Circulating Cell-Free DNA Level in Prediction of COVID-19 Severity and Mortality: Correlation of with Haematology and Serum Biochemical Parameters

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Abstract Lymphocyte dysregulation in coronavirus disease-19 (COVID-19) is a major contributing factor linked to disease severity and mortality. Apoptosis results in the accumulation of cell-free DNA (cfDNA) in circulation. COVID-19 has a heterogeneous clinical course. The role of cfDNA levels was studied to assess the severity and outcome of COVID-19 patients and correlated with other laboratory parameters. The current case series included 100 patients with mild COVID-19 (MCOV-19) and 106 patients with severe COVID-19 (SCOV-19). Plasma cfDNA levels were quantified using SYBR green quantitative real-time PCR through amplification of the β-actin gene. CfDNA level was significantly higher in SCOV-19 at 706.7 ng/ml (522.6–1258) as compared to MCOV-19 at 219.8 ng/ml (167.7–299.6). The cfDNA levels were significantly higher in non-survivor than in survivors ($p = 0.0001$). CfDNA showed a significant correlation with NLR, ferritin, LDH, procalcitonin, and IL-6. The diagnostic sensitivity and specificity of cfDNA in the discrimination of SCOV-19 from MCOV-19 were 90.57% & 80%, respectively. CfDNA showed a sensitivity of 94.74% in the differentiation of non-survivors from survivors. CfDNA levels showed a significant positive correlation with other laboratory and inflammatory markers of COVID-19. CfDNA levels, NLR, and other parameters may be used to stratify and monitor COVID-19 patients and predict mortality. CfDNA may be used to predict COVID-19 severity with higher diagnostic sensitivity.

Keywords COVID-19 · Diagnosis · Prognosis · Cell-free DNA · Immune dysregulation

Introduction:

Coronavirus disease 2019 (COVID-19) is a major clinical problem that requires an in-depth analysis of the forecasting factors for the prognostication of patients at a higher risk of respiratory failure. The presence of comorbidities, immune system hyper activation, and other cardiovascular disorders are associated with poor outcomes in SARS-CoV-2 infected patients [1, 2]. Systemic inflammation is associated with an unfavorable clinical course of the disease and the development of severe COVID-19.

Circulating cell-free DNA (cfDNA) is an extracellular nucleic acid that circulates freely in the blood [3]. Circulating free DNA has been reported in healthy individuals, persons with non-malignant diseases, and various types of malignancies. In addition, trauma and therapeutic procedures may also lead to the release of circulating free DNA into the bloodstream. Theoretically, circulating DNA is predominantly released from degrading cells after cleavage by endonucleases that cut chromatin into the basic nucleosomes, which conserves them from proteolytic degradation in the blood [4].

In a healthy person, it is believed that cfDNA enters the circulation via apoptosis of lymphocytes and other nucleated cells [5]. During pathological conditions, the primary

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sources of cfDNA are apoptotic and necrotic cells, even though the level of input of the other cell death types over the other has been a subject of controversy [6]. Studies have demonstrated that circulating cfDNA exists at steady-state levels and sometimes increases dramatically with cellular injury or necrosis. A supplementary mechanism for the circulating nucleosome is NETosis, a recently recognized mode of neutrophil antimicrobial defense [7]. In NETosis, neutrophils release extracellular traps (NETs), including DNA, both nuclear and mitochondrial, antimicrobial peptides, and histones. However, pathogen clearance is frequently related to NETosis and has been implicated in the pathogenesis of thrombosis and systematic lupus erythematosus (SLE) [8, 9].

In COVID-19 patients, apoptotic and dying cells (leukocytes & lymphocytes) are major sources of cfDNA. Lymphopenia is observed in 60% of COVID-19 patients and is a major source of cfDNA [10]. Phagocytic cells play an important role in removing apoptotic debris and thereby decreasing the consequences of dead cell material [11]. In COVID-19 disease, cell death surpasses clearance capacity; and the phagocytic system is dazed [12]. There is a clear link between inflammation and cfDNA levels released from dying and damaged cells suggesting that cfDNA is a biomarker of COVID-19 severity. It has been reported that the concentration of cfDNA in healthy subjects ranges between 0 and 100 ng/ml with an average of 30 ng/ml [13].

Studies have reported lymphocyte levels as a means of early identification of risk factors for severe COVID-19 (SCOV-19). The neutrophil to lymphocyte ratio (NLR) is associated with the systematic inflammatory response to COVID-19 [14]. A high level of NLR indicates a predominance of inflammatory factors. Changes in hematological and serum biochemical markers were also been reported in COVID-19 patients [15, 16]. However, there is still a need to explore the association between peripheral lymphocyte alterations and cfDNA levels in COVID-19 patients.

