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Levosimendan reduces plasma cell-free DNA levels in patients with ischemic cardiomyopathy

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Abstract Heart failure (HF) is a condition associated with the apoptosis and cell death of both cardiac myocytes and cardiac non-myocytes. DNA fragments released from programmed cell death or acute cellular injury are the main sources of disease-associated elevation of cell-free (cf) DNA. We hypothesized that cfDNA could be a relevant marker of cardiac apoptosis in HF patients that could be affected by the improvement of myocardial performance. To test our hypothesis, we measured plasma cfDNA in 19 patients with ischemic HF and severe left ventricular (LV) systolic dysfunction before and 12 h after completion of levosimendan infusion. Echocardiographic and biochemical markers of LV diastolic pressure and LV systolic function were also assessed. In accordance with previous observations levosimendan improved echocardiographic and biochemical indices of LV function. Plasma cfDNA was significantly reduced in HF patients post-levosimendan treatment (median: 89.4, interquartile range: 87.1 to median: 25.9, interquartile range: 12.3, $P = 0.028$). Notably, in 15/19 patients there was a reduction in cfDNA levels post-levosimendan infusion; while in 12/19 patients, a more than 50% reduction in plasma cfDNA was observed. Since cfDNA is a marker of tissue injury and apoptosis these results indicate that improvement of LV function has a potential impact on cell preservation and survival. Further studies are needed to substantiate our promising results regarding the role of plasma cfDNA as a marker of HF.

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Introduction

During the last two decades, significant advances in the quantitative analysis of plasma cell-free (cf) DNA have emerged. Technical innovations have resulted in the increased interest in the potential applications of plasma or serum cfDNA measurement. Research has focused on two major fields: diagnosis and prognosis of malignancies and early detection of pregnancy-associated disorders. In cancer patients, the levels of plasma or serum cfDNA have been correlated with metastasis, response to therapy and disease relapses [1–5]. Additionally, fetal cfDNA in the maternal circulation has been used for the detection and monitoring of fetal diseases and pregnancy-associated disorders [6, 7]. However, elevated levels of plasma cfDNA have been also detected in a variety of conditions. cfDNA levels have been associated with myocardial infarction, stroke and the activity of autoimmune diseases [8–11]. DNA fragments released from programmed cell death or acute cellular injury are presumed to be the main sources of disease-associated elevation of cfDNA [12–14].

Heart failure (HF) is a condition associated with low cardiac output, tissue hypoperfusion apoptosis and cell death of both cardiac myocytes, cardiac non-myocytes and extra-cardiac tissue [15–17]. Plasma cfDNA levels have yet to be assessed in patients with advanced stage HF.

It was postulated that cfDNA could be a clinical relevant marker of HF affected by alteration of left ventricular (LV) systolic function that can be the result of inotropic treatment. To test this hypothesis, we measured plasma cfDNA in patients with advanced stage of HF before and after levosimendan infusion.

Materials and methods

Patients and controls

Ninety patients were recruited from the outpatient HF clinic of the Army Veterans Hospital of Athens during a 1 year recruitment period. Patients with advanced stage HF are routinely followed up at the outpatient clinic and receive inotrope infusion for symptom relief when every other therapeutic option has been considered. Despite the fact that this is a common practice currently under investigation by the LevoPro study [18], there are no definite evidence-based data on the efficacy of intermittent infusions of inotropic agents in this severe clinical condition. Thus, the use of levosimendan in non-decompensated end-stage HF patients should be considered off-label. Inclusion criteria considered were: history of stable stage III or IV (according to the New York Heart Association classification) HF due to associated coronary artery disease and ejection fraction (EF) <0.35. Exclusion criteria included creatinine clearance (Clcr) <30, recent decompensation, and age <18 or >80 years. All patients were considered eligible for therapy with inotropes and were scheduled for levosimendan infusion. An echocardiographic assessment of LV and right ventricular (RV) function was performed before any intervention. Plasma NT-proBNP-levels were also measured in all patients before any intervention. Levosimendan was administered at an 8 μg/kg loading dose over 10 min followed by 0.1 μg/kg/min in a continuous infusion over 24 h. Echocardiographic and biochemical markers (NT-proBNP) of myocardial function were re-assessed 12 h after the completion of levosimendan infusion. Plasma NT-proBNP levels were measured using the Pathfast® immunoassay analyzer (Mitsubishi Chemical Europe). Height and weight (at discharge) of the case subjects were determined, and body mass index (BMI) was calculated. Basic biochemical indices were assessed on admission including serum creatinine. Clcr was calculated using the Cockroft–Gault formula. Twenty gender-matched subjects, with normal myocardial function, were recruited from the department's staff and formed the control group. Informed consent was obtained from all the individuals participating in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by a priori approval by the ethics committee of the Army Veterans Hospital of Athens.

