THE ROLE OF CFDNA CONCENTRATION IN VARIOUS CARDIAC DISEASES AND IT'S CORRELATION WITH COVENTIONAL BIOMARKERS

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ABSTRACT
Objective: To quantify the concentration of plasma cfDNA in patients with cardiovascular diseases and healthy volunteers at different stages, analyze the correlationship between plasma cfDNA and traditional biomarkers with clinicopathological features of cardiovascular patients. Additionally, evaluate its significance in early clinical diagnosis of cardiac disease state. In recent years cell free DNA’s are coming under spotlight while establishing it’s significance in the field of precision medicine. Over the past decades it has been affirmed that larger amount of cell free DNA’s can be detected in the circulation with pathological condition which plays a significant role suggesting a noteworthy clinical part in cancer, Diabetes mellitus, Rheumatoid Arthritis, prenatal screening, inflammation, stroke, myocardial infarction and variety of cardiac diseases. Method: We purified cfDNA directly from peripheral blood using magnetic beads by modifying binding and lysis conditions followed by a spectrometric DNA concentration test. Result: The serum from cardiac patients showed significant cfDNA elevation (p<0.01). The interquartile value of cfDNA (median 136.79 µg/ml, range 10.54–263.04 µg/ml), (median 36.62 µg/ml, range 17.58-57.67 µg/ml), (median 8.18 µg/ml range 1.83-14.53 µg/ml), (median 12.65 µg/ml range 4.67-20.63 µg/ml) account respectively for Myocardial infarction, Cardiac angina, Atrial fibrillation, and cardiac failure vs. (median 5.46 µg/ml, range 1.38-9.54 µg/ml) indicating healthy controls patients were shown. In our study we didn’t find any significant correlation of cfDNA concentration with CRP and CK-MB respectively even though noteworthy elevation of cfDNA was observed in selected cardiac patients. Conclusion: These results enlighten us to brief that plasma cfDNA in cardiac patients may originate from apoptosis and suggest an impactful diagnostic validity.

KEYWORD: cfDNA, MI, biomarkers, CVD.

1. INTRODUCTION
Cardiac diseases are the number one growing worldwide pandemic that results in notable morbidity and mortality in the aging population. World Heart Federation Roadmap for Heart Failure claimed One in nine deaths includes heart failure as contributing cause. Due to the aging population, increased cardiovascular risk factors, and improved survival of cardiovascular infirmities, the predominance of cardiovascular diseases is expanding globally to an estimated number of millions, with additional millions of undiagnosed cases. Because of its high prevalence rate, cardiovascular abnormalities are an essential contributor to national healthcare expenditures’ burden and cost. More older people are hospitalized for cardiovascular diseases than for any other medical condition. Besides, the application of several targeted drugs in the past 15 years and the 5-year survival rate of last-stage cardiovascular patients is still associated with a poor prognosis. The incidence increases with age, rising from approximately 65–69 years of age to >80 years of age. Due to the aging population, the increase in cardiovascular diseases will be tremendous for older people. Approximately half of the people who develop last-stage cardiovascular diseases die within 5 years of diagnosis. The search for better treatments for CVD's is one of the major challenges in cardiology. A greater understanding of the molecular dynamics and humoral perturbation will lead to newer treatments. It is evident that early diagnosis is possible to achieve a curative effect. Moreover, for limited and advanced diseases, better predictive and prognostic biomarkers are needed.

1.2 Traditional Biomarkers
Biomarkers have incredibly added to improving the analysis of heart failure. These biomarkers can be protein-based, such as Natriuretic peptides, especially N-terminal
proE-type natriuretic peptide (NT-proBNP), Cardiac specific troponins (cTn), and CK-MB, an invasive surgical technique such as endomyocardial biopsy and nucleic acid-based, circulating cell-free DNA (cfDNA), and microRNA. Cardiac-specific troponin has been the most commonly used circulating biomarker for decades, but its sensitivity and specificity are limited. EMB (Endomyocardial biopsy) is used as the gold standard invasive technique for the differential diagnosis of many primary and secondary CV diseases, including cardiomyopathies, myocarditis, infiltrative lesions, arrhythmias, and drug toxicities, as well as to monitor allograft rejection after heart transplantation. However, due to its invasive procedures and high risk of complications, it is often difficult for patients to accept this routine screening technique. Therefore, it is a problem to screen people with a high risk of developing CVD’s by minimally invasive means for Endomyocardial biopsy.