The current study was designed to assess plasma cfDNA and NLR levels in COVID-19 patients to answer the following clinically relevant questions related to severity and outcome: Whether cfDNA may serve as a prognostic and predictive marker for SCOV-19? Are the levels of cfDNA different in patients with mild COVID-19 (MCOV-19) and SCOV-19? Whether the level of these biomarkers at hospital admission is different between patients who recovered (survivor) and those who died (non-survivor) with the disease and whether this information is of potential clinical relevance (e.g., identifying patients within the clinically 'severe' group, who are at higher risk of an adverse outcome/mortality). The cfDNA level was also correlated and compared with other commonly used laboratory parameters such as C-reactive protein (CRP), procalcitonin (PCT), neutrophil to lymphocyte ratio (NLR), absolute lymphocyte count (ALC), and absolute neutrophil count (ANC) to ascertain any added advantage of testing cfDNA parameters.

Material and Method

Patients and Methods

COVID-19 Patients

This prospective cohort study was conducted at the Chemical Pathology Laboratory, Department of Pathology, King Georges Medical University, Lucknow. Patients were recruited from a dedicated COVID-19 hospital, the RALC campus, a wing of KGMU, Lucknow. Patients who were consecutively admitted to the COVID-19 ward from March to June 2021 were prospectively included in the study if they met the following inclusion criteria (1) confirmed COVID-19 infection as confirmed by a positive reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay of a specimen collected on a nasopharyngeal swab; (2) bilateral pulmonary interstitial opacities on chest imaging that were not fully explained by congestive heart failure or other forms of volume overload; and (3) acute respiratory distress syndrome, showing at least one of the following conditions: respiratory rate ≥ 30 breaths/min; peripheral capillary oxygen saturation (SpO_2) $\leq 94\%$ while breathing ambient air or ratio of the partial pressure of oxygen in arterial blood to the fractional concentration of oxygen in inspired air (PaO_2/FiO_2) ≤ 300 mmHg. Cases were categorized as mild COVID-19 (MCOV-19) & severe COVID-19 (SCOV-19) according to WHO guidelines 2020. Patients requiring a ventilator at admission to the wards were excluded from the study. The study was carried out according to the declaration of Helsinki and its later amendments and was approved by the Institutional Ethical Committee, and written informed consent was obtained. Pregnant women and patients taking immunosuppressant until the time of sampling were excluded. Cases were categorised as mild/ MCOV-19 & severe/ SCOV-19 as to WHO guidelines.

All the study patients were followed until the end of the clinical observation, defined as death or complete recovery and discharge from the hospital with $SpO_2 > 94\%$ while breathing in ambient air. The clinical profiles of each patient were recorded, and each patient's age, sex, comorbidities, date of 1st symptoms, and previous drug treatment were assessed. The following laboratory parameters were also evaluated at hospital admission: circulating white blood cells, granulocytes, monocytes, lymphocytes and hemoglobin, c-reactive protein (CRP), procalcitonin, ferritin, D-dimer, Fibrinogen, Ferritin (U/L), Lactate dehydrogenase (LDH, U/L), procalcitonin (PCT, ng/ml), Interleukin-6 (IL-6), and creatinine (mg/dl). Absolute lymphocyte count

(ALC), Absolute neutrophil count (ANC), Absolute monocyte count (AMC), and neutrophil–lymphocyte ratio (NLR) was calculated.

Sample Collection for Cell-Free DNA (cfDNA) Isolation

At the time of admission, 4 ml of peripheral blood was also collected into an EDTA vial for plasma isolation before the start of any treatment/medication. Plasma was separated by centrifugation at 2000xg for 10 min. The second step of high-speed centrifugation at 16000 g for 10 min was also performed, and plasma was stored at −80 °C for further processing. To remove potential contamination by leukocytes, all the samples were processed within 1 h of collection. The cfDNA was isolated using the QIAmp circulating nucleic acid kit (Qiagen, Germany) as per the manufacturer's instructions, using 1 ml of plasma. Purified cfDNA was stored at −80 °C for further processing. The β-actin gene primers were used for quantitative real-time PCR [17].

Study Tools for Hematology and Biochemistry Parameters Analysis

Whole blood EDTA samples, sent at admission, were run on 3 part cell counter for CBC. First reading of prothrombin time (PT) and D-dimer values were obtained from citrated samples carried out on Stago (Satellite analyzer) sent within the first two days of admission. Values for serum CRP, and lactate dehydrogenase (LDH) were obtained. Serum biochemistry parameters were done on a biochemistry auto analyzer as per the kit insert using the samples collected in a plain vial. All the test parameters were performed in National Accreditation Board for Testing and Calibration Laboratories (NABL) certified chemical pathology laboratory, department of pathology, KGMU. Calibrations performed with standard controls were tested. To avoid the effect of interpolation, if any, only the first sent investigation was included.

Real-Time PCR for cfDNA Quantification

CfDNA quantification in cases was performed by quantitative SYBR Green real-time PCR (QPCR) and compared with a standard curve plotted by control human genomic DNA with known concentration at 10 ng/ml (Applied Biosystem, USA). qPCR was performed on the Rotor-Gene Q (Qiagen, Hilden, Germany). qPCR reaction components for the SYBR Green detection approach were as follows: 10 μl of SYBR Green Supermix (Applied Biosystems, USA), 500 nM of each primer, and 1 μl of extracted DNA, and the volume was brought to 20 μl by nuclease-free water. Thermal cycling started with a first denaturation step of 10 min at 95 °C,

followed by 35 cycles of 95 °C for 30 s, 55 °C for the 30 s, and 72 °C for 30 s.

