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The Expression of miRNA223 that related with lipid profile in chronic kidney disease patients

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Abstract

Chronic kidney disease (CKD) is a major public health concern and a leading cause of mortality worldwide. This study aimed to explore the relationship between the expression of microRNA-223 (miRNA-223) and the prevalence of chronic renal disease, with a focus on identifying potential biomarkers in Iraqi patients. The study found a correlation between blood lipid profiles and the risk of developing CKD. A total of 250 blood samples were collected, 150 samples from CKD patients and 100 samples control group were collected from healthy individuals. Clinical biomarkers showed that there was a significant increase (p-value ≤ 0.05) in white blood count, neutrophil, lymphocyte, blood urea and creatinine, while the increase in platelet count was not significant (p-value≥ 0.05). Additionally, analysis of serum lipid levels revealed a significant difference between the two study groups according to triglycerides, high-density lipoprotein and very low-density Lipoprotein (p-value < 0.01) which was 219.3±141.2, 36.16±11.8 and 42.77±26.33 respectively. However, there were no significant differences in total cholesterol and low-density Lipoprotein levels, which were 186.5±63.45 and 96.37±37.44 (p-value ≥ 0.05) in the patient group compared to the healthy controls group. A molecular study was done through analysis by (RT-qPCR) quantifying miRNA-223 expression as a novel biomarker for the disease. The folding expression of mi-RNA 223 showed down-regulation in the patient compared to the control group (0.322±0.32vs 1.00).

Keywords: chronic kidney disease; microRNA-223; Biomarker; diagnosis**;** Dyslipidemias

التعبير الجيني لرنا223 المرتبط مع الدهون لدى مرضى أمراض الكلى المزمنة

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الخلاصة

ان مرض الكلى المزمن من أهم الشواغل الصحية العامة والسبب الرئيسي للوفيات في جميع أنحاء العالم. تهدف هذه الدراسة إلى الكشف عن العلاقة بين التعبير (miRNA−223) microRNA−223 وانتشار مرض الكلى المزمن، مع التركيز على تحديد المؤشرات الحيوية المحتملة لدى المرضى العراقيين. وجدت الدراسة وجود علاقة بين مستويات الدهون في الدم وخطر الإصابة بمرض الكلى المزمن. تم جمع ما مجموعه 250 عينة

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دم، تتضمن 150 عينة من مرضى مرض الكلى المزمن و 100 عينة من مجموعة السيطرة من أفراد أصحاء. أظهرت المؤشرات الحيوية السريرية وجود زيادة معنوية كبيرة (قيمة $p \leq 0.05$) في تعداد خلايا الدم البيضاء والعدلات واللمفاويات واليوريا في الدم والكرياتينين، في حين لم يكن الزيادة في عدد الصفائح الدموية ذات دلالة إحصائية (قيمة $p \geq 0.05$). بالإضافة إلى ذلك، كشف تحليل مستويات الدهون في المصل عن وجود فرق معنوي كبير بين مجموعتي الدراسة للدهون الثلاثية والبروتين الدهني عالي الكثافة والبروتين الدهني منخفض الكثافة جدًا (القيمة الاحتمالية < 0.01) والتي كانت 219.3 ± 141.2 و 36.16 ± 11.8 و 42.77 ± 26.33 على التوالي. اضافة الى لم تكن هناك فروق معنوي في مستويات الكوليسترول الكلي والبروتين الدهني منخفض الكثافة، والتي كانت 186.5 ± 63.45 و 96.37 ± 37.44 (القيمة الاحتمالية ≤ 0.05) في مقارنة مجموعة المرضى مع مجموعة السيطرة. أجريت الدراسة الجزيئية من خلال التحليل بواسطة (RT–qPCR) لتحديد كمية التعبير عن miRNA–223 كعلامة حيوية جديدة للمرض. أظهر التعبير عن mi–RNA 223 انخفاضًا في مستوى التعبير الجيني لدى المرضى مقارنةً بمجموعة السيطرة (0.322 ± 0.32 مقابل 1.00).

Introduction

Chronic kidney disease (CKD) has emerged as one of the leading causes of mortality and suffering in the twenty-first century. This rise is partly attributed to an increase in risk factors linked to unhealthy lifestyles in the ageing population in both industrialized and developing nations, such as obesity, diabetes mellitus, and hypertension. Often referred to as a "silent disease," CKD typically presents no overt clinical symptoms in its early stages [1].

CKD is a growing global concern characterized by a gradual decline in renal function, which leads to increased morbidity and mortality [2]. In 2017, approximately 1.2 million fatalities related to CKD, with a 95% uncertainty interval (UI) ranging from 1.2 to 1.3 million. Additionally, impaired kidney function led to approximately 1.4 million deaths caused by cardiovascular disease, with a range of 1.2 to 1.6 million. By 2030, the number of people requiring renal replacement therapy is projected to increase fourfold to reach 5.4 million. Currently, over 2.5 million persons are receiving this treatment [3]. Numerous miRNAs have been associated with kidney disorders, and have provided evidence supporting the notion that miRNAs play a key role in the area of chronic kidney disease (CKD). Altered expression of miRNA has been shown to affect the onset and progression of various pathological processes, including kidney injury, renal malignancy, and diabetic nephropathy [3]. Moreover, serum miRNAs are recognized for their exceptional stability in blood and serve as valuable diagnostic and prognostic markers for a diverse array of conditions. Research using cellular and animal models of CKD have demonstrated a clear association between the levels of microRNA (miRNA) and the development of the disease [3].

