Research Article

The Role of *EDN-1* and *iNOS* genes expression in Cardiovascular Diseases

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Abstract

One of the key regulators that contributes to several pathologies, including cardiovascular diseases, is the peptide known as endothelin-1 (ET-1), along with nitric oxide (NO); both of these molecules orchestrate the vasoconstriction and vasodilation of blood vessels, which play a pivotal role in CVD.In this regard, many research laboratories have studied the functions of endothelin-1 and nitric oxide in relation to CVD. The aim of this study was to investigate the role of endothelin-1 and nitric oxide in CVD patients at the gene expression levels. A case-control research was performed in Kirkuk Governorate from December 2024 to April 2025, with 75 participants aged 50 to 80 years, comprising 50 patients with cardiovascular disease under the supervision of a specialist physician for treatment and clinical monitoring. A control group including 25 persons devoid of cardiac disease was established. The result of the present study investigating endothelin-1 protein concentrations was markedly lower in cardiovascular disease patients than in healthy controls. The mean Endothelin-1 level was (356.63 ± 106.89, 452.07± 86.53 pg/mL, respectively, at a p-value ≤ 0.001. Nitric oxide protein concentrations were markedly higher in cardiovascular-disease patients than in healthy controls. The mean nitric oxide level was (110.39 ± 43.89, 86.40 ± 27.78) pg/mL, at a p-value ≤ 0.001. Gene expression analysis revealed significant differences between the CVD patient group and the control group. *EDN1* gene expression was significantly lower in the patients compared to the control group, with a mean normal expression of 0.070 versus 1.00 in the control group, representing a 14.25-fold decrease. In contrast, iNOS gene expression was significantly higher in the CVD patients, with a mean expression of 2.287 compared to the control reference value (1.00), representing a 2.29-fold increase. The present study demonstrated endothelin-1 highly decreased protein levels, while NO was shown to be overexpressed in cardiovascular disease. In contrast, downregulation of *EDN-1* gene expression and upregulation of *iNOS* gene expression are indicative of promising approaches in CVD applications and clinical trials.

Keywords:

Cardiovascular disease, Endothelin-1, Nitric oxide, *EDN-1* gene, Inducible nitric oxide synthase *(iNOS)* gene

Introduction:

Cardiovascular diseases (CVD) are a significant contributor to morbidity and mortality. The endothelium contributes to atherogenesis, and endothelial dysfunction is implicated in the initiation and progression of CVD. Endothelial cells which form the vascular layer synthesize, then release into circulation a variety of bioactive molecules working on vascular smooth muscle cells in an autocrine and paracrine way[1]. These bioactive molecules, which are primarily sourced from the endothelial layer, collectively regulate inflammation in the blood vessels[2]. Endothelial dysfunction leads to diminished availability of nitric oxide and prostacyclin, vasoconstriction, oxidative stress, inflammation, and platelet activation [3].

ET-1 is a powerful vasoconstrictor composed of 21 amino acids. ET-1 is encoded by the *EDN1* gene and is predominantly produced by vascular endothelial cells [4]. ET-1, a vasoconstrictive factor, attaches to its receptor to elicit its biological effects. ET-1 has been identified as a predictor of negative outcomes, including mortality, in patients following acute myocardial infarction [5]. Higher ET-1 levels are also related to augmented peripheral vascular resistance and are involved in the vascular remodelling in conditions such as hypertension and CAD [6, 7].

Nitric oxide (NO), also known as endothelial-derived relaxing factor (EDRF), is among the few gaseous signalling chemicals synthesised by the endothelium. It stimulates guanylatecyclase and promotes vasodilation by

dephosphorylating the myosin light chains in smooth muscle cells [8]. It facilitates vessel homeostasis by inhibiting vascular smooth muscle contraction and proliferation, platelet aggregation, and leukocyte adhesion. Phagocytes, including monocytes, macrophages, and neutrophils, also generate it as part of the human immune response. Phagocytes contain inducible nitric oxide synthase (iNOS). In healthy conditions, endothelial stimulation induces the production and release of nitric oxide (NO), which diffuses to neighbouring tissues and cells. It serves its cardiovascular preventative role by relaxing medial smooth muscle cells, reducing leukocyte adherence and migration into the arterial wall, preventing muscle cell proliferation, platelet adhesion and aggregation, and the synthesis of adhesion molecules[9].