Standard Curve for Absolute cfDNA Quantification

Five-fold serial dilutions of control genomic DNA were prepared at 100, 10, 1, 0.1, and 0.01 ng to construct the calibration curve on each plate. Linear amplification down to the last dilution point representing 0.01 ng of target DNA was obtained in each experiment (equation: efficiency $= 10(-1/\text{slope}) - 1$), and all correlation coefficients (R^2) were 0.99 to 1.00. Melt curve analyses were also performed to check the generation of specific PCR products (Fig. 1). A negative control (without template) was performed on each plate. All samples were performed in duplicate, and the mean values were used for quantification. The machine-generated Ct values and amplification plots were used to create a standard curve, which was employed to quantify the cfDNA content in the samples.

Follow-Up

In the SCOV-19 group (n = 106), 38 patients (35.84%) died during hospital admission. All surviving patients were re-evaluated after discharge. Outcomes were assessed either at the end of the period of admission to the hospital (COVID-19-related death or complete recovery and discharge from the hospital) or after 2–3 months on average (re-evaluation with follow-up).

Statistical Analysis

Data were analyzed with SPSS version 25.0 (Chicago, IL, USA). The categorical variables are reported as numbers (%) and continuous variables as median and interquartile ranges. Statistical significance was assessed using the chi-square test for dichotomous variables using the two independent sample *t*-test or by Mann–Whitney *U* test when appropriate. The correlations between quantitative variables were done using the Spearman correlation coefficient. Receiver operating characteristic (ROC curve) was constructed, with area under curve (AUC) analysis performed to detect the best cut-off value of different parameters for differentiating SCOV-19 and MCOV-19 infections. A *P* value < 0.05 was considered statistically significant. The primary endpoint was the survival rate in the different subgroups.

Results

A total of 206 (n = 206) COVID-19 positive cases were included in the study and were divided into two groups: Group1 comprised 100 (n = 100) cases of MCOV-19, and
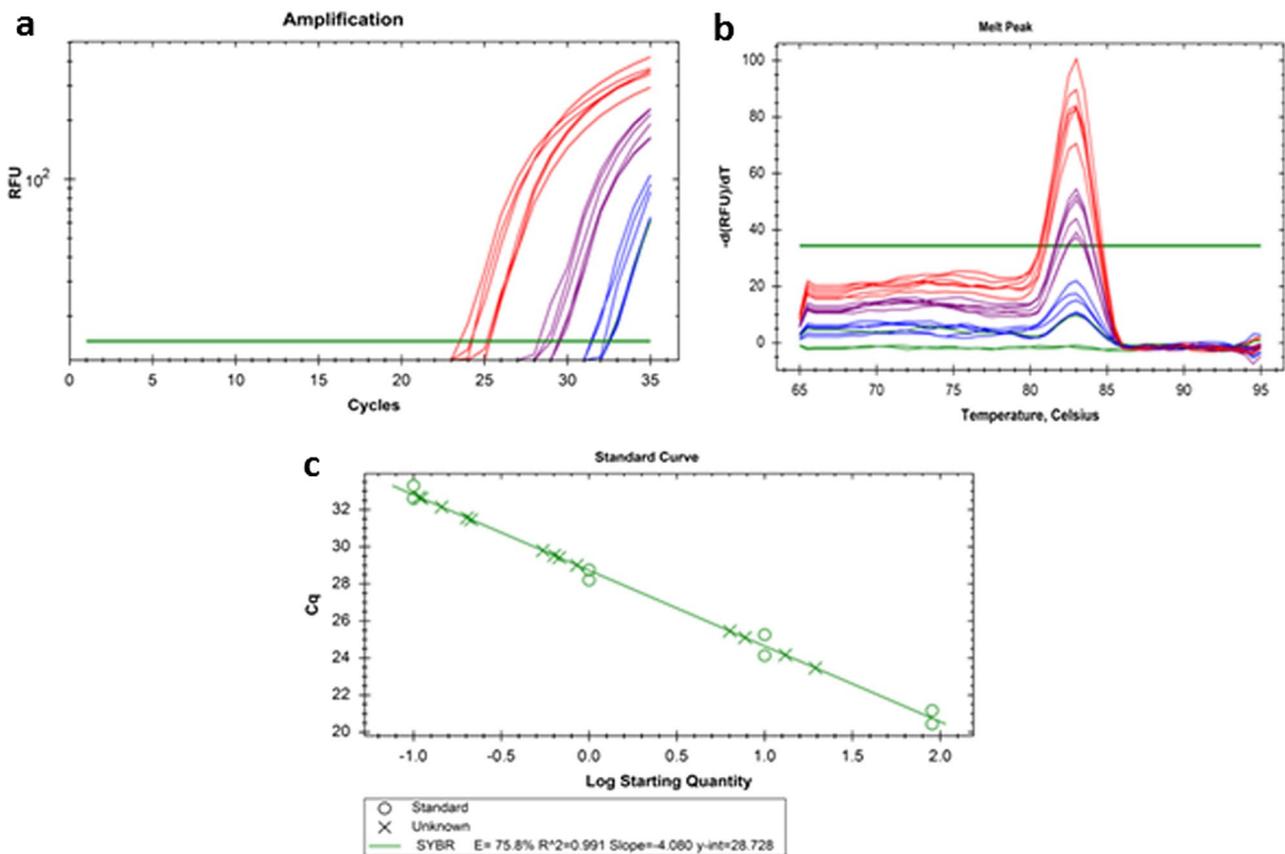
Fig. 1 Sybr green Quantitative real time PCR (**a**) Amplification plot, (**b**) Melt Curve and (**c**) standard curve