1.3 Liquid Biopsy/CfDNA Concerning Cardiovascular Diseases

The fluid biopsy/Liquid biopsy is a minimally invasive procedure based on simple venipuncture. It can be safely performed in a large area and can be repeated for patients with minimal risk. Also, liquid biopsy can illustrate the molecular diversity of potential disease processes, and serial tests can help monitor their genomic evolution while avoiding the need for biopsy. Liquid biopsy is turning into a brilliant sample obtaining technique, substituting the invasive strategies for the conventional diagnostic protocol. Advances in fluid-based assays may give novel, useful non-invasive markers of cardiovascular (CVD) diseases. This technique incorporates the likelihood of isolating cell-free nucleic acids (cfDNAs) for diagnostic (cfDNAs) or screening reasons. Over the previous decade, cell-free nucleic acids (cfDNAs) have become a point of concentration for molecular pathology research such as early cancer detection, genetic and epigenetic monitoring, recurrence prediction, therapeutic resistance assessment of cancer, and preclinical testing since cfDNA is effectively and non-invasively distinguishable in body fluids, can be assayed multiple times, and above all might be utilized to survey the seriousness of the infection and predict the viability of its treatment. cfDNA is derived from normal or tumor cells and can be detected in healthy people. It plays a vital role in the inflammatory process and infection.

Circulating cell-free DNA (cfDNA) was first announced by Mandel and Metais, who noticed free DNA and RNA in blood plasma. Most proof has stated that cfDNA can be found in plasma, urine, or cerebrospinal fluid originating from the apoptosis or necrosis of all cell types. Even though, apoptosis is the most prominent event that decides the amount of cfDNA. On the other hand, cfDNA that is conveyed from tumor cells, which is called circulating tumor-cells DNA (ctDNA), is the outcome of both apoptosis and necrosis. Physiologically, low degrees of fragmented nucleosome-size cell-free DNA (cfDNA) can instantly circulate in the plasma of healthy subjects before being cleared by the liver. cfDNA is known to augment due to work out, extended age, similarly as in neurotic conditions, for instance, cardiovascular sicknesses (CVD), including hypertension, myocardial dead tissue (MI), and cardiovascular breakdown. In any case, its symptomatic worth is totally high in certain CV illnesses. For sure, under pathological conditions prompting its overproduction, larger amounts of cfDNA can be distinguished in the circulation, exhorting a putative clinical part in stroke, myocardial infarction, namely widespread cardiac diseases.

| Table 1: Baseline characteristics, plasma cfDNA concentration, cardiovascular patients and controls. |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                | Controls (n = 40) | Myocardial infarction (n = 12) | Cardiac angina (n = 10) | Atrial Fibrillation (n = 5) | Cardiac failure (n = 5) |
| Age (years)                    | 37.5 (16-59)     | 75.5 (57-94)   | 89 (84-94)     | 78.5 (71-86)   | 66 (38-94)     |
| Male/Female                    | 10/30            | 4/8            | 4/6            | 2/3            | 3/2            |
| cfDNA concentration (µg/ml)    | 5.46 (1.38-9.54) | 136.79 (10.54-263.04) | 37.62 (17.58-57.67) | 8.18 (1.83-14.53) | 12.65 (4.67-20.63) |

Values are median (interquartile range)

2. MATERIALS AND METHODS

2.1 Study population

This study was conducted at the Medical Biochemistry laboratory and Cardiology Departments, Faculty of Medicine, Yangzhou University affiliated Hospital. From November 2020 to January 2021, 32 patients with cardiovascular diseases were selected for data analysis, including 13 males and 19 females. Their age ranged from 38 to 94 years old. 32 patients were diagnosed with various cardiovascular diseases by two or more pathologists while 40 healthy individuals were selected including 10 male and 30 female and their age ranged from 16 to 59 years old Inclusion criteria: previous or concurrent myocardial infarction, hypertension, heart block, atrial fibrillation, vulvar injury, arrythmia, cardiomyopathy, atherosclerosis, coronary artery disease, angina, cardiac failure, serious or uncontrollable medical diseases Exclusion criteria: good cardiac health, no hypertension, no myocardial damage, heart block or heart related complications.