ET-1and NO, vasoactive molecules produced by endothelial cells, exert promoting and protective effects on CVD, respectively[10, 11]. The gene expression of ET-1and NO plays a central role in the pathophysiology of CVD. Dysregulated ET-1 gene expression contributes to impaired vasomotor control in CVD. Moreover, upregulation of iNOS gene expression under inflammatory pathological conditions may lead to the excessive production of NO. Although NO is generally considered vasoprotective, its overproduction can promote the formation of reactive nitrogen species, such as peroxynitrite, thereby inducing oxidative stress and endothelial cell damage rather than exerting protective effects. This mechanism has been implicated in the progression of endothelial dysfunction and vascular inflammation in various cardiovascular diseases [9, 12].Several intracellular signalling pathways particularly the PI3K pathway are activated in stressful and inflammatory conditions such as hypoxia and exposure to proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin1β(IL-1β), when turned on, the transcription factor nuclear factor- kappa B (NF-κB) moves into the nucleus and makes iNOS gene more active. The iNOS enzyme turns L- arginine into NO and L-citrulline, which makes more NO. Elevated NO decrease activity and expression of *EDN-1*gene a strong vasoconstrictor, which cause vasodilatation and anti-inflammatory effects [13], as shown in Figure (1).

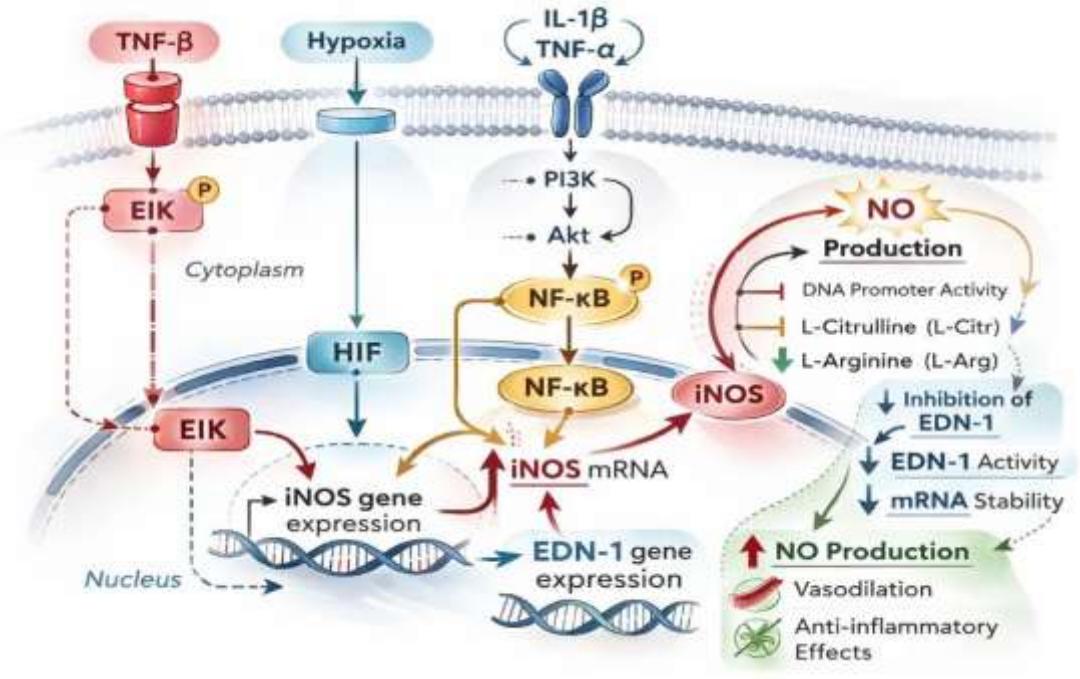

Figure (1): The role of nitric oxide in inflammation and vasodilation

Materials and Methods

Study Design

A case-control research was undertaken in Kirkuk Governorate from December 2024 to April 2025, with 75 participants aged 50 to 80 years, including 50 patients with cardiovascular disease, who were under the care of a specialist physician for treatment and clinical monitoring. A control group including 25 persons devoid of cardiac disease was established.