Clinical definitions

Clinical characteristics including age, smoking habits, history of diabetes mellitus, dyslipidemia, and hypertension are reported in Table 1. Smoking was defined as a current, or a prior history of tobacco use. Diabetes was defined as a fasting blood glucose level higher than 126 mg/dl, or treatment with dietary modification, oral hypoglycaemic agents or insulin at the time of the study. Hypertension was defined as systolic blood pressure greater than 140 mmHg and/or diastolic pressure higher than 90 mmHg on at least three occasions, or if such a diagnosis had been made in the past and the patient was being treated with medication, or lifestyle modification. Dyslipidemia was defined as treatment with lipid lowering medication or dietary modification or lipid levels greater than those recommended by the Third Joint Task Force of European and other Societies on Cardiovascular Disease Prevention in Clinical Practice [19].

Echocardiographic assessment of myocardial performance

Left ventricular function was quantified utilizing the myocardial performance index (MPI), the left ventricular outflow tract velocity time integral (LVOTVTI) and the tissue Doppler systolic velocity of the mitral annulus (SmLV). Right ventricular function was quantified by the right ventricular outflow tract velocity time integral (RVOTVT) and the tissue Doppler systolic velocity of tricuspid annulus (SmRV). Ejection fraction (EF) was determined using the Simpson's biplane technique. All measurements were performed by the same operator and the same equipment (General Electric Vivid 7). Echocardiographic data obtained by the echo operator were evaluated independently by two experienced echocardiographers who were not aware of the medical status (pre- or post-levosimendan) of the patients. Measurements were made off-line and reported independently. No inconsistencies were observed in the reports.

Assessment of plasma cfDNA

Several publications indicate the strong influence of blood sampling, handling, storage and plasma preparation on DNA yield and DNA quality [20]. Therefore, the blood sampling procedure, handling, storage and plasma preparation method were kept constant in order to achieve the highest reproducibility. Venous blood samples (5.0 ml) were collected in K_3EDTA glass tubes; within 15 min of collection, the plasma was separated (by centrifugation at $2{,}000 \times g$ for 10 min) and stored in propylene tubes at –80°C until use. The plasma samples were thawed and re-centrifuged ($\geq 11{,}000 \times g$ for 3 min) to remove residual cells, cell debris, and particulate matter. cfDNA was extracted from 480 μl of the supernatant in two subsequent steps in the same column, with the use of the NucleoSpin Plasma XS kit (Macherey–Nagel, Duren, Germany), following the "high sensitivity" protocol and addition of proteinase K. cfDNA was eluted in 15 μl final volumes of water to result in highly concentrated cfDNA, followed by a heat incubation of the eluate at 70°C for not more than 5 min, in a heat block

Table 1 Clinical data of heart failure patients and fold increase/decrease in plasma cell-free DNA levels post-levosimendan treatment

ID	Age	Diabetes	Dyslipidemia	Hypertension	Smoking	Clcr	EF	NYHA	ACEI	ARB	Statin	ASA	BB	AA	Fold change of cfDNA
9	68				●	102	0.3	III					●	●	1.73
10	61	●		●	●	72	0.25	III		●		●	●	●	1.71
8	70	●	●	●		41	0.21	III		●	●	●	●		1.37
7	70	●	●	●	●	35	0.25	III		●		●	●		1.25
13	50	●	●	●	●	53	0.2	III	●		●	●	●	●	0.91
2	64	●	●	●	●	95	0.25	III	●		●			●	0.80
11	70	●	●	●		96	0.3	III		●	●		●	●	0.68
12	70			●	●	70	0.24	III		●		●		●	0.50
5	65	●	●	●	●	85	0.31	III	●		●	●	●	●	0.35
14	52	●	●	●	●	122	0.3	III		●	●	●	●	●	0.34
1	62		●	●	●	77	0.3	III	●					●	0.31
6	68	●			●	85	0.23	III	●			●		●	0.25
16	62	●	●	●		144	0.33	III		●	●	●	●	●	0.25
18	63	●		●	●	67	0.18	III	●			●	●	●	0.16
3	70	●		●		81	0.34	III	●			●			0.13
4	70		●	●	●	81	0.34	III			●	●			0.11
15	70			●		64	0.29	III		●		●	●	●	0.08
19	43		●	●	●	124	0.26	III	●		●	●	●	●	0.03
17	59				●	90	0.26	III	●			●	●	●	0.03

