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Background: Mitochondrial DNA (MT-DNA) are intrinsically inflammatory nucleic acids released by damaged solid organs. Whether circulating cell-free MT-DNA quantitation could be used to predict the risk of poor COVID-19 outcomes remains undetermined.

Methods: We measured circulating MT-DNA levels in prospectively collected, cell-free plasma samples from 97 subjects with COVID-19 at hospital presentation. Our primary outcome was mortality. ICU admission, intubation, vasopressor and renal replacement therapy requirements were secondary outcomes. Multivariate regression analysis determined whether MT-DNA levels were independent of other reported COVID-19 risk factors. Receiver operating characteristics and area under-the-curve assessment were used to compare MT-DNA levels to established and emerging inflammatory markers of COVID-19.

Results: Circulating MT-DNA levels were highly elevated in patients who eventually died, required ICU admission, intubation, vasopressor use or renal replacement therapy. Multivariate regression revealed that high circulating MT-DNA is an independent risk factor for these outcomes after adjusting for age, sex, and comorbidities. We also found that circulating MT-DNA levels have a similar or superior area-under-the curve when compared against clinically-established measures of inflammation and emerging markers currently of interest as investigational targets for COVID-19 therapy.

Conclusions: These results show that high circulating MT-DNA […]

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Circulating Mitochondrial DNA is an Early Indicator of Severe Illness and Mortality from COVID-19

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

ABSTRACT

Background: Mitochondrial DNA (MT-DNA) are intrinsically inflammatory nucleic acids released by damaged solid organs. Whether circulating cell-free MT-DNA quantitation could be used to predict the risk of poor COVID-19 outcomes remains undetermined.

Methods: We measured circulating MT-DNA levels in prospectively collected, cell-free plasma samples from 97 subjects with COVID-19 at hospital presentation. Our primary outcome was mortality. ICU admission, intubation, vasopressor and renal replacement therapy requirements were secondary outcomes. Multivariate regression analysis determined whether MT-DNA levels were independent of other reported COVID-19 risk factors. Receiver operating characteristics and area under-the-curve assessment were used to compare MT-DNA levels to established and emerging inflammatory markers of COVID-19.

Results: Circulating MT-DNA levels were highly elevated in patients who eventually died, required ICU admission, intubation, vasopressor use or renal replacement therapy. Multivariate regression revealed that high circulating MT-DNA is an independent risk factor for these outcomes after adjusting for age, sex, and comorbidities. We also found that circulating MT-DNA levels have a similar or superior area-under-the curve when compared against clinically-established measures of inflammation and emerging markers currently of interest as investigational targets for COVID-19 therapy.

Conclusions: These results show that high circulating MT-DNA levels are a potential early indicator for poor COVID-19 outcomes.

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INTRODUCTION

Coronavirus Disease 2019 (COVID-19) is a respiratory tract infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that has resulted in a global health emergency, causing considerable strain on economic, social and medical systems (1). COVID-19 presents in a wide spectrum of severity. Although most patients develop only mild or uncomplicated illness, others require prolonged hospitalization, ICU care and intubation for respiratory support. In severe cases, patients can develop acute respiratory distress syndrome (ARDS), cytokine storm, multi-organ failure and death (1). Although the underlying mechanisms of severe COVID-19 illness remain unclear, it appears to be exacerbated by an over-exuberant innate immune response (2). These observations have led to several ongoing clinical trials targeting components of the innate immune response such as inflammatory cytokine signaling and complement activation (3-5).

Previous work has established that viral infection can trigger cellular necrosis, which in turn inhibits viral replication along with amplifying anti-viral immune responses through the release of damage associated molecular patterns (DAMPs) (6). DAMPs in particular are potent triggers of innate responses through their engagement of pattern recognition receptors such as toll-like receptors (TLR) that drive the expression of inflammatory cytokines and presentation of viral antigens (7). Mitochondrial DNA (MT-DNA) is a member of a group of mitochondrial DAMPs (MT-DAMPs) released by injured or dying cells and is recognized by TLR9 due to encoded hypomethylated CpG motifs reminiscent of an ancestral bacterial origin (8). MT-DNA levels have been previously

shown to be elevated in the plasma of patients that develop ARDS and multi-organ dysfunction during sepsis, as well as during sterile injury including trauma, hemorrhagic shock and ischemia-reperfusion (9-14). The release of MT-DNA is often accompanied by the release of other MT-DAMPs, such as N-formylated peptides, cytochrome c and cardiolipin, which collectively engage multiple TLRs and the N-formylated peptide receptor-1 that in turn not only induce inflammatory cytokine expression (15-17) but also the generation of reactive oxygen species and the facilitation of neutrophil trafficking and activation (14, 18, 19). Through these effects, MT-DAMPs can directly contribute to acute lung injury and systemic inflammation (8). Given that ARDS secondary to SARS-CoV-2 infection is also linked with lung tissue injury and immune cell activation (20), we asked if elevated levels of circulating MT-DNA could be used as a risk indicator for the development of severe illness.

Here, we demonstrate that COVID-19 patients with high circulating levels of cell-free MT-DNA at the time of hospital presentation are more likely to require ICU level care and intubation and are at heightened risk of death. In addition, MT-DNA quantitation as predictor of poor COVID-19 outcomes is comparable or better than inflammation indicators commonly used in current clinical practice, as well as certain emerging immune markers.

RESULTS

Participants

A total of 107 subjects were assessed for eligibility from 3/26/2020 to 4/26/2020. Of these, 97 adult subjects with laboratory-confirmed COVID-19 were included in our study (**Table 1**, **Figure 1**). 10 subjects were excluded as they did not have samples from the day of presentation. The median age of the population in our study was 65 (54-73). Among these subjects, 55.7% (54/97) were male and 44.3% (43/97) were female; 77.3% (75/97) were African American, 20.6% (20/97) were white, 1% (1/97) was Middle Eastern and 1% (1/97) was Indian; 46.4% (45/97) were obese (BMI ≥ 30), while 49.5% (48/97) were nonobese (BMI 18.5-29.9) and 4.1% (4/97) were underweight (BMI<18.5). A positive smoking history was reported in 46.4% (45/97) of subjects. The median follow-up time was 81 days (74-87).

Comorbidities and Outcomes

The primary outcome of mortality was observed in 25.8% (25/97) subjects in our study (**Table 1**). Those who died were older [76.2 versus 61.1 years, OR 1.08 (1.04-1.13), p = 0.0003], were more likely to be smokers [64% versus 40.3%, OR 2.63 (1.04-7), p = 0.04] and have a higher proportion of type 2 diabetes mellitus [72% versus 45.8%, OR 3.04 (1.17-8.64), p = 0.02], coronary artery disease [48% versus 19.4%, OR 3.8 (1.44-10.34), p = 0.007], and 2 or more comorbidities [84% versus 56.9%, OR 5.54 (1.72-24.91, p = 0.009] on univariate analysis (**Table 1**). The time to discharge for subjects who survived was mean 12.4 days, median 7 days (3 - 18) and range (1 - 56).

56.7% of subjects with COVID-19 (55/97, **Table 2**) required an ICU admission. These subjects were older [71 versus 54.4, OR 1.1 (1.06-1.15), p <0.0001] and were more likely to have type 2 diabetes mellitus [65.5% versus 35.7%, OR 3.41 (1.49-8.07), p = 0.004], and 2 or more comorbidities [72.7% versus 52.4%, OR 2.66 (1.14-6.38), p = 0.02]. As expected, the time to discharge was higher for patients requiring ICU [15 days (5 - 28), n=27 versus 5 days (3 - 12.5), n=36, p=0.0012]. 25.8% (25/97) required invasive mechanical ventilation as a treatment for acute respiratory failure. The main clinical characteristic associated with higher risk for intubation was age, with a median of 70 years for intubated subjects compared with 61 years for non-intubated subjects [OR 1.05 (1.01-1.09), p = 0.005] on univariate analysis, **Table 3]**. As expected, the time to discharge was higher for patients that required intubation [27.5 days (18.5 - 38.5), n=12 versus 5 days (3 - 13), n=51, p=<0.0001].

COVID-19 patients with high circulating MT-DNA are at higher risk for mortality

To assess MT-DNA levels in COVID-19 patients we utilized a previously established in-situ quantitative PCR method (14) to measure the accumulation of fragments derived from the mitochondrial encoded gene cytochrome b (MT-CYTB) within cell-free circulating plasma. Plasma MT-CYTB levels were elevated in those subjects who died from COVID-19 [7.56 (7.15 - 7.81, n=25)] compared to those who survived [7.23 (7.05 – 7.485), n=72, p=0.008, **Figure 2A**] and were associated with an increased risk for mortality on univariate logistic regression (OR 2.24, 95% CI 1.29 – 4.16, p = 0.006). For MT-CYTB, area under the curve (AUC) for mortality was 0.68 (95% CI

0.54 – 0.81, **Figure 2B**). On multivariable logistic regression, plasma MT-CYTB levels remained an independent risk factor for mortality when adjusted for age, sex and two or more comorbidities (OR_{adj}, 2.19, 95% CI 1.19 – 4.28, p = 0.015, **Table 4**). Levels of MT-COX3, another mitochondrial DNA encoded gene, MT-COX3 [**Figure S1B**] revealed a an increased trend in subjects who died [5.63 (5 - 6.46), n=25] relative to those who survived [5.32 (4.9 - 5.83), n=72] but did not reach statistical significance (p=0.1).

COVID-19 patients with high circulating MT-DNA are more likely to require ICU admission and intubation

Plasma MT-CYTB levels were elevated in those subjects with COVID-19 who required an ICU admission [7.52 (7.14 – 7.72), n=55] compared to those who were not admitted to the ICU [7.13 (7.0 – 7.31), p<0.0001, n=42, **Figure 3A**]. MT-CYTB levels were associated with ICU admission on univariate logistic regression (OR 4.25, 95% CI 2.15 – 9.59, p = 0.0001). For MT-CYTB, AUC for ICU admission was 0.75 (95%CI 0.65 – 0.85, **Figure 3B**). On multivariable logistic regression, plasma MT-CYTB levels remained an independent risk factor for ICU admission after adjusting for age, sex and 2 or more comorbidities (OR_{adj}, 3.97, 95% 1.83 – 10.34, p = 0.002, **Table 5**). Notably, we also made similar findings for MT-COX3 [**Figure S1C**, OR_{adj} for age, sex and 2 or more comorbidities, 1.47, 95% CI 1.14 – 1.95, p = 0.005), AUC of COX3 for ICU admission 0.69 (95% CI 0.58 - 0.79)].