Cardiovascular disease (CVD) is primarily driven by chronic renal disease, with patient morbidity and mortality worsened by cardiometabolic risk factors, irrespective of race or ethnicity [4]. Dyslipidemia is a well-known risk factor for cardiovascular disease. In terms of morbidity and mortality, total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) are independent predictors of cardiovascular disease, as numerous observers have documented on a broad scale. In patients with CKD, the development of CVD is influenced by multiple risk factors, with dyslipidemia and modified risk variables being particularly significant [5]. Abnormal levels of lipid and lipoprotein in patients with chronic kidney disease increase the likelihood of atherosclerosis and cardiovascular disease [6]. Deficiencies in lipoprotein transport, combined with proteinuria, contribute to the progression of renal failure. Patients who have proteinuria may experience elevated cholesterol levels. The primary indicator of dyslipidemia is typically elevated serum triglyceride levels, high levels of VLDL, low levels of HDL, and elevated HDL levels. CKD regulates the primary metabolic

pathways [7]. miRNA-223, initially recognized as a regulator of blood cell lineage development, is also found to be abnormally regulated in several cancer types and is increasingly recognized for its involvement in inflammatory and metabolic illnesses, specifically muscle diseases, type II diabetes, atherosclerosis, and vascular calcification [8]. Elevated levels of miRNA-223 have been observed in kidney biopsies of individuals experiencing progressive chronic renal failure in comparison to those with stable CKD, suggesting that miRNA-223 may contribute to the worsening of renal dysfunction [3]. Additionally, miRNA-223 possesses antiangiogenic properties and inhibits the proliferation of newly obtained endothelial cells by specifically targeting β1integrin and growth factor signaling pathways in these cells. miR-223 appears to be essential for preserving the state of rest in endothelial cells [9]. This study aims to evaluate various biochemical parameters including complete blood count, renal function test and lipid profile and estimate the level of miRNA-223 in the blood of Iraqi patients with CKD as a biomarker.

Materials and Methods

Sample collection

This study covered the period from October to March 2024. Blood samples were collected from al-kindi Teaching Hospital and al-alami Hospital in Baghdad, Iraq. For this study in total 150 samples were collected for patients' group, patients with a mean age of 50±5 years old, including 72 males and 78 females. In addition, 100 samples were collected for the healthy control group. All samples (patient and control) were examined for urinary tract infection and the presence of game-negative bacteria. From those samples were taken blood samples divided as follows:

1. For CBC, a volume of 1.5 ml of blood samples was added into an ethylene diamine tetra acetic acid (EDTA) tube.
2. For biochemical parameters, a volume of 4.5 ml of blood samples was transferred to a gel tube and centrifuged at 4000 rpm for 10 minutes to obtain serum and storage at (-20 °c) until used.
3. For the molecular study, the samples that diagnosed infection with gram-negative bacteria were selected, and a volume of 600µl of serum was added to 400 µl of Trizol and stored at (-20 °c) until used.

Primers

All primers used in this study were obtained from macrogen® (Korea). The name and sequence are provided in Table 1.

Table 1: The name, sequence and product size of primers used in this study

Name of Primer	5`-Sequence-3`	Reference
miR-223RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGG ATACGACGGGGTA	[10]
miR-223F	TGTCAGTTTGTCAAA	[10]
miR-223R	CAGTGCGTGTCGTGGAGT	
U6-FP	CTCGCTTCGGCAGCACA	[11]
U6-RP	AACGCTTCACGAATTTGCGT	[11]

Hematology study

Complete blood count (CBC) test

The Complete Blood Count test was utilized to determine the cell count for each cell type and platelets in the blood and detect the concentration of haemoglobin. This test provides the healthcare provider with details about the blood and general health. CBCs assist medical professionals in the diagnosis, surveillance, and screening of a variety of illnesses, ailments,

disorders, and infections. Results were recorded based on age and sex using an automated device and according to manufacture protocol by DxH 520 Beckman Coulter's Compact.

Biochemical tests

The renal function test and lipid profile test were conducted using a chemistry analyzer with electrolyte (IMT) and photometric testing capabilities. This test involves analyzing blood samples for certain substances according to manufacture protocol and automated device by Spin 200 E throughput instrument.

Molecular study

RNA extraction by TRIzol™

A volume of 400µl of serum was added to 600 µl of TRIzol™, the lysate multiple times to homogenized, incubated for 5 minutes to completely dissociate the nucleoprotein complex and stored in deep freeze (-20°c) until use. The samples were mixed vigorously, and then 150 µl of chloroform was added to the TRIzol™ for lysis. The mixture was vortexed again and incubated for 15 minutes in a freeze. After that, the mixture was centrifuged at a speed of 12,000 rpm for 15 minutes, which was separated into three layers, a lower layer containing red phenol-chloroform, an intermediate layer, and an upper layer consisting of colorless aqueous solution. The aqueous phase (upper layer) containing RNA was subsequently transferred to a new tube and 300 µl of isopropanol to precipitate RNA, mixed using a vortex and the resulting combination was kept in a freezing environment for a duration of 25 minutes. Subsequently, apply centrifugation to the mixture at a rate of 12,000 rpm for 10 minutes for precipitation of total RNA leads to the creation of a dense, gelatine mass that settles at the bottom of the tube and has a white appearance. The supernatant above was taken out using a micropipette, and the RNA was washed by adding 600 µl of 75% ethanol, the pellet was dissolved by vertexing and centrifuge at 7500 × g for 5 minutes, emptying the supernatant with the micropipette and the tube was opened for 25-30 minutes to dry. The pellet was resuspended in 25 µL of RNase-free water, and incubated at 60°c for 15 minutes using a thermomixer, the RNA that collected was kept at -20 °c until processing.

miRNA Quantitation

The Qubit® working solution was prepared by diluting the Qubit® miRNA HS reagent at a ratio of 1:200 in Qubit® miRNA HS buffer. A volume of 190 µL of Qubit® working solution was added to the tube intended for use as a standard. Subsequently, 10 µL of specified standard solution was added to the same tubes, followed by vertexing. Each tube prepared for the sample was supplemented with 197 µL of the Qubit® working solution, followed by the addition of 3 µL of sample to each tube individually. The entire mixture was vigorously mixed and left to sit at room temperature for 3 minutes. Standards tubes were placed into the Qubit device to generate a concentration curve. The tubes containing the samples were sequentially inserted to measure the concentration of miRNA in each sample.

RT-qPCR protocol

The RT-qPCR experiment was divided into two steps, the first step was cDNA synthesis from miRNA through a specific primer for miRNA-223 as mentioned in Table 1 and protoscript cDNA synthesis kit. This procedure was performed as follows:

A volume of 5 µL of samples that contained total RNA was transferred into a fresh PCR tube. A Protoscript reaction mix consisting of (dNTPs, buffer, and other necessary components) was added at a volume of 10 µL for each sample tube. The MuLV Enzyme was added with a volume of 3 µL for each sample and a specific primer of 1.5 µL was added, then the volume was completed to 25 µL by adding 4 µL of nuclease-free water. The mixture was incubated at 42 ºC for 1 hour in a thermocycler and the enzyme was deactivated at 80 ºC after this. The reaction

mixture was formed of components listed in Table 2 below, along with their respective quantities.

