Evaluation of the expression of miRNA126 in chronic kidney disease patients infected with Gram-negative bacteria

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ABSTRACT

Background: Chronic kidney disease (CKD) is a major health problem and a killer in the modern era, and has become a cause of death worldwide, caused by different types of microbial agents, from the skin or rectum, that enters the urethra and infects the urinary tract. **Aim of study:** This study was designed to investigate the relationship between gene expression of miRNA-126 and the incidence of chronic kidney disease as biomarkers in a sample of Iraqi patients. **Methodology:** In total, 200 blood and urine samples were collected:150 samples from CKD patients and 50 samples from the control group were collected from healthy individuals. All samples were subjected to microbiological examination. Bacterial isolates from urine samples of all patients with UTI were identified by microscopy and phenotypic characteristics on two selective media, MacConkey and EMB, and subsequently confirmed by the Analytical Profile index and Vitek diagnostic system. **Results:** the results showed that the 110 isolates gave typical phenotypic characteristics and biochemical tests belonging to the genus *Escherichia coli*, accounting for 54.9%, 50 isolates (25.3%) were *Klebsiella spp.*, 28 isolates (12.7%) were *Shigella spp.*, and 12 isolates (7%) were *Proteus spp.*, clinical biomarkers were determined for all patients group that infected with CKD, the results showed that there was significant increasing (p-value ≤ 0.05) in white blood cell, neutrophil, lymphocytes, blood urea and creatinine while there was non-significant increasing (p-value ≥ 0.05) in platelet count. A molecular study was done through analysis by RT-qPCR to quantify miRNA-126 expression as a biomarker for the disease. **Conclusion:** The fold change for miRNA126 is down-regulated in the patient compared to the control group (0.227).

Keywords: chronic kidney disease, CKD, microRNA-126, Biomarkers, diagnosis, urinary tract infections.
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INTRODUCTION

Chronic Kidney Disease (CKD) has become a leading cause of mortality and morbidity in the 21st century. The increasing prevalence of risk factors, such as obesity, diabetes mellitus, and hypertension, among the elderly population in both industrialised and developing countries has contributed to the emergence of a silent condition known as hypertension. This condition is characterised by the absence of noticeable clinical symptoms, especially in its early stages (1).

The urinary system comprises the kidneys, ureters, bladder, and urethra. Its primary role is to purify the blood by eliminating waste substances and surplus water. The system also plays a crucial role in maintaining the balance of ions and solutes in the blood, as well as controlling blood volume and blood pressure. Urinary tract infections (UTIs) are prevalent infections that can happen in the urethra (urethritis), bladder (cystitis), or kidneys (pyelonephritis). They are considered among the most globally prevalent infectious diseases, affecting approximately 150 million individuals annually. UTIs are categorised into two types: uncomplicated UTIs (uUTIs) and complex UTIs (cUTIs). UTIs are primarily caused by bacteria, with other species, such as fungi and viruses, being extremely uncommon. *Candida albicans* is the predominant fungal species responsible for UTIs.(2) Urinary retention, vesicoureteral reflux, frequent sexual activity, prostate gland enlargement, vulvovaginal atrophy, and familial history all increase UTI risk. Spermicides may increase UTI risk in women (3)**.** The pathogenesis of UTIs begins when uropathogens from the intestines colonise the urethra and eventually the bladder, via specific adhesions. If the host's inflammatory response fails to eliminate all bacteria, the bacteria begin to proliferate,