Inclusion criteria

Patients experience stroke, acute or recent (within past six months), myocardial infarction, or another acute cardiovascular event.

Exclusion Criteria

1.Patients with thyroid problems.

2.Smokers.

3.Individuals who have had cancer or are currently receiving radiation or chemotherapy.

Samples Collection

Samples were collected from patients admitted to the cardiac care unit and controls. 5 ml blood samples were obtained in the morning between 8 and 9 a.m. and then distributed into two separate tubes.The first portion was transferred into a gel separator tube and allowed to clot at room temperature for 20 minutes. All samples were centrifuged at 3000 rpm for 15 minutes. Then the serum was then separated and aliquoted into two Eppendorf tubes (500 µl per aliquot) and stored at -20°C until the time of analysis, which included ET-1 and NO.The second portion (2.5 ml) was collected in an EDTA tube to prevent coagulation. The blood was gently mixed and agitated for 5 minutes to ensure homogeneity. The samples were then stored at -20°C for subsequent RNA extraction and molecular diagnostic procedures.

Ethical Approval

Ethical approval for this study was secured from Kirkuk General Hospital in Kirkuk city. The Scientific Council of the Faculty of Science at Kirkuk University officially approved the research protocol, which had earlier accepted the technique. All participants in the study were apprised of its goal, methodology, inherent risks and benefits, and objectives. Before data and sample collection, each subject submitted signed consent. The study rigorously upheld the confidentiality and anonymity of participants as per document number 167 (dated 24/2/2024).

Gene Expression

Primer selection and preparation:

The primers for *EDN-1* and *iNOS* genes were designed using Primer 3plus V4 and double-checked by the University of California, Santa Cruz (UCSC) programs and with their reference sequences in the database of the National Center for Biotechnology Information (NCBI) as illustrated in Table 1. The expression of B2M was used as an internal control to normalize the data.

RNA Extraction

Ribonucleic acid (RNA) was extracted following manufacturer's instructions (GeneAll® Hybrid-R™ Blood RNA kit-Korea) (Cat. No. 315-150). The purity and concentration of the extracted RNA were measured using a Quintus Fluorimeter (Promega, USA) [14].

Synthesis of Complementary DNA

After RNA (total RNA) was extracted from both cardiovascular and control samples, the mRNA was reverse transcribed to synthesize complementary DNA (cDNA) using the AddScript RT Master (2x Conc.) kit. The total RNA containing mRNAs was converted into cDNA by the reverse transcriptase enzyme. The cDNA was performed to quantify the expression level of ET-1 and iNOS genes, which were associated with perfect yield, reflecting efficient reverse transcription using specific primers by using qRT-PCR.

Quantitative Reverse Transcription Real Time PCR

Levels of gene expression of ET-1 and NO were estimated by quantitative reverse transcription polymerase chain reaction (qRT–PCR) for confirming the expression of target genes, using RealQ Plus 2x Master Mix Green (Without ROX™) from Ampliqon (Made in Denmark). The gene expression levels and fold change were quantified by estimating the threshold cycle (Ct) employing the qPCR Kits components. The gene expression, high Ct value indicate low gene expression and low Ct-value indicates a high gene expression. The lower Ct-value indicates the presence of higher copies of the target gene and vice versa (Nolan et al., 2006). As shown in Table (1).

Table 1: Designed primers used in this study gene.

Primer name	Sequences	Annealing Temp (° C)	Reference
ET-1-F	5′ CTACTCTGGCACCTGGACATC 3′	65	Design in this study
ET-1-R	5′ TCACGGCTGTTGCCTTTGTGG 3′		
iNOS -F	5'GCTCTACACCTCCAATGTGACC 3'	63	
iNOS -R	5' CTGCCGAGATTTGAGCCTCATG 3'		
B2M	5' GTGCAGGATTCAGACTTGTCTTT 3'	65	
B2M	5' TGCTTACATGTCTCGATCCCAC 3'		

The qRT–PCR reaction condition for ***EDN-1*** and ***iNOS*** genes was programmed according to the thermal profile shown in Table (2).