Table 2: component of cDNA mixture

Component	25 µL Reaction
Enzyme	3
Extracted total RNA	5
Nuclease-free Water	4
Random Primer	1.5
Reaction mix	10
RT223	1.5

Using Qubit 4.0, the cDNA product was quantified and then kept until the second step (relative quantitative PCR).
The second step was done by the following:
The cDNA sample was chosen from both the patient and control groups concurrently. Each sample requires the use of two PCR tubes: one for the target miRNA (miRNA223) and another tube for U6 snRNA which serves as a housekeeping gene in this experiment. The quantification was done by assessing the fluorescence intensity of SyberGreen. The reaction mixture consists of the components indicated in Table 3, along with their corresponding amounts:

Table 3: component of the reaction mix

Component	20 µL Reaction
Forward primer (10 µM)	1
Luna Universal qPCR Master Mix	10
Nuclease-free Water	3
Reverse primer (10 µM)	1
Template DNA	5

After that, adjust the Real-Time PCR software based on the selected thermocycling methodology provided in Table 4.

Table 4: RT PCR setup

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95°C	8 minutes	1
Denaturation Extension	95°C 60°C	15 seconds 30 seconds (+plate read)	50
Melt Curve	60-95°C	20 minutes	1

The result from RT-PCR (Ct) was collected and analyzed according to Livak formula as following:
ΔCt patient = Ct patient – Ct U6
ΔCt control = Ct control – Ct U6
$\Delta\Delta$Ct = ΔCt patient – ΔCt control
Folding expression = $2^{-(\Delta\Delta CT)}$

Statistical investigation

The SPSS (2016) software (IBM Corp, 2016) was utilized to analyze the effects of variation in study parameters. The least significant difference (LSD) test, part of the analysis of variance (ANOVA), or the T-test was used for significant comparisons between means. The Chi-square test was employed to identify significant associations among percentages. In this investigation, a confidence interval (CI), an odd ratio, and a P value between 0.05 and 0.01 were projected in all statistical analyses.

Results and discussion

Subjects' data

The identification of biochemical parameters, epigenetic markers and their effects influences on human health may provide insights into the correlation between genetic background and susceptibility to developing CKD disease. The study involved a total of 250 participants, with 150 persons diagnosed with CKD and 100 healthy individuals serving as the control group. The data were collected through various criteria acquired from the questionnaire forms completed by both patients and control individuals. The demographic characteristics utilized in this investigation are presented in Table 5.

Table 5: Demographic characteristics of the study groups

Criteria		Control (100 samples)	CKD (150 samples)
Age (year)	<40	34 (34%)	30 (20%)
	40-50	50 (50%)	57 (38%)
	>50	16 (16%)	63(42%)
Sex	Male	46(46%)	72 (48%)
	Female	54 (54)	78 (52%)
Family history	Yes	16 (16%)	135 (90%)
	No	84 (84%)	15 (10%)
Hypertension	No	86 (86%)	60 (40%)
	Yes	14 (14%)	90 (60%)
Body mass index	18.5 – 25	30 (30%)	36 (24%)
	>30	70 (70%)	114 (76%)

The findings for both healthy subjects and CKD patients are shown in Table 5, including age, sex, family history, hypertension, and body mass index. The results indicate that 34% of the participants were reported to be in good health, while 20% were diagnosed with CKD. Specifically, among individuals under the age of 40, 50% of individuals were reported to be in good health, whereas 38% had been diagnosed with CKD. Among those over 50 years old, 42% of individuals diagnosed with CKD were over 50 years old, while 16% appeared to be in good health. Additionally, the study found that 46% of the male participants were ostensibly in good health, while 48% had been diagnosed with CKD. The proportion of women was 54% and 52%, respectively. The prevalence of a family history of CKD was noted to be 16% among ostensibly healthy subjects and 90% among CKD patients. Hypertension was present in 14% of apparently healthy subjects and 60% of CKD patients, respectively. Regarding body mass index indicated that 30% and 24% of healthy and patients with CKD, respectively, were within the 18.5-25 range, while 70% and 76% of healthy and patients with CKD, respectively were within more than >30 range. The current study found a significant association between obesity (as assessed by BMI, waist circumference, and W/H ratio) and CKD in the current study, in addition to other established risk factors [12].

The hematology analysis

The results indicated a significant increase (p-value ≤ 0.0001) in white blood cells, lymphocytes and neutrophils with values of 11.12, 2.8 and 6.77 respectively while there were non-significant increases (p-value ≥ 0.001) in platelet count that was 264.9. The haematological test of all Patients who had CKD was shown in Table 6.

Table 6: Hematological parameter test for Patients with CKD and control group

Group / Parameter	Normal Range for Healthy Cases	Healthy Control Group	Patient Group	Sig.	p-Value
WBC $10^3/\mu L$	3.60-10.20	7.29 ± 1.38	11.12±3.387	**	<0.0001
LYM $10^3/\mu L$	1.00-3.20	2.0±0.82	2.8±1.06	*	0.0272
NET $10^3/\mu L$	1.85-5.94	3.28 ± 1.7	6.77±2.11	**	<0.0001
PLT $10^3/\mu L$	152.4-3547.9	232.3±46.83	264.9±72.38	NS	0.0702

NS: non-significant *: significant **: highly significant

Chronic kidney disease (CKD) affects over 10% of the population and has substantial social and economic consequences [13]. CKD is particularly prevalent among the elderly [14]. Laboratory data from urinalysis included assessments of white blood cells, positive nitrite test, bacteria and red blood count. The white blood cell count was evaluated for the minimal, median and maximal values, with the complete blood count in which the median value of WBC was within normal limits [15].

In CKD patients, the observed white blood cell count was (11.12 $10^3/\mu L$), compared to the control group (6.76 $10^3/\mu L$). The results showed a highly significant difference between patients (who had CKD) and healthy control group (p-value ≤ 0.001)

White blood cells, often known as WBCs, are a type of immune cells that play a crucial role in major diseases such as cancer, infections, and inflammatory disorders [16].