group 2 comprised 106 SCOV-19 patients. The median (Q1–Q3) age of SCOV-19 and MCOV-19 was 55 (45–65) and 50 (40–64) years, respectively. The majority of the patients were male (127/206). The patients suffering from the mild disease were admitted to the isolation ward (49%), while the remaining 51% with severe disease were admitted to the high dependency unit or intensive care unit, depending on availability. We have included only two moderate COVID-19 cases and hence excluded them from the cohort and final analysis. Hypertension was observed in 51% and 46%, type II diabetes mellitus in 33% and 30%, coronary artery disease in 9% and 5%, and chronic kidney disease in 7% and 4% of SCOV-19 and MCOV-19, respectively. Other comorbid conditions were malignancy and tuberculosis recorded in less than 2% of cases of SCOV-19 and none in MCOV-19. Alcoholic liver disease was found in 3% of SCOV-19 and none in MCOV-19. No significant difference was found in the between-group comparison. In SCOV-19 cases, the median time between the first symptoms to admission to the hospital was 5.6 days. The laboratory parameter values of ANC, NLR ($p = <0.0001$), D-dimer ($p = 0.0012$), fibrinogen, ferritin, LDH, PCT ($p = <0.0001$), and IL-6 ($p = 0.0008$) were significantly different and higher

in SCOV-19 cases as compared to MCOV-19 cases. The cfDNA level was 3.23 times higher in SCOV-19 cases as compared to the MCOV-19 cases ($p = <0.0001$) (Fig. 2). The comparison of laboratory parameter values between the groups is depicted in Table 1. The cfDNA level among SCOVID & MCOVID with and without comorbidities is depicted in Table 2. There was no significant difference in median cfDNA level among SCOVID & MCOVID with comorbidities as well as with no comorbidities ($p = 0.897$, $p = 0.092$). Moreover, among nonsurvivors, cfDNA levels in
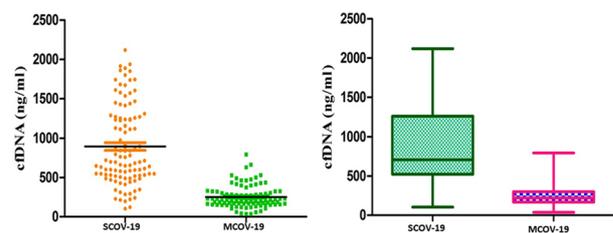

Fig. 2 CfDNA level in MCOV-19 and SCOV-19 at time of hospital admission

Table 1 Comparison of laboratory parameters in MCOV-19 and SCOV-19

Variable	SCOV-19 (n = 106) Median (Q1-Q3)	95%CI Lower–upper	MCOV-19 (n = 100) Median (Q1-Q3)	95%CI Lower–upper	P value
Age	55 (45–65)	51.39–56.93	50 (40–64)	48.08–53.78	0.097
ANC (X10^3/mm^3)	8.36 (5.06–12)	8.07–9.99	3.03 (0.32–4.38)	2.33–3.18	<0.0001
ALC (X10^3/mm^3)	0.6 (0.3–1.2)	0.7–1.3	1.91 (0.86–4.67)	2.67–3.96	<0.0001
AMC (X10^3/mm^3)	0.38 (0.19–0.61)	0.37–0.49	0.59 (0.45–0.73)	0.58–0.75	<0.0001
NLR	8.5 (5.7–23)	8.69–17.50	3.7 (3–8.6)	4.12–7.55	<0.0001
Creatinine (mg/dl)	0.98 (0.80–1.23)	0.87–1.96	0.90 (0.77–1.24)	0.84–1.87	0.6020
CRP (mg/ml)	54.40 (20.55–108.4)	51.95–106.7	40.60 (6.60–105.9)	47.83–68.67	0.0692
D-Dimer	2.27 (0.67–6.70)	2.86–4.14	0.92 (0.51–2.16)	1.52–2.52	0.0012
Fibrinogen	603.2 (474.5–671.9)	543.4–626.3	388.0 (324.0–499.0)	383.6–436.1	<0.0001
Ferritin (ug/l)	863.8 (541.7–1428)	914.6–1252.0	215.0 (146.3–680.0)	373.6–618.3	<0.0001
LDH (u/l)	1312 (931.2–1816)	1250–1458	721.2 (521.2–996.4)	681.5–818.1	<0.0001
Procalcitonin (ng/ml)	0.50 (0.20–1.10)	0.73—1.29	0.10 (0.05–0.30)	0.03–0.86)	<0.0001
IL6	13.27 (10.80–68.71)	32.01–63.81	10.80 (10.71–33.87)	17.84–47.18	0.0008
cfDNA (ng/ml)	706.7 (522.6–1258)	797.7–990.0	219.8 (167.7–299.6)	224.4–278.8	<0.0001