Additionally, plasma MT-CYTB levels were elevated in those subjects with COVID-19 who required intubation [7.69 (7.54 – 8.07, n=25)] versus those who did not require

intubation [7.18 (7.033 – 7.438), n=72, p<0.0001, **Figure 3C**]. Plasma MT-CYTB levels were associated with intubation on univariate logistic regression (OR 9.12, 95% 3.77 – 28.56, p < 0.0001). For MT-CYTB, AUC for intubation was 0.86 (95% CI 0.76 – 0.95, **Figure 3D**). On multivariable logistic regression, plasma MT-CYTB levels remained an independent risk factor for intubation (OR_{adj}, 8.47, 95% CI 3.49 – 27.33, p < 0.0001, **Table 6**). Similar findings were observed with MT-COX3 [**Figure S1D**, OR_{adj} for age, sex and 2 or more comorbidities, 2.69, 95% CI 1.77 – 4.74, p < 0.0001), AUC for intubation 0.80 (95% CI 0.69 - 0.91)].

COVID-19 patients with high circulating MT-DNA are more likely to require vasopressors and renal replacement therapy (RRT)

Plasma MT-CYTB levels were also elevated in those subjects with COVID-19 who ultimately had end-organ dysfunction requiring vasopressors [7.63 (7.35 – 8.02), n=29] compared to those who did not require vasopressors [7.20 (7.04 – 7.43), p<0.0001, n=68, **Figure 4A**]. For MT-CYTB, AUC for vasopressor requirement was 0.80 (95%CI 0.69 – 0.91, **Figure 4B**). Similarly, plasma MT-COX3 levels were elevated in subjects requiring vasopressors [5.98 (5.25 - 6.68), n=29] compared to [5.25 (4.85 – 5.70), p=0.0001, n=68] (**Figure S1E)**, AUC of COX3 for vasopressor requirement was 0.74 (95% CI 0.62 - 0.86). Notably, plasma MT-CYTB levels on admission were also elevated in subjects who had end-organ dysfunction requiring RRT while in the ICU [7.75 (7.64 – 8.08), n=14] compared to those who did not require RRT [7.2 (7.04 – 7.47), p<0.0001, n=81, **Figure 4C**]. For MT-CYTB, AUC for RRT requirement was 0.95 (95%CI 0.91 – 0.99, **Figure 4D).** Similarly, plasma MT-COX3 levels were elevated in

subjects who required RRT [6.59 (6.36 – 7.12), n=14] compared to those who did not require RRT [5.24 (4.81 – 5.69), p<0.0001, n=81] (**Figure S1F**), AUC of COX3 for RRT requirement was 0.94 (95%CI 0.88 – 0.99).

Circulating MT-DNA levels show similar or improved sensitivity over clinically established measurements of inflammation used in COVID-19 patients

Plasma MT-CYTB levels had a similar AUC for mortality when compared to LDH, ferritin or D-dimer levels, and were better than CRP drawn within the first 24 hours of presentation (**Figure 5A**). Importantly, the AUC for plasma MT-CYTB levels was superior to CRP, LDH, ferritin and D-dimer levels when predicting the need for an ICU admission (**Figure 5B**) or intubation (**Figure 5C**). A similar pattern was identified for MT-COX3 when compared to CRP, LDH, ferritin and D-dimer levels for predicting the need for an ICU admission (**Figure S2B**) but was superior to these clinically utilized markers for the need for intubation (**Figure S2C**).

Circulating MT-DNA levels correlate with other emerging markers of COVID-19 severity

Plasma MT-CYTB levels moderately correlated with concurrently measured levels of IL-6, which has been implicated in the pathogenesis of COVID-19 [r=0.39, 95% CI 0.196 – 0.555, p = 0.0001, n=92, **Figure 6A**]. Similarly, MT-CYTB levels highly correlated with plasma sC5b-9, which is a marker of complement activation and suggests the formation of a membrane attack complex (MAC) [r=0.49, 95% CI 0.32 – 0.64, p < 0.0001, n=95, **Figure 6B**]. MT-CYTB also correlated with the neutrophil-to-lymphocyte ratio [r=0.37, 95% CI 0.17 – 0.54, p = 0.0003, n=90, **Figure 6C]**. Plasma MT-CYTB levels

had a similar or/improved accuracy compared to IL-6 for mortality (**Figure S3A**), ICU admission (**Figure S3B**) and intubation (**Figure S3C**).

We also observed significant correlations with other biomarkers that have been implicated in the pathogenesis of COVID-19 such as CXCL9 [r=0.31, 95% CI 0.11 – 0.49, p = 0.002, n=93, **Figure 6D**], CCL2 [r=0.25, 95% CI 0.05 – 0.44, p = 0.01, n=93, **Figure 6E**], CXCL10 [r=0.25, 95% CI 0.05 – 0.44, p = 0.01, n=94, **Figure 6F**], IL-1RA [r=0.43, 95% CI 0.25 – 0.59, p < 0.0001, n=94, **Figure 6G**] and IL-2R [r=0.27, 95% CI 0.07 – 0.46, p = 0.007, n=93, **Figure 6H**]. Similarly, the levels of HGF highly correlated with MT-CYTB in COVID-19 [r=0.47, 95% CI 0.29 –0.62, p < 0.0001, n=94, **Figure 6I**].

DISCUSSION

Here we observe that high levels of circulating MT-DNA are an early independent risk factor for severe illness and mortality in hospitalized patients with COVID-19, after adjusting for age, sex and comorbidities. Importantly, we made similar findings for two MT-DNA genes, MT-CYTB and MT-COX3, although the latter target did not reach significance for mortality risk. The reasons for this incongruence are not clear. One possibility is the accumulation of heteroplasmic mitochondrial genome deletions or point mutations that could prevent or attenuate PCR primer recognition (21).Heteroplasmic DNA deletions have been repeatedly demonstrated to accumulate with age within the MT-COX3 gene (22-24), and to a lesser degree, within the MT-CYTB locus, due to age-related defects of mitochondrial DNA polymerase proofreading capability (25, 26).

Although our studies were not specifically designed to identify the mechanisms that drive peripheral blood MT-DNA accumulation, significant correlations between LDH and IL-6 with MT-DNA levels point to a potential deleterious role for cellular necrosis in COVID-19 pathophysiology. LDH release and IL-6 production are reported indicators of cellular necrosis (27, 28). A specific form of necrosis, necroptosis, has been demonstrated to induce the release of damaged mitochondria (29). Necroptosis is characterized by the eventual loss of plasma membrane integrity due to the kinase activity of the receptor-interacting proteins (RIPK)1 and RIPK3 (30) and has been reported to act as a host defense mechanism when apoptotic death pathways are disabled by viral infection (31). For example, in a mouse model of influenza infection, necroptosis was shown to inhibit viral replication but with deleterious consequences to

bronchial epithelial integrity (32). In humans, both H5N1 and H1N1 influenza-induced acute respiratory distress syndrome have been shown to be associated with necrotic cell death within the distal pulmonary epithelia (33, 34). Intriguingly, the accessory protein open reading frame 3a (Orf3a) expressed within the highly related SARS-CoV-1 genome has been recently demonstrated to induce necroptosis through activating RIPK3 (35). SARS-CoV-2 also expresses an Orf3a accessory protein (36). However, whether SARS-CoV-2 Orf3a promotes necroptosis has yet to be determined. Some MT-DNA release may also result from innate immune cell activation. For example, human neutrophils following activation extrude their MT-DNA due to an inability to complete mitophagy (37). Additionally, neutrophil extracellular traps (NETs), which have been found in the peripheral circulation of COVID-19 patients (38), are rich in MT-DNA (39). Neutrophils have also been identified as undergoing metabolic reprogramming in the context of SARS-CoV-2 (40). In addition to neutrophils, activated platelets also release mitochondria (41), which in turn can induce NET generation, possibly contributing to pulmonary pro-thrombotic complications observed in patients with severe COVID-19 illness (42). Additionally, MT-DNA has been shown to directly promote NETosis (43, 44). The source and mechanisms of MT-DNA release in response to SARS-CoV-2 infection is a subject of future investigation.

Multiple clinically established biomarkers such as LDH, Ferritin, CRP and D-dimers are currently being evaluated to assess the risk of clinical deterioration from a COVID-19 diagnosis (40, 45-49). However, as products of gene expression in response to both acute and chronic stimuli they tend to be largely non-specific measures of systemic

inflammation with the exception of LDH, a marker of cell death. Nevertheless, LDH can also be released by cells undergoing apoptosis, a predominantly anti-inflammatory form of cell death (50). Ferritin is primarily produced by the liver, and serves as an acute phase reactant. Similarly, D-dimer is a non-specific test, and provides us minimal insight into the underlying pathophysiology of the disease or events occurring at the cellular level. In contrast, there are accumulating observations that high amounts of MT-DNA release are specifically generated by necrotic cells (51). Additionally, high MT-DNA levels have been shown to be associated with acute lung injury, in multiple, independent cohorts (12, 13, 52). In our cohort, although CYTB performed similar to ferritin/D-dimer for mortality, it performed better than these markers in the context of identifying those at risk for an ICU admission or intubation, which are important resource utilization metrics. Moreover, we observed that MT-DNA levels are approximately five-fold higher in COVID-19 patients who developed severe pulmonary dysfunction or eventually died suggesting it is at least as sensitive a biomarker as other clinically established and exploratory indicators used in prediction models. To conduct plasma MT-DNA measurements we employed a rapid PCR assay technique that takes about 60 minutes to complete due to the elimination of the DNA purification step. In resource-limited settings, this can be especially important given the current necessity to identify subjects at a higher risk of clinical deterioration. Additionally, PCR-based assays measuring MT-DNA tend to be less cost prohibitive and do not need specialized equipment, facilitating easy implementation. For the abovementioned reasons, we suggest that MT-DNA be investigated as an adjunct clinical biomarker in COVID-19, Nevertheless, whether MT-

DNA levels are equivalent or better measures than routinely obtained laboratory measurements will require additional validation with independent cohorts.

