cut-off of ≥ 17.31 ng/µl and AUC of 0.96 yielded a specificity of 73% and sensitivity of 93% with $P < 0.001$. The NLR at cut-off of ≥ 3.1 and AUC of 0.98 yielded a specificity of 93% while sensitivity was 91%, with $P < 0.001$. ALC at cut-off of $\geq 1.5 \times 10^3/mm^3$ and AUC of 0.092 yielded the lowest sensitivity of 76% with specificity of 93% and $P < 0.001$ to differentiate severe from non-severe cases (Table 3, Figure 4).

Discussion

The current coronavirus pandemic is the most dramatic healthcare crisis linked to acute and highly infectious disease. The main goal of all the predictive and monitoring strategies is the challenge to adapt the number of severely sick people to the limited capacity of corresponding

Table 2. Correlation of cfDNA with other study parameters.

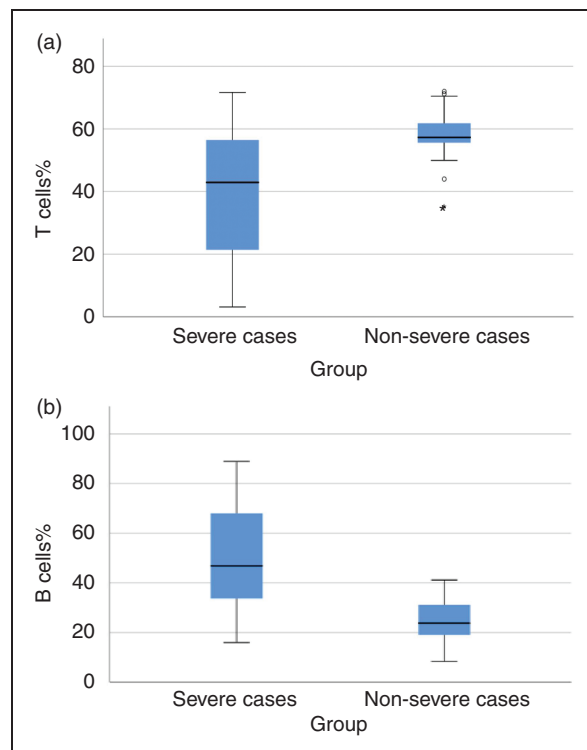|	cfDNA (ng/µl)	
CRP (mg/l)	Correlation coefficient	0.577
	P value	<0.001
Ferritin (ng/ml)	Correlation coefficient	0.58
	P value	<0.001
TLC	Correlation Coefficient	0.276
($\times 10^3$/mm^3)	P value	<0.05
ANC	Correlation coefficient	0.482
($\times 10^3$/mm^3)	P value	<0.001
ALC	Correlation coefficient	−0.683
($\times 10^3$/mm^3)	P value	<0.001
PLTs	Correlation coefficient	−0.279
($\times 10^3$/mm^3)	P value	<0.05
T cells (%)	Correlation coefficient	−0.438
	P value	<0.001
NK (%)	Correlation coefficient	−0.317
	P value	<0.05
TNK (%)	Correlation coefficient	−0.153
	P value	0.226
B cells (%)	Correlation coefficient	0.556
	P value	<0.001
NLR	Correlation coefficient	0.698
	P value	<0.001

$P > 0.05$ is not significant (NS). ANC, absolute neutrophil count; ALC, absolute lymphocyte count; CRP, C-reactive protein; Hb, haemoglobin; NK, natural killer; NLR, neutrophil-to-lymphocyte ratio; PLTs, platelets; TLC, total leukocyte count; TNK T-natural killer.
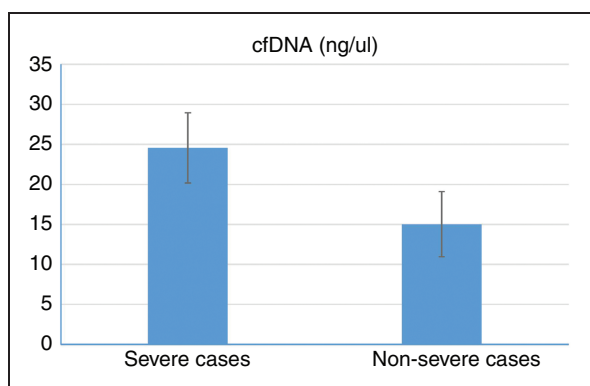
Figure 2. Comparison of mean values of cfDNA in severe and non-severe groups. The cfDNA was significantly higher in severe cases than in non-severe cases ($P < 0.001$).