The investigation, including the usage of plasma and
DNA collected from patients and control subjects, was reviewed and embraced by the Human ethics Advisory committee of Yangzhou University-affiliated hospital. Also, informed consent was signed from all members.

2.2 Blood sampling
First, the blood sample was centrifuged to separate plasma. The specific operation steps were as follows: first, the blood sample was centrifuged at room temperature and low speed for 10 min, then the supernatant was drawn and placed in the centrifuge tube, next the supernatant was centrifuged at 4000 rpm, 4 °C and high speed for 10 min, then the upper plasma was drawn and placed in the sample storage tube, labeled (indicating the patient’s name, age and other information), and stored in the refrigerator at - 38°C.

2.3 CfDNA extraction
Circulating cell-free DNA (cfDNA) filtration from peripheral blood requires centrifugation to isolate plasma from whole blood. We conceived a strategy to purify cfDNA straightforwardly from peripheral blood using magnetic beads (TIANGEN magnetic serum/Plasma Dna Kit) by modifying, binding and lysis conditions followed by a spectrometric DNA concentration test equipped with smart dsDNA 50 software. Our strategy incorporates a purification step to eliminate heme and different foreign substances to get the quality outcome.

Table 2: Tiangen Magnetic serum/ Plasma DNA purification manual.

<table>
<thead>
<tr>
<th>Sample volume(µl)</th>
<th>Consumables Specification</th>
<th>Pyrolysis solution</th>
<th>Proteinase K (µl)</th>
<th>Magnetic bead WD (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>1.5 ml centrifuge tube</td>
<td>1.5 µl sample volume</td>
<td>0.1 µl sample volume</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

This kit is based on 0.4ml/400 µl sample

2.5. Statistical Analysis
The differences in cfDNA concentration between controls and patient groups were investigated and expressed as mean and standard deviation (x ± s). Variances were analyzed with Student t-test resulted in a statistically significant (p<0.01). Regression analyses were performed to estimate the correlation between cfDNA concentration and CK-MB as well as CRP. These statistics were directed using the program both IBM SPSS statistics 26.0 v and Microsoft Excell programming to reverify.

3. RESULT AND DISCUSSION
3.1. Significant elevation of cfDNA concentration in different cardiac disease patients
Our Objective was to analyze the relationship between plasma cfDNA concentration and clinical characteristics of patients with main stream cardiac diseases with the corresponding features such as gender, age, severity of the diseases, cardiac failure class, stage and infarct size. The serum from various cardiac patients showed significant cfDNA elevation (p<0.01) (Fig 1). Our study shows an elevation of cfDNA (median 136.79 µg/ml, range 10.54–263.04 µg/ml), (median 36.62 µg/ml, range 17.58–57.67µg/ml), (median 8.18µg/ml range 1.83-14.53µg/ml), (median 12.65 µg/ml range 4.67-20.63µg/ml) account respectively for Myocardial infarction, Cardiac angina, Atrial fibrillation, and cardiac failure vs. (median 5.46 µg/ml, range 1.38–9.54 µg/ml) indicating healthy controls (Table 1) which agree with the previous conducted studies. The outcomes are displayed in (Fig. 1). Patients who had endured cardiac abnormalities had mean cell free DNA significantly higher (p<0.01) than that of control subjects. Altogether higher cfDNA concentration were seen in cardiac patients (p < 0.01) when contrasted with control subjects that drew a convincing relationship with plasma DNA concentration. Studies conducted in the past have detailed ischaemic injuries promote apoptosis in AMI /HF patients. Therefore we assume that significantly elevated level of cfDNA might be the case of apoptosis in the patients with different cardiac diseases. We have found age over 59 basically female patients had more elevated level of cfDNA when contrasted with healthy individuals. We canceme with a fact that age plays an important role concerning sex. Cardiac disease patients had gone through the tests of Troponins, CK-MB, CRP, we have found those who have higherpercentage of cfDNA tend to show the severity of the disease condition which again may complement traditional biomarkers such as...

