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Correlation between the insertion-deletion variant of the angiotensin-converting enzyme gene and various classes of heart failure

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ABSTRACT

The angiotensin-converting enzyme (ACE) genetic variation for insertion/deletion (I/D) is located at the 16th intron of the ACE gene. A number of studies investigated the homozygous deletion genotype of ACE and its association with cardiovascular diseases. However, ACE's genetic variation and its association with heart failure (HF) is yet to be confirmed. We examined the possibility of the association between the ACE I/D gene variant with the severity of HF. The ACE genotypes were determined by polymerase chain reactions using samples derived from 150 patients with HF and 90 healthy subjects which were age and gender-matched. These patients included those of all four of the New York Heart Association (NYHA) classes. Echocardiography was performed on all HF patients and ejection fraction (EF), left ventricular systolic and diastolic diameters were measured. The HF patients were redistributed to systolic where EF is equal and less than 45% and non-systolic HF where EF is more than 45%. We demonstrate a statistically significant difference in DD genotype in NYHA class IV in comparison to the control group. The values of odds ratio (OR) (95%CI) of the DD genotype (DD vs ID and II) were 3.37 (1.01-11.19) (p value = 0.039) and the OR (95%CI) of the D allele (D vs I) was 2.55 (0.98-6.65) (p value = 0.049). Higher frequencies of D allele compared to I allele is linked to severity of HF. DD variant of the ACE gene is associated with NYHA class IV heart failure. This could have a profound impact on risk stratification and prognosis of HF in the management of this condition.

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Introduction

Heart failure (HF) is a multifactor disease that is primarily hereditary and environmental (1, 2). Our knowledge of the environmental factors that contribute to the occurrence of HF in individuals has advanced recently. This has resulted in the development of preventative measures and/or treatment of the disease (2). However, as far as genetic factors are concerned, except for uncommon monogenic diseases, our knowledge is limited to the importance of genetic factors related to HF (3-5). A finding of a link between the Angiotensin Converting Enzyme (ACE) gene variant and some cardiovascular diseases has been reported in the literature (6-8).

The pathophysiology of heart failure is molecularly associated with the renin-angiotensin system. Angiotensin-converting enzyme inhibitors are a class of pharmaceuticals that have been used routinely in the management of heart failure (9). Polymorphism such as insertion or deletion, i.e., the presence or absence of a 287-base pair (bp) alu repeat in the intron 16 on the ACE gene on chromosome 17 in humans have been found. Higher concentration of ACE in the circulation has been linked with deletion allele (10, 11) and this has ultimately been associated with multiple cardiovascular diseases such as left ventricular hypertrophy (LVH), myocardial infarction, and essential hypertension as well as HF (6, 12-15). However, other studies showed a lack of correlation between the ACE genotype and HF (16, 17).

The recognition of variation in the ACE gene plays a crucial role in the optimum management of HF in the future. Therefore, the aim of the current study is to investigate the association of ACE genetic variants with the severity of HF. In addition, this study aims to identifies the relation of insertion/deletion (I/D) variations of the ACE gene according to the NYHA classification of HF (18). Moreover, the same identification of I/D variations is going to be employed for systolic and non-systolic HF patients. Furthermore, the association of echocardiographic results such as ejection fraction (EF), left ventricular systolic diameter (LVSD) and left ventricular diastolic diameter (LVDD) will be investigated in DD, ID and II ACE genotyped patients.

Materials and Methods

Study design and sample

In order to determine the connection of ACE I/D gene polymorphism with the severity of HF, we conducted a case-control study. Samples were consecutively collected in the period between January 2021 to September 2022 from a total of 240 subjects selected from cardiac centre hospitals and cardiologist clinics in Erbil city/Iraq. These were comprised of 90 healthy participants and 150 patients

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diagnosed with heart failure. Both groups were recruited by a cardiologist during routine check-up visits.

Ethical considerations

The study procedure was approved on 25th November 2020 by Hawler Medical University/ College of Pharmacy's committee for ethics under the approval number HMU-PH-EC 25112020-119.