Table 2: The qRT-PCR reaction conditions for amplification of *EDN-1* gene and *iNOS*gene

Steps	Duration of cycle	Temperature	Cycle
Initial denaturation	15 minutes	95 °C	1

Denaturation	30 seconds	95 °C	40
Annealing	30 seconds	55 – 65 °Cd	
Extension	30 seconds	72 °C	

Gene Expression Analysis

Relative quantification:

$$Folding = 2^{-\Delta\Delta CT}$$

$$\Delta CT = CT_{gene} - CT_{House\ Keeping\ gene}$$

$$\Delta\Delta CT = \Delta CT_{Treated\ or\ Control} - \Delta CT_{Average\ Control}$$

Determination of Human Endothelin 1(ET-1) and Nitric Oxide by ELISA

Serum Human ET-1 and Nitric Oxide were determined by using ELISA kit (SUNLONG, CHINA) according to manufacturer instructions.

Bioinformatic and Statistical Analysis.

All statistical analyses were performed using IBM SPSS Statistics (version 26.0). continuous variables were expressed as mean ± standard deviation (SD). The normality of data distribution was assessed using the Shapiro–Wilk test. For comparisons between two independent groups (CVD patients vs. healthy controls), the independent-samples t-test was applied normality assumptions were satisfied; otherwise, the Mann–Whitney U test was used as a non-parametric alternative. Gene expression data were analyzed using the 2^-ΔΔCt method, and comparisons between groups were performed using an independent-samples t-test after appropriate data transformation where necessary. Effect sizes had been calculated using Cohen's d for parametric comparisons. No correction for multiple testing was applied due to the limited number of target genes analyzed; however, results were interpreted with appropriate caution. The sample size was determined based on available clinical recruitment during the study period; a formal power analysis was not conducted. A p value < 0.05 was considered statistically significant for all tests.

Results

Endothelin-1 (END1) and Inducible nitric-oxide synthase (iNOS) expression

EDN1 expression was markedly reduced in the patients with cardiovascular disease. Relative to controls, patients exhibited a mean normalized expression of 0.070 (95% CI, 0.043-0.116) versus the control reference of 1.00 (95% CI, 0.716-1.397), corresponding to a 14.25-fold decrease. This down-regulation was statistically significant (independent-samples t-test, $p < 0.001$). although the Shapiro-wilk test indicated borderline non-normality in the patient group ($p = 0.047$), the independent-samples t-test was retained due to its robustness at the present sample size (n = 50 patients, n = 25 controls); results were confirmed with the non-parametric Mann–Whitney U test, which yielded a consistent finding ($p < 0.001$). Visual summaries, including bar plots and volcano plots, consistently highlighted EDN1 as a strongly down-regulated marker in patients, and the expression heatmap showed a broad suppression of EDN1 signal across the patient sample block. As shown in Table (3) and Fig (1).

Table (3): Endothelin-1 *(EDN1)* normalized gene expression levels in the different studied groups (2^-ΔΔCt)

Group	Means Ct of value *EDN1*	Means Ct value of *B2M*	ΔCt	ΔΔCt	Fold of gene expression
Patients	22.38	23.34	0.96	4.90027	0.070
Control	27.34	23.46	3.88	0.06027	1.00

In contrast to *EDN1, iNOS* genes expression was elevated in patients with CVD. The mean normalized expression was 2.287 (95% CI, 1.564-3.344) in patients compared with the control reference of 1.00 (95% CI, 0.601-1.665), yielding a 2.29-fold increase. Group differences reached statistical significance (independent-samples t-test, p = 0.012) as shown in in Table (4) and Fig (1).

Table (4): inducible nitric oxide synthase *(iNOS)* normalized gene expression levels in the different studied groups (2^-ΔΔCt)

Group	Means Ct value of *iNOS*	Means Ct value of *B2M*	ΔCt	ΔΔCt	Fold of gene expression
Patients	23.41	18.39	5.02	-1.10	2.29
Control	27.34	23.46	3.88	0.06027	1.00