Table 2 Comparison of cfDNA level in patients with co morbidities and no comorbidities

	Median (Q1-Q3) cfDNA level	P value
SCOV with Comorbidities (n = 46)	741.8 (562.8–1429.0)	0.897
SCOV with no Comorbidities (n = 60)	675.4 (493.6–1205.0)	
MCOV with Comorbidities (n = 49)	255.3 (193.5–463.5)	0.092
MCOV with no Comorbidities (n = 51)	247.2 (177.2–476.0)	
Non-survivor with comorbidities (n = 30)	861.2 (518.2–2426.0)	0.305

Table 3 Correlation of cfDNA with other studied laboratory parameters

Parameter	Correlation coefficient p Value	
ANC (X10^3/mm^3)	0.451 <0.0001	Spearman correlation for cfDNA
ALC (X10^3/mm^3)	−0.593 <0.001	
AMC (X10^3/mm^3)	−0.565 <0.001	
NLR	0.701 <0.001	
Ferritin (ug/l)	0.620.001	
LDH (u/l)	0.6540.001	
Procalcitonin (ng/ml)	0.6750.001	
IL6	0.589 <0.001	

those with co-morbidities and those with no comorbidities were not different ($p = 0.305$).

Correlation of cfDNA with Laboratory Parameters

The correlation of cfDNA level with the other established laboratory parameters is depicted in Table 3. The cfDNA level showed a significant correlation with ANC

($p = <0.0001$), ALC, AMC, NLR, ILC ($p = <0.001$), and with ferritin, LDH, and PCT ($p = 0.001$).

Diagnostic Performance of Laboratory Parameters and Circulating Cell-Free DNA

The output data of the ROC curve analysis revealed the highest AUC for ALC (0.98) with sensitivity and specificity of 97.17% & 96.0%, respectively, in the differentiation of MCOV-19 and SCOV-19. The AUC for cfDNA was 0.92 with diagnostic sensitivity and specificity of 90.57% and 80%, respectively. For the other laboratory parameters, the AUC was in the range of 0.63–0.88 (Table 4).

Outcome Prediction

We have not observed the mortality in the MCOV-19 group, whereas 38 SCOV-19 patients expired (38/106). We compared the difference in biomarkers at hospital admission between COVID-19 patients who recovered (survivor) vs. those who died due to the disease (non-survivor), as depicted in Table 5. The higher values for ANC ($p = <0.0001$), NLR ($p = 0.0009$), CRP (0.01), fibrinogen ($p = 0.001$), LDH ($p = 0.0013$), and PCT (<0.0001) were noted in non-survivor

Table 4 Receiver operator characteristic curve (ROC) analysis for significant laboratory parameters to differentiate between MCOV-19 and SCOV-19

Diagnostic	Cut-off value	AUC	p value*	Sensitivity (95% CI)	Specificity (95% CI)
ANC (X10^3/mm^3)	> 3.28	0.886	< 0.0001	90.57 83.33–95.38%	53.06 42.71–63.22%
ALC (X10^3/mm^3)	> 0.364	0.988	< 0.0001	97.17 91.95%–99.41%	96.00 90.07–98.90%
AMC (X10^3/mm^3)	< 0.296	0.702	< 0.0001	42.45 32.91–52.43%	90.0 82.38–95.10%
NLR	< 4.07	0.997	< 0.0001	98.11 93.35–99.77%	100.0 96.38–100.0%
D-Dimer	> 0.990	0.630	0.0012	65.09 55.22–74.10%	53.00 42.76–63.06%
Fibrinogen	> 431.5	0.768	< 0.0001	82.08 73.43–88.85%	68.00 57.92–76.98%
Ferritin (ug/l)	> 443.1	0.764	< 0.0001	80.19 71.32–87.30%	65.00 54.81–74.27%
LDH (u/l)	> 852.0	0.822	< 0.0001	82.08 73.43–88.85%	65.00 54.81–74.27%
Procalcitonin (ng/ml)	> 0.175	0.799	< 0.0001	92.45 85.67–96.69%	56.00 45.72– 65.92%
IL6	> 12.76	0.634	0.00087	54.72 44.75–64.41%	61.00 50.73– 70.60%
cfDNA (ng/ml)	> 324.8	0.921	< 0.0001	90.57 83.33–95.38%	80.00 70.82– 87.33%

