Survival outcomes correlate with the level of cell-free circulating DNA in ST-elevation myocardial infarction

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Background: Myocardial infarction (MI) can lead to higher cellular damage, making cell-free DNA (cfDNA) a potential biomarker for assessing disease severity. The aim of this study is to evaluate survival predictions using cfDNA measurements and assess its correlation with MI. **Materials and Methods:** A direct fluorescence assay was employed to measure cfDNA content in the blood samples of participants. The inclusion criteria included patients who gave informed consent, suffering from ST-elevation myocardial infraction (STEMI) based on established diagnostic criteria (joint ESC/ACC guidelines), between the age of 18 and 80 years old, and had elevated troponin biomarker levels. The study included 150 patients diagnosed with STEMI and 50 healthy volunteers as controls. Serial monitoring of patients was conducted to track their postdisease status. The rate of change of cfDNA was calculated and daily measurements for 7 days were recorded. **Results:** Mean levels of cfDNA were found to be 5.93 times higher in patients with STEMI compared to healthy controls, providing clear evidence of a clinical correlation between cfDNA and STEMI. Patients were further categorized based on their survival status within a 90-day period. The study observed a strong predictive relationship between the rate of change of cfDNA during daily measurements and survival outcomes. To assess its predictive capability, a receiver operating characteristics (ROC) curve analysis was performed. The ROC analysis identified an optimal cutoff value of 2.50 for cfDNA, with a sensitivity of 81.5% and specificity of 74.0% in predicting disease outcomes. **Conclusion:** This study demonstrates a robust association between cfDNA and STEMI, indicating that cfDNA levels can be a valuable early prognostic factor for patients. Serial measurements of cfDNA during early disease onset hold promise as an effective approach for predicting survival outcomes in MI patients.

Key words: Biomarkers, cardiovascular diseases, cell-free nucleic acids, prognosis

INTRODUCTION

Patients with acute myocardial infarction (AMI) experience cardiomyocyte death in the infarct area.[1] This leads to loss of contractile function and significant cell death through apoptosis, which is observed in several studies.[2] An inherent strong link exists in cardiac cellular damage and disease outcomes. Early predictions will be valuable to guide treatment strategies.[3,4] The gold standards for detection rely heavily on biomarkers such as troponin[5] and electrocardiograms (ECG) to measure ST-segment elevation.[6] These tests are useful in AMI detection but do not suffice to have effective prognostic predictions. Cell-free DNA (cfDNA) that exists within the circulatory system is a by-product of necrotic and apoptotic cells. Following cardiac failure, widespread cellular damage can occur and directly impact cfDNA quantities. cfDNA can serve as a surrogate biomarker that measures the extent of cellular damage to evaluate the outcomes of cardiac patients.

The uses of cfDNA are demonstrated in many biomedical disciplines. These include oncology,[7,8] obstetric disease, autoimmune disease, and noninvasive

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prenatal tests (NIPT).[9] There is increasing interest to explore how cfDNA can substitute or complement conventional assays. Its' utility is attractive as specimen extraction is noninvasive and patient compliant. The presence of cfDNA was first observed in human blood by Mandel and Metais in 1948[10] during a cancer patient autopsy. Recent studies demonstrated that cfDNA is highly specific to cancer staging,[11] reflective of tumor burden,[12] and sensitive to detect genetic abnormalities.[13] For cardiology, consistent declines in cfDNA levels monitored over an extended period were associated with less risk of heart transplant rejections.[14] The use of cfDNA in AMI was demonstrated by Destouni *et al.*[15] who observed higher cfDNA levels in AMI patients compared with healthy volunteers. The changes in nucleic acid levels were significant to associate with further disease complications. In another study, Antonatos *et al.*[16] collected peripheral blood samples for 5 days and claimed prognostic utility in measuring cfDNA levels. These clinical applications greatly impact the management of patients to improve survivability and quality of life. The quantity of cfDNA within plasma is a function of the degradation by nuclease activities,[17] and its rate of removal by the liver and kidney.[18] Its half-life in circulation has been determined to be within a couple of hours.[19] These characteristics make it ideal to measure the real-time status of cardiac patients and for serial monitoring to gauge disease changes.