Variables

Patients were diagnosed with HF and further classified into groups according to NYHA classification based on heart function (18). Moreover, HF patients in this study were redistributed into systolic and non-systolic HF based on EF from echocardiograph measurements. Systolic HF is identified when EF is ≤ 45% according to Maisel et al, (19). Other parameters of echocardiograph results such as LVSD and LVDD were ordered for all HF patients. Siemens Medical Solutions USA, (made in Korea, model number 10133170) was the echocardiograph device used. ACE II, ID and DD genotypes were compared between each NYHA class and control. Three models analysis; regressive model (DD vs ID and II), dominant model (DD and ID vs II) and allelic model (D allele vs I allele) were chosen. The same comparisons were carried out for systolic and non-systolic HF. The models of the ACE gene have been carried out in previous studies (20). In addition, three echocardiographic parameters were individually compared between ACE II, ID and DD genotypes.

Experimental procedure

Genomic DNA was used from whole peripheral blood (200 µl) for genotype analysis. AddPrep Genomic DNA Extraction Kit (made in Korea, www.addbioinc.com) was used to extract the genomic DNA in accordance with the manufacturer's instructions. The concentration and purity of the eluted genomic DNA were measured by using Nanodrop (NANODROP 1000 Thermo Scientific). The region of insertion and deletion of the ACE gene was amplified using oligoprimers. 319 and 597 bp amplicons for alleles of deletion and insertion, respectively were produced from 5'GCCCTGCAGGTGTCTGCAGCATGT-3' (sense primer) and 5' GGATGGCTCTCCCCGCCTTGTCTC-3' (antisense primer) (21, 22). 0.5 µM primers were used in 25 l total volume reaction. 100 g of human DNA sample was also added to the reaction in a polymerase chain reaction (PCR) plate. The plate was placed in a thermal cycler (Applied Biosystems 2720 thermal cycler) and ran for 35 cycles. Cycles included denaturation, annealing, and extension at 94° C for 30 sec, 56°C for 45 sec, and 72°C for 2 min respectively. A final extension step was performed at 72°C for 7 min. The amplification products of D and I alleles were examined and semi-quantified applying 1.5% agarose gel electrophoresis. Samples that had the DD genotype, due to their heterozygous nature and consequent D allele's preferential amplification rates (21), were amplified again using an independent PCR reaction. Sense 5'TGGGACCACAGCGCCCGCCACTAC-3' and antisense 5'TCGCCAGCCCTCCCATGCCCATAA-3' primer pairs that recognise an insertion-specific sequence were used in the second PCR reaction identical to the first rection with an exception to the annealing step which was carried out at 70°C. The reaction resulted in a 335 bp band only when I allele was present but not in the homozygous DD sample. The four to five percent of samples with the heterozygous ID variant that are usually mischaracterised as homozygous DD were correctly identified with the insertion-spanning primers (22).

Statistical analysis

Statistical analysis was conducted using SPSS 27.0 statistical package. To determine statistical variances in ACE II, ID and DD genotypes between all HF patients and control, each NYHA class and control, either systolic or non-systolic HF and control, a chi-square test was performed. Odds ratios (OR), confidence intervals (CI) and P-values were calculated. An independent sample t-test was applied to compare age and weight characteristics between HF groups and control. All the echocardiographic outcomes were analysed by ANOVA. The data were presented as mean ± SEM. Differences were considered significant if the P-value was less than 0.05.

Results

A total of 240 consecutive subjects participated in the current study which comprised 90 healthy subjects and 150 HF cases. Healthy Participants aged 60.39±0.51 years and were a combination of 58 males and 32 females. The patient group aged 61.7±0.96 years and were a combination of 106 male and 44 female. The mean weight of HF patients and the control group were 77.22±0.86 and 75.98±0.62 kg, respectively. The number of smokers in the HF patient group was 66 and non-smokers were 84 while the control group was comprised of 42 smokers and 48 non-smokers. 47% of the control group were smokers whereas 44% of HF patients were smokers. Furthermore, there was not a significant difference between the age of the control and patient group (P=0.312). The 150 HF patients were classified according to the NYHA classification. Further baseline characteristics such as the number of patients, age, smoking, co-present diabetes mellitus (DM), gender, left ventricular ejection fraction (LVEF), LVSD and LVDD of NYHA-classified HF patients' subgroups are illustrated in Table 1. The number of patients recorded in NYHA I, II, III and IV subgroups were 19, 62, 56 and 13, respectively. Average age was recorded as 56.16, 60.52, 63.82 and 66.38 years in each of NYHA I, II, III and IV subgroups, respectively. The percentage of men in NYHA I, II, III and IV subgroups were 68, 81, 64 and 54, respectively.