Table 5 Comparison of laboratory parameters in survivor and non-survivor in COVID-19

Variable	Non-survivor Median (Q1–Q3)	95%CI Lower–upper	Survivor Median (Q1–Q3)	95%CI Lower–upper	P value
Age	58.50 (48.75–65.25	50.65–60.67	55. 9 (43.0–65.0)	49.98–56.46	0.2963
ANC (X10^3/mm^3)	8.24 (3.87–10.69)	6.68–10.33	4.02 (2.54–5.59)	3.50–5.30	< 0.0001
ALC (X10^3/mm^3)	0.13 (0.05–1.05)	0.43–1.25	1.28 (0.73–4.50)	1.79–4.06	< 0.0001
AMC (X10^3/mm^3)	0.40 (0.19–0.61)	0.35–0.55	0.54 (0.29–0.67)	0.47–0.69	0.0897
NLR	9.68 (5.42–23.67)	12.86–36.46	5.31 (3.81–13.01)	4.42–17.78	0.0009
Creatinine (mg/dl)	1.08 (0.85–1.33)	1.02–1.54	0.90 (0.75–1.27)	0.61–2.29	0.0995
CRP (mg/ml)	91.50 (51.23–126.7)	50.34–197.1	51.0 (21.30–110.1)	52.74–77.30	0.0170
D-Dimer	1.51 (0.62–7.25)	2.44–4.82	1.25 (0.59–3.16)	1.62–2.77	0.1946
Fibrinogen	613.2 (496.0–669.0)	524.7–656.5	419.0 (281.0–566.0)	395.2–484.9	0.0001
Ferritin (ug/l)	757.3 (267.2–1879.0)	817.1–1572.0	625.0 (181.7–1026.0)	576.4–997.8	0.0556
LDH (u/l)	1352.0 (766.3–1916.0)	1150.0–1574.0	866.2 (548.1–1253.0)	821.7–10,174.0	0.0013
Procalcitonin (ng/ml)	0.61 (0.27–1.12)	0.64–1.43	0.20 (0.05–0.40)	0.21–0.72	< 0.0001
IL6	74.47 (10.71–70.81)	25.69–94.111	10.80 (10.7–33.7)	18.83–67.29	0.1149
cfDNA (ng/ml)	839.1 (491.1–1272.0)	737.7–1049.0	235.6 (173.8–546.1)	305.2–483.5	0.0001

at admission ($p = < 0.001$). The cfDNA level ($p = 0.0001$) was also 3.5 times higher in non-survivors than survivors (Fig. 3).

The ROC curve analysis between the survivor and non-survivor revealed that the cfDNA level at a cut-off point of > 287.3 ng/ml and an AUC of 0.820 yielded a sensitivity of 94.74% with a specificity of 58.21% ($p = < 0.0001$). The AUC of NLR was 0.696 with a sensitivity of 86.84% and specificity of 52.22% ($p = 0.0008$) (Table 6).

Discussion

COVID-19 has emerged as the most intense healthcare disaster linked with acute to high transmissible disease. Which biomarker needs to be evaluated when and in whom, and how best this information can contribute to patient care are questions that currently lack convincing answers. The circulating biomarkers are currently being used to monitor and predict COVID-19 severity. Studies
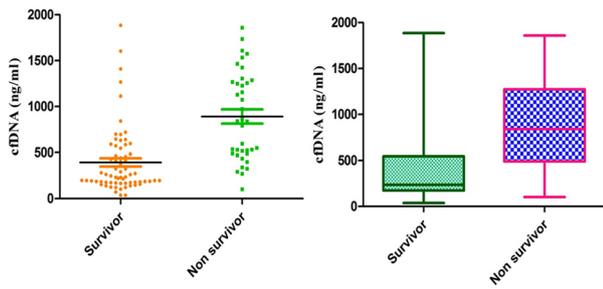
Fig. 3 CfDNA level in survivor and non-survivor of COVID-19

have found that severe COVID-19 is associated with a significant increase in hematological parameters, including leukocytes, neutrophils, infection biomarkers [such as CRP, PCT, and ferritin], and cytokine levels [IL-2R, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)-α] and decreased lymphocyte counts [18].

The presence of cfDNA has been reported in serum, plasma, induced sputum, bronchial lavage, milk, urine, and stool [19]. The release of cfDNA in circulation occurs via active release from diseased cells, apoptosis and necrosis, and the interface of the tumor & adjacent non-tumor cells; however, its release into the circulation is still a debate [20]. CfDNA serves as non-invasive tests for molecular analysis in several malignancies, and quantification may help optimize medical practice personalized medicine and improve quality of life [20]. The analysis of circulating DNA, ribonucleic acid (RNA), and protein markers hold great hope for diagnostic and screening purposes.

We have hypothesized that massive cell destruction and inflammation in COVID-19 patients may accumulate plasma cfDNA, which may help assess the severity

and mortality. We have also investigated the possible link between the cfDNA and other established laboratory parameters.