The current study aims to address the use of cfDNA in AMI patients. Specifically, we hope to test the predictive abilities by analyzing the trends in cfDNA during early disease onset. The results demonstrated clear specific associations of cfDNA to AMI patients and provided the basis to incorporate cfDNA measurements to complement current disease management.

MATERIALS AND METHODS

Study setting and patient population

Written informed consent was sought from all participating individuals. The study was conducted in The First Affiliated Hospital, Lanzhou University and the research protocol was approved by the local ethics institutional review committee (18–621). A total of 150 patients were recruited alongside 50 healthy individuals whom provided baseline comparison. Exclusion criteria for patients included individuals <18 years of age, pregnant, or patients who were transferred to other hospitals for postdisease care. Patients were confirmed with MI using the joint ESC/ACC guidelines[20] that included a combination of chest pain, elevation in cardiac troponin I (TnI), and ECG deviations that form the inclusion criteria for patients. These included cases with TnI above the 99th percentile and with 0/3 delta TnI measurements. All cases were ST-segment elevation

myocardial infarction (STEMI) and primarily determined with TnI levels above critical cutoff.

Trial participants' characteristics are provided in Table 1. Baseline cfDNA measurement was taken during hospital admission and daily for up to a week. Similar test regimes were performed for healthy volunteers. Healthy volunteers were selected based on a health questionnaire that confirmed that they did not have any conditions related to cardiovascular disease (CVD), cancer, pregnancy, and/or inflammation due to injury that might present with elevated cfDNA levels. Postbiochemistry results indicated one with diabetes and three others hyperlipidemic. As they do not have clear indications of CVD, these volunteers' results were included.

Blood extraction and purification of cell-free DNA

The first blood samples were collected within 2 h on admission and subsequently during morning rounds at the wards. Five microliters of blood was extracted through the peripheral vein into EDTA blood tubes. Samples were transported at 4°C. To separate plasma from whole blood, the sample tube was placed in a centrifuge with settings at 1000 g, 4°C for 30 min. The top plasma layer was carefully removed to a new DNase-free tube and centrifuge at 1000 g, 4°C for 5 min to remove any residual contaminants. DNA in the plasma fraction was purified using the QIAamp Circulating Nucleic Acid kit (QIAGEN Inc., MD, USA). Briefly, extraction of DNA was achieved using spin columns with settings stipulated in the manufacturer's instructions. The eluate was recovered in nuclease-free water (10 uL) and stored before being quantified. Approximately 3 mL of plasma was processed from each specimen.

Cell-free DNA measurements for plasma specimens

cfDNA was detected directly using Quant-iT PicoGreen dsDNA kit (Thermo Fisher Scientific, USA). The procedures followed the manufacturer's recommendations. Briefly,

Table 1: Baseline characteristics of acute myocardial infarction patients and healthy volunteers who participated in the study

Parameter	AMI (*n*=150)	Healthy (*n*=50)	*P*
Age	63±6	59±5	0.38
Gender (male/female)	92/58	26/24	0.27
Cardiovascular risk factors*			
BMI (kg/m²), mean±SD	28.7±2.9	21.6±2.4	0.46
Diabetes mellitus (%)	31	2	<0.01
Hyperlipidemia (%)	75	6	<0.01
Hypertension (%)	53	0	<0.01
Smoking/tobacco (%)	52	46	0.18
Genetic background/family history (%)	45	6	<0.01

*Univariate analysis was conducted to understand significant risk factors among this cohort. AMI=Acute myocardial infraction; SD=Standard deviation; BMI=Body mass index

each 50 uL reaction consisted of input DNA from plasma specimens together with the working solution. Five microliter of purified cfDNA eluate was used. All reagents were prepared fresh as suggested to ensure the accuracy of the tests. The readout was taken by scanning the sample plate in a fluorometer. All readings were taken in duplicates and mean values were registered. Standard curves for comparison were produced by serially diluting lambda DNA.