The identification of insertion and deletion of the ACE genotype was carried out by gel electrophoresis. D allele was located at 319 bp while the I allele appeared at 597 bp. Bands appeared on the 297 bp position considered homozygous for DD whereas bands appeared on the 597 bp position considered homozygous for II. If bands appeared on both 319 and 597 bps positions, they were considered heterozygous for ID of the ACE genotype (Figure 1). In the control group, D and I alleles recorded frequencies of 0.567 and 0.433, respectively. While in the patient group, these values were 0.65 and 0.35, respectively, which were both consistent with Hardy-Weinberg equilibrium. ACE genotype percentage of II, ID and DD in control recorded 19%, 49% and 32%, respectively. However, in the patient group the percentage of II, ID and DD were 14%, 42% and 44%, respectively. These frequencies of D and I alleles, and genotype percentages of II, ID and DD were determi-

Table 1. Baseline characteristics of NYHA classified subgroups of heart failure patients.

	Heart Failure Patients				
	Subgroup 1	**Subgroup 2**	**Subgroup 3**	**Subgroup 4**	**All HF cases**
NYHA classification	I	II	III	IV	
LVEF (%)	50.05	42.61	42.7	34.23	42.86
LVSD (mm)	35.74	42.02	42.75	46.31	41.87
LVDD (mm)	49.73	54.56	55.39	56.92	54.47
Male (%)	68	81	64	54	71
DM (%)	42	42	54	62	48
Smoker (%)	21	48	46	46	44
Age (year)	56.16	60.52	63.82	66.38	61.7
Weight (kg)	78.47	79.24	75.09	74.92	77.22
Patients (number)	19	62	56	13	150

NYHA: The New York Heart Association, LVEF: left ventricular ejection fraction, LVSD: Left ventricular systolic diameter, LVDD: Left ventricular diastolic diameter, DM: diabetes mellitus.

Table 2. Record of ACE genotypes and frequencies of alleles of the insertion and deletion variations in control, heart failure patients and NYHA-classified patients' subgroups.

	ACE Genotypes No. (%)			Alleles No. (Frequency)	
	II	**ID**	**DD**	**I**	**D**
Control Subjects (n = 90)	17 (19)	44 (49)	29 (32)	78 (0.433)	102 (0.567)
all HF patients (n = 150)	21 (14)	63 (42)	66 (44)	105 (0.35)	195 (0.65)
NYHA class I (n = 19)	2 (11)	9 (47)	8 (42)	13 (0.342)	25 (0.658)
NYHA class II (n = 62)	11 (18)	24 (39)	27 (43)	46 (0.371)	78 (0.629)
NYHA class III (n = 56)	7 (13)	26 (46)	23 (41)	40 (0.357)	72 (0.643)
NYHA class IV (n = 13)	1 (8)	4 (31)	8 (61)	6 (0.231)	20 (0.759)

ACE: angiotensin-converting enzyme, NYHA: The New York Heart Association, I: insertion, D: deletion, n: number. DD: homozygous deletion for the ACE gene. ID: heterozygous insertion/deletion for ACE gene. II: homozygous insertion for the ACE gene.

ned more specifically for NYHA classes as shown in Table 2. Frequencies of the D allele were 0.658, 0.629, 0.643 and 0.759, in NYHA class I, II, III and IV, respectively. Moreover, percentages of the DD genotype in NYHA class I, II, III and IV were 42, 43, 41 and 61, respectively. Furthermore, Gene models selected for the current work were the regressive model (DD vs ID and II), dominant model (DD and ID vs II) and allelic model (D vs I). All statistics were carried out by OR, 95% CI and P value as shown in Table 3. The comparison of the three models was carried out as it has been done previously (20) for all HF patients including HF NYHA classes against control. Two models (DD vs ID and II) and (D vs I) were statistically significant in NYHA class IV in comparison to the control group. The value of OR (95%CI) of the regressive effect of the DD genotype (DD vs ID and II) was 3.37 (1.01-11.19) and P value was 0.039. Moreover, the OR (95%CI) of the allelic effect of the D allele (D vs I) recorded 2.55 (0.98-6.65) and the P value was 0.049. However, the three models were statistically non-significant when all HF patients were compared to the control. Furthermore, these values were also non-significant in NYHA class I, II and III against control as shown in Table 3.