producing toxins and enzymes that aid their survival (4). Urine retention, vesicoureteral reflux, frequent sexual intercourse, prostate gland growth, vulvovaginal atrophy, and familial history all contribute to the risk of a UTI. Additionally, the utilisation of spermicides may increase the susceptibility to UTIs in females (3). Asymptomatic UTIs should only be treated in certain circumstances, such as in pregnant women, neutropenic patients, and those undergoing genitourinary surgery. This is because antibiotic treatment can potentially lead to the emergence of bacterial resistance (5). On the other hand, when UTIs have noticeable symptoms, they are typically treated with medications. However, these antibiotics may alter the bacterial flora in the intestines and vagina, thereby increasing the risk of spreading drug-resistant pathogens (6). Multiple studies have reported that microRNAs (miRNAs) play a crucial role in CKD, since certain miRNAs appear to be implicated in kidney disorders. Dysregulation of miRNAs has been demonstrated to have a role in the onset and advancement of various pathological conditions, including diabetic nephropathy, renal cancer, and renal damage. Furthermore, serum miRNAs exhibit exceptional stability in the bloodstream and have been proposed as valuable diagnostic and prognostic indicators for a wide range of disorders (7). miRNAs are a substantial group of preserved, diminutive, non-coding RNAs that are 19-25 nucleotides in length. They inhibit the translation and/or breakdown of their target mRNAs. (8). This regulation mechanism relies on the principle of base-pair complementarity between miRNAs and target sequences, which are primarily found in the 3' untranslated region (UTR) of messenger RNAs (mRNAs). Additionally, functional miRNA binding sites might be present in the 5' UTR and open reading frame regions (ORFs) (9). This characteristic enables a single miRNA to potentially target hundreds of mRNAs, whereas a single mRNA can be controlled by many miRNAs. Cooperative suppression is done through the binding of closely spaced target sites (10). Moreover, the problem of standardising miRNA expression levels during the investigations remains. Real-time polymerase chain reaction (RT-PCR) is a highly effective technique for analysing miRNA levels, due to its simplicity and precision. U6 and miR-1249 were previously employed before their substantial heterogeneity was uncovered. microRNA 126 (miRNA 126) can be found either within its own open reading frame (ORF) or within the intron of a host gene. miRNAs located within introns rely on the transcriptional control of their host genes (11). miR-126 is located on chromosome 9, specifically inside intron 7 of the EGFL7 gene in the human genome. miR-126 is an angiogenesis-promoting microRNA found in endothelial cells, and it is abundantly expressed in blood vessels (12). So this study aims to investigate miR-126 levels in patients with CKD and end-stage renal disease (ESRD), and investigate its correlation with well-established indicators of endothelial function and myocardial infarction (13).

METHODOLOGY

Sample collection

This study covered the period from October 2023 to March 2024. The samples collected from patients were urine and blood, from Al-Kindi Teaching Hospital and Al-Alami Hospital in Baghdad, Iraq. For this study, a total of 150 samples were collected from patients with a mean age of 50±5 years, which included 72 males and 78 females. In addition, 50 samples were collected for the healthy control group; patients with chronic diseases such as diabetes and autoimmune disease were excluded from the current study.

All samples were collected in accordance with institutional regulations, safety guidelines, and ethical standards, following ethical approval (Approval No.1090/2023, dated 01 October 2023), with written informed consent obtained from all participants before enrollment.

Inclusion criteria of chronic kidney disease patients

All samples (patient and control) were examined for the presence of UTI. The urine samples were collected in special tubes, then cultured on nutrient agar, MacConkey agar, and EMB, followed by microscopic examination, biochemical tests, and VITEK® 2 COMPACT to isolate and characterize Gram-negative bacteria

The blood samples were divided as follows:

1. For the complete blood account test (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 stored at (-20 °c) until used.
3. For molecular study, a volume of 600µl of serum is added to 400 µl of Trizol and stored at (-20 °c) until used.

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

Table (1): The name and sequence of primers used in this study

Name of Primer	5`-Sequence-3`	Reference
miR-126RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACT GGATACGACCGCATT	(14)
miR-126F	GGGCATTATTACTTTTGG	(14)
miR-126R	TGCGTGTCGTGGAGTC	
U6-FP	CTCGCTTCGGCAGCACA	(15)
U6-RP	AACGCTTCACGAATTTGCGT	(15)

Complete blood count (CBC) test

CBC was used to count each cell type and platelets in the blood and to detect the concentration of haemoglobin. It provides the healthcare provider with details about the blood and general health. CBCs aid medical professionals in the diagnosis, surveillance, and screening of a variety of illnesses, ailments, disorders, and infections. Results were recorded by the DxH 520 Beckman Coulter Compact according to the manufacturer's protocol, by age and sex.

Renal function test

Renal function testing was performed using a chemistry analyzer with electrolyte (IMT) and photometric capabilities. A test in which blood samples are checked for the amounts of certain substances according to the manufacturer's protocol, using an automated device by Spin 200 E throughput instrument. Results were recorded according to sex and age.