Clcr creatinine clearance, *EF* ejection fraction, *NYHA* New York Heart Association, *ACEI* angiotensin converting enzyme inhibitors, *ARB* angiotensin receptor blockers, *ASA* acetylsalicylic acid, *BB* beta blockers, *AA* aldosterone antagonists. *Fold change* refers to value post-treatment/value pre-treatment

without shaking. 5 μl of the appropriate dilutions were used for downstream quantitative polymerase chain reaction (qPCR) application. The β-globin gene was used to determine the amount of total cfDNA, using the Plexor qPCR System (Promega, Madison, WI, USA), as previously described [21]. qPCR analysis was performed with a Mx3000P Sequence Detector (Stratagene, La Jolla, CA, USA) and the raw data were further analyzed by the Plexor qPCR software (Promega, Madison, WI, USA). β-globin primer sequences were designed with the Plexor Primer Design Software, using all of the necessary parameters required for use with the Plexor qPCR System: 5′-ROX™-iso-dC-GGTCTCCTTAAACCTGTCTTGTAACC-3′ and 5′-GGGCAAGGTGAACGTGGATGAA-3′. "No template" and "positive control" reactions were run in parallel to ensure the absence of contamination of the DNA and to verify that the reagents and instrumentation performed consistently. 5 μl of the extracted plasma cfDNA and dilutions was used for amplification. The thermal profile was carried out using a 2 min incubation at 95°C, followed by an initial denaturation step at 95°C for 5 s, followed by 40 cycles of 35 s at 60°C. The reaction ended with a Melt–Curve Analysis in which the temperature was increased from 60 to 95°C at a linear rate of 0.2°C/s. The melting curves of qPCR were reviewed and did not show signs of non-specific amplification (Fig. 1). Each sample was analyzed in duplicate, and the average of the two measurements was used for analysis. To determine the number of copies of cfDNA present in the plasma sample, a standard dilution curve was run using a known concentration of a commercially available genomic DNA (Promega, Madison, WI, USA). We used eight serial fivefold dilutions, which ranged from 460 to 0.02944 ng (Fig. 2). A conversion factor of 6.6 pg of DNA per cell was used to express the results as copy numbers or genome equivalents (genome equivalents/milliliter), as previously described [19]. One genome-equivalent was defined as the quantity of a particular DNA sequence present in one diploid male cell. Strict precautions were taken pre-analytically, as well as during the experimental procedures in order to avoid qPCR contamination.

Statistical analysis

Normally and not normally distributed numerical values are expressed as the mean-standard deviation or the median-interquartile range, respectively. Kolmogorov–Smirnov test, was applied to asses normality of data sets. For normally distributed parameters differences between means were compared either using the independent sample *t*-test (for independent variables) or the paired sample *t*-test (for