Figure 3. Comparison showing lymphocytes alteration in severe and non-severe groups. (A) Frequency of circulating T lymphocytes was significantly decreased in severe cases compared with non-severe cases ($P < 0.001$). (B) Frequency of B cells was significantly higher in severe cases than in non-severe cases ($P < 0.001$).

health systems, providing adequate care and therefore keeping the morbidity level at a minimum.[13]

The fact that Egypt is facing this COVID-19 pandemic surge with limited hospital beds, resources, and health care personnel, highlights the importance of biomarkers in predicting severity to prevent the pandemic scenario of China and Europe.

Inflammatory responses contribute to immune response imbalance. Therefore, circulating biomarkers which are able to define inflammation and immune status are potential predictors for the severity of COVID-19.[8] Accumulation of cfDNA in plasma may be the result of excess release from massive cell destruction, and inflammation.[2] This justifies our aim to investigate the utility of plasma cfDNA in assessment of COVID-19 severity. Besides, our objective was to investigate the presence of a link between cfDNA level, NLR, alterations in lymphocyte subsets and COVID-19 severity.

The current study showed a significant increase in cfDNA level among severe cases, with mean ± SD = 24.562 ± 4.387 as compared with non-severe cases with mean ± SD = 15.018 ± 4.068 ($P < 0.001$). This was in line with Zuo et al., who reported that cfDNA was higher in hospitalized patients receiving mechanical ventilation as compared with hospitalized patients breathing room air.[14] This can be explained by cfDNA that can be released to the circulation as a result of inflammatory process,[2] either due to cellular breakdown mechanisms, as apoptosis, or due to active DNA-release mechanisms by neutrophils.[3] cfDNA can also be a reason for amplification of the inflammatory process. Liu et al. reported that cfDNA can produce

Table 3. The output data of ROC curve for discriminative power of cfDNA, NLR and ALC to differentiate between severe and non-severe infections with COVID-19.

	AUC	P Value	Cut-off	Sensitivity	Specificity
cfDNA (ng/μl)	0.96	< 0.001	17.31 ng/ul	97%	73%
NLR	0.98	< 0.001	3.1	91%	93%
ALC (×10^3/mm^3)	0.92	< 0.001	1.5 (×10^3\mm^3)	76%	93%

ALC, absolute lymphocyte count; AUC; area under the curve; cfDNA, cell-free DNA; NLR, neutrophil-to-lymphocyte ratio.

severe inflammation mediated by C-type lectin receptors encoded within the NK complex family, MHC class I genes, and type I interferons, and some DNA sensors, and those inflammations will end with cytokine storm.[6] Similarly, Wu et al. reported that cfDNA can circulate and activate different immune cells to produce a huge amount of cytokines, leading to cytokine storm.[15] cfDNA was also reported to destroy vascular endothelial cells directly, which will add to the dysfunctions of multiple organs by cytokine storm.[16]

The present study revealed a significant increase in CRP in severe cases when compared with non-severe cases, with a median of 29.5 in severe cases and a median value in non-severe cases of 1.5 ($P < 0.001$). This was in line with Ali and Chen et al., who reported that patients with severe disease courses had a far elevated level of CRP than non-severe patients.[17,18] Wang et al. proposed CRP as a predictive marker for the aggravation of non-severe COVID-19 patients, with an optimal threshold value of 26.9 mg/l.[19] Huang et al. reported that CRP can also be used to monitor disease improvement beside being used as prognostic marker in COVID-19.[20]