Statistical analysis

Continuous variables determined from cfDNA measurements were presented as mean and standard deviation, with appropriate confidence intervals (CI). Normality for these variables was assessed using Shapiro–Wilks statistics. Parametric tests such as Student's *t*-test were used when normality was established, while nonparametric tests such as the Mann–Whitney test were applied otherwise. Univariate analysis was performed to ascertain the significant risk factors associated as shown in Table 1. A $P < 0.05$ was considered statistically significant for all comparison tests. For comparing cfDNA against conventional TnI, we performed a Spearman's correlation analysis to establish the correlation coefficient, rho. To determine if the proposed cfDNA assay was clinically relevant, we plotted receiver operating characteristics (ROC) curves and determined the area under the ROC curves. The corresponding 95% CI was provided. Accompanying optimal cutoffs with corresponding sensitivity and specificity were reported and hazard ratios (HR) from Cox regression were established. Survival curves were constructed using Kaplan–Meier analysis to show differences between two distinctive groups. All statistical analyses were performed using the Prism version 6.0 software (GraphPad Inc., CA, USA).

RESULTS

Clinical association of cell-free DNA in acute myocardial infarction patients at baseline measurements

A total of 150 patients diagnosed with AMI participated. Of these patients, the gender ratio was 1.6 (male/female) and the mean age was 63 ± 6 years. Table 1 presents the clinical and demographic characteristics. Univariate analysis was used to determine any significant continuous or categorical parameters. Baseline epidemiological characteristics among patients, such as diabetes and hypertension, were not significantly different, similar to age and gender in this cohort. The patients' survival status was confirmed with postdischarge follow-up with bi-weekly phone calls for 90 days. For healthy individuals who acted as controls in this study, the gender ratio was 1.1 (male/female) with a mean age of 59 ± 5 years. The study design of the current investigation is presented in Figure 1a.