The study by [17-18] noted significant differences in leukocyte counts in patients infected with CKD, which aligns with the findings of this study. Lymphocytes are a type of agranulocytes that make up 20 to 40% of the total leukocyte count. They are an integral part of the adaptive immune system. Lymphocytes are immune cells that are capable of recognizing and reacting to antigens. They circulate between different lymphatic tissues [19]. A correlation has been established between the severity of illness in different conditions, such as infections, and the fluctuations in neutrophil and lymphocyte cell counts. In this study, significant increase in lymphocyte counts, with values of 2.8 $10^3/\mu L$ for the patient group and (2.0 $10^3/\mu L$) for the healthy control group in this study (p-value ≤ 0.0272) [20].

Lymphocytes are significant in CKD, due to their diverse and adaptable nature allows them to have multiple functions in causing renal injury and fibrosis. The interaction between innate and adaptive lymphocytes in the immune response follows a sequential pattern of reactions throughout time. This underscores the importance of targeting certain subsets of lymphocytes with different therapeutic approaches. Research into lymphocyte interferences within living organisms and the transfer of these cells has opened new possibilities for therapeutic techniques connected to lymphocytes. Additionally, the regulation of gut microbiota and metabolites to regulate lymphocyte immunological responses associated with CKD demonstrates promising therapeutic promise for the development of drugs targeting kidney illnesses in the future [21].

T-lymphocyte subsets serve as indicators of a patient's immunological state and have been linked to negative outcomes in many diseases. However, the relationship between T-lymphocyte subsets and primary infection as well as renal prognosis in patients with CKD, has not been thoroughly investigated [22]. In this study, there was a non-significant difference

between the general group for patients with CKD and the healthy control group (p-value >0.005) and that is in line with this study.

The result of this study demonstrated a highly significant increase in neutrophile count, recorded at 6.77 10^3/µL. Neutrophils, also known as NET, are phagocytes in an immature state that have a brief lifespan. These cells are known for their ability to secrete proteolytic enzymes and oxygen free radicals, which actively contribute to the harm caused during inflammatory processes [23]. They play a crucial role in inflammation and tissue damage, especially in cases of acute renal failure, by releasing a combination of cytotoxic substances such as reactive oxygen intermediates, enzymes, and microbicidal polypeptides. NET depletion prevents or reduces these effects. In this study, we examined the correlation between the number of neutrophil extracellular traps (NETs) and the level of kidney dysfunction in individuals diagnosed with CKD [24]. The results indicated a significant difference between the general group of patients with CKD and healthy control group (p-value ≤ 0.05).

The platelet count results indicated no significant difference between the patient group and the healthy control group, recorded at 264.9 10^3/µL. Platelets are the smallest blood cells resulting from bone marrow megakaryocyte cells in the bone marrow and play a crucial role in hemostasis and coagulation [25].
Research has examined the role of platelet parameters in a variety of diseases, with information regarding these parameters also accessible for urinary tract infections [26]. Platelets may contribute to the inflammatory process by increasing the recruitment of leukocytes and the prevention of apoptosis in neutrophils and monocytes. Additionally, there was an increase in the production of inflammatory mediators, including cytokines and chemokines [27]. During the acute phase response, platelet count rises, continuing for the duration of the inflammatory process [28].

Biochemical analysis

Renal function test (urea and creatinine)

The result of this study showed significant increases in urea and creatinine levels, recorded at 62.71 mg/dl and 1.38 mg/dl respectively as shown in Table 7. Various biochemicals are available in both blood and urine to evaluate renal function. Many of these biomarkers have advantages and disadvantages that are important to consider when requesting and employing them in a therapeutic context. An ideal marker should be capable of quickly detecting the onset of acute kidney injury and be applicable for both the initial diagnosis and continuous monitoring and treatment of kidney disease [29].

Table 7: biochemical parameter (Urea and Creatinine test) for Patients with CKD and control group

Group / Parameter	Normal Range for Healthy Cases	Healthy Control Group	Patient Group	Sig.	p-Value
Urea (mg/dl)	15-45	32.33±5.9	62.71±59.51	*	0.0267
Creatinine (mg/dl)	0.55-1.25	0.90	1.38	**	0.0019

*: significant **: highly significant

The isolation of urea from human urine in 1797 marked the identification of the first biomarker associated with renal failure. For over a century, the term uremia, which denotes to the presence of urine in the blood, has been employed as a synonym for kidney failure [30]. Five factors influence serum urea levels: (I) hepatic urea generation rate; (II) protein intake; (III) urea tubular reabsorption intensity; (IV) some medications; and (V) the presence of

gastrointestinal hemorrhage. Urea is a straightforward test to administer, with a high sensitivity but low specificity for the diagnosis of kidney disease. It is not employed to quantify GFR [31]. Creatinine is the most frequently employed and extensively accessible biomarker of renal function. It is derived from creatine, a substance that serves as a rapid-acting energy reservoir in muscles. Creatine endures a spontaneous, irreversible conversion to its anhydride form, creatinine. Although creatinine is readily filtered and minimally reabsorbed, the proximal tubule also secretes 20–30% of it [32].

Biological markers are essential for the accurate diagnosis, risk assessment, and implementation of therapy to enhance the clinical product. Serum analysis of renal function markers, including urea, creatinine, electrolytes, uric acid, and blood urea nitrogen, is now routinely employed instead of urine analysis, which was relatively uncomfortable for the patient. Creatinine, a byproduct of the breakdown of creatine phosphate in muscle, is eliminated by the kidneys and serves as a primary nitrogenous waste product resulting from protein and amino acid metabolism. Urea and creatinine are reliable indicators of normal kidney function, and an increase in serum creatinine levels is indicative of kidney dysfunction. Serum creatinine is the most widely accepted and commonly used parameter for measuring renal functions [33].

In this study, a significant increase in urea levels was observed in both the CKD patient group and healthy control group (p-value 0.0267); (62.71±59.51) and (32.33±5.9) respectively. Conversely, there was a highly-significant difference in the creatinine level between CKD patient and healthy control group (p-value 0.0019); which is (1.38) and (0.90) respectively.