There was a significant hyperferritinemia ($P < 0.001$) in severe cases when compared with non-severe cases, which was in agreement with Chen et al.[21] In a meta-analysis performed by Taneri et al. based on combined estimates from 29 studies and 13,620 individuals, serum ferritin was higher in severe COVID-19 individuals compared with moderate cases.[22] Daher et al. reported that hyperferritinemia is linked to systemic level of inflammation, as during the inflammatory state IL-6 stimulates ferritin and hepcidin synthesis.[23]

Kernan and Carcillo claimed that ferritin is a direct mediator of the immune system, and reported a feedback mechanism between ferritin and cytokines, as cytokines can induce ferritin expression, and ferritin can induce the expression of pro- and anti-inflammatory cytokines, as well.[24] On top of that, Gómez-Pastora et al. added that ferritin is a mediator for the inflammatory process, stimulating inflammatory pathways, which will initiate a vicious pathogenic immune loop.[25]

The current study revealed a significant increase in TLC and ANC ($P = 0.002$ and ($P < 0.001$, respectively) in severe cases, while there was a significant decrease in ALC in severe cases ($P < 0.001$). This was in agreement with Chen et al. and Henry et al., who reported that patients with severe COVID-19 had significantly increased TLC, ANC and decreased lymphocyte compared with non-severe disease.[21,26] A comparison of platelet count showed a non-significant difference between severe cases and non-severe cases ($P = 0.07$); this was in disagreement with Henry et al.[26] and Zhao et al.,[27] who reported that early decrease in blood platelet count is associated with poor prognosis.

The NLR was significantly higher in severe cases ($P < 0.001$). This was in line with Liu et al., who further added that NLR was an independent risk factor of the in-hospital mortality for COVID-19 patients.[28] A meta-analysis performed by Lagunas-Rangel stated that increased NLR levels reflect an enhanced inflammatory process which suggests poor prognosis.[29] This increase in NLR may be due to increased ANC in severe cases, as neutrophils get activated and migrate from the venous system to the immune system. Neutrophils release large amounts of reactive oxygen species (ROS) that can free the virus from the cells. Thus, Ab-dependent cell-mediated cytotocicity (ADCC) may kill the virus directly, expose virus Ag, and stimulate cell-specific and humoral immunities.[30] Neutrophils can also be triggered by virus-related inflammatory factors, such as IL-6 and IL-8, TNF-α and IFN-γ factors, produced by lymphocytes. The human immune response triggered by viral infection mainly relies on lymphocytes, whereas systematic inflammation significantly decreases T lymphocytes. Thus, virus-triggered inflammation increased NLR.[8]