Baseline reference measurements for cfDNA were established by comparing diseased individuals to healthy volunteers. Mean concentrations of cfDNA of healthy volunteers and AMI patients were 1112 ± 113 ng/mL and 6598 ± 1915 ng/mL, respectively [Figure 1b], and translate to approximately 5.93 folds higher. We observed a smaller cfDNA level dispersion within healthy volunteers. The measured coefficient of variation (CV) was 10.2%. In contrast, CV for AMI patients showed a larger dispersion at 29.0%. Using a Mann–Whitney test, this was statistically significant ($P < 0.05$). We performed a ROC analysis to ascertain the sensitivity and specificity. The optimal cutoff was determined at 2255 ng/mL. Given the clear separation of data points between the diseased individuals and healthy cohort, this corresponded to 100% specificity and sensitivity, achieving perfect separation of cohorts. Correlating to
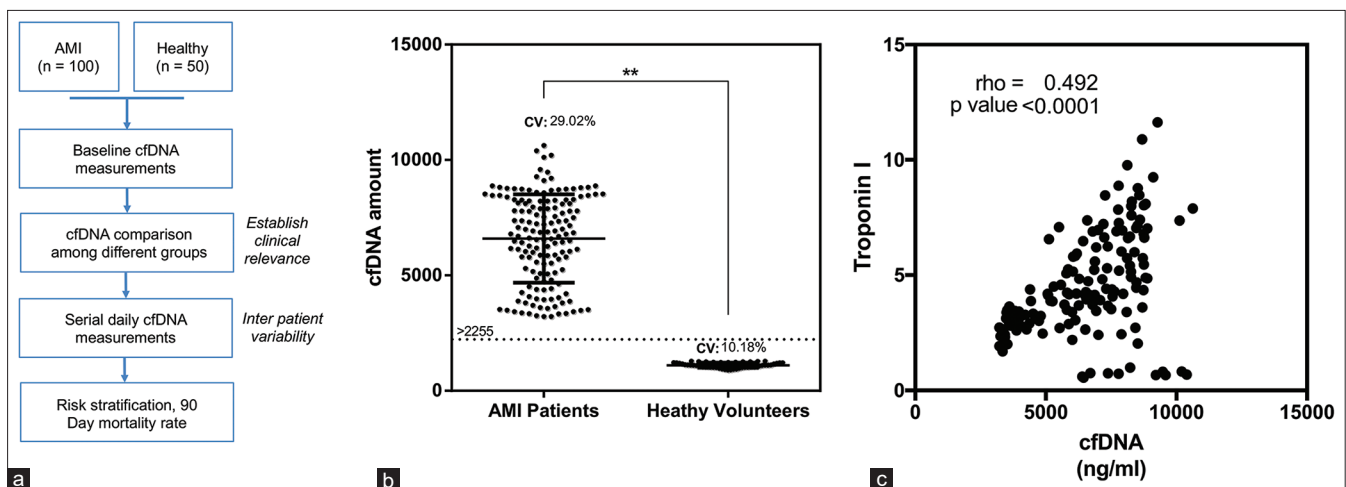
Figure 1: Distinction of cell-free DNA (cfDNA) within acute myocardial infarction (AMI) patients and healthy volunteers. Baseline reference measurements were taken from February 2016 to March 2017. (a) Study design comparing different groups of participants. (b) cfDNA concentration extracted from plasma showed higher content in AMI patients. (c) Correlation of cardiac troponin I with cfDNA for each patient using the Spearman's correlation coefficient, rho. AMI = Acute myocardial infarction, cfDNA = Cell-free DNA, CV = Coefficient of variation. **denotes *p* value < 0.0001

well-established biomarkers such as cardiac TnI [Figure 1c], we observed statistical significance ($P < 0.0001$, Spearman's rho = 0.49) comparing it with recovered cfDNA. Troponin results are typically gender specific and we compared results of both male and female data points. Mean cfDNA levels of the AMI group were 6952 ± 1835 ng/mL and 6437 ± 1980 ng/mL for female and male groups, respectively. The results were not statistically different using a Student's *t*-test [$P = 0.143$, Supplementary Figure 1]. The cfDNA levels within the healthy cohorts were 1045 ± 136 ng/mL and 1233 ± 96 ng/mL for female and male groups, respectively.

Daily variations and trends of cell-free DNA among acute myocardial infarction patients

We are interested in cfDNA variations to understand early disease onset. Daily measurements for 7 days were recorded. Figure 2a shows the absolute cfDNA measurements for all patients and healthy volunteers. Interestingly, we observed limited fluctuations within the 7-day period for healthy volunteers and this was affirmed using the Kruskal–Wallis test ($P = 0.717$). Healthy volunteers

Figure 2: Serial measurements and trend analysis for acute myocardial infarction patients and healthy volunteers. (a) Monitoring of cell-free DNA (cfDNA) variations daily showed an initial increasing trend upon manifestation of the disease. (b) Coefficient of variation measurements of the study cohort that addressed variability during the 7-day measurements. (c) The maximum rate of change in cfDNA measurements for each individual showing good separation with healthy volunteers. AMI = Acute myocardial infarction, cfDNA = Cell-free DNA, CV = Coefficient of variation

registered <2255 ng/mL of cfDNA levels. For AMI patients, we observed an increasing mean cfDNA concentration for 4 days before registering a drop thereafter. The Kruskal–Wallis test showed that the mean concentrations were different during this period ($P < 0.05$). Absolute mean cfDNA concentrations of disease individuals were also persistently higher than healthy volunteers. We attempted to understand the trends in cfDNA variations. The CV comparisons for different time points are provided in Figure 2b. This measurement showed the dispersion of data points across the entire measurement time points for each patient or volunteer. A stark contrast in trending was observed between healthy and diseased individuals. Within AMI patients, we registered a maximal CV of 37.2% and a minimum of 19.2% CV value. The mean CV value was 27.3% (95% CI: 26.8%–27.9%). We hypothesize that the rate of change in cfDNA will be important, as this reflects the extent of cellular damage. Figure 2c shows the maximal rate of change with respect to baseline. A maximum value of 3.39 was registered and the minimum was 1.75. The mean recorded value was 2.48 (95% CI: 2.43–2.53).