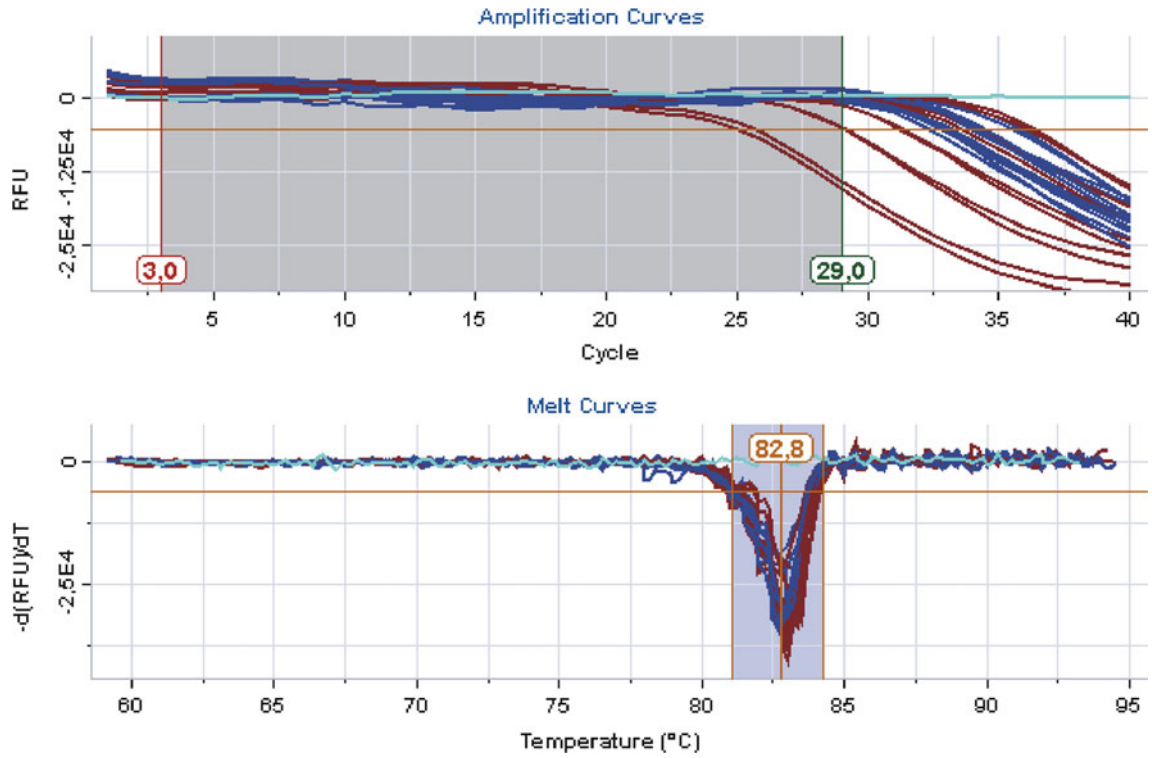

Fig. 1 Amplification curves (*up*) and melting curves (*down*) of the qPCR amplicons. *Brown color* denotes samples used in order to construct the standard curve (serial fivefold dilutions); *blue color* denotes unknown samples and *aqua color* denotes the "non-template control"

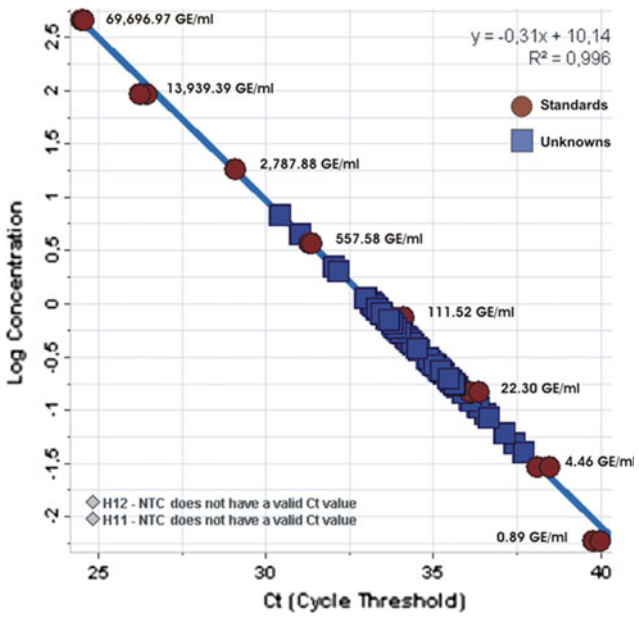

Fig. 2 To determine the number of copies of cfDNA present in the plasma sample, a standard dilution curve was run using a known concentration of a genomic DNA. We used eight serial fivefold dilutions, which ranged from 69,696.97 to 0.89 GE/ml. A conversion factor of 6.6 pg of DNA per cell was used to express the results as copy numbers or genome equivalents (genome equivalents/milliliter). The *Y*-axis shows the Log2 concentration, and the *X*-axis depicts the cycle threshold (*Ct*) values of each qPCR reaction

interpreting results before and after intervention). For not normally distributed parameters (plasma levels of NTproBNP and cfDNA) the Mann–Whitney *U* test (for unpaired variables) or the Wilcoxon Signed-Rank test (for interpreting results before and after intervention) was utilized. Pearson product-moment correlation coefficient was used to identify positive or negative linear correlation between variables. Linear regression analysis using plasma cfDNA levels or fold change of plasma cfDNA change as dependent variables and indices of myocardial performance as independent variables was also performed to identify the ability of plasma cfDNA to predict baseline myocardial function or treatment outcome. In all cases,a two-tailed probability value <0.05 was considered significant.