There was a significant decrease, in severe cases, in circulating T lymphocytes, TNK and NK cells ($P < 0.001$), $P = 0.04$, and $P < 0.01$, respectively), while the frequency of B cells was significantly higher in severe cases than in non-severe cases ($P < 0.001$). This was in line with Chen et al.[21] Qin et al. reported that the number of T cells decreased in severe cases of COVID-19. The increase in the frequency of B cells could be due to the more significant decrease in
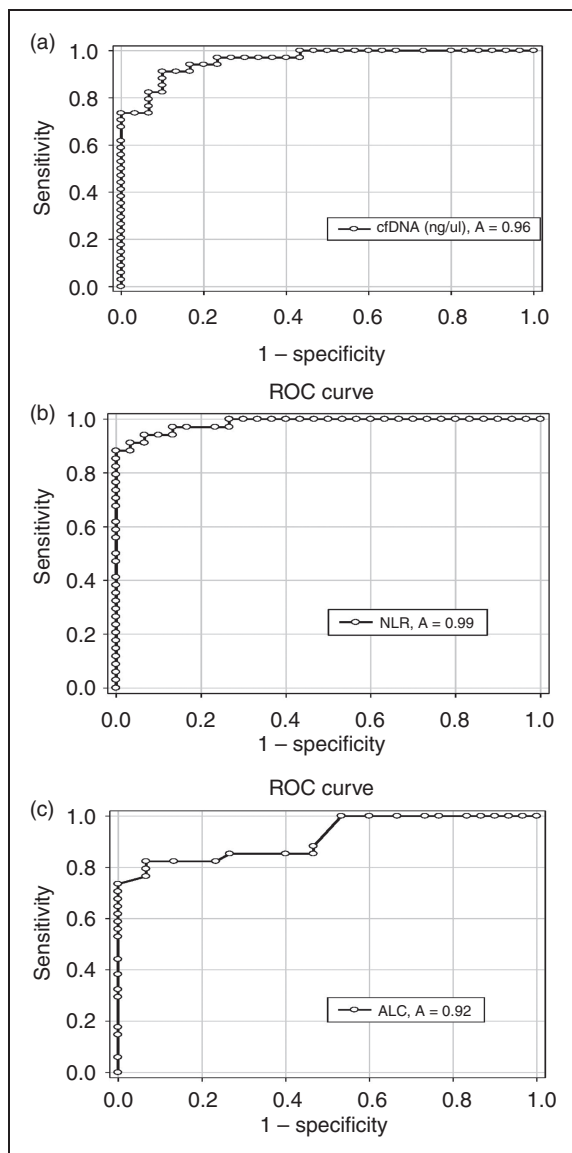
Figure 4. Shows output data of ROC curves of the discriminating power of cfDNA, NLR and ALC to differentiate between severe and non-severe COVID-19 infections. (A) cfDNA at cut-off $\geq$17.31 ng/µl and AUC of 0.96 yielded a sensitivity of 93% and specificity of 73% ($P < 0.001$). (B) The NLR at cut-off of $\geq$ 3.1 and AUC of 0.98 yielded a sensitivity of 91% while specificity was 93% ($P < 0001$). (C) ALC at cut-off of $\geq$1.5 × 10^3/mm^3 and AUC of 0.0.92 yielded 76% sensitivity with 93% specificity ($P < 0.001$) to differentiate severe from non-severe cases. cfDNA, cell-free DNA; NLR, neutrophil-to-lymphocyte ratio; ALC, absolute lymphocyte count.

T lymphocytes in severe cases, as well.[31] He et al. reported that all lymphocyte subsets, including T-, B- and NK cells, were significantly lower in the severe group, but emphasized that only T-lymphocyte count correlated with the disease course of patients in COVID-19 pneumonia.[32] Wang et al. also reported that peripheral lymphocyte subset alteration was associated with clinical characteristics of COVID-19.[33]

There was a significant decrease of haemoglobin (Hb) in severe cases ($P < 0.001$), which could be explained by the fact that angiotensin II regulates normal erythropoiesis and stimulates early erythroid proliferation. Binding of SARS-CoV-2 to the host ACE2 increases dysregulation of erythropoiesis through the downstream angiotensin II pathway.[34] Cheng et al. also reported that there was an increased erythroid turnover in COVID-19 patients and that a portion of plasma cfDNA was derived from destruction of erythroid cells. They added that possible causes of anaemia in COVID-19 patients included: (a) excessive inflammation and cytokine storm, (b) haemophagocytosis in relation to inflammation, and (c) consumption in microthrombi.[18]

Correlation studies revealed that the cfDNA positively correlated with inflammatory marker CRP ($r = 0.577$; $P < 0.001$), which highlights the role of cfDNA in the inflammatory state. Endothelial cell inflammation is documented in patients with COVID-19. Rauch et al.'s study revealed the presence of vascular inflammation and severe endothelial injury as a direct consequence of SARS-CoV-2 infection and ensuing host inflammatory response in COVID-19.[35] Ng et al., in addition, reported that levels of the neutrophil extracellular traps (NETs) contributing to circulating cfDNA formation correlated with CRP as well.[36] The cfDNA positively correlated with ferritin ($r = 0.58$; $P < 0.001$). This could be explained by excess intracellular iron interacting with molecular oxygen, generating ROS which contributes to oxidative damage of cellular components of different organs and apoptosis which is a source of cfDNA. Programmed cell death mediated by iron-dependent peroxidation mechanisms in inflammatory pathologies is called ferroptosis.[37] On top of that, it was documented that hyperferritinemia was linked to coagulopathy, as oxidized iron accelerates serum coagulation by interacting with proteins of the coagulation cascade.[38]