Based on our data from this study, it is not possible to clearly determine if circulating MT-DNA contributes to the pathogenesis of COVID-19 disease. Nevertheless, cell-free MT-DNA is itself a signatory marker for the release of other MT DAMPs, which collectively drive pro-inflammatory cytokine expression through the engagement of PRRs on innate immune cells (7). MT-DAMPs drive IL-6 expression by macrophages (29) and stimulate IL-8 release by neutrophils (9). Notably, IL-6 suppresses lymphopoiesis (53) while IL-8 promotes neutrophil release from the bone marrow (54) and therefore, could possibly explain our observed correlation between MT-DNA levels and elevations in the neutrophil to lymphocyte ratio. We additionally noted a significant correlation between membrane attack complex and MT-DNA levels. Extracellular mitochondria have been reported to activate complement. Mannose-binding lectin has been observed to bind to cell free mitochondria resulting in C3 consumption in the peripheral blood of mice (55). Therefore, it is interesting to note that C3 consumption is a general sign of C3 convertase activity, which would be a prerequisite for the downstream generation of membrane attack components (56). Along those lines, complement activation has been implicated in the pathogenesis of COVID-19-related end-organ damage, including acute lung injury (57, 58), with potential therapeutic implications (5, 59). Finally, MT-DAMPs, unlike other inflammatory markers linked to poor outcomes of COVID-19 afflicted patients, have been reported to directly cause acute pulmonary dysfunction and tissue damage (60). Administration of MT-DAMPs into

the blood stream or pulmonary airways of rodents promotes acute lung injury mediated by neutrophil chemotaxis and reactive oxygen species generation to mitochondrial formylated peptides (9, 14). In these studies, the formylated peptide receptor-1 inhibitor cyclosporine H was shown to inhibit MT-DAMP-mediated acute lung injury. Given ongoing clinical trials that target the effects of IL-6, complement activation and NETs in COVID-19 patients (3-5, 61, 62) future approaches that block necroptosis or MT-DAMP recognition could also be warranted.

Our study has several limitations. First, we were unable to concurrently enroll a COVID-19 negative group with severe respiratory disease. Our center, like many others, experienced a sharp decline in hospital presentations for illnesses other than COVID-19 making it difficult to collect samples for an adequately matched control group. Additionally, as our samples were drawn as a part of a prospective study of SARS-CoV-2 PCR positive patients, any other adequately matched control group would not be entirely comparable, as not drawn concurrently. However, it is unlikely that MT-DNA levels are considerably different between patients with COVID-19 and acute respiratory diseases arising from other etiologies. For example, reports have shown that increased MT-DNA levels in critically-ill patients are significantly associated with a higher risk of developing ARDS regardless of the underlying etiology (12, 13, 52). Nakahira et al also showed that high levels of plasma MT-DNA is a general marker of mortality risk in ICU patients (13). Additionally, more recent work from Huang et al. (52) shows that high plasma MT-DNA levels in ARDS patients are associated with increased risk for mortality regardless of admission diagnosis. Indeed, several emerging studies suggest that

biomarkers may be no different in subjects with COVID-19-induced lung injury versus those due to other etiologies (63). However, the strength of our study lies in an assay that can potentially identify subjects presenting with COVID-19 who are likely to develop adverse outcomes during the course of their hospitalization, which becomes increasingly important when resources are constrained as has been unfortunately too common during this pandemic (64-66). Second, this study does not assess how MT-DNA correlates with viral load in COVID-19, which is especially important in the context of deciding when to intervene with targeted therapeutics (67-69). Therefore, further studies will be necessary to evaluate whether it may be a predictor of response to treatment (70). Third, decision-making in the ICU changed over the course of our enrollment. For example, there was a tendency towards intubating earlier in the course of the critical illness in the initial months of the COVID-19 pandemic. We also lack data to support objective measurement of disease severity at the time of presentation as can be reflected in SOFA, APACHE or CURB65 scores. However, we identified MT-DNA levels as a predictor of severity utilizing mortality as a primary endpoint, and then requirement of ICU admission and the requirement for intubation as independent, secondary endpoints. Additionally, we identified MT-DNA levels as a predictor of disease severity utilizing requirement of vasopressors and renal replacement therapy while in the ICU as independent secondary endpoints of end-organ dysfunction.

It also remains possible that our findings may not generally apply to all patients who contract COVID-19. Members of underrepresented minority communities are at significantly more risk for COVID-19 infection as well as suffering its most severe outcomes. Our analysis was conducted in an urban medical center that serves a large

African American community who may have a tendency to inherit MT-DNA variations that differ from other groups. This maybe most exemplified in a recent report that shows certain point mutations within the MT-DNA of aging African Americans is associated with decreased pulmonary maximal inspiratory pressures (MIP) (71). However, this study also found other point MT DNA mutations in African Americans were associated with increased MIP suggesting a more complex picture for MT-DNA variation and its link to respiratory function. Nevertheless, this MT-DNA variation was not within the primer target sequences used in our assays. Finally, prior studies that analyzed other diseases that involve respiratory distress, such as ARDS and primary lung allograft dysfunction on populations predominantly composed of Caucasians, and to lesser extent, underrepresented minority groups, demonstrated significant associations with cell free circulating MT DNA (12-14) Taken together with our findings, these reports support a robust association with MT DNA levels and poor outcomes from COVID-19 infection that is likely applicable to all racial groups.

In summary, we demonstrate that MT-DNA measured early in the disease course can predict survival status, requirement for intensive level care, the need for endotracheal intubation, as well as end-organ dysfunction requiring vasopressors and renal replacement therapy. We also show that MT-DNA levels are associated with exploratory biomarkers implicated in the pathogenesis of COVID-19-related morbidity and mortality. Further studies will be needed to discern the contribution of MT DAMPs such as MT-DNA to COVID-19 pathogenesis, as well as to understand whether MT-DNA and other inflammatory mediators, such as activated complement, work synergistically to promote cellular injury.

METHODS

Study Design, Settings and Participants

This prospective cohort study utilized cell-free plasma samples that had been prospectively collected from 97 adult patients with confirmed COVID-19 presenting to the Barnes-Jewish Hospital from March 15, 2020, to April 24, 2020. Eligible participants included consented adults, greater than 18yo, presenting with COVID-19 symptoms who received a COVID-19 test at the time of hospital presentation and resulted in a COVID-19 diagnosis. Diagnosis of COVID-19 was based on a positive nasopharyngeal swab test. Excluded participants were COVID-19 diagnosed patients who did not have plasma sample collected within 24hrs of time of hospital presentation.

Outcome Definition

Subjects were followed through June 29, 2020. The primary outcome was mortality. The secondary outcomes included (a) the need for an ICU admission, (b) endotracheal intubation, as well as the development of end-organ dysfunction while in the ICU requiring (c) vasopressors and (d) renal replacement therapy. Subjects that were on dialysis prior to admission were excluded from the RRT outcome analysis. These outcomes were abstracted utilizing an honest broker system from electronic medical records.

Sample Collection and Processing

MT-DNA and other cytokines were measured in cell-free plasma of subjects within the first 24 hours of emergency department presentation. Blood samples were collected in

EDTA-containing vacutainers (BD Biosciences, San Jose, CA) and subjected to 2 rounds of centrifugation to generate platelet poor plasma. First at 2,500 x g for 20 mins to generate plasma. The plasma was removed from vacutainers and centrifuged at 13,000 x g in sterile nuclease-free eppendorf tubes (ThermoFisher Scientific, Waltham, MA) for 10 min to remove platelets. These platelet poor specimens were then immediately stored at −80°C until further analysis. Concurrently measured clinical markers (I.e., C-reactive protein, ferritin, lactate dehydrogenase, D-dimer) were obtained from the electronic medical record.

MT-DNA quantification

MT-DNA quantification Real-Time PCR was performed in a BioRad CFX-Connect machine using reaction mixture containing 0.1 µL of cell-free plasma, 10 µL iQ SYBR Green Supermix (Bio-Rad), 0.5 µL of 5µM forward and reverse primers and 8.9 µL H2O. Assays were performed in triplicate under the following conditions: 1 cycle at 95°C for 3 min, then up to 40 cycles at 95°C for 10 sec and 55°C for 30 sec and then a melt curve was performed from 65°C to 95 °C (0.5 °C every 5 sec). Primers for Human cytochrome B (CYB; forward 5′-ATGACCCCAATACGCAAAAT-3′ and reverse 5′-CGAAGTTTCATCATGCGGAG-3′), Human cytochrome C oxidase subunit III (COX3: forward 5′-ATGACCCACCAATCACATGC-3′ and reverse 5′-ATCACATGGCTAGGCCGGAG-3′) were synthesized by Integrated DNA technologies (IDT, Coralville, IA). Copy number was estimated by comparison to a real-time PCR standard amplification curve. To generate the standard curve, selected regions of human purified MT-DNA containing the target sequences for MT-CYTB

(forward 5'-ATGACCCCAATACGCAAAAT-3' and reverse 5'-GACGGATCGGAGAATTGTGT 3') and MT-COX3 (forward 5'-ATGACCCACCAATCACATGC-3' and reverse 5'-ATCAATAGATGGAGACATAC-3') were amplified by PCR under the following conditions: initial denaturation at 94°C for 2 min, then up to 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72°C for 1 minute. After amplification, MT-DNA was gel purified, extracted, and quantified by nano-drop. Serial dilutions were then used to calibrate the real-time PCR standard curves. Final results were expressed as copy number of MT-DNA, and their absolute values converted to $\log_{10}$ before statistical analysis.

Quantification of cytokines and complement activation

Cell-free plasma was analyzed using a Cytokine 35-Plex Human Panel, which provide simultaneous measurement of 35 cytokines (ThermoFisher Scientific, Waltham, MA). The assay was performed in accordance with manufacturer's instructions with each subject sample performed in duplicate and then analyzed on a Luminex FLEXMAP 3D instrument. Internal validation control with every plate also was run in duplicate. Complement activation was assessed in cell-free plasma specimens (not previously thawed) using the soluble C5b-9 (sC5b-9) assay (BD OptEIA Human C5b-9 ELISA set, Franklin Lakes, NJ, USA).