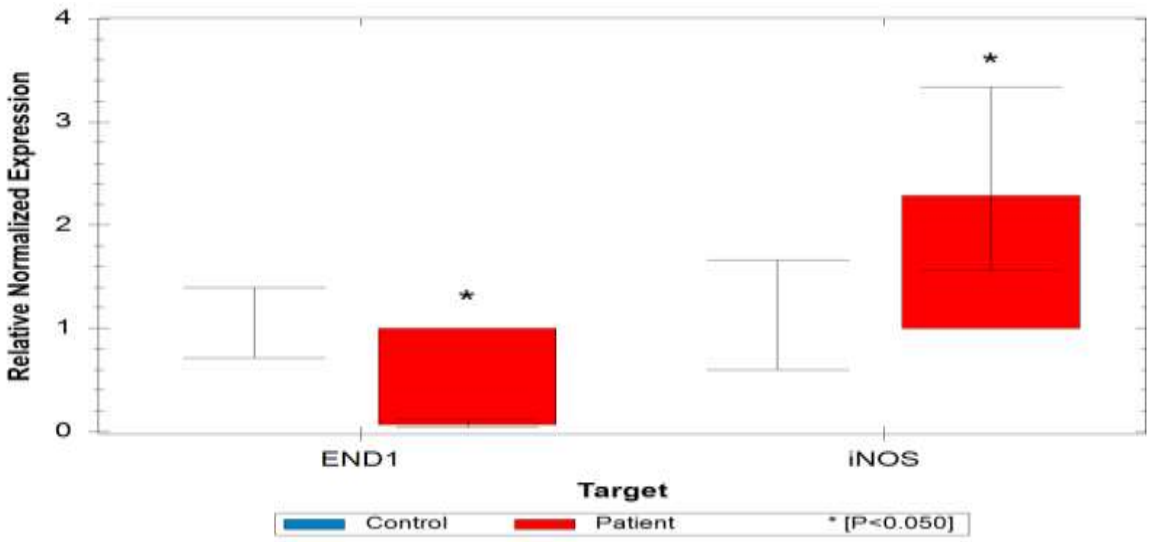

Figure 1: Relative normalized expression levels of *EDN1* and inducible nitric oxide synthase (iNOS) genes in cardiovascular disease patients and healthy controls. Bars represent mean ± standard deviation. Asterisks (*) indicate statistically significant differences between groups (p < 0.05) *.

Summary expression estimates and 95% CIs are shown in Fig. 2 (“bar chart”), and global significance and effect sizes are visualized in Fig 3 (“volcano plot”).