We have found higher cfDNA in SCOV-19 patients with a median (Q1–Q3) of 706.7 ng/ml (522.6–1258) as compared to the MCOV-19 patients with a median (Q1–Q3) of 219.8 ng/ml (167.7–299.6). A study by Zuo et al. also reported a higher cfDNA level in COVID-19 patients on mechanical ventilation compared to the patients with mild symptoms and breathing in room air. The comorbidities such as hypertension, diabetes, cardiovascular diseases, and liver disease results in vascular damage in COVID-19 patients. The increased accumulation of free DNA in the vessels of these patients may be found. A study by Liu et al. 2020 suggested free DNA as a useful biomarker in predicting COVID-19 severity and reported that free DNA-induced damage plays an essential role in the etiology of cytokine storm [11]. Similarly, Wu et al. 2013 found that the presence of free DNA is responsible for the cytokine storm by activating the immune cells for the production of cytokines [21]. Kostyuk et al. 2018 reported the role of cell-free DNA in the depletion of endothelial cells resulting in multi-organ non-functioning due to the cytokine storm [22].

The severe COVID-19 cases are mostly reported with lymphopenia, a higher level of NLR, CRP, D-Dimer, and PCT than mild COVID-19 patients. Studies have also found a higher IL-2, IL-6, and TNF-α in ICU admitted patients [23]. We have also found a significantly high level of ANC, PCT, NLR, and CRP and a lower level of ALC, and AMC in severe patients compared to mild COVID-19 patients.

Higher CRP in COVID-19 patients may not be attributed to COVID-19 infection alone and may represent the associated pathology such as secondary bacterial associated pneumonia. In our study, the CRP level between the SCOV-19

Table 6 Receiver operator characteristic curve (ROC) analysis for significant laboratory parameters to differentiate between survivor and non-survivor with COVID-19	Diagnostic	Cut-off value	AUC	*p* value*	Sensitivity (95% CI)	Specificity (95% CI)
	ANC (X10³/mm³)	> 3.287	0.739	< 0.0001	84.21 68.75–93.98%	33.00 22.20–46.01%
	ALC (X10³/mm³)	> 0.384	0.760	< 0.0001	86.84 71.91–95.59%	62.69 50.01–74.20%
	NLR	< 50.28	0.696	0.0008828	86.84 71.91–95.59%	52.22 42.58–67.40%
	CRP (mg/dl)	> 42.50	0.640	0.01686	86.84 71.91–95.59%	43.28 31.22–55.96%
	Fibrinogen	> 470.0	0.728	0.0001069	81.58 65.67–92.26%	61.19 48.50–72.86%
	LDH (u/l)	> 852.0	0.690	0.001257	71.05 54.10–84.58%	47.76 35.40–60.33%
	Procalcitonin (ng/ml)	> 0.190	0.760	< 0.0001	89.47 75.20–97.06%	47.76 35.40–60.33%
	cfDNA (ng/ml)	> 287.3	0.820	< 0.0001	94.74 82.25–99.36%	58.21 45.52–70.15%

and MCOV-19 was not different (median 54.40 vs. 40.60, $p = 0.60$). Moreover, in survivors and non-survivor among SCOV-19 patients, the CRP level was different and higher in non-survivors compared to the survivor ($p = 0.0170$). However, other studies have found a significantly higher CRP level in severe COVID-19 patients than in non-severe patients. In contrast to our finding, Wang et al. used CRP level as a predictive marker in monitoring disease severity [24]. Huang et al. proposed CRP as a marker for disease improvement besides its use as a prognostic marker [25]. The time for serum CRP measurement is important in light of the timely manner of serum CRP increment, which accumulates 72 h after in blood. Despite its value in forecasting a meager outcome in COVID-19, various factors such as age, gender, smoking status, weight, lipid levels, blood pressure, and liver injury may affect the CRP level.

We have found a significant decrease in serum ferritin level in MCOV-19 cases compared to the SCOV-19 cases (< 0.0001) and was concordant with the Chen et al. Taneri et al. analyzed the ferritin level from the 29 studies and have found significantly higher level in severe patients as compared to moderate cases [26]. There was hyperferritinemia in non-survivor compared to survivors; however, the difference was significant ($p = 0.05$). Studies have also reported the association of higher ferritin with systematic inflammation [27]. The diagnostic sensitivity of ferritin (80.19%) in discrimination of SCOV-19 from MCOV-19 was higher than the IL6 (54.72%), D-dimer (65.09%), and AMC (42.45%).