Statistical Analysis

Continuous variables were reported as median (interquartile range). The predictive value of each biomarker was expressed as area under the curve (AUC) derived from

the relative ROC curve. Spearman r coefficient was calculated to estimate the correlation between two continuous variables. Univariate logistic regression analysis was used to calculate the unadjusted odds ratios. Independent variables known to have a biological relationship with the outcomes were selected a priori based on existing literature for the multivariate logistic model to calculate the adjusted odds ratios (72-74). Model diagnostics were performed with Corrected Akaike Information Criterion (AICc), log-likelihood, and model deviance. The MT-DNA was log (base 10) transformed prior to data analysis to better meet model assumptions. To facilitate interpretation for this variable, the odds ratio estimates were raised to the 0.5 power which is interpreted as the increase in the odds of the outcome for each 0.5 change in MT-DNA on the log scale. All the statistical analyses were calculated, and all figures were prepared using Graphpad Prism 8.4.2 (GraphPad Software Inc., La Jolla, CA). For all the analyses, a p value <0.05 was considered significant.

Study Approval

The study was approved by the Washington University School of Medicine Institutional Review Board (ID#202004091 and #202003085). Written informed consent was obtained from all subjects.

AUTHOR CONTRIBUTIONS

Conceptualization: H.S.K., A.E.G., M.I., Methodology: D.S., M.C., C.G., H.S.K., A.E.G., Software: D.S., M.C., H.S.K., Validation: J.O., A.A.R., C.G., Formal Analysis: D.S., M.C., V.P., M.R., A.R., R.A., L.A., H.S.K., A.E.G., Investigation: L.M., D.Z., J.Z., Data Curation: L.M., D.S., M.C., H.S.K., A.E.G., Writing – Original Draft: D.S., M.C., H.S.K., A.E.G., Writing – Review & Editing: D.S., M.C., L.M., J.H., C.G., A.R., Z.L., R.H., M.I., D.K. H.S.K., A.E.G., Visualization: D.S., M.C., Supervision: H.S.K., A.E.G. , Project Administration: H.S.K., A.E.G., Funding Acquisition: H.S.K., R.R.H., D.K., A.E.G.

[12]Co-first authors D.S. and M.C. equally contributed to methodology, data and software analysis. They also helped write, reviewed and edited the original draft. The decision to order them as listed was due to D.S.'s novel contribution to the PCR method used in this study.

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REFERENCES

1. Lipman M, Chambers RC, Singer M, and Brown JS. SARS-CoV-2 pandemic: clinical picture of COVID-19 and implications for research. *Thorax.* 2020;75(8):614-6.

2. Vabret N, Britton GJ, Gruber C, Hegde S, Kim J, Kuksin M, Levantovsky R, Malle L, Moreira A, Park MD, et al. Immunology of COVID-19: Current State of the Science. *Immunity.* 2020;52(6):910-41.

3. Maes B, Bosteels C, De Leeuw E, Declercq J, Van Damme K, Delporte A, Demeyere B, Vermeersch S, Vuylsteke M, Willaert J, et al. Treatment of severely ill COVID-19 patients with anti-interleukin drugs (COV-AID): A structured summary of a study protocol for a randomised controlled trial. *Trials.* 2020;21(1):468.

4. Rilinger J, Kern WV, Duerschmied D, Supady A, Bode C, Staudacher DL, and Wengenmayer T. A prospective, randomised, double blind placebo-controlled trial to evaluate the efficacy and safety of tocilizumab in patients with severe COVID-19 pneumonia (TOC-COVID): A structured summary of a study protocol for a randomised controlled trial. *Trials.* 2020;21(1):470.

5. Smith K, Pace A, Ortiz S, Kazani S, and Rottinghaus S. A Phase 3 Open-label, Randomized, Controlled Study to Evaluate the Efficacy and Safety of Intravenously Administered Ravulizumab Compared with Best Supportive Care in Patients with COVID-19 Severe Pneumonia, Acute Lung Injury, or Acute Respiratory Distress Syndrome: A structured summary of a study protocol for a randomised controlled trial. *Trials.* 2020;21(1):639.

6. Nailwal H, and Chan FK. Necroptosis in anti-viral inflammation. *Cell Death Differ*. 2019;26(1):4-13.

7. Kulkarni HS, Scozzi D, and Gelman AE. Recent advances into the role of pattern recognition receptors in transplantation. *Cellular immunology.* 2020;351(104088.

8. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, and Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature.* 2010;464(7285):104-7.

9. Hauser CJ, Sursal T, Rodriguez EK, Appleton PT, Zhang Q, and Itagaki K. Mitochondrial damage associated molecular patterns from femoral reamings activate neutrophils through formyl peptide receptors and P44/42 MAP kinase. *J Orthop Trauma.* 2010;24(9):534-8.

10. Simmons JD, Lee YL, Mulekar S, Kuck JL, Brevard SB, Gonzalez RP, Gillespie MN, and Richards WO. Elevated levels of plasma mitochondrial DNA DAMPs are linked to clinical outcome in severely injured human subjects. *Ann Surg.* 2013;258(4):591-6; discussion 6-8.

11. Puskarich MA, Shapiro NI, Trzeciak S, Kline JA, and Jones AE. Plasma levels of mitochondrial DNA in patients presenting to the emergency department with sepsis. *Shock.* 2012;38(4):337-40.

12. Faust HE, Reilly JP, Anderson BJ, Ittner CAG, Forker CM, Zhang P, Weaver BA, Holena DN, Lanken PN, Christie JD, et al. Plasma Mitochondrial DNA Levels Are Associated With ARDS in Trauma and Sepsis Patients. *Chest.* 2020;157(1):67-76.

13. Nakahira K, Kyung SY, Rogers AJ, Gazourian L, Youn S, Massaro AF, Quintana C, Osorio JC, Wang Z, Zhao Y, et al. Circulating mitochondrial DNA in patients in the ICU as a marker of mortality: derivation and validation. *PLoS Med.* 2013;10(12):e1001577; discussion e.

14. Scozzi D, Ibrahim M, Liao F, Lin X, Hsiao HM, Hachem R, Tague LK, Ricci A, Kulkarni HS, Huang HJ, et al. Mitochondrial damage-associated molecular patterns released by lung transplants are associated with primary graft dysfunction. *Am J Transplant.* 2019;19(5):1464-77.

15. Grazioli S, and Pugin J. Mitochondrial Damage-Associated Molecular Patterns: From Inflammatory Signaling to Human Diseases. *Front Immunol.* 2018;9(832.

16. Wu G, Zhu Q, Zeng J, Gu X, Miao Y, Xu W, Lv T, and Song Y. Extracellular mitochondrial DNA promote NLRP3 inflammasome activation and induce acute lung injury through TLR9 and NF-κB. *J Thorac Dis.* 2019;11(11):4816-28.

17. Zhang JZ, Liu Z, Liu J, Ren JX, and Sun TS. Mitochondrial DNA induces inflammation and increases TLR9/NF-κB expression in lung tissue. *Int J Mol Med.* 2014;33(4):817-24.

18. Dorward DA, Lucas CD, Doherty MK, Chapman GB, Scholefield EJ, Conway Morris A, Felton JM, Kipari T, Humphries DC, Robb CT, et al. Novel role for endogenous mitochondrial formylated peptide-driven formyl peptide receptor 1 signalling in acute respiratory distress syndrome. *Thorax.* 2017;72(10):928-36.

19. Nakayama H, and Otsu K. Mitochondrial DNA as an inflammatory mediator in cardiovascular diseases. *Biochem J.* 2018;475(5):839-52.

20. Mangalmurti N, and Hunter CA. Cytokine Storms: Understanding COVID-19. *Immunity.* 2020;53(1):19-25.

21. Wallace DC, and Chalkia D. Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. *Cold Spring Harb Perspect Biol.* 2013;5(11):a021220.

22. Müller-Höcker J. Cytochrome-c-oxidase deficient cardiomyocytes in the human heart--an age-related phenomenon. A histochemical ultracytochemical study. *The American journal of pathology.* 1989;134(5):1167-73.

23. Kraytsberg Y, Kudryavtseva E, McKee AC, Geula C, Kowall NW, and Khrapko K. Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. *Nat Genet.* 2006;38(5):518-20.

24. He L, Chinnery PF, Durham SE, Blakely EL, Wardell TM, Borthwick GM, Taylor RW, and Turnbull DM. Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. *Nucleic Acids Research.* 2002;30(14):e68-e.

25. Lujan SA, Longley MJ, Humble MH, Lavender CA, Burkholder A, Blakely EL, Alston CL, Gorman GS, Turnbull DM, McFarland R, et al. Ultrasensitive deletion detection links mitochondrial DNA replication, disease, and aging. *Genome Biology.* 2020;21(1):248.

26. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly-Y M, Gidlöf S, Oldfors A, Wibom R, et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature.* 2004;429(6990):417-23.

27. Vanden Berghe T, Kalai M, Denecker G, Meeus A, Saelens X, and Vandenabeele P. Necrosis is associated with IL-6 production but apoptosis is not. *Cell Signal.* 2006;18(3):328-35.

28. Chan FK, Moriwaki K, and De Rosa MJ. Detection of necrosis by release of lactate dehydrogenase activity. *Methods Mol Biol.* 2013;979(65-70.

29. Maeda A, and Fadeel B. Mitochondria released by cells undergoing TNF-α-induced necroptosis act as danger signals. *Cell Death Dis.* 2014;5(7):e1312.

30. He S, Wang L, Miao L, Wang T, Du F, Zhao L, and Wang X. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell.* 2009;137(6):1100-11.

31. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, and Chan FK. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell.* 2009;137(6):1112-23.

32. Rodrigue-Gervais IG, Labbé K, Dagenais M, Dupaul-Chicoine J, Champagne C, Morizot A, Skeldon A, Brincks EL, Vidal SM, Griffith TS, et al. Cellular inhibitor of apoptosis protein cIAP2 protects against pulmonary tissue necrosis during influenza virus infection to promote host survival. *Cell Host Microbe.* 2014;15(1):23-35.

33. Gu J, and Korteweg C. Pathology and pathogenesis of severe acute respiratory syndrome. *Am J Pathol.* 2007;170(4):1136-47.

34. Mauad T, Hajjar LA, Callegari GD, da Silva LF, Schout D, Galas FR, Alves VA, Malheiros DM, Auler JO, Jr., Ferreira AF, et al. Lung pathology in fatal novel human influenza A (H1N1) infection. *Am J Respir Crit Care Med.* 2010;181(1):72-9.