Our study showed weak correlation of cfDNA with TLC, while a moderate positive correlation was seen with ANC ($r = 0.276$, $P < 0.05$) and ANC ($r = 0.482$; $P < 0.001$). This was in line with Ng et al., who stated that NET markers which are cfDNA, citrullinated histone H3, and neutrophil elastase correlated with TLC and neutrophils.[36]

During the inflammatory process there is activation of neutrophils and an increase in the production of NETs, which are microbicidal proteins, and oxidant enzymes, those released by neutrophils to contain infections.[2] Still, NETs have the potential to propagate inflammation and microvascular thrombosis. Zuo et al. and Becker previously reported that the

concentration of circulating histones is directly proportional to the degree of inflammation and end organ dysfunction in trauma or sepsis-like conditions.[14,39]

Our study revealed an inverse correlation of cfDNA with ALC ($r = -0.683$; $P < 0.001$); this was in line with Laurent et al., who stated that haematopoietic cells, including lymphoid and erythroid cells, are the major components of cfDNA.[40] There was a positive correlation of cfDNA with NLR ($r = 0.698$; $P < 0.001$), which can be attributed to activation and migration of neutrophils to the bloodstream during COVID-19 and their release of large amounts of ROS which can induce cell DNA damage and release of circulating cfDNA. Likewise, associated lymphopenia due to lymphocyte apoptosis contributes to more release of cfDNA. NLR was considered as an independent biomarker for indicating poor clinical outcomes by Yang et al.[8]

There was a negative correlation of cfDNA with percentage of T cell ($r = -0.438$; $P < 0.001$) and NK ($r = -0.317$; $P < 0.05$), while no correlation was seen with TNK. Interestingly, there was a significant positive correlation with B cells ($r = 0.556$; $P < 0.001$). It is worth mentioning that He et al. claimed T cells as being an independent predictor for COVID-19 severity. The study revealed an inverse weak correlation of cfDNA with platelets (PLTs) ($r = -0.279$; $P < 0.05$).[32] This can be explained by the virtue of the fact that COVID-19 disease is characterized by hyper-inflammation and endothelial injury as a direct consequence of intracellular SARS-CoV-2 infection and ensuing host inflammatory response. Injury to endothelial cells thus contributes to the release of cfDNA, which then promotes coagulation leading to the widespread formation of microthrombi, provoking microcirculatory failure or large-vessel thrombosis leading to consumption of PLTs in thrombi.[35] The cfDNA at cut-off ≥ 17.31 ng/μl and AUC of 0.96 yielded a specificity of 73% and sensitivity of 93% with $P < 0.001$ to differentiate severe from non-severe cases. The NLR at cut-off of ≥ 3.1 and AUC of 0.98 yielded a specificity of 93% and sensitivity of 91%, with $P < 0.001$; ALC at cut-off of $\geq 1.5 \times 10^3$/mm^3 and AUC of 0.0.92 yielded the lowest sensitivity of 76% with specificity of 93% and $P < 0.001$, which indicates that cfDNA is the most sensitive among the studied markers in discriminating severity, proposing it as a monitoring marker and a target of therapy.

This study was limited due to lack of determination of tissues of origin of the cfDNA; this needs to be addressed in future studies. Despite this limitation, our study proposed a minimally invasive severity indicator which can provide immediate insights into the dynamics of COVID-19. The sample size may not be enough to generalize our findings. Nonetheless, we compared non-hospitalized with hospitalized patients. More comprehensive investigations are therefore needed to confirm and further refine the observations reported herein. Nevertheless, the conclusions of this study are consistent with the conclusions of other scholars.

Conclusion

Excessive release of cfDNA can serve as a sensitive COVID-19 severity predictor. There is an association between excessive release of cfDNA and NLR increase and T-cell percentage down-regulation. cfDNA level can be used as a strategy for stratification and monitoring of COVID-19 patients. Altered distribution of lymphocyte subsets is a feature of COVID-19. The study proposes cfDNA as a marker for personalized prediction, a monitoring biomarker for COVID-19 severity, and as a target for novel therapeutic interventions in COVID-19. The output data of the ROC curve showed an accepted discriminative power of cfDNA, NLR and ALC to differentiate between severe and non-severe infections with COVID-19.

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