The present study demonstrated the increase in the ANC ($p = < 0.0001$) in SCOV-19 cases compared to the MCOV-19 cases. The ANC level was also higher in non-survivors than in the survivor ($p = < 0.0001$). Previous studies have also found a significant increase in TLC, ANC and a decrease in lymphocyte count in severe cases compared to mild-moderate COVID-19 patients [23, 28]

There was a significant increase in NLR in SCOV-19 cases compared to the MCOV-19 cases ($p = < 0.0001$). The NLR level was also significantly higher in non-survivors as compared to the survivors ($p = 0.0009$). The study of Liu et al. found NLR as an independent risk predictor for mortality in hospitalized patients [29]. In a meta-analysis, the NLR was found to present the inflammatory process and has been suggested as a marker for predicting poorer prognosis [30]. The inflammatory conditions have also been shown to increase the cfDNA level. The virus-associated inflammatory markers such as IL-6 and IL-8, TNF-α, and IFN-c factors produced by the lymphocytes can also trigger neutrophil production. The immune response to the viral infection depends on the lymphocytes, and systematic inflammation decreases T lymphocytes. We have found the highest diagnostic sensitivity and specificity for NLR in discriminating SCOV-19 from MCOV-19. The diagnostic sensitivity of NLR was 86.84% and was higher than the LDH (71.05%),

fibrinogen (81.58%), and ANC (84.21%) and was in agreement with the ALC (86.84%). The increased NLR suggests virus-associated inflammation in COVID-19 patients [31].

The correlation of cfDNA with the other laboratory and inflammatory parameters showed a significant correlation as depicted in Table 2 and thus highlights the value of cfDNA in inflammatory conditions. The study by Rauch et al. demonstrated that vascular inflammation and severe endothelial injury directly result from COVID-19 infection and ensuing host inflammatory response to COVID-19. The study of Ng H et al. 2020 analyzed the neutrophil extracellular traps (NETs) as a contributing factor for cfDNA accumulation and also correlated with the CRP level. We have found significant positive correlation of cfDNA with the ferritin (r = 0.62, $p = 0.001$) [32]. The generation of reactive oxygen species may explain this by interacting intracellular iron with the molecular oxygen, increasing apoptosis and damage to the cellular component.

There was an inverse correlation of cfDNA level ALC (r = −0.593, $p = < 0.001$) and AMC (r = −0.565, $p = < 0.001$). In concordance with Laurent et al., This finding concluded that the lymphoid and erythroid cells are the main components of cfDNA. We have also demonstrated a positive correlation of cfDNA with NLR (r = 0.701, $p = < 0.001$). This may be explained by the activation and migration of neutrophils and the generation of ROS that may be a reason behind the cell damage and release of cfDNA in circulation. Moreover, lymphopenia is a characteristic feature of SCOV-19 due to the lymphocyte apoptosis results in higher cfDNA levels. Yang et al. demonstrated the NLR as an independent risk predictor [33].

The diagnosis of cfDNA in discrimination of MCOV-19 form SCOV-19 reveals the sensitivity and specificity of 90.57% & 80.0%. The diagnostic value of cfDNA was higher when compared to the other laboratory and inflammatory parameters such as AMC (42.45%), D-dimer (65.09%), fibrinogen (82.08%), and ferritin (80.19%) LDH (82.08%) and IL-6 (54.72%). In discriminating survivors from non-survivor, the diagnostic sensitivity was highest for cfDNA (94.74%) compared with the other test parameters; however, the specificity was only 58.21%. The current study reveals the highest sensitivity and specificity for NLR (98.11%, 100%) respectively in discrimination of MCOV-19 from the SCOV-19.

The world health organization (WHO) used ordinal scales in 1 to 8 scales in COVID-19 patients using cfDNA to stratify the severity and found the correlation of cfDNA with the COVID-19 disease severity [34]. The cfDNA and mtcfDNA have also been used as markers to measure the severity and identify the patients requiring ICU care at admission [35]. Our cfDNA findings showed that cfDNA quantification at the time of hospital admission could be used to identify patients requiring ICU care and predict mortality. The

cfDNA can be added with the NLR to assess higher sensitivity's severity and risk of mortality. The applicability of cfDNA as a biomarker is limited by the intra- and interindividual variations even from day to day and within a day; thus, cfDNA change over the time and reference interval should be evaluated [36].

The current study is limited by the analysis of cfDNA in plasma samples only; determination of tissue origin of cfDNA is warranted in future studies. In the current COVID-19 scenario, the studied sample size may not be sufficient to generalize our findings. However, we have proposed cfDNA as an indicator for monitoring severity and outcome.

Conclusion

A higher level of cfDNA in COVID-19 patients can be used in severity prediction. There was a significant association of cfDNA level with the other laboratory parameters. The assessment of cfDNA level can be used as a tool for monitoring and stratification of MCOV-19 and SCOV-19 patients. The dysregulated lymphocytes are the feature of COVID-19 patients. The current study findings propose cfDNA as a marker for monitoring and prediction of COVID-19 severity. The ROC curve analysis showed a high discrimination power of cfDNA and ANC, ALC & NLR between MCOV-19 & SCOV-19 as well as in the prediction of survivor and non-survivor in SCOV-19. Early monitoring of cfDNA may have an important basis to guide the treatment plan, and early evaluation of cfDNA for assessment of patients' severity may be of great value.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Consent to Participate Informed consent was obtained from all the study participants or from legal guardians.

Human Participants and Animals Approval was obtained from the ethics committee of the King George Medical University, Lucknow. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Informed Consent A written informed consent was obtained from the study participant or legal guardian and was explained about the study procedure.

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