35. Yue Y, Nabar NR, Shi CS, Kamenyeva O, Xiao X, Hwang IY, Wang M, and Kehrl JH. SARS-Coronavirus Open Reading Frame-3a drives multimodal necrotic cell death. *Cell Death Dis.* 2018;9(9):904.

36. Bojkova D, Klann K, Koch B, Widera M, Krause D, Ciesek S, Cinatl J, and Münch C. Proteomics of SARS-CoV-2-infected host cells reveals therapy targets. *Nature.* 2020;583(7816):469-72.

37. Caielli S, Athale S, Domic B, Murat E, Chandra M, Banchereau R, Baisch J, Phelps K, Clayton S, Gong M, et al. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. *J Exp Med.* 2016;213(5):697-713.

38. Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, Blair C, Weber A, Barnes BJ, Egeblad M, et al. Neutrophil extracellular traps in COVID-19. *JCI Insight.* 2020;5(11).

39. Yousefi S, Mihalache C, Kozlowski E, Schmid I, and Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* 2009;16(11):1438-44.

40. McElvaney OJ, McEvoy N, McElvaney OF, Carroll TP, Murphy MP, Dunlea DM, O NC, Clarke J, O'Connor E, Hogan G, et al. Characterization of the Inflammatory Response to Severe COVID-19 Illness. *Am J Respir Crit Care Med.* 2020.

41. Boudreau LH, Duchez AC, Cloutier N, Soulet D, Martin N, Bollinger J, Paré A, Rousseau M, Naika GS, Lévesque T, et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood.* 2014;124(14):2173-83.

42. Middleton EA, He XY, Denorme F, Campbell RA, Ng D, Salvatore SP, Mostyka M, Baxter-Stoltzfus A, Borczuk AC, Loda M, et al. Neutrophil Extracellular Traps (NETs) Contribute to Immunothrombosis in COVID-19 Acute Respiratory Distress Syndrome. *Blood.* 2020.

43. Itagaki K, Kaczmarek E, Lee YT, Tang IT, Isal B, Adibnia Y, Sandler N, Grimm MJ, Segal BH, Otterbein LE, et al. Mitochondrial DNA released by trauma induces neutrophil extracellular traps. *PLoS One.* 2015;10(3):e0120549.

44. Mallavia B, Liu F, Lefrançais E, Cleary SJ, Kwaan N, Tian JJ, Magnen M, Sayah DM, Soong A, Chen J, et al. Mitochondrial DNA Stimulates TLR9-Dependent Neutrophil Extracellular Trap Formation in Primary Graft Dysfunction. *Am J Respir Cell Mol Biol.* 2020;62(3):364-72.

45. Messner CB, Demichev V, Wendisch D, Michalick L, White M, Freiwald A, Textoris-Taube K, Vernardis SI, Egger AS, Kreidl M, et al. Ultra-High-Throughput Clinical Proteomics Reveals Classifiers of COVID-19 Infection. *Cell Syst.* 2020;11(1):11-24.e4.

46. Song JW, Lam SM, Fan X, Cao WJ, Wang SY, Tian H, Chua GH, Zhang C, Meng FP, Xu Z, et al. Omics-Driven Systems Interrogation of Metabolic Dysregulation in COVID-19 Pathogenesis. *Cell Metab.* 2020.

47. Luo M, Liu J, Jiang W, Yue S, Liu H, and Wei S. IL-6 and CD8+ T cell counts combined are an early predictor of in-hospital mortality of patients with COVID-19. *JCI Insight.* 2020;5(13).

48. Cummings MJ, Baldwin MR, Abrams D, Jacobson SD, Meyer BJ, Balough EM, Aaron JG, Claassen J, Rabbani LE, Hastie J, et al. Epidemiology, clinical course, and outcomes of critically ill adults with COVID-19 in New York City: a prospective cohort study. *Lancet.* 2020;395(10239):1763-70.

49. Wu Y, Huang X, Sun J, Xie T, Lei Y, Muhammad J, Li X, Zeng X, Zhou F, Qin H, et al. Clinical Characteristics and Immune Injury Mechanisms in 71 Patients with COVID-19. *mSphere.* 2020;5(4).

50. Kumar P, Nagarajan A, and Uchil PD. Analysis of Cell Viability by the Lactate Dehydrogenase Assay. *Cold Spring Harb Protoc.* 2018;2018(6).

51. Vringer E, and Tait SWG. Mitochondria and Inflammation: Cell Death Heats Up. *Front Cell Dev Biol.* 2019;7(100.

52. Huang L, Chang W, Huang Y, Xu X, Yang Y, and Qiu H. Prognostic value of plasma mitochondrial DNA in acute respiratory distress syndrome (ARDS): a single-center observational study. *J Thorac Dis.* 2020;12(4):1320-8.

53. Maeda K, Baba Y, Nagai Y, Miyazaki K, Malykhin A, Nakamura K, Kincade PW, Sakaguchi N, and Coggeshall KM. IL-6 blocks a discrete early step in lymphopoiesis. *Blood.* 2005;106(3):879-85.

54. Terashima T, English D, Hogg JC, and van Eeden SF. Release of polymorphonuclear leukocytes from the bone marrow by interleukin-8. *Blood.* 1998;92(3):1062-9.

55. Brinkmann CR, Jensen L, Dagnæs-Hansen F, Holm IE, Endo Y, Fujita T, Thiel S, Jensenius JC, and Degn SE. Mitochondria and the lectin pathway of complement. *J Biol Chem.* 2013;288(12):8016-27.

56. Leslie RG, and Nielsen CH. The classical and alternative pathways of complement activation play distinct roles in spontaneous C3 fragment deposition and membrane attack complex (MAC) formation on human B lymphocytes. *Immunology.* 2004;111(1):86-90.

57. Java A, Apicelli AJ, Liszewski MK, Coler-Reilly A, Atkinson JP, Kim AH, and Kulkarni HS. The complement system in COVID-19: friend and foe? *JCI Insight.* 2020.

58. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, Baxter-Stoltzfus A, and Laurence J. Complement associated microvascular injury and thrombosis in the

pathogenesis of severe COVID-19 infection: A report of five cases. *Transl Res.* 2020;220(1-13.

59. Kulasekararaj AG, Lazana I, Large J, Posadas K, Eagleton H, Lord Villajin J, Zuckerman M, Gandhi S, and Marsh JCW. Terminal complement inhibition dampens the inflammation during COVID-19. *Br J Haematol.* 2020.

60. Lee YL, Obiako B, Gorodnya OM, Ruchko MV, Kuck JL, Pastukh VM, Wilson GL, Simmons JD, and Gillespie MN. Mitochondrial DNA Damage Initiates Acute Lung Injury and Multi-Organ System Failure Evoked in Rats by Intra-Tracheal Pseudomonas Aeruginosa. *Shock.* 2017;48(1):54-60.

61. Kulkarni S, Fisk M, Kostapanos M, Banham-Hall E, Bond S, Hernan-Sancho E, Norton S, Cheriyan J, Cope A, Galloway J, et al. Repurposed immunomodulatory drugs for Covid-19 in pre-ICu patients - mulTi-Arm Therapeutic study in pre-ICu patients admitted with Covid-19 - Repurposed Drugs (TACTIC-R): A structured summary of a study protocol for a randomised controlled trial. *Trials.* 2020;21(1):626.

62. Desilles JP, Gregoire C, Le Cossec C, Lambert J, Mophawe O, Losser MR, Lambiotte F, Le Tacon S, Cantier M, Engrand N, et al. Efficacy and safety of aerosolized intra-tracheal dornase alfa administration in patients with SARS-CoV-2-induced acute respiratory distress syndrome (ARDS): a structured summary of a study protocol for a randomised controlled trial. *Trials.* 2020;21(1):548.

63. Sinha P, Matthay MA, and Calfee CS. Is a "Cytokine Storm" Relevant to COVID-19? *JAMA Intern Med.* 2020.

64. Barrett K, Khan YA, Mac S, Ximenes R, Naimark DMJ, and Sander B. Estimation of COVID-19-induced depletion of hospital resources in Ontario, Canada. *Cmaj.* 2020;192(24):E640-e6.

65. Litton E, Bucci T, Chavan S, Ho YY, Holley A, Howard G, Huckson S, Kwong P, Millar J, Nguyen N, et al. Surge capacity of intensive care units in case of acute increase in demand caused by COVID-19 in Australia. *Med J Aust.* 2020;212(10):463-7.

66. Moghadas SM, Shoukat A, Fitzpatrick MC, Wells CR, Sah P, Pandey A, Sachs JD, Wang Z, Meyers LA, Singer BH, et al. Projecting hospital utilization during the COVID-19 outbreaks in the United States. *Proc Natl Acad Sci U S A.* 2020;117(16):9122-6.

67. Catanzaro M, Fagiani F, Racchi M, Corsini E, Govoni S, and Lanni C. Immune response in COVID-19: addressing a pharmacological challenge by targeting pathways triggered by SARS-CoV-2. *Signal Transduct Target Ther.* 2020;5(1):84.

68. Du SQ, and Yuan W. Mathematical modeling of interaction between innate and adaptive immune responses in COVID-19 and implications for viral pathogenesis. *J Med Virol.* 2020.

69. Maggi E, Canonica GW, and Moretta L. COVID-19: Unanswered questions on immune response and pathogenesis. *J Allergy Clin Immunol.* 2020;146(1):18-22.

70. Sinha P, Delucchi KL, McAuley DF, O'Kane CM, Matthay MA, and Calfee CS. Development and validation of parsimonious algorithms to classify acute respiratory distress syndrome phenotypes: a secondary analysis of randomised controlled trials. *Lancet Respir Med.* 2020;8(3):247-57.

71. Vaz Fragoso CA, Manini TM, Kairalla JA, Buford TW, Hsu FC, Gill TM, Kritchevsky SB, McDermott MM, Sanders JL, Cummings SR, et al. Mitochondrial DNA variants and pulmonary function in older persons. *Exp Gerontol.* 2019;115(96-103.

72. Lederer DJ, Bell SC, Branson RD, Chalmers JD, Marshall R, Maslove DM, Ost DE, Punjabi NM, Schatz M, Smyth AR, et al. Control of Confounding and Reporting of Results in Causal Inference Studies. Guidance for Authors from Editors of Respiratory, Sleep, and Critical Care Journals. *Ann Am Thorac Soc.* 2019;16(1):22-8.

73. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, Tobin KA, Cerfolio RJ, Francois F, and Horwitz LI. *BMJ (Clinical research ed).* 2020:m1966.

74. Williamson EJ, Walker AJ, Bhaskaran K, Bacon S, Bates C, Morton CE, Curtis HJ, Mehrkar A, Evans D, Inglesby P, et al. OpenSAFELY: factors associated with COVID-19 death in 17 million patients. *Nature.* 2020.

FIGURES AND FIGURE LEGENDS

Figure 1.

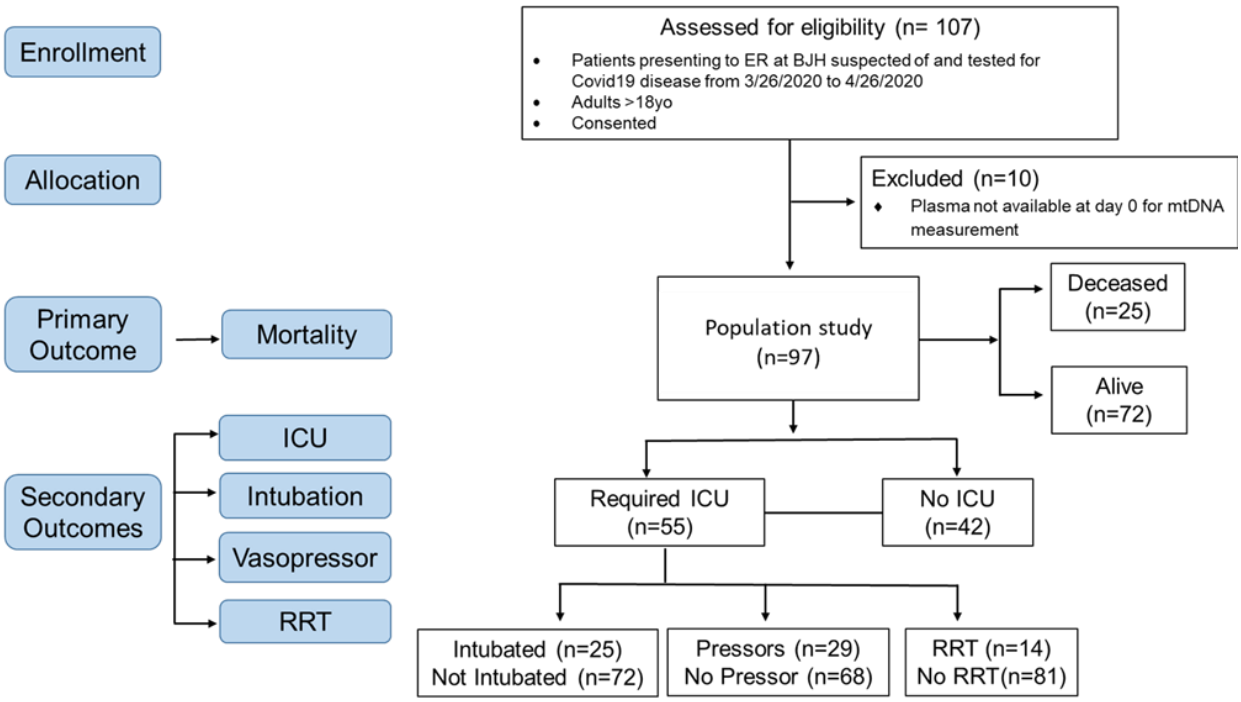


Figure 1. Consort flow diagram showing enrollment of patients, allocation, and outcomes

Figure 2.

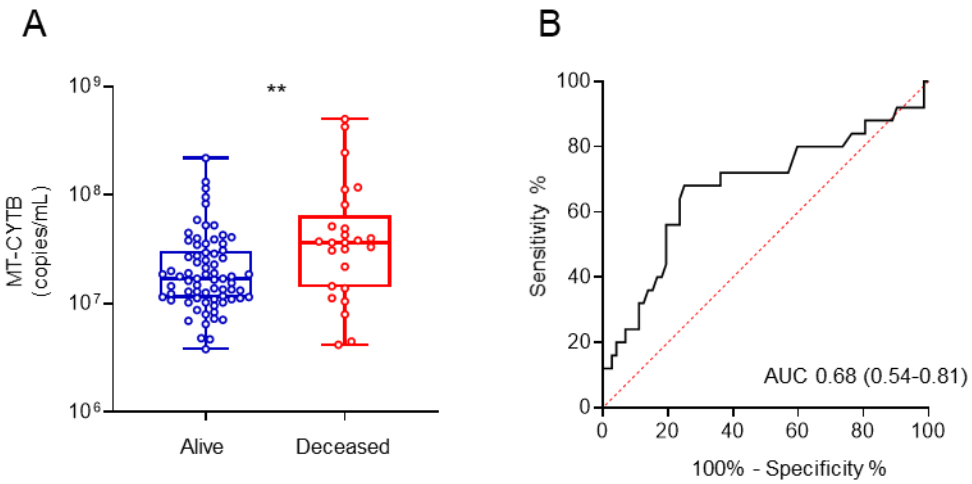


Figure 2. High Circulating MT-DNA levels predict a higher risk of mortality in COVID-19 patients. Plasma for determination of circulating levels of MT-CYTB was obtained at time of hospital presentation (A) Box and whiskers plots of MT-CYB levels in relation to mortality status in COVID-19 patients. (B) Receiver operating characteristic (ROC) curves in predicting the outcome mortality based on MT-CYTB levels. Statistical significance was determined using Mann-Whitney U Test (**P < .01).

Figure 3.

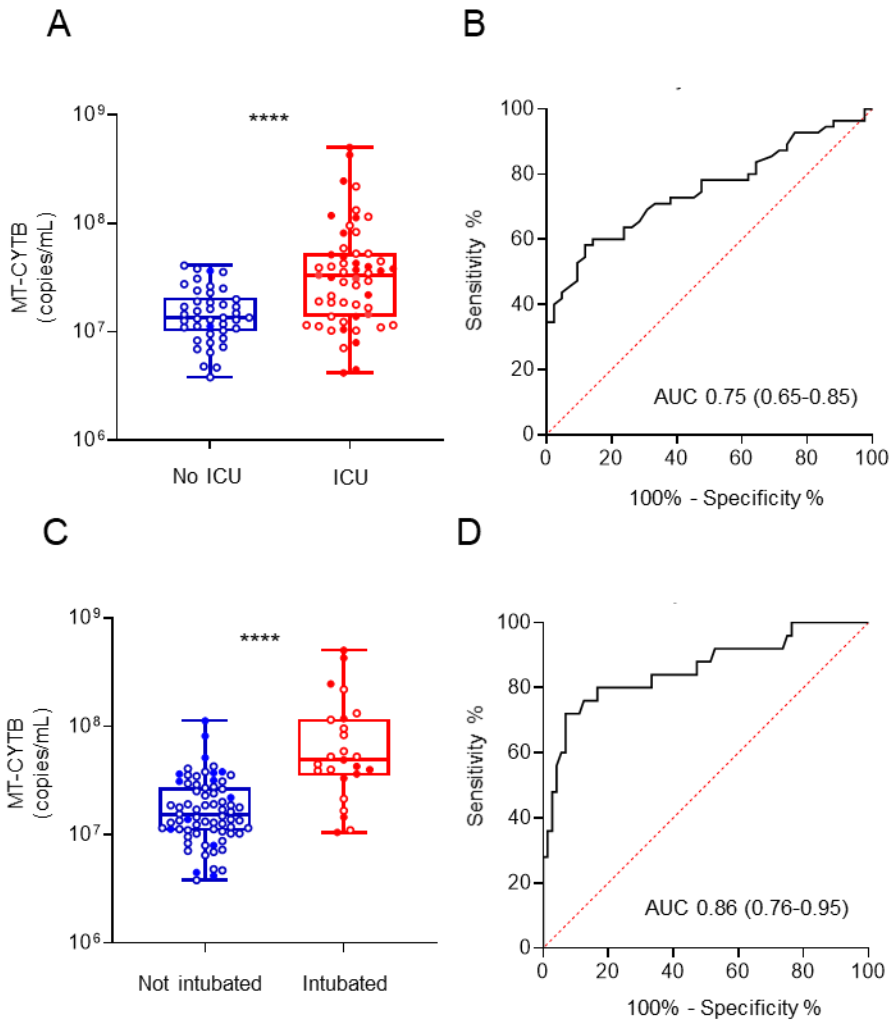


Figure 3. High circulating MT-DNA levels predict a higher risk of ICU requirement and intubation in COVID-19 patients. Plasma for determination of circulating levels of MT-CYTB was obtained at time of hospital presentation. Box and whiskers plots of MT-CYB levels in relation to (A) ICU admission and (C) intubation in COVID-19 patients.
Empty dots indicate alive patients and shaded dots indicate deceased patients.
Receiver operating characteristic (ROC) curves in predicting the outcome (B) ICU and (D) intubation based on MT-CYTB levels.
Statistical significance was determined using Mann-Whitney U Test (****P < .0001).

Figure 4.