Clinical correlates of acute myocardial infarction using cell-free DNA measurements

By addressing the 90-day mortality rate, we attempted to demonstrate cfDNA's clinical utility. The study group was divided based on their survival status and correlated to absolute cfDNA content, CV measurements, or rate of change in cfDNA. Figure 3a presents the result comparing all three parameters. We observed higher mean quantities in the nonsurvival group. Using a Student's *t*-test, we observed statistical significance in using CV ($P < 0.05$) or rate of change in cfDNA ($P < 0.05$). In each case, the ratios of the nonsurvival over survival groups for absolute cfDNA, CV, and rate of change in cfDNA were 1.02, 1.11, and 1.13, respectively. The ROC analyses were subsequently conducted to determine suitable cutoff values using these parameters. Figure 3b shows the results with the area under the curve for absolute cfDNA concentrations, CV, and rate of change of cfDNA to be 0.515, 0.720, and 0.806, respectively.

Using these cutoffs, survival analyses were performed to investigate the prognostic value. Figure 4a and c show the results using all three different parameters to stratify patients. Using the cutoff of 16,649 ng/mL for absolute cfDNA concentrations, the HR was 1.06 [Figure 4a, 95% CI: 0.625–1.816, $P = 0.82$]. Using the CV cutoff value to separate the patient cohort, we determined that the HR was 1.99 [Figure 4b, 95% CI: 1.18–3.43, $P = 0.01$]. For the last parameter that measures the maximal rate of change in cfDNA, we computed the HR to be 3.95 [Figure 4c, 95% CI: 1.98–5.76, $P < 0.0001$]. The results demonstrated a good separation of patient groups to identify high-risk individuals that had poorer survival outcomes.
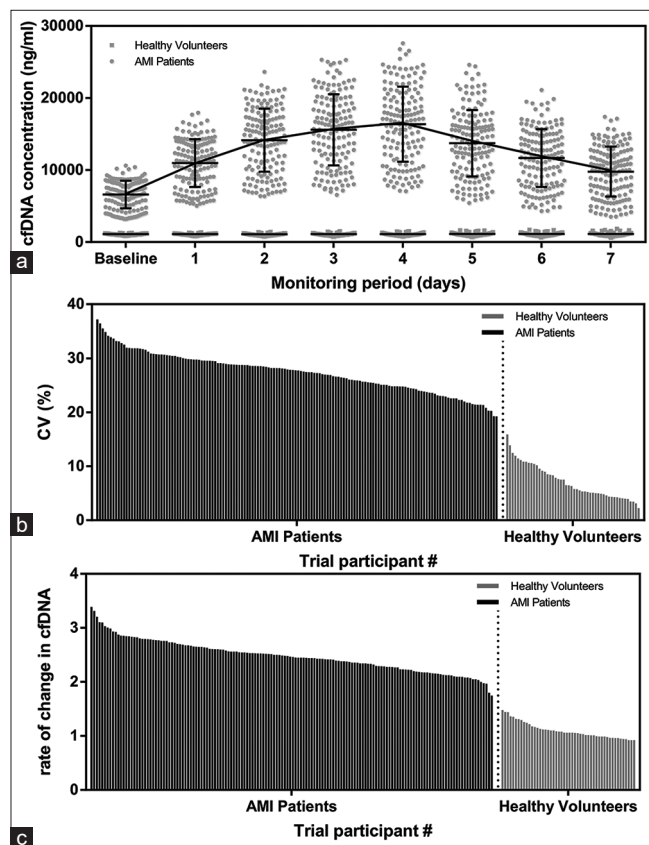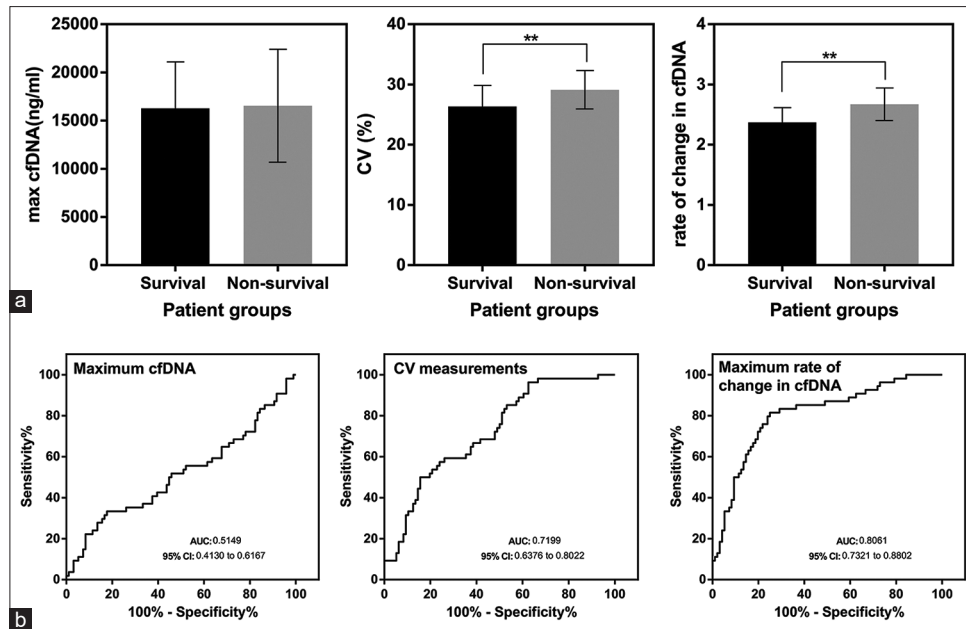
Figure 3: Analysis of the variations in cell-free DNA (cfDNA) and its implication for 90-day survival in acute myocardial infarction patients. (a) Measurements of maximum cfDNA concentration, coefficient of variation (CV), and rate of change in cfDNA in the survival vs nonsurvival group. (b) Receiver operating characteristics analysis for the parameters maximum cfDNA concentration, CV, and rate of change in cfDNA to determine the optimal cutoff value for each test. cfDNA = Cell-free DNA, CV = Coefficient of variation, AUC = Area under the curve, CI = Confidence interval. **denotes *p* value < 0.0001
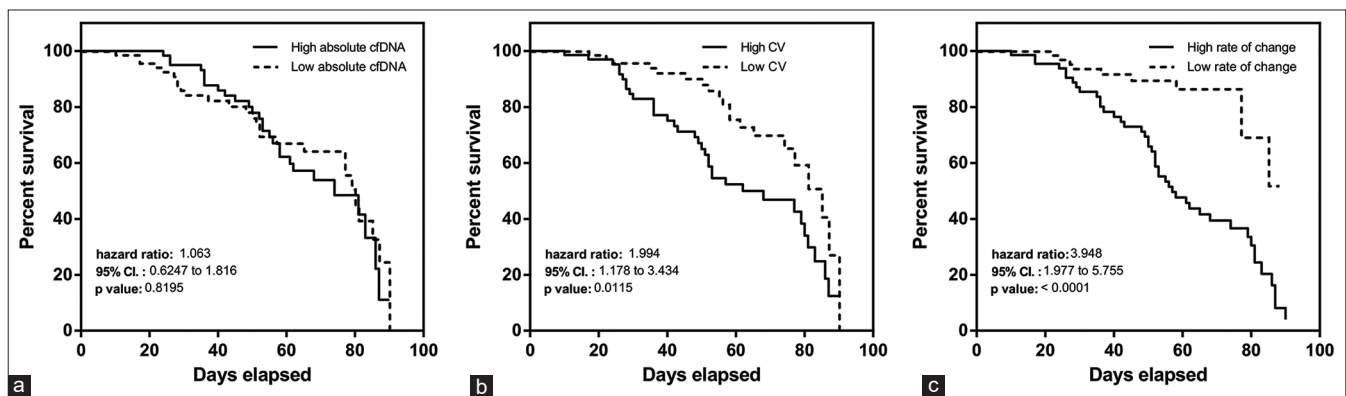