To determine the association of ACE genotype with systolic and non-systolic HF groups, we redistributed all HF patients into systolic and non-systolic HF subgroups. The percentage of II, ID and DD genotypes were 18%, 38% and 44%, respectively in systolic HF cases. These percentages were 8%, 49%, and 43%, respectively for the non-systolic HF group as shown in Table 4. The same regressive model (DD vs ID and II), dominant model (DD and ID vs II) and allelic model (D vs I) analysis were employed in systolic and non-systolic HF groups against control. There were no significant differences in the three models between the groups tested against control group

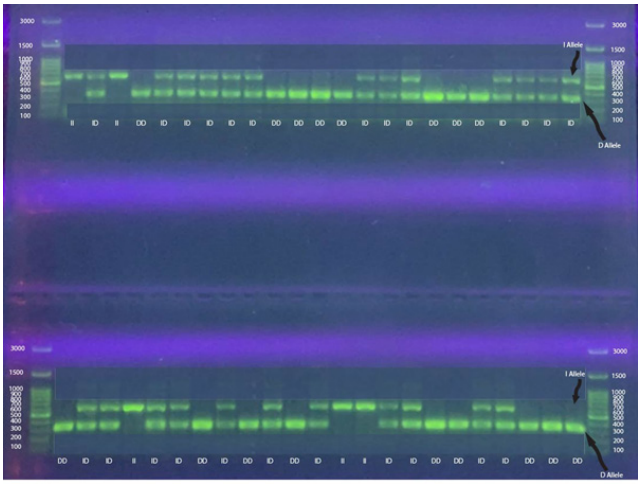

Figure 1. Identification of angiotensin-converting enzyme variation by PCR amplification and 1.5% agarose gel electrophoresis. DD: homozygous deletion for the ACE gene. ID: heterozygous insertion/deletion for ACE gene. II: homozygous insertion for the ACE gene.

Table 3. Correlation of ACE gene polymorphism to the severity of HF based on ACE genotypes for all HF samples or each NYHA class against control.

	Odds Ratio (95% CI)	P values
All HF case vs control		
DD vs ID and II	1.65 (0.96 - 2.86)	0.071
DD and ID vs II	1.43 (0.71 - 2.88)	0.315
D allele vs I allele	1.42 (0.97 - 2.07)	0.069
NYHA class I vs Control		
DD vs ID and II	1.53 (0.56 - 4.21)	0.408
DD and ID vs II	1.98 (0.42 - 3.39)	0.383
D allele vs I allele	1.47 (0.707 - 3.06)	0.3
NYHA class II vs Control		
DD vs ID and II	1.62 (0.83 - 3.19)	0.155
DD and ID vs II	1.08 (0.47 - 2.50)	0.858
D allele vs I allele	1.30 (0.81 - 2.07)	0.277
NYHA class III vs Control		
DD vs ID and II	1.66 (0.84 - 3.29)	0.147
DD and ID vs II	1.63 (0.63 - 4.22)	0.311
D allele vs I allele	1.38 (0.85 - 2.29)	0.197
NYHA class IV vs Control		
DD vs ID and II	3.37 (1.01 - 11.19)	0.039*
DD and ID vs II	2.80 (0.34 - 12.98)	0.32
D allele vs I allele	2.55 (0.98 - 6.65)	0.049*

*Statistically significant in P values. ACE: angiotensin-converting enzyme, HF: heart failure, CI: confidence interval, NYHA: The New York Heart Association, DD: homozygous deletion for the ACE gene, ID: heterozygous insertion/deletion for ACE gene, II: homozygous insertion for the ACE gene.

Table 4. Record of ACE genotypes and frequencies of alleles of the insertion and deletion variations in control, heart failure patients, and systolic and non-systolic classified patient subgroups.

	ACE Genotypes No. (%)			Alleles No. (Frequency)	
	II	ID	DD	I	D
Control Subjects (n = 90)	17 (19)	44 (49)	29 (32)	78 (0.433)	102 (0.567)
All HF patients (n = 150)	21 (14)	63 (42)	66 (44)	105 (0.35)	195 (0.65)
Systolic HF (n = 90)	16 (18)	34 (38)	40 (44)	66 (367)	114 (0.633)
Non-systolic HF (n = 60)	5 (8)	29 (49)	26 (43)	39 (0.325)	81 (0.675)

ACE: angiotensin-converting enzyme, I: insertion, D: deletion, n: number. DD: homozygous deletion for the ACE gene. ID: heterozygous insertion/deletion for ACE gene. II: homozygous insertion for the ACE gene.