Blood creatinine and urea are the primary parameters used to evaluate kidney function and are essential in assessing its severity [34]. Among minors, there was a discrepancy in creatinine levels between men and females, with males exhibiting lower creatinine values and females experiencing reduced muscle mass. Serum creatinine level serves as an indirect indicator of glomerular filtration, as the glomerulus filtered creatinine. In comparison to urea, serum creatinine has a superior prognostic capacity for predicting adverse outcomes, and this increase is indicative of the progression of kidney disease [35]. The management of CKD involves the use of serum creatinine. It was contingent upon the variables of race, age, and sex. Additionally, following kidney disease [33].

The gastrointestinal (GI) tract excretes the remaining portion of the urea, while the kidneys are responsible for eliminating approximately 85% of it. Serum urea levels increase in acute and chronic renal failure as renal clearance decreases. Enhancements observed in this study may also lead to an increase in urea in conditions that are not associated with renal diseases, such as upper gastrointestinal haemorrhage, catabolic states, high protein diets, and dehydration. Individuals who are experiencing severe liver disease, malnutrition, or a low-protein diet may find that their urea levels are diminished. Measurement of renal function was more precise with serum creatinine than with urea. However, renal disease indicated an earlier elevation of urea, which is in accordance with the results of this investigation [36].

Lipid profile

This study included an analysis of serum lipid levels in two study groups (patients and control) by using a conventional method. The result of this study revealed that there was a significant difference between the two study groups according to TG, HDL and VLDL (p-value ≤ 0.05) and there are non-significant differences in the level of TC and LDL (p-value ≥ 0.05) in the patient group comparison to healthy controls group. The mean triglyceride level for the patient group was (219.3±141.2) and for control group was (101.3±20.27) that indicated there was highly significant differentiation between two studied groups (p-value < 0.05) and the mean of VLDL for the patient's group was (42.77±26.33) and for control group was

(20.38±4.05) that indicated there was highly significant differentiation between two studied groups (p-value < 0.05). In addition, the mean of HDL for patients' group was (36.16±11.8) and for control group was (43.75±8.2) which indicated there was significant differentiation between two studied groups (p-value < 0.05). While the mean of total cholesterol for patients' group was (186.5±63.45) and for control group was (156.5±33.99) that indicated there was non-significant differentiation between two studied groups (p-value > 0.05). The mean of LDL for patients' group was (96.37±37.44) and for control group was (96.50±32.39) which also indicated no significant differentiation between two studied groups (p-value > 0.05), as shown in Table 8.

Table 8: biochemical parameter (lipid profile test) for Patients with CKD and control group

Parameter	Mean ± SD		Sig.	*p*-Value
	Control	Patients		
Cholesterol (mg/dl)	156.5±33.99	186.5±63.45	NS	0.0858
Triglycerides (mg/dl)	101.3±20.27	219.3±141.2	**	0.0019
HDL (mg/dl)	43.75±8.2	36.16±11.8	*	0.0267
LDL (mg/dl)	96.50±32.39	96.37±37.44	NS	0.9905
VLDL (mg/dl)	20.38±4.05	42.77±26.33	**	0.0016

NS: non-significant *: significant **: highly significant

Based on Roc analysis, the cut-off values, sensitivity, specificity, and area under the Roc curve (AUC), between the patient lipid profile that includes (TC, TG, HDL, LDL, VLDL) and healthy control, the AUC for (TG, HDL, VLDL) was (0.8313, 0.7135, 0.8417) respectively, the sensitivity was (80.0%, 77.0%, 80.0%) respectively and specificity (69.0%,44.0%,75.0%) respectively. The AUC values for TC, LDL were (0.6531 and, 0.5094) respectively, with sensitivity of 63.0%, and 50.0% and specificity (57.0%, 44.0%) as shown in Figure 1 if the AUC value > from (0.7), it was effective and considered as a novel diagnostic biomarker panel in the CKD.

The results of this study indicated that TG and VLDL test for lipid profile are considered the most effective marker and raised with CKD which was highly significant differences and had 89% for AUC value.

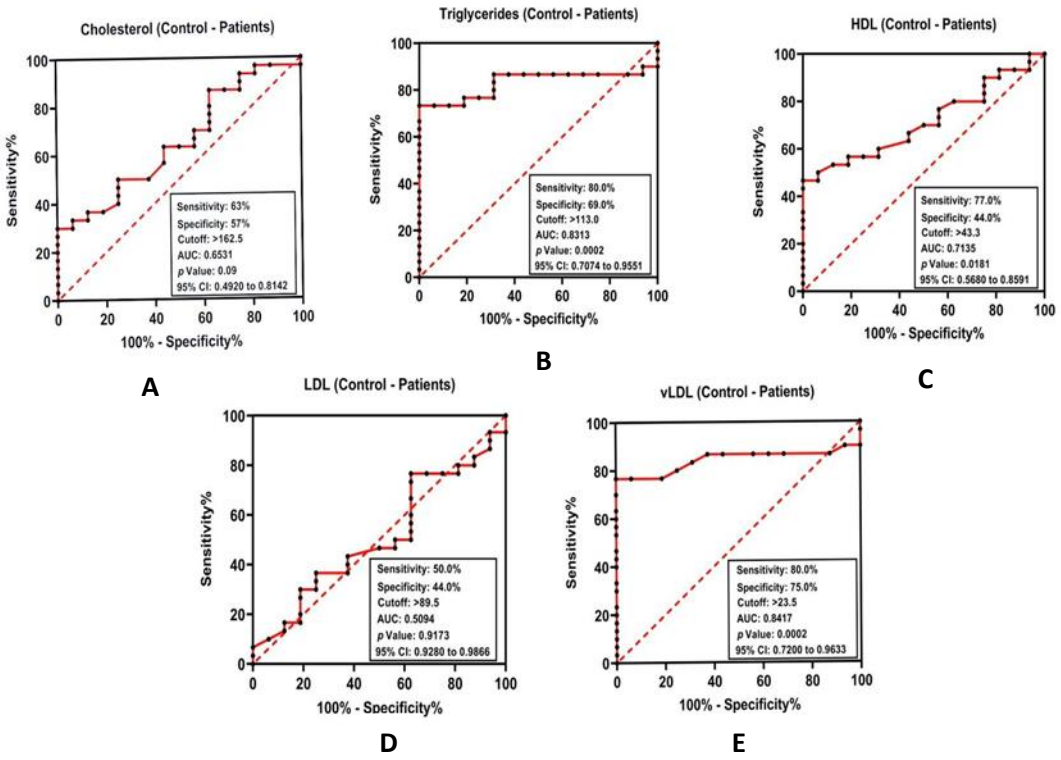

Figure 1: AUC of markers, (A) combination between TC and CKD, (B) combination between TG and CKD, (C) combination between HDL and CKD, (D) combination between LDL and CKD, (E) combination between VLDL and CKD, for patients and healthy control groups.