![Graph showing cfDNA concentrations in plasma from cardiac disease patients and healthy control subjects. Data were analyzed by student t test and are expressed as mean ± S.D. Significantly higher levels of cfDNA were detected in myocardial infarction and cardia angina and cardiac failure patients when compared to those in normal controls. **p < 0.01 when compared with the control group.](image-url)
CVDs are the primary source of death worldwide: a more significant number of individuals die yearly from CVDs than from any other cause. More than 523.2 million cases of cardiovascular cases in 2019, an increment of 26.6% compared with 2010 and 17.9 million death every year, and the trend is increasing year by year. With the improvement of living standards and lifestyle, the incidence rate is still high. It will increase year by year. Therefore, conventional treatment after diagnosing can increase the chance of cure. Certainly, prognostic factors are likely to achieve curative effect; for limited and advanced diseases, therefore better predictability and accuracy, prognostic biomarker is needed in the field of cardiology medicine.

CK-MB, and Troponin I screening has been shown to reduce disease-specific mortality. It has been a clinical hotspot for decades. Conventional use of circulating biomarkers efficacy is limited meaning sensitivity and specificity is not good. Endomyocardial biopsy is considered the gold standard, and It can effectively reduce the incidence and mortality of heart failure. However, because of the method’s invasiveness, the high risk of complications is often challenging to accept for routine screening patients. It is a problem that needs to be actively studied to select people with a high risk of developing cardiac disease such as heart failure for endomyocardial biopsy. Liquid Biopsy is a minimally invasive procedure based on simple venipuncture, which can be safely carried out in a wide range and can be used for the diagnosis of the disease people repeat it with minimum risk. Also, fluid biopsy can demonstrate the molecular diversity of underlying disease processes, and A series of tests help to monitor its spatial genome evolution while avoiding the need for re-biopsy. Besides, CfDNA is derived from normal or tumor cells and can be detected in healthy people. It plays a vital role in the inflammatory process and infection. Necrotic and apoptotic cells can passively release DNA fragments, which is one of the most important factors., the level of cfDNA in cardiac patients depends on the cardiac status and kinetics of the patient. The concentration of cell-free circulating DNA (cfDNA) in the plasma of cardiac patients has been reported. It is significantly higher than that in normal people. Even in the early stage of cardiovascular disease, it is easy to detect cfDNA concentration in various body fluids such as plasma and serum. In addition, elevated plasma cfDNA levels were associated with heart failure stages, classes, and progression and were correlated with burden, differentiation status, and survival rate. These circulating molecules are also considered to be research components Non-invasive sources of biomarkers. As a result, some studies emphasize quantitative analysis.

The purpose of this study was to quantify the plasma cfDNA in patients with variety of cardiovascular diseases and to evaluate the clinical significance of the diagnosis. After total DNA was extracted from plasma, cfDNA was purified by serum magnetic plasma kit and the concentration was determined by spectrophotometer. Our data showed that the mean concentration of cfDNA in the cardiac diseased group MI (64.57 ± 77.56) ng/ml, (35.09 ± 15.27) ng/ml, (6.06 ± 4.94) ng/ml, (15.93 ± 6.65) ng/ml accounts respectively for MI, Cardiac angina, Atrial fibrillation and cardiac failure was significantly higher than that in healthy control group (3.56 ± 1.69) ng / ml (P < 0.01). we found there were no significant correlation of cfDNA concentration with CK-MB, C reactive protein respectively. Alternatively we did not find any outcomes which correlate cfDNA concentration with cardiac biomarker cTnI (cardio selective Troponin I) Similarly,
the results of Chang et al. showed that the concentration of cfDNA did not correlate with the cardiac marker Troponin I, even though the circular cell-free DNA concentration was significantly higher in all patients endured myocardial infarction / heart failure. J Fujihara et al. also showed high levels of cell-free DNA elevation in variety of cardiac disease condition. The high level of plasma cfDNA in patients with cardiac disease are thought to be released from the mechanism called programmed cell death / apoptosis which is the primary factor along with the necrotic mechanism. The rapid proliferation and cell lysis of infarcted cell lead to large amount of cfDNA entering the blood circulation. Additionally cfDNA can rise due to other clinicopathological features, such as inflammation, infection, Hypertension, DM, Rheumatoid Arthritis and strokes. Therefore, it is speculated these disease condition mentioned above can also help to release DNA from Cardiac cell/myocardium into the bloodstream. Schwarzenbach et al. reported that the average concentration range of serum cfDNA in cancer patients was wide or higher than that in healthy subjects. It is reported that the serum level of cfDNA in patients with liver cancer is significantly higher than that in normal people. In addition we have found in our study increased age difference plays an important role in the elevation of cfDNA of the disease patients particularly in the Heart failure/myocardial infarction patients that can be a future prospect study for upcoming researches.