(Table 5).

Echocardiographic measurements such as EF, LVSD and LVDD have been measured for ACE genotyped groups. Figures 2 and 3 demonstrate the echocardiographic data of all HF patients. The mean ± SEM values of EF percentage for each of the II, ID and DD genotype groups were 42.38±2.18, 44.33±1.39 and 42.64±1.44, respectively which were statistically non-significant (P value 0.384) (Figure 2). Moreover, the LVSD of II, ID and DD groups were statistically non-significant (p-value = 0.397) with a mean ± SEM of 42.29±1.65 mm, 40.83±1 mm and 42.73±1.02 mm, respectively (Figure 3A). Furthermore, LVDD of II, ID and DD groups were statistically non-significant (p-value = 0.675) with mean ± SEM values of 54.57±1.38 mm, 53.9±0.86 mm and 54.97±0.86 mm, respectively (Figure 3B).

Discussion

Heart failure is a chronic condition where hereditary and environmental factors contribute to its progression. The presence of the D allele on the ACE gene and its association with a higher risk of developing heart failure is an interesting concept (13, 23) and requires investigation. Therefore, past research in this area has focused on the relationship of the ACE DD genotype with HF (13, 16, 17, 24). The results have been mixed so far and currently, there is no consensus whether there is a significant relation of D allele polymorphism to HF. The reason for that could partially be due to the fact that these studies have looked at all HF patients with different signs, symptoms and degrees of severity similarly rather than subgrouping them based on the aforementioned factors. With this approach, the relation between ACE gene polymorphism and subclasses of HF patients could be missed. Therefore, in the current study, we asked whether the ACE DD genotype would be related to a subclass of HF rather than the occurrence of HF.

Therefore, we classified all HF patients according to the severity of HF into NYHA classes. Our results confirm

Table 5. Correlation of ACE gene polymorphism to the severity of HF based on ACE genotypes for all HF samples or systolic and non-systolic HF against control.

	Odds Ratio (95% CI)	P values
All HF case vs control		
DD vs ID and II	1.65 (0.96 - 2.86)	0.071
DD and ID vs II	1.43 (0.71 - 2.88)	0.315
D allele vs I allele	1.42 (0.97 - 2.07)	0.069
Systolic HF vs Control		
DD vs ID and II	1.68 (0.92 - 3.09)	0.092
DD and ID vs II	1.08 (0.51 - 2.29)	0.847
D allele vs I allele	1.32 (0.87 - 2.02)	0.197
Non-systolic HF vs Control		
DD vs ID and II	1.61 (0.82 - 3.16)	0.167
DD and ID vs II	2.56 (0.89 - 7.37)	0.73
D allele vs I allele	1.59 (0.98 - 2.57)	0.059

ACE: angiotensin-converting enzyme, HF: heart failure, CI: confidence interval, DD: homozygous deletion for the ACE gene, ID: heterozygous insertion/deletion for ACE gene, II: homozygous insertion for the ACE gene.

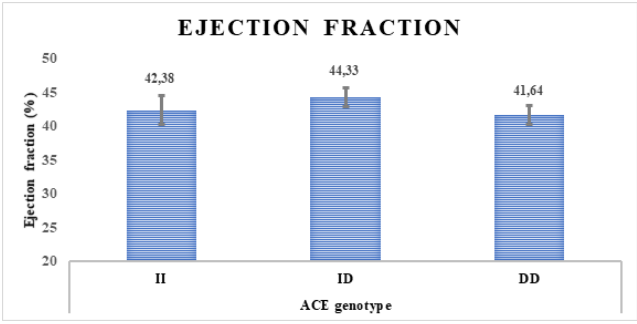

Figure 2. Ejection fraction (%) of II, ID and DD genotyped groups. The differences in mean values are not significant. II: homozygous insertion for the ACE gene, ID: heterozygous insertion/deletion for the ACE gene, DD: homozygous deletion for the ACE gene.