Results

Nineteen NYHA stage III male HF patients (median age: 65, range: 27) and 20 gender-matched healthy controls (median age: 35.5, range: 38) were recruited in the study. Successful cfDNA isolation and quantification were performed in 19 patients and 20 controls. Most relevant clinical data of the HF patients recruited in the study are summarized in Table 1.

Baseline plasma cfDNA levels in cases and controls

In accordance to previous observations no association was established between age and levels of plasma cfDNA in both case and control subjects ($r < 0.1$ in all data sets). Moreover, in our case sample no association was established between renal function assessed as Clcr or BMI and plasma cfDNA ($r < 0.1$ in both data sets).

Baseline plasma cfDNA levels and indices of myocardial performance

Higher levels of cfDNA were observed in HF patients compared to normal controls nevertheless the difference did not reached the cut off point of statistical significance (median: 89.4, interquartile range: 87.1 vs. median: 47.9, interquartile range: 57.7, $D = 0.28$, $P = 0.3$) (Fig. 3a). In a linear regression model including five echocardiographic markers of myocardial function a strong positive correlation was established between baseline MPI and baseline plasma cfDNA levels (Table 2). No association was established between baseline biochemical markers (NTproBNP) of LV function and levels of plasma cfDNA.

Plasma cfDNA levels before and after levosimendan treatment

In accordance to previous observations levosimendan significantly improved myocardial function assessed by MPI, $Sm_{LV,}$ and $Sm_{RV.}$ However, no significant changes were demonstrated in the VTI of either left or right ventricular outflow tract (Table 3). In a logistic regression model including five indices of myocardial performance, MPI was mostly affected by levosimendan infusion. Echocardiographic improvement in global myocardial performance assessed by MPI was observed in all patients treated with levosimendan. Both NTproBNP and plasma cfDNA levels were significantly reduced post-treatment (median: 3616, interquartile range: 7332 to median: 2730, interquartile range: 1630, $w = 140$, $P = 0.005$ and median: 89.4 interquartile range: 87.1 to median: 25.9, interquartile range: 12.3, $w = 110$, $P = 0.028$, respectively). Notably, in 15/19 patients there was a reduction of cfDNA levels post-levosimendan infusion; while in 12/19 patients, a more than 50% reduction in plasma cfDNA was observed (Fig. 3b).

Finally, in a linear regression model no association was established between plasma cfDNA reduction (fold change) and the degree of improvement (fold change) of echocardiographic markers of cardiac contractility (Table 4).

Discussion

Heart failure is the common outcome of diverse etiologies. It is characterized by impairment of ventricular systolic and diastolic dysfunction to various degrees. These functions are energetically and tightly coupled [22]. At the cardiomyocyte level, adenosine triphosphate is a substance essential for both active contraction and relaxation while at the myocardial level, regional wall motion asynchrony influences global LV diastolic function. Hemodynamically systolic function indirectly affects LV filling pressure [23].

The pathophysiology of HF involves multiple pathways and mechanisms, but the progressive loss of cardiac myocytes is the most prominent pathogenic component. The traditional explanation for myocyte loss is cell necrosis. However, over the last decade, there has been a surge of evidence affirming the role of apoptosis in the genesis of HF [24]. In the end-stage, HF apoptosis does not occur solely in myocardiac muscle. In humans and experimental models of HF, programmed cell death has been found in extra-cardiac tissue such as skeletal muscle and interstitial cells [15–17]. This cell death has been attributed to cytokine-induced pathways mostly involving tumour necrosis factor alpha (TNF-α) and growth hormone/insulin-like growth factor (IGF) [24]. The induction of apoptosis has been described and associated with the over-expression of programmed cell death-specific genes. Moreover, in atherosclerotic plaques, genetic alterations have been reported to occur, such as microsatellite instability and loss of heterozygosity [25]. Cell-free DNA concentrations are sensitive indicators of cellular damage originating from