Among patients with CKD, cardiovascular diseases are the leading cause of mortality. Kidney and cardiovascular disease are interrelated and exacerbate each other's severity. For patients with CKD, dyslipidemia is a significant contributor to cardiovascular disease, in addition to hypertension and diabetes. CKD and dyslipidemia reinforce each other; the term "dyslipidemic profile" refers to the lipoprotein profile of patients identified as a risk factor for CKD [37]. The "dyslipidemic profile" is produced by an increase in triglycerides, very low-density lipoprotein, and oxidized low-density lipoprotein, as well as a decrease in high-density lipoprotein and alterations in the composition of lipoproteins [38].

In this large Chinese cohort, higher levels of blood triglycerides (TG), blood high-density lipoprotein (HDL), the total cholesterol to HDL ratio (TC: HDL), and the TG to HDL ratio were associated with an increased risk of CKD. Conversely, a lower risk of CKD was associated with a higher blood HDL level. In addition, prior epidemiologic investigations have identified positive correlations between the risk of CKD and blood TG, HDL, TC: HDL, and TG:HDL ratios [39-47]. Conversely, a reduced blood HDL level [39,40,45,46,48,49,50] was linked to an increased risk of CKD. Additionally, the majority of studies did not identify any statistically significant correlations between the risk of CKD and blood TC and LDL levels [39-41,45,46,49]. In both conventional and Mendelian randomization (MR) analyses, we were unable to identify any significant associations between blood TC or LDL-c levels and the risk of CKD. The MR analysis only suggested a potential causal relationship for blood TG levels, which was weaker than the observed relationship, despite the conventional analysis determining that both HDL and TG levels were associated with CKD risk [16].
Patients with CKD display a dyslipidemic phenotype [51]. This constellation includes varying amounts of low-density lipoprotein (LDL), reduced high-density lipoprotein (HDL), and increased levels of triglyceride (TG) and very low-density lipoprotein (VLDL). The atherogenicity of these substances is affected by their altered composition and their levels in

individuals with CKD [52]. A study has shown that high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), and total cholesterol (TC) are all independent predictors of cardiovascular disease [53]. Dyslipidemia and altered risk factors are among the multiple factors that impact the development of cardiovascular disease (CVD) in patients with CKD [54]. The dyslipidemic profile is characterized by elevated levels of triglycerides, very low-density lipoprotein, and oxidized low-density lipoprotein, as well as reduced levels of high-density lipoprotein. Additionally, there are alterations in the composition of lipoproteins [55].

The influence of triglycerides on chronic renal disease is significant, as hypertriglyceridemia is the primary lipid abnormality observed in CKD patients. This condition arises from the impaired breakdown of triglycerides and increased levels of triglycerides within lipoproteins [6]. CKD has a good impact on HDL, which is a lipoprotein that has features that defend against inflammation, oxidation, and blood clotting. It is a constituent of the Reverse Cholesterol Transport mechanism, which includes the movement of cholesterol molecules from the peripheral tissues to the liver for elimination by the liver. In CKD, the structure of HDL can be changed, leading to the conversion of HDL into substances that might harm blood vessels and affect the beneficial functions of HDL [56].

In regular clinical practice, LDL levels are the primary criterion evaluated; however, the distribution of LDL subfractions also plays a role in determining cardiovascular risk. Patients with CKD display exhibit a broad range of low-density lipoprotein (LDL) values. However, alterations in the structure and composition of LDL associated with CKD can worsen cardiac risk factors in this population [57].
The impact of CKD on the metabolism of very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL), VLDL was secreted by the liver and then undergoes conversion into IDL, LDL, and HDL. In patients with CKD, the levels of triglycerides, VLDL, and IDL are elevated due to a deficiency of lipoprotein lipase (LPL), the enzyme responsible for the hydrolysis of triglycerides into VLDL and chylomicrons. Consequently, patients with CKD experience delayed VLDL catabolism [58]. An increase in the levels of VLDL and HDL was associated with an increased risk of incident CKD patients and control cases in the study [59,7]. Hypertriglyceridemia was identified as the most common plasma condition in patients with CKD [60,7]. Cholesterol concentrations and HDL levels were reduced, and the total synthetic rate and catabolic fractional rate were both reduced in correlation with HDL levels. The cholesterol levels of CKD patients were not significantly altered in another study [61,7]. Lipid abnormalities may be observed in patients with CKD as an increase in LDL, TG, and very low-density lipoprotein (VLDL) and a decrease in HDL [62-65]. Proteinuria appears to be a substantial factor, despite the complexity of the mechanisms accountable for these metabolic abnormalities. Additionally, CKD may lead to increased hepatic VLDL secretion and an impaired clearance of VLDL and its remnants from serum. This results in hypertriglyceridemia and an increase in HDL catabolism [66].

Gene expression of miRNA-223 genes

The real-time PCR amplification program was conducted using melting curve analysis for all evaluated gene expressions, following the manufacture stimulation programs, using relative gene expression.
Total RNA extracted from blood samples were converted to cDNA for gene expression level analysis using Real-time PCR (qPCR) for miRNA-223 gene.
The gene expression was standardized to the level of the housekeeping gene (U6) and measured using the Ct value, as depicted in Figure 2. Each color in the curve displayed in these images refers to a certain Ct value.