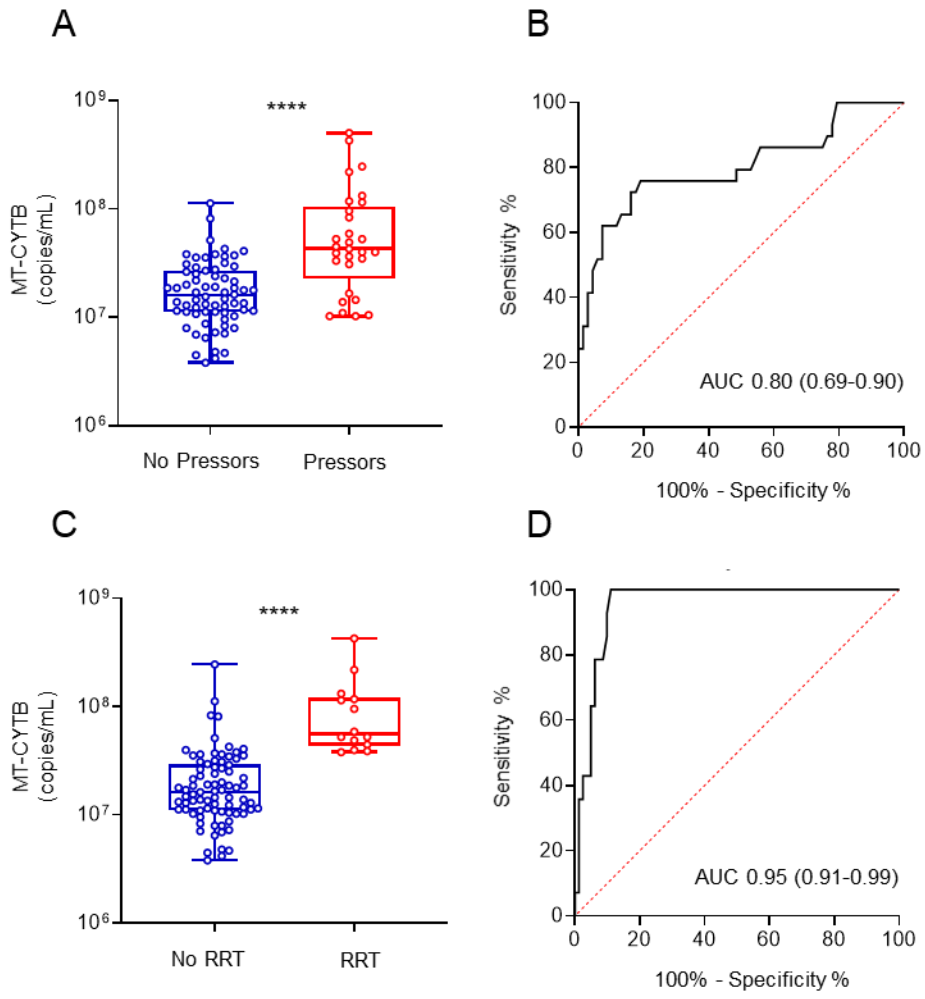
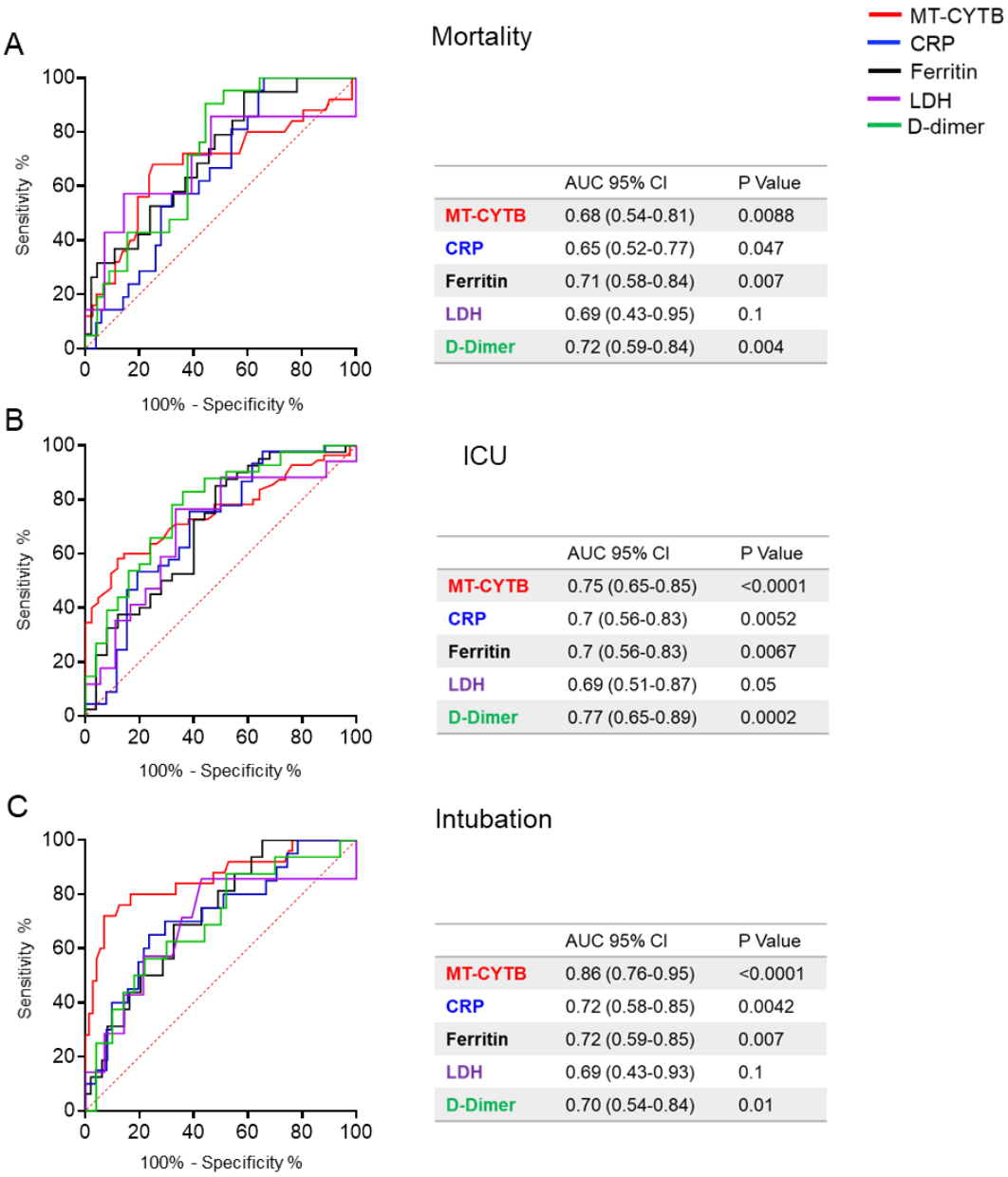


Figure 4. High circulating MT-DNA levels predict a higher risk for end-organ dysfunction requiring vasopressors and renal replacement in COVID-19 patients. Plasma for determination of circulating levels of MT-CYTB was obtained at time of hospital presentation. Box and whiskers plots of MT-CYB levels in relation to (A) requirement for pressors and (C) renal replacement in COVID-19 patients. Receiver operating characteristic (ROC) curves in predicting the outcome (B) requirement for pressors and (D) renal replacement based on MT-CYTB levels. Statistical significance was determined using Mann-Whitney U Test (****P < .0001).

Figure 5.



	AUC 95% CI	P Value
MT-CYTB	0.68 (0.54-0.81)	0.0088
CRP	0.65 (0.52-0.77)	0.047
Ferritin	0.71 (0.58-0.84)	0.007
LDH	0.69 (0.43-0.95)	0.1
D-Dimer	0.72 (0.59-0.84)	0.004

	AUC 95% CI	P Value
MT-CYTB	0.75 (0.65-0.85)	<0.0001
CRP	0.7 (0.56-0.83)	0.0052
Ferritin	0.7 (0.56-0.83)	0.0067
LDH	0.69 (0.51-0.87)	0.05
D-Dimer	0.77 (0.65-0.89)	0.0002

	AUC 95% CI	P Value
MT-CYTB	0.86 (0.76-0.95)	<0.0001
CRP	0.72 (0.58-0.85)	0.0042
Ferritin	0.72 (0.59-0.85)	0.007
LDH	0.69 (0.43-0.93)	0.1
D-Dimer	0.70 (0.54-0.84)	0.01

Figure 5. Circulating MT-DNA levels have a similar or improved accuracy over clinically utilized biomarkers for outcomes of severity in COVID-19.
Blood samples for determination of biomarkers levels were collected within 24 hours from hospital presentation. Receiver operating characteristic (ROC) curves in predicting the outcome (A) mortality, (B) admission to ICU and (C) Intubation based on MT-CYTB (red), reactive C protein (CRP) (blue), Ferritin (black), Lactic acid dehydrogenase (LDH) (purple) and D-Dimer (green) levels. Area under the curve (AUC) with 95% CI and P values for the different biomarkers are summarized in the corresponding tables.

Figure 6.