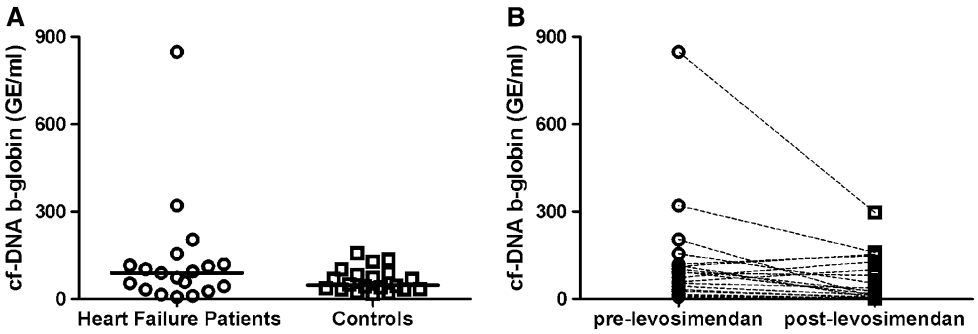

Fig. 3 Graphic illustration indicating plasma cfDNA levels in cases and controls (**a**) and levosimendan-induced plasma cfDNA alteration (**b**) *Gray lines* indicate medians

Table 2 Linear regression analysis correlating baseline levels of cfDNA plasma levels (depending variable) with fold change of myocardial performance indices before and after intervention

Model	Unstandardized coefficients		Standardized coefficients	*T*	*P*
	B	Std. error	Beta		
1(constant)	3213.313	1994.670	–	1.611	0.142
MPI	−1794.937	435.006	−0.819	−4.126	0.003
Sm_{LV}	8.567	22.746	0.099	0.377	0.715
$LVOT_{VTI}$	−6.816	9.774	−0.132	−0.697	0.503
Sm_{RV}	−2.927	17.925	−0.041	−0.163	0.874
$RVOT_{VTI}$	−74.876	130.117	−0.103	−0.575	0.579

cfDNA cell-free DNA, *MPI* myocardial performance index, *SmRV* systolic velocity of tricuspid annulus, *SmLV* systolic velocity of mitral annulus, $LVOT_{VTI}$ left ventricular outflow track velocity–time integral, $RVOT_{VTI}$ right ventricular outflow tract velocity–time integral

Table 3 Median values (interquartile range) of myocardial indices assessed in the study

	Pre levosimendan	Post-levosimendan	Two-tailed *P* value (Wilcoxon Signed-Rank Test)
MPI	1.08(0.08)	0.56(0.28)	0.001
Sm_{LV} (cm/s)	5(3)	8(4)	0.002
$LVOT_{VTI}$ (cm)	14.4(6.1)	15(6)	0.293
Sm_{RV} (cm/s)	10(5)	11(4)	0.04
$RVOT_{VTI}$ (cm)	14.2(0.48)	12(6.4)	0.096
EF Simpson	0.26(0.05)	0.35(0.08)	0.0001
HR (min^{-1})	86(23)	75(15)	0.02
$LVOT_{VTI}$ X HR	1155(572)	1050(435)	0.9

MPI myocardial performance index, *SmRV* systolic velocity of tricuspid annulus, *SmLV* systolic velocity of mitral annulus, $LVOT_{VTI}$ left ventricular outflow track velocity–time integral, $RVOT_{VTI}$ right ventricular outflow tract velocity–time integral, *EF* ejection fraction, *HR* heart rate

Table 4 Linear regression analysis correlating fold change of cfDNA plasma levels (depending variable) with fold change of myocardial performance indices before and after intervention

Model	Unstandardized coefficients		Standardized coefficients	*t*	*P*
	B	Std. error	Beta		
1(constant)	0.386	1.364	–	0.283	0.784
MPI	−0.964	1.008	−0.310	−0.956	0.364
Sm_{LV}	0.547	0.742	0.251	0.738	0.479
$LVOT_{VTI}$	−0.223	0.452	−0.168	−0.493	0.634
Sm_{RV}	0.595	1.066	0.236	0.559	0.590
$RVOT_{VTI}$	−0.491	0.808	−0.254	−0.608	0.558

cfDNA cell-free DNA, *EFLV* left ventricular ejection fraction, *BNP* brain natriuretic peptide, *MPI* myocardial performance index, *SmRV* systolic velocity of tricuspid annulus, *SmLV* systolic velocity of mitral annulus, $LVOT_{VTI}$ left ventricular outflow track velocity–time integral, $RVOT_{VTI}$ right ventricular outflow tract velocity–time integral

apoptosis or necrosis and thus are expected to differ among HF patients and healthy controls.