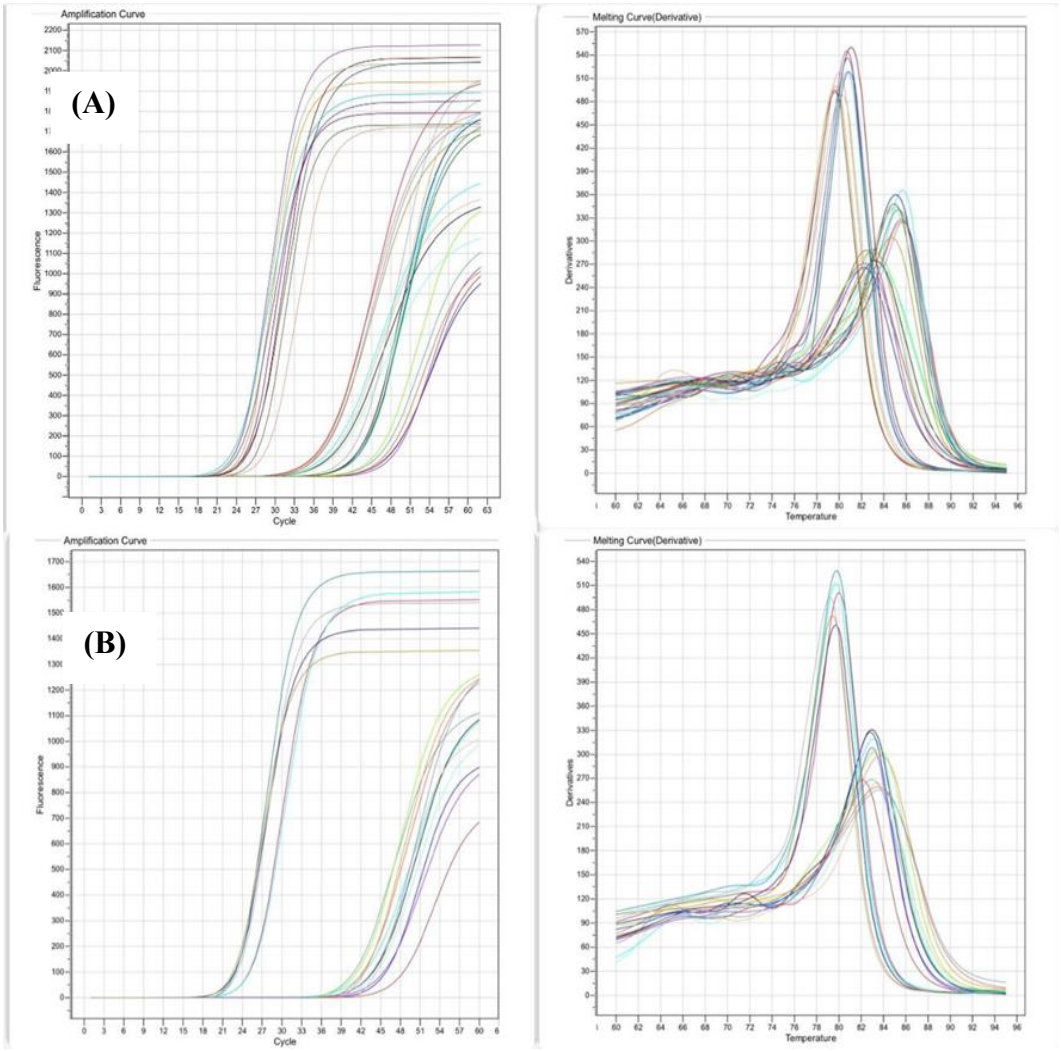

Figure 2: (A) amplification plots for miRNA 223 and U6 obtained by RT-qPCR, (B) melting curve analysed for miRNA-223 and U6 expression.

MiRNA-223 gene expression was quantified using reference gene U6 and compared between patient and healthy control groups in different stages (Ct, ΔCt, ΔΔCt, and fold change) using qRT-PCR, as shown in Table 9.

Table 9: Comparison between the studied groups (patients and healthy control) in different stages of gene expression level Ct, ΔCt, ΔΔCt and fold.

Parameters	Mean Ct for 50 patients	Mean Ct for 50 healthy control	Ct U6 patients	Ct U6 control	ΔCt miRNA patient	ΔCt miRNA control	ΔΔCt	Fold
miRNA223	44.42	42.92	24.80	24.80	19.62	18.12	1.5	0.322± 0.32

The Ct value is inversely related to gene expression, with a lower Ct value indicating a higher number of copies of the target gene.
Specifically, a high Ct value indicated low gene expression and a low Ct value indicated high gene expression [66]. The results showed that Ct value for miRNA 223 were 44.42 for patients' group and 42.92 for the healthy control and Ct of U6 was 24.80 for patient group and healthy control.

The ΔCt value was calculated as the difference between the Ct value of miRNA 223 gene in patient and control with the Ct of U6 gene. The analysis revealed that the expression level of miRNA 223 was 19.62 in the patient group and 18.12 in the control group, dependent on $2^{-\Delta\Delta Ct}$ and livak equation the result of folding showed to indicated there was downregulation in miRNA 223 gene (0.322±0.32) that occurred in a patient with CKD. Furthermore, there was a highly significant difference *p*- *p*-value ≤ 0.0011 between patients with CKD and healthy control groups in the levels of miRNA 223 as shown in Table 10.

Table 10: The correlation between patients with CKD and healthy control groups in the levels of miRNA-223 depending on $2^{-\Delta\Delta Ct}$ method.

Parameter	Mean ± SD		Sig.	*p*-Value
	Control	Patients		
miRNA-223	1.0	0.322±0.32	**	0.0011

A variety of microRNAs (miRNAs) have been linked to the progression and mortality of CKD (CKD) [67]. Recently, miRNAs have gained interest as possible biomarkers for evaluating the severity and/or cause of renal disease [68]. miRNAs are a suitable biomarker that can be detected without the need for intrusive procedures. Their steric stability is a key advantage of using them as biomarkers [69]. Therefore, these short RNAs may exhibit a degree of reliability that may be beneficial in future therapeutic applications [70]. The discovery of miRNAs marks a significant advancement in gene expression regulation, as these molecules are adept at controlling gene expression post-transcription. miRNAs are short, single-stranded RNAs that form base pairs with specific areas of target mRNAs, usually inside the 3′ untranslated region. It is now understood that miRNAs play a role in the pathophysiology of the kidney. The roles of miRNAs in the molecular mechanisms of kidney disease, as well as in individuals with acute renal injury and chronic kidney illness [71].
However, miRNAs can serve as novel biomarkers in the field of nephrology. Prior to incorporating them into the daily clinical routine, it will be important to standardize measuring procedures and choose the most appropriate tissues and biofluids. Additionally, it is important to assess their expression in extensive groups of individuals. Besides their potential as biomarkers, the modulation of miRNAs expression offers a new therapeutic strategy for treating renal problems [72]. MiRNAs are important in human disease pathophysiology, therefore their diagnostic and therapeutic potential has received interest. MiRNA modification affects several signalling pathway components. Antisense methods, antagomirs, and Decoy or sponge technologies can inhibit miRNA activity [8]. Silencing miRNAs related to albuminuria, extracellular matrix buildup, EMT, and podocyte dysfunction, or restoring miRNA activity in renal disorders with downregulated miRNAs, may be a therapeutic option [73].