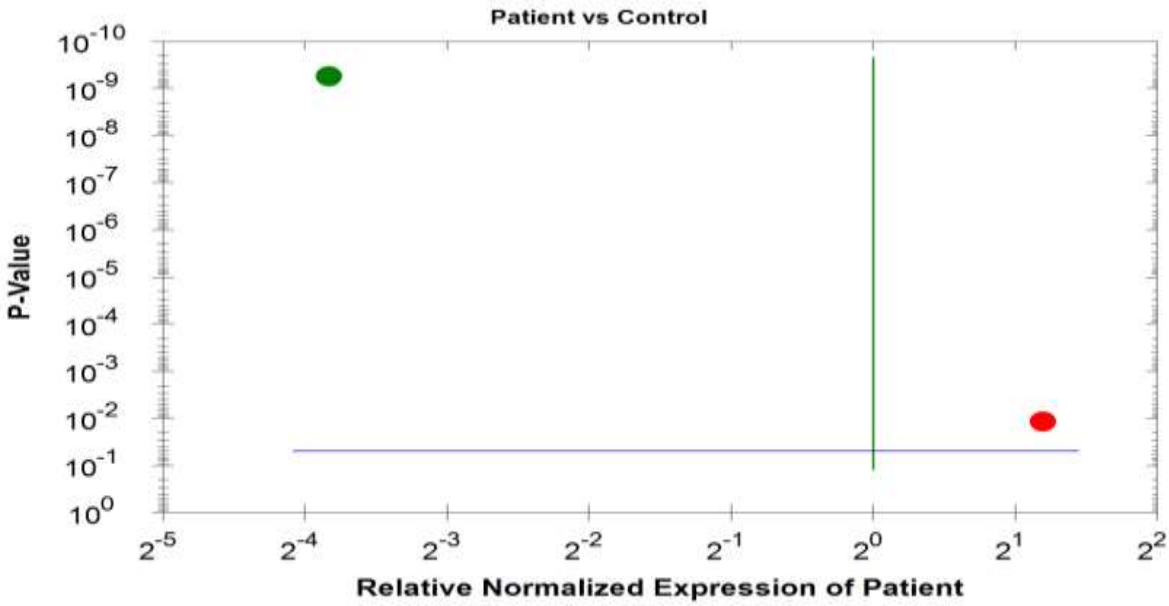

Figure 2 P-value vs. relative normalized expression in patients compared to controls for *EDN1* and *iNOS* gene targets. The x-axis represents log-scaled relative expression in patients, and the y-axis shows the -log10-transformed p-values. Colored dots represent gene targets: green (significantly downregulated) and red (significantly upregulated) based on statistical threshold lines.

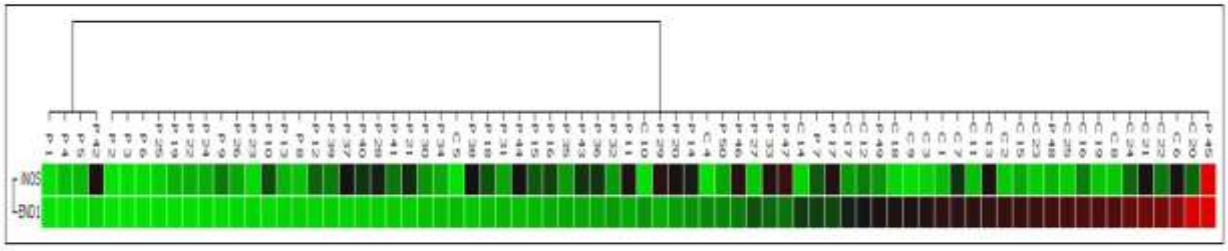

Figure 3. Unsupervised heatmap and hierarchical clustering of *EDN1* and *iNOS* gene qPCR expression. Columns represent individual samples labeled P1-P50 (patients) and C1-C25 (controls); rows are genes *(EDN1, iNOS).* Values are ΔΔCt-normalized to B2M with the control group as calibrator (relative expression = 1.0), then standardized per gene for visualization. The color scale reflects relative abundance across samples (green = lower, black = mid, red = higher expression). The top dendrogram depicts sample-level similarity; the left brackets label genes.

Mean ET-1 protein level in studied group

The result of present study investigate that ET-1 concentrations were markedly lower in cardiovascular-disease patients than in healthy controls. The mean ET-1 level was (356.63 ± 106.89pg/mL). In controls, the mean was (452.07± 86.53 pg/mL). (independent-samples t-test, $p < 0.001$). As shown in Table (5).

Table 5: Mean ET-1 protein level in studied group

Group	n	Mean ± SD
Patients	50	356.63 ± 106.89

Controls	25	452.07 ± 86.53

Mean NO molecules level in studied group

The result of present study showed that NO concentrations were markedly higher in cardiovascular-disease patients than in healthy controls. The mean NO level was (110.39 ± 43.89pg/mL). In controls, the mean was (86.40 ± 27.78pg/mL). (independent-samples t-test, $p < 0.001$). As shown in Table (6).

Table 6: Mean NO molecules level in studied group

Group	**n**	**Mean ± SD**
Patients	50	110.39 ± 43.89
Controls	25	86.40 ± 27.78

Discussion

The present study showed a decreased level of ET-1 in patients with cardiovascular disease, this result may be due to the use of cardiovascular medications. Several drugs commonly prescribed for cardiovascular disease are known to suppress endothelin-1 synthesis and expression, thereby improving endothelial function and reducing vasoconstrictive activity, regardless of whether this decrease is a positive indicator of illness. A study done by [6]showed a decreased level of ET-1 in patients with CVD who were under treatment, which agrees with the present study. Another study[15]also demonstrated a decrease in ET-1, which is a marker for macrophage activation, in heart failure patients when using ACE inhibitors. ACE inhibitors, angiotensin receptor blockers, and statins are examples of treatments that are known to stop the production and release of endothelin-1 and make the endothelium work better. Therefore, this decrease may reflect a systemic therapeutic effect aimed at reducing vascular constriction and limiting the progression of endothelial dysfunction rather than being solely attributable to the disease's pathology. A study by [6] demonstrated an increase level of ET-1 in CVD patients as compared with the control. ET-1 as a potential biomarker for cardiovascular risk, particularly in populations with combined risk factors[16]

In addition expression of ET-1 protein also decrease in the present study. The present study agree with[17] that showed that ET-1 expression at the transcriptional level decreased in vascular endothelial cells in patients after treatments. Besides plasma ET-1 measurement, urinary ET-1 levels; they confirmed a significant reduction of urinary ET-1 during therapy [18].