In the present study, quantitative real-time PCR was performed in order to evaluate plasma cfDNA in healthy individuals and patients with stage III HF scheduled for inotrope therapy. Levosimendan is a calcium sensitizer with rapid hemodynamic and clinical effects in patients with severe HF [26]. It exerts its inotropic effect by increasing the affinity of troponin-C for Ca^{2+}, directly stabilizing the Ca^{2+}-induced conformation of troponin-C, or acting distal to the troponin-C molecule. It binds in a Ca^{2+}-dependent manner to the N-terminal domain of troponin-C, thus magnifying the extent of the contraction produced by troponin-C when it is Ca^{2+}-activated. This leads to a positive inotropic effect without impairing diastolic relaxation or causing cytosolic Ca^{2+} ion overload,

which might provoke cardiac myocyte dysfunction, arrhythmogenesis, and cell death. Levosimendan's binding to troponin-C is dependent on cytosolic Ca^{2+}, and it is relatively weak (causing a minimum Ca^{2+} sensitization) during diastole, when intracellular Ca^{2+} levels are low. This is the reason why levosimendan enhances myocardial contractility and improves LV diastolic function. [27].

MPI is a surrogate of both diastolic and systolic function. A number of studies have documented that the MPI is independent of arterial pressure, heart rate, ventricular geometry, atrioventricular valve regurgitation, afterload, and preload in patients who are in a supine position [23]. In the present study MPI was the parameter most significantly affected by levosimendan infusion. The latter observation is in agreement with the previously known beneficial effects of levosimendan in both the diastolic and systolic function of the human heart.

Furthermore, levosimendan has a unique alternative mechanism of action including peripheral vasodilatation [27]. The anti-apoptotic properties of levosimendan may also be an additional mechanism which further inhibits the cytotoxic and hemodynamic consequences of abnormal immune and neurohormonal responses in HF [27]. It has been proposed that levosimendan exerts its cardioprotective effects through its antioxidant properties. It further seems to be a potent inhibitor of H_2O_2-induced cardiomyocyte apoptotic cell death [27, 28], Levosimendan administration causes a significant reduction in the circulating proinflammatory cytokine interleukin-6 and soluble apoptotic mediators, such as soluble FAS (sFAS) and Fas ligand in patients with decompensated HF [27–30].

In the present study we assessed a potential association between plasma cfDNA levels and cardiac performance, using two different approaches. Firstly, we tested whether HF patients have significantly higher cfDNA levels compared to healthy controls. Secondly, we demonstrated that improved cardiac function (systolic and diastolic), induced by levosimendan infusion, results in a reduction in plasma cfDNA levels in HF patients, suggesting that plasma cfDNA is possibly a marker of improved myocardial performance. On the other hand levosimendan improved cardiac contractility increased stroke volume but significantly reduced heart rate resulting in a non-significant alteration of cardiac output. The latter observation indicates a potential direct anti-apoptotic effect rather than an anti-apoptotic effect related to improved tissue perfusion.

We failed to demonstrate a statistically significant difference in plasma cfDNA levels between HF patients and controls. Nevertheless, in the absence of statistical power it was difficult to identify whether this was true lack of association or a type II error. Studies with greater statistical power need to address this point. Moreover, no association was established between cfDNA reduction and the degree of improvement in cardiac contractility. The study was also limited by the fact that only advanced stage HF patients were recruited; thus, no association could be made between baseline plasma cfDNA concentration and the clinical stage of HF. Finally, in order to avoid biases associated with the different etiology of HF, we limited our sample to ischemic cardiomyopathy patients. Thus, we can only assume that these findings can be expected in other forms of HF (dilated CM, hypertensive CM, valvular heart disease).

Conclusion

Our results provide promising evidence that plasma cfDNA is a potential marker of response to treatment in HF patients. However, further studies involving a greater number of patients, a variety of disease stages and a variety of disease etiologies are certainly needed. Besides an association with myocardial performance indices a marker should further provide prognostic information. Thus, any novel marker in HF should prove its ability to predict hard end-points against already established markers such as BNP and NTproBNP. Issues of cost effectiveness and bedside accessibility need to be further addressed.

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