It has been suggested that the expression of these disorders could be reduced by adjusting the activity of dysregulated miRNAs in living organisms [74]. MiRNAs play a crucial role in governing the gene response to various pathophysiological stimuli and have the potential to be therapeutically impactful [75]. To achieve an effective therapeutic response in CKD, it is necessary to deliver miRNA precursors (to increase expression) and miRNA inhibitors (to decrease expression) at a precise time and in a specific tissue [76]. Quantitative PCR is a commonly employed technology in clinical laboratories for accurately measuring miRNA levels in blood samples, and it can be easily applied in other settings. Given the relatively low quantities of miRNA in the bloodstream, it is crucial to select a suitable reference gene for quantitative PCR. Some research teams have utilized small endogenous RNAs, such as U6, to control the quantities of miRNAs in circulation. The results, however, are considered

untrustworthy because to the significant heterogeneity in the expression of these RNAs in connection to disease situations [77].

MiRNA-223 exhibits strong expression in freshly isolated endothelial cells, but its levels decrease fast during cell cultures [78,79]. These observations imply that miRNA-223 is not produced within endothelial cells, but rather acquired from other cell types such as macrophages and monocytes [80,81]. Furthermore, the authors stated that miRNA-223 is present in endothelial cells that have been recently obtained from the brain microvasculature [71, 82]. The expression of the inflammatory miRNA-223 significantly increases in both the intima and media as CKD progresses, leading to the development of chronic low-grade inflammation and atherosclerosis [83].

MiRNA-223 was found to be downregulated in this study, as illustrated in Figure 3. In serum samples from the general population with CKD, this miRNA was consistently downregulated [67, 84-86], and that is consistent with the findings of this investigation.

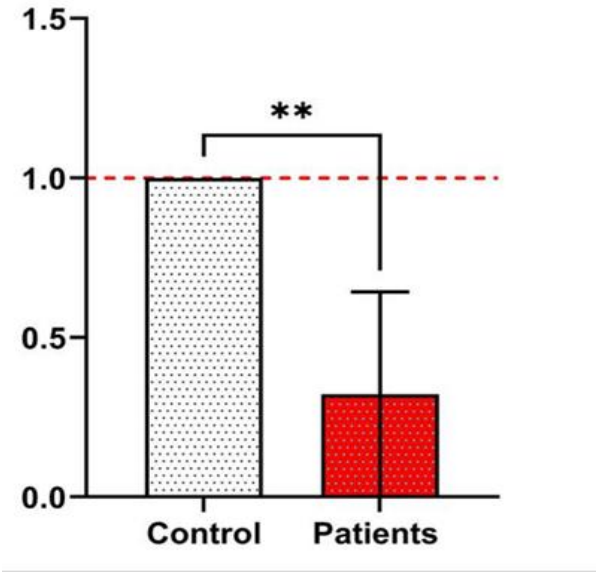

Figure 3: fold of change ($2^{-\Delta\Delta ct}$) miRNA223 expression of the two study groups.

Furthermore, the presence of prevalent CKD was associated with lower levels of this specific miRNA [67]. Additionally, it has a role in regulating the growth and division of vascular smooth muscle cells (VSMCs) [87]. While miRNA 223 is commonly associated with inflammatory pathways, some data suggest that it may also exert a beneficial influence on vascular smooth muscle cells (VSMCs) by modulating the interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) pathway, which prevents calcification. The findings suggest that increased levels of miRNA-223 could enhance kidney function and potentially serve as a treatment approach to enhance outcomes for CKD (CKD) in the general population [88]. The renal biopsies of patients undergoing increasing chronic renal failure demonstrated elevated levels of miRNA-223 compared to those with stable CKD. It is suggested that miRNA-223 may have a role in the advancement of renal failure [89,3].

The relationships observed between the miRNA levels and other symptoms of severe kidney failure, such as hyperparathyroidism and hyperphosphatemia, were influenced by the impairment in glomerular filtration in the univariate analyses. A strong correlation was found between the levels of sex, leukocyte count, platelet count, blood haemoglobin, cholesterol, and the level of miRNA-223. Previous research has demonstrated a link between blood cell counts and miRNA-223, a molecule that is highly abundant in granulocytes, platelets, and red blood cells. Furthermore, miRNA-223 has been discovered to play a role in the process of

haematopoiesis [90-95]. Prior investigations linked miRNA-223 to cholesterol control. Additionally, miRNA-223 has been shown to play a role in hematopoiesis. However, miRNA-223 is often associated with inflammation [96,67]. Disease-related comorbidities like CKD-MBD may include these miRNAs in key pathways. They could serve as biomarkers and targets for CKD diagnostic and therapeutic studies [97]. CKD-MBD is a systemic condition of mineral and bone metabolism that arises from chronic renal failure, characterized by abnormalities in the metabolism of calcium, phosphorus, parathyroid hormone (PTH), and vitamin D. It also involves irregularities in bone turnover, bone mineralization, bone volume, and calcification outside of the skeletal system, which is relevant to the cardiovascular system [98].

Conclusions

This study examined the relationship between the gene expression of miRNA-223 and the prevalence of CKD (CKD) as a potential biomarker in a cohort of Iraqi patients. The findings indicate that the altered expression of miRNA-223, along with changes in specific serum lipid parameters and clinical biomarkers, may serve as valuable diagnostic and prognostic indicators for CKD. Further investigation is needed to validate the utility of these biomarkers and explore their potential applications in CKD risk assessment and management.

Conflict of interest

There are no Conflicts of interest.

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