High levels of big ET-1 are predictive of death and worsening in patients with hypertrophic cardiomyopathy[19]. Furthermore, modifying the balance between nitric oxide and endothelin-1 may significantly contribute to improving the vascular condition of patients, as increased nitric oxide activity is an indicator of improved endothelial function and a reduction in the effects of constrictors such as endothelin-1[20].

The present study also demonstrated an increased the level of nitric oxide in CVD patients, while decreasing the level of gene expression

EDN1 is downregulated in blood and iNOS is upregulated, because the ET-1 peptide is primarily due to vascular and endothelial sources as opposed to transcription from leukocytes, and iNOS (NOS2) in leukocytes is elevated due to

inflammatory and oxidative-nitrosative stress in hypertension and ischemia[21, 22]. This cross-layer agreement is in the same direction as the major reviews and position papers on the distribution of endothelin across the cardio-renal axis and the translational discussion on antagonism of endothelin receptors.
The growing expression of iNOS (NOS2) gene in whole blood has been shown, in prior evidence, to mirror the activation of the inducible NO-pathway in cardiac failure and vascular inflammation." According to Haywood et al. [23] and Schulz et al. [24], studies performed on human tissue and leukocytes in heart failure have shown and characterized *iNOS* gene expression and down stream nitrosative stress in a responsive inflammatory and cytokine dominative environment. In this situation, patients with a higher expression of *iNOS* gene can be viewed as possessing endothelial dysfunction and an oxidative load in a compensatory or maladaptive response driven by ET-1 mediated vasoconstriction and adrenergic/RAAS(renin-angiotensin-aldosterone system) circuitry.

The relationship between ET-1 and NO is not solely antagonistic. ET-1 inhibits endothelial NOS via ETA receptor-dependent mechanisms, and, in turn, NO reduces *EDN1*gene transcription and somehow participates in an endothelial function feedback loop. Disruption of such a mechanism, particularly those changes involving altered gene regulations such as mutations or dysregulated gene expression, drives the development of vascular stiffness, increased peripheral resistance, and, ultimately, the development of arterial hypertension and atherosclerosis. Increased expression of EDN1 is associated with the clinical worsening of patients with hypertension, whereas uncontrolled endothelial NOS gene *(NOS3)* down regulation is associated with impaired endothelial repair and increased cardiovascular risk [25] or may be due to nuclear factor kappa b (NF-kB) and MAPK pathway which leads to the production of iNOS[26]

Conclusion

The current study concluded that CVD is associated with a clear dysregulation of gene expression, characterized by severe suppression of the *END1* gene and activation of the *iNOS* gene. The significant decrease in END1 may reflect a potential role for this gene in maintaining normal cardiac function, while the increase in iNOS may indicate activation of inflammatory pathways and oxidative stress associated with the development of CVD Therefore, *EDN1gene* can be considered a low-expression molecular marker associated with disease, while *iNOS gene* represents a high-expression genetic marker that may contribute to the pathophysiological mechanisms of CVD.

LIMATIMATION OF STUDY

This study has several limitations that should be considered when interpreting the findings. First, the relatively small sample size may have limited the statistical power to detect *EDN-1* and *iNOS* gene expression levels. Second, the single-center design may restrict the generalizability of the results to other populations within Iraq. Future studies with larger sample sizes, multicenter recruitment, and comprehensive genetic analyses are required to validate these findings and further clarify the role of *EDN-1* and *iNOS* genes expression in cardiovascular diseases

Conflict of Interest Statement
The author declares that there are no conflicts of interest regarding the publication of this paper.

Author Contributions
Almass Ahmed and Shilan Khairalla Jabbar were solely responsible for the study conception and design, data collection, data analysis and interpretation, manuscript drafting, revision, and final approval of the submitted version.
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