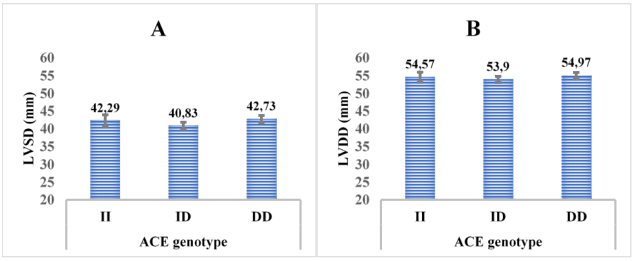

Figure 3. Left ventricular systolic (A) and diastolic (B) diameters of II, ID and DD genotyped groups. The differences in mean values are not significant for both LVSD and LVDD. LVSD: Left ventricular systolic diameter, LVDD: Left ventricular diastolic diameter, II: homozygous insertion for the ACE gene, ID: heterozygous insertion/deletion for ACE gene, DD: homozygous deletion for the ACE gene.

that the ACE genotype is correlated to the severity of HF. The percentage of DD genotype vs ID and II was significantly higher in NYHA class IV patients with the highest severity of HF compared to the control group. Therefore, these results suggest that the risk of developing NYHA class IV HF is three times higher in individuals carrying the AEC DD genotype compared to individuals carrying ID and II gene polymorphism. Moreover, the difference in DD genotype was not significant in other NYHA classes with mild and moderate severity of HF in comparison to control subjects. Studies that have not been able to show a significant difference in DD genotype between HF patients and control might be due to having a higher number of patients in NYHA class I, II and III collectively compared to NYHA class IV. Furthermore, this effect could be explained by pathophysiological mechanisms. Individuals carrying DD gene polymorphism have significantly higher serum levels of ACE (25, 26) which in turn have been linked to developing cardiovascular conditions such as hypertension, myocardial infarction, atherosclerosis including HF (27-30) and consequently developing NYHA IV HF. Studies have shown at least a two-fold increase in plasma ACE levels in individuals carrying DD genotypes in comparison to those carrying II and ID ACE gene polymorphism (26, 31).In addition, higher levels of plasma ACE have been linked to inflammatory responses indicated by an increase in Interleukin-6 which in turn cause coronary plaque vulnerability and worsening cardiovascular conditions (25).

We further classified all HF patients to systolic and non-systolic HF to determine their correlation to ACE genotypes. The same three ACE gene models were analysed in systolic and non-systolic HF groups compared to the control. The comparison analysis in regressive, dominant and allelic models was not statistically significant in both systolic and non-systolic HF compared to control. Our results are consistent with previous studies (32).

In addition, the classification of all HF patients based on ACE genotypes such as II, ID and DD to determine the difference in echocardiographic measurements was carried out. Although the worst echocardiographic outcomes were associated with the ACE DD genotype compared to ACE II and ID genotypes, the echocardiographic results of EF, LVSD and LVDD were not significant between groups of ACE genotypes. Albuquerque et al. conducted a study on 111 Brazilian HF patients and concluded the same outcomes (33). Another study was conducted to examine the effect of ACE gene variation on the echocardiographic findings of 103 patients with HF and a lack of association between them was revealed (34-36).

To the best of our knowledge, this is the first study to determine a significant relation of DD gene polymorphism to a group of HF patients amongst other HF patients based on the NYHA classification. These results suggest that

there could be a correlation between DD gene polymorphism and the severity of HF. This research opens avenues to further investigate the correlation between DD gene polymorphism to HF based on specific factors such as gender and ethnicity rather than randomly looking at HF as a single-angled condition.

In conclusion, our findings reveal the association of the ACE DD genotype with NYHA class IV HF. Establishing this connection would advance the management protocol of HF, particularly in its risk stratification and prognosis. Future research should concentrate on reinforcing the current study in order to be able to reach the point where we can make better decisions in the management of NYHA class IV HF based on early detection of DD genotype.

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Competing interests
The authors declare that the research was conducted in the absence of any financial or non-financial interests that could be considered as potential conflicts of interest.

Data availability
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The request for data should be forwarded to Rawaz D. Tawfeeq.

Author Contributions
Rawaz Tawfeeq (RT), Ava Ismael (AI) and Mohammed Alwan (MA) contributed to the conception and initiation of the research. RT, AI and MA designed the experiments. AI and MA supervised the study. RT, AI and MA conducted the experiments. RT, AI and MA collected and analysed the data. RT wrote the first manuscript AI and MA reviewed the manuscript. All authors approved the submitted version of the manuscript.

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