Figure 4: Survival analysis to gauge the prognostic utility to use cell-free DNA (cfDNA) to stratify acute myocardial infarction (AMI) patients. (a) Separation of patient cohort using maximum cfDNA concentrations. (b) Coefficient of variation measurements within AMI patients showed good prognostic utility to predict mortality with a hazard ratio of 1.99. (c) The maximum rate of change in cfDNA in each patient showed good prognostic value to identify high-risk patients. cfDNA = Cell-free DNA, CI = Confidence interval, CV = Coefficient of variation

DISCUSSION

The use of alternative detection methods to profile infarction events can lead to improve disease management. Our results demonstrated that cfDNA was a specific marker that differentiates AMI patients from healthy volunteers. In Supplementary Figure 2, we noted that there was an insignificant difference in baseline cfDNA levels for survivors and nonsurvivors. It is interesting that cfDNA levels showed close correlation to AMI patients and time-dependent results could be utilized for prognostic purposes. De Vlaminck *et al.*[14] demonstrated that variations in cfDNA measured over a week correlated to heart transplant rejections. This provides a significant

advantage over invasive biopsy to assess a rejection episode for patients. In our study, the appeal of this assay is that this closely relates to cellular damage, which is one of the hallmarks of the disease.[21] No gender-specific influences were seen as shown in Supplementary Figure 1. Due to its relatively short half-life within the body,[19] it provides a relatively up-to-date reflection of changes within the body. From these considerations, we hypothesize that serial measurements will be useful to track disease manifestation.

The utility and profiling of circulating nucleic acid have been predominantly used in NIPT[9] and oncology.[22,23] The sample collection is straightforward and attractive as opposed to tradition means of invasive sampling. In the

case of AMI, blood sampling is a routine procedure and measurements of cfDNA do not increase the burden of these patients. Baseline measures can be taken from the same tube of blood where Troponin measurements are established and subsequent sampling from daily routine blood tests. In the current study, we observed a distinctive difference in cfDNA levels between patients and healthy controls. This demonstrated clear evidence of the clinical association of cfDNA to the disease. We also showed its utility in stratifying AMI patients. Our results are consistent with prior work done by Cui *et al.*,[24] who found value in using cfDNA for diagnosis. In their work, a significant increase of more than 40 folds in plasma cfDNA was observed with MI. We focused our analyses on both plasma DNA quantity and inter-patient variability. Concentrations of cfDNA were observed to decrease after day 4 [Figure 2a]. This could be closely related to the stabilization phase for most patients and translates to reduced cfDNA levels into the circulatory system. Given that the extent of tissue damage can be closely related to cfDNA release, the concentration of measured cfDNA may affect prognosis. In another study by Destouni *et al.*,[15] it was found that substantial differences exist between cfDNA levels during disease complications that arose in their patients. Our study further addressed the benefits of close monitoring of these patients that showed patients with worse outcomes.