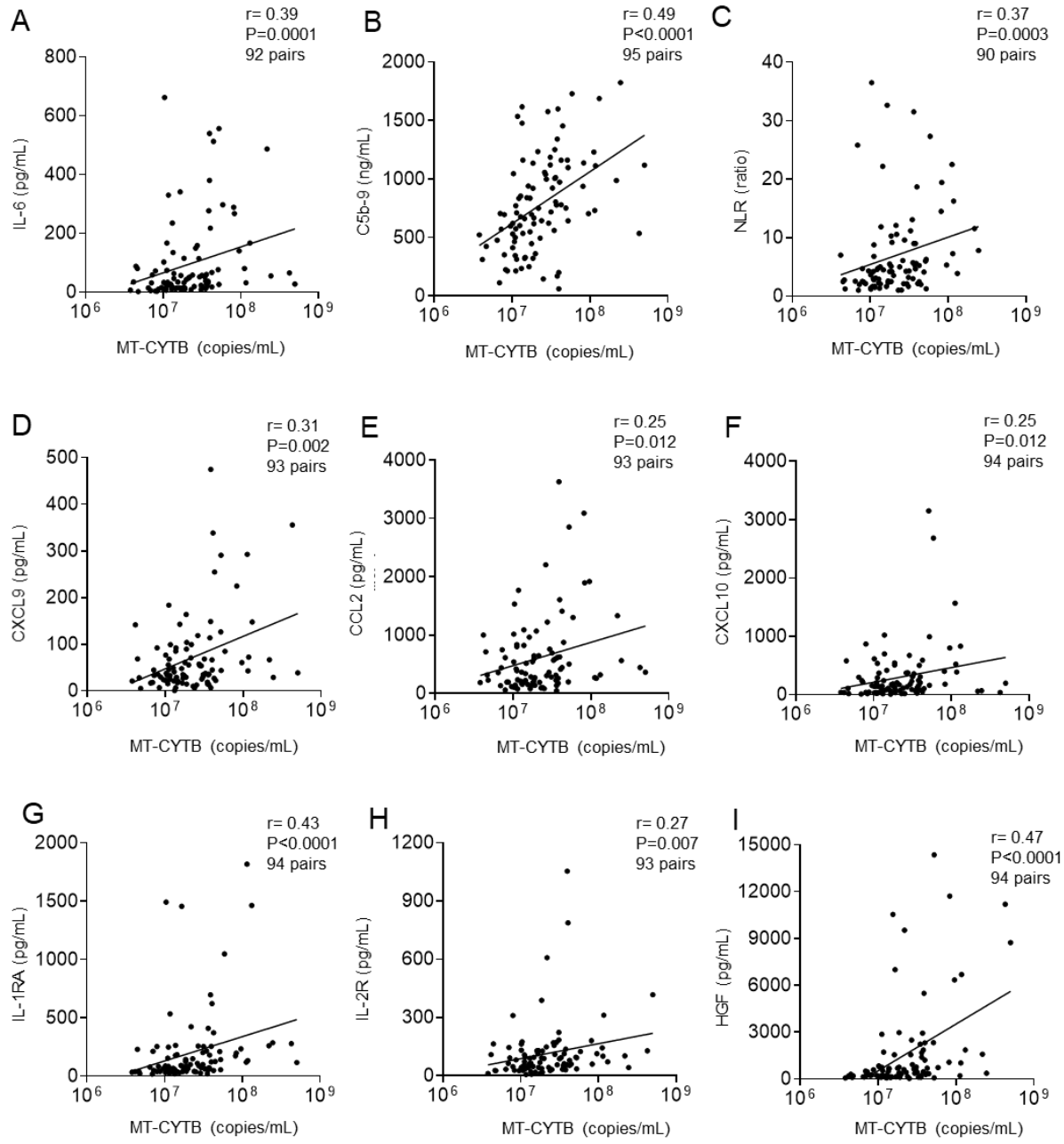


Figure 6. Circulating MT-DNA levels correlate with other emerging markers of inflammation and cytokines in COVID-19 patients. Scatter plots showing the correlation between MT-CYTB and (A) IL-6, (B) C5b-9 (terminal complement complex and (C) Neutrophil to Lymphocyte ratio (NLR), (D) CXCL9, (E) CCL2, (F) CXCL10, (G) IL-1RA, (H) IL-2R, (I) HGF.
The degree of correlation was assessed using Spearman`s Rank Correlation Coefficient test.

TABLES

Table 1. Demographic and Clinical characteristics associated with mortality

	Total (n=97)	Alive (n=72)	Deceased (n=25)	OR for mortality (95% CI)	P value
Demographics					
Age, yr[a]	65 (54-73)	61.1 (50.3-70.5)	76.2 (65.3-84.9)	1.08 (1.04-1.13)	0.0003
Male n (%)[b]	54 (55.6)	39 (54.17)	15 (60)	1.26 (0.5-3.27)	0.6
African-American[b]	75 (77.3)	57 (79.2)	18 (72)	0.6 (0.24-2)	0.4
BMI[a]	29.1 (24.7-34.4)	28.9 (24.7-33.3)	30.9 (24.5-37.6)	1.02 (0.96-1.08)	0.4
Smoking history[b]	45 (46.3)	29 (40.3)	16 (64)	2.63 (1.04-7)	0.04
Comorbidities					
HTN[b]	73 (75.2)	51 (70.8)	22 (88)	3.02 (0.91-13.71)	0.09
DMIII[b]	51 (52.5)	33 (45.8)	18 (72)	3.04 (1.17-8.64)	0.02
COPD[b]	13 (14.4)	7 (9.7)	6 (24)	2.93 (0.85-9.89)	0.08
CKD>2[b]	37 (38.1)	24 (33.3)	13 (52)	2.16 (0.85-5.53)	0.1
ESRD[b]	8 (8.2)	4 (5.6)	4 (16)	3.23 (0.71-14.80)	0.1
CAD[b]	26 (26.8)	14 (19.4)	12 (48)	3.8 (1.44 -10.34)	0.007
CVA[b]	21 (21.6)	14 (19.4)	7 (28)	1.61 (0.54-4.53)	0.3
VTE[b]	16 (16.4)	14 (19.4)	2 (8)	0.36 (0.05-1.42)	0.2
2 or more comorbidities[b]	62 (63.9)	41 (56.9)	21 (84)	5.54 (1.72-24.91)	0.009
3 or more comorbidities[b]	50 (51.5)	32 (43.8)	18 (75)	3.21 (1.23-9.15)	0.02

Definition of Abbreviations: HTN= Hypertension; DMII= Diabetes Mellitus Type II; COPD= Chronic Obstructive Pulmonary Disease; CKD>2= Chronic Kidney Disease; ESRD= End-Stage Renal Disease; CAD= Coronary Artery Disease; CVA= Cerebrovascular Accident; VTE= Venous Thromboembolism. Continuous variables are reported as median (interquartile range).

P values indicate differences between alive and deceased COVID 19 patients.

[a]Values presented as median and interquartile range

[b]Values presented as number and % of the column total

Table 2. Demographic and Clinical characteristics associated with ICU admission

	No ICU (n=42)	ICU (n=55)	OR for ICU (95% CI)	P value
Demographics				
Age, yr[a]	54.4 (38.5-64.2)	71 (63.8-78.9)	1.1 (1.06-1.15)	<0.0001
Male n (%)[b]	19 (45.2)	35 (63.6)	2.11 (0.93-4.86)	0.07
African-American[b]	33 (78.6)	42 (76.4)	0.88 (0.32-2.29)	0.8
BMI[a]	30.2 (25.3-34.8)	28.4 (24.4-33)	0.98 (0.92-1.03)	0.5
Smoking history[b]	15 (35.7)	30 (54.5)	2.16 (0.95-5)	0.067
Comorbidities				
HTN[b]	29 (69)	44 (80)	1.79 (0.7-4.61)	0.2
DMIII[b]	15 (35.7)	36 (65.5)	3.41 (1.49-8.07)	0.004
COPD[b]	6 (14.3)	7 (12.7)	0.87 (0.26-2.93)	0.8
CKD>2[b]	12 (28.6)	25 (45.5)	2.08 (0.89-5)	0.09
ESRD[b]	4 (9.5)	4 (7.3)	0.74 (0.16-3.33)	0.7
CAD[b]	8 (19.1)	18 (32.7)	2.06 (0.81-5.61)	0.1
CVA[b]	7 (16.7)	14 (25.5)	1.7 (0.63-4.94)	0.3
VTE[b]	8 (19.1)	8 (14.6)	0.72 (0.24-2.15)	0.5
2 or more comorbidities[b]	22 (52.4)	40 (72.7)	2.66 (1.14-6.38)	0.02
3 or more comorbidities[b]	16 (38.1)	34 (61.8)	2.63 (1.16-6.11)	0.02

Definition of Abbreviations: HTN= Hypertension; DMII= Diabetes Mellitus Type II; COPD= Chronic Obstructive Pulmonary Disease; CKD>2= Chronic Kidney Disease; ESRD= End-Stage Renal Disease; CAD= Coronary Artery Disease; CVA= Cerebrovascular Accident; VTE= Venous Thromboembolism. Continuous variables are reported as median (interquartile range).

P values indicate differences between not ICU and ICU admitted COVID 19 patients.

[a]Values presented as median and interquartile range

[b]Values presented as number and % of the column total

Table 3. Demographic and Clinical characteristics associated with intubation

	Not Intubated (n=72)	Intubated (n=25)	OR for Intubation (95% CI)	P value
Demographics				
Age, yr[a]	61 (50.35-71.9)	69.8 (63.9-78.5)	1.05 (1.01-1.09)	0.005
Male n (%)[b]	38 (52.8)	16 (64)	1.59 (0.63-4.19)	0.3
African-American[b]	56 (77.8)	19 (76)	0.9 (0.31-2.81)	0.8
BMI[a]	28.9 (24.4-34.8)	29.7 (25-32.9)	1.0 (0.94-1.06)	0.9
Smoking history[b]	32 (44.5)	13 (52)	1.35 (0.54-3.4)	0.5
Comorbidities				
HTN[b]	53 (73.6)	20 (80)	1.43 (0.49-4.70)	0.5
DMIII[b]	36 (50)	15 (60)	1.5 (0.6-3.87)	0.4
COPD[b]	8 (11.1)	5 (20)	2.0 (0.55-6.71)	0.3
CKD>2[b]	25 (34.7)	12 (48)	1.73 (0.68-4.39)	0.2
ESRD[b]	5 (6.9)	3 (12)	1.82 (0.35-8.07)	0.4
CAD[b]	17 (23.6)	9 (36)	1.82 (0.66-4.82)	0.2
CVA[b]	14 (19.4)	7 (28)	1.61 (0.54-4.53)	0.4
VTE[b]	13 (18)	3 (12)	0.61 (0.13-2.14)	0.5
2 or more comorbidities[b]	44 (61.1)	18 (72)	2.01 (0.74-6.08)	0.2
3 or more comorbidities[b]	34 (47.2)	16 (64)	1.98 (0.79-5.25)	0.2

Definition of Abbreviations: HTN= Hypertension; DMII= Diabetes Mellitus Type II; COPD= Chronic Obstructive Pulmonary Disease; CKD>2= Chronic Kidney Disease; ESRD= End-Stage Renal Disease; CAD= Coronary Artery Disease; CVA= Cerebrovascular Accident; VTE= Venous Thromboembolism. Continuous variables are reported as median (interquartile range).

P values indicate differences between not intubated and intubated COVID 19 patients.

[a]Values presented as median and interquartile range

[b]Values presented as number and % of the column total

Table 4. Multivariate analysis associated with mortality

	Adjusted OR for Mortality (95% CI)	P value
MT-CYTB	2.24 (1.28 – 4.16)	0.015
Age	1.074 (1.026 - 1.131)	0.003
Sex, (male)	1.380 (0.460-4.362)	0.570
2 or more comorbidities	2.578 (0.611-14.200)	0.225

N =97 (25 mortality events)

Table 5. Multivariate analysis associated with ICU admission

	Adjusted OR for ICU Admission (95% CI)	P value
MT-CYTB	3.97 (1.83 – 10.34)	0.002
Age	1.113 (1.062 - 1.180)	<0.0001
Sex, (male)	2.060 (0.688 - 6.478)	0.201
2 or more comorbidities	0.465 (0.113 – 1.713)	0.264

N =97 (55 ICU events)

Table 6. Multivariate analysis associated with intubation

	Adjusted OR for Intubation (95% CI)	P value
MT-CYTB	8.48 (3.48 – 27.33)	<0.0001
Age	1.059 (1.008 - 1.119)	0.03
Sex, (male)	1.339 (0.381 - 4.920)	0.649
2 or more comorbidities	0.831 (0.166 - 4.368)	0.821

N =97 (25 intubation events)