3.4 Future prospective
Circulating levels of cfDNA can possibly help in cardiovascular disease management, much remaining parts to be done prior to considering interpretation of these examination discoveries to clinical application. To begin with, the issue of the fast clearout of cfDNA by the liver must be thought of. Although current examinations support the biomarker worth of cfDNA in ischemic cardiomyopathy, it is intriguing to decide if circling profiles of cfDNA vary as per cardiovascular breakdown etiology. The prognostic value of cfDNA would merit exploring tentatively. For instance, the current model standard biomarker of heart failure, NT-proBNP, neglects to precisely fail to accurately predict left ventricular remodelling leading heart failure after acute myocardial infarction which happens in one fifth of patients. cfDNA may offer incremental predictive value in this clinical setting, as shown beforehand for RNA's. Additionally sex differences should be an integral part. As a generally new biomarker, cfDNA has shown incredible promise in clinical work, including during pre-natal testing and as a fluid biopsy for cancer; however, very little is understood with respect to the mechanism of action in CVD. Further investigation of the relationship of CVD and cfDNA will upgrade its clinical utility to more readily address patients' requirements and treatment. To identify cfDNA with high specificity and reactivity, approaches have been created including, capillary based electrophoresis, droplet digital PCR and molecular index-based next genera- tion sequencing technologies. Every one of them have advantages and limitation related with increased sample variation during sample preparation steps, such as extraction and purification.

3.5 Limitation of the study
Limitation of this study is the small number of patients. Therefore, further confirmation of these data using more patient samples is needed before employing this ratio in the diagnosis of cardiovascular diseases.

4. CONCLUSION
In conclusion, this study found that plasma cfDNA has high efficacy in distinguishing apoptotic scenarios of Myocardial infarction from healthy individuals. Compared with Troponin I, plasma cfDNA may have higher sensitivity and diagnostic efficiency since Elevated concentrations of high-sensitivity troponin I or T have been independently associated with the risk of cardio- vascular death and hospitalization due to heart failure. It was considered that both cardiac troponins and cfDNA originate from the damaged cells in heart failure. However, it is not clear whether the concentration of troponin actually correlates with the degree of cardiomyocyte death. Because cardiac troponin levels are increased in renal failure, their diagnostic performance and interpretation are problematic when renal insufficiency coexists in the patients. In addition, troponin elevation may occur during reversible myocardial injury. In contrast, the copy numbers of cfDNA can be considered as those of the originating cells, indicating that content of DNA per cell may allow a quantitative inference of the actual rate of cfDNA shows a very short half-life of an hour or offering the advantages of real-time detection for car- diomyocyte death.

We suggest similar studies regarding cfDNA needs to be verified by an independent study with a larger sample. The auxiliary diagnostic model based on plasma cfDNA needs to be optimized with larger sample size. In recent years, with the progress of molecular biology technology, the detection level of cfDNA is also constantly improving, and its various detection method showing promising result, which can be used as an effective, non-invasive and sensitive detection method. But at the same time, we realize that there is still room for improvement in cfDNA. For example, cfDNA is low in blood concentration, easy to degrade and difficult to detect. Its detection method is unstable, there is no unified standard, and the quality is difficult to guarantee. The lack of clinical standardization has brought difficulties for the popularization of cfDNA. However, cfDNA detection has many advantages, such as real-time, minimally invasive, comprehensive, accurate and overcoming cardiac heterogeneity. It shows a good prospect in the clinical application of Cardiovascular diseases such as Myocardial infarction, cardiac angina, atrial fibrillation and last but not least in other cardiac abnormalities. With the continuous progress of liquid biopsy technology, combined with conventional markers and imaging
methods, the diagnosis and treatment of cardiac patients will be more accurate and individualized.

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REFERENCES