Addressing the dynamic changes during early disease onset is important. A study conducted by Antonatos *et al.*[16] showed distinct differences for patients with various outcomes. Our study further addressed the dynamic changes of cfDNA during the monitoring period of 7 days to understand how these changes were correlated. At baseline, CV was 29% and dispersion remained constant thereafter. The change could be reflective of the relative tissue damage sustained by these patients and higher CV linked to more extensive cellular damage. We also registered a higher mean cfDNA count postinfarction that further supported some patients may have more cellular damage. This could be linked to postdisease complications observed in some patients and prior studies had established a close correlative link.[15,16] Our study further analyzes its trends for the utility to identify patients with the likelihood of better survival outcomes. Early prediction of prognosis is difficult for AMI patients as postdisease complications and status change quickly. In earlier studies, there existed little correlations to other risk factors such as creatine kinase-MB or TnI.[25] In our serial measurements, cfDNA showed interesting trends. For patient survival within a 90-day period, we observed nonsurvival group having higher cfDNA levels within the 7-day monitoring period, the highest CV changes and the greatest rate of change of cfDNA with respect to the baseline measurements. These parameters were considered as they were deemed to be highly associative to the extent of the

cellular damage. Both CV measurements and rate of change in cfDNA were statistically significant to differentiate survival and nonsurvival groups. Our results showed that measuring the rate of change and variations were important clinicopathologic factors that associated with patients' likelihood for better survival outcome. We observed that the HR in both cases were higher than when stratifying patients using absolute cfDNA measurements. This could be due to the fact that variations in cfDNA readings better reflect the instantaneous changes in the body than the absolute reading that could be biased due to body mass and injury. One current limitation of the study is the analysis by age bands as the risk of cardiac events increases with age. This will be beneficial in future investigations. To gain traction in clinical use, assay performance must be thoroughly tested, inexpensive, and simple to implement.

In conclusion, the present study found an association between cfDNA and patients with AMI. We observed variations within cfDNA had prognostic utility. It represented possible measures of the extent of cellular damage occurring in the body. The predictive values of cfDNA were tested using patients' survival data within a 90-day period. The results demonstrated the potential to measure cfDNA to stratify patients and identify high-risk individuals based on their changing patterns in cfDNA levels.

Conflicts of interest
There are no conflicts of interest.

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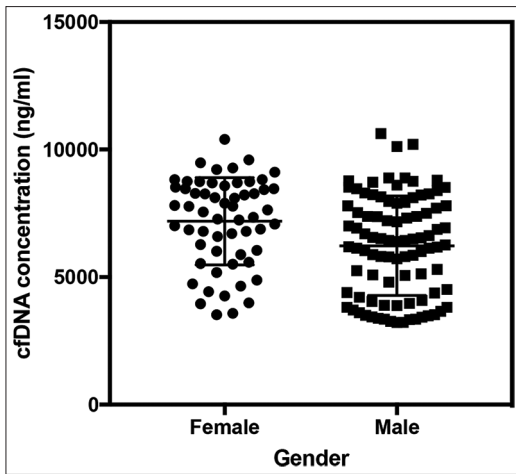
Supplementary Figure 1: Gender-specific cell-free DNA comparison shows no significant difference using a Student's *t*-test. cfDNA = Cell-free DNA
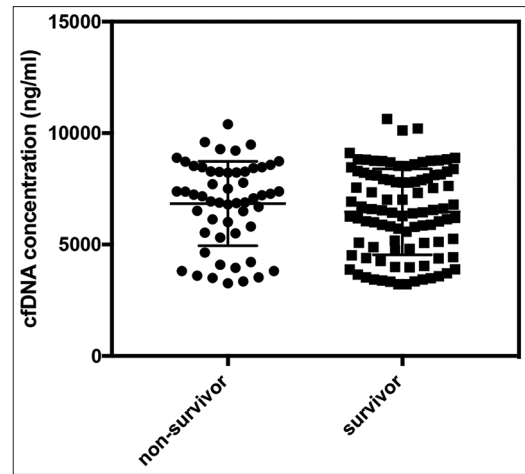
Supplementary Figure 2: Comparison of baseline cell-free DNA levels among nonsurvivors and survivors. cfDNA = Cell-free DNA