RNA extraction by TRIzol™(16)

1. A volume of 400 µL serum was added to 600 µL of TRIzol™. Pipette the lysate multiple times to homogenize. After that, it was incubated for 5 minutes to completely dissociate the nucleoprotein complex and stored in deep freeze (-20°c) until use.
2. The samples were mixed vigorously, then 150 µL of chloroform was added to the TRIzol™ Reagent for lysis. The mixture was vortexed again and incubated for 15 minutes in a freezer.
3. The samples were centrifuged at a speed of 12,000 rpm for 15 minutes. The mixture was separated into a lower layer containing red phenol-chloroform, an intermediate layer, and an upper layer consisting of colorless aqueous solution. The aqueous phase containing RNA was subsequently transferred to a new tube.
4. The RNA was precipitated by adding 300 µL of isopropanol to the aqueous phase. The samples were mixed using a vortex, and the resulting mixture was kept in a freezing environment for duration of 25 minutes. Subsequently, apply centrifugation to the mixture at 12,000 rpm for 10 minutes. Precipitation of total RNA yields a dense, gelatinous white mass that settles to the bottom of the tube.
5. The supernatant above was taken out using a micropipette, the RNA was precipitated by adding 600 µl of 75% ethanol; the pellet was dissolved by vortexing, and centrifuged at 7500 × g for 5 minutes.
6. The tube was opened for 25-30 minutes to dry after emptying the supernatant with the micropipette. The pellet was incubated at 60°c for 15 minutes, then resuspended in 25 µL of RNase-free water using a thermomixer. The collected RNA was kept at -20 °c until processing.

miRNA Quantitation

1. A volume of 190 µL of Qubit® working solution was added to each tube intended for use as a standard. Subsequently, 10 µL of each specified standard solution was added to the same tubes, followed by vortexing.

2. Each tube prepared for the sample was supplemented with 197 µL of the Qubit® working solution, followed by the addition of 3 µL of the sample to each tube individually.

3. The entire mixture was vigorously mixed and left to sit at room temperature for 3 minutes.

4. Standards tubes were placed into the Qubit device to generate a concentration curve.

5. The tubes containing the samples were sequentially inserted to measure the concentration of miRNA in each sample.

The same procedure was used for cDNA quantification, except that different standards and reagents (dyes) were used; otherwise, the remaining steps were identical.

RT-qPCR protocol

This step was divided into two parts: the first was performed by synthesizing cDNA from miRNA using a specific miRNA-126 primer (listed in Table 2) with the Protoscript cDNA synthesis kit. This procedure was performed as follows:

1. A volume of 5 µL of samples that contain total RNA was transferred into a fresh PCR tube.

2. A Protoscript reaction mix that consisted of (dNTPs, buffer, and other necessary components) was applied at a volume of 10 µL for each sample.

3. The MuLV enzyme was added with a volume of 3 µL for each sample.

4. A specific primer of 1.5 µL was added, and then the volume was completed to 25 µL by adding 4 µL of nuclease-free water.

The reaction mixture comprises the components listed in Table (2), each with its specified quantity.

Table (2): component of the cDNA mixture

Component	25 µL Reaction
Enzyme	3
Extracted total RNA	5
Nuclease-free Water	4
Random Primer	1.5
Reaction mix	10
RT126	1.5

5. The mixture was incubated at 42 ºC for an hour in a thermocycler. The enzyme was deactivated at 80 ºC, and thereafter, using a Qubit 4.0, the cDNA product was quantified and kept until the second part (Relative quantitative PCR).

The second part was done by following:

The cDNA sample was chosen from both the patient and control groups concurrently. Each sample requires two PCR tubes: one for the target miRNA (miRNA-126) and another for U6 snRNA, which serves as a housekeeping gene in this investigation. The quantification was done by assessing the fluorescence intensity of SybrGreen. 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

Perform high-speed centrifugation of the PCR tubes for 1 minute at 2000g to remove any air bubbles and collect the liquid. Afterward, adjust the Real-Time PCR software according to the selected thermocycling methodology (17) listed 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 was collected and analyzed by the Livak formula (18) as follows:

ΔCt patient = Ct patient – Ct U6

ΔCt control = Ct control – Ct U6

$\Delta\Delta Ct = \Delta Ct$ patient – ΔCt control

Folding expression = $2^{-(\Delta\Delta CT)}$

Statistical investigation:

The SPSS (2016) (IBM Corp, 2016) program was employed to observe the impact of variation in study parameters. The least significant difference (LSD) test (Analysis of variance (ANOVA) or T-test was employed to conduct a significant comparison between means. The Chi-square test was employed to identify significant associations among percentages. In this investigation, a confidence interval (CI), an odds ratio, and a P-value between 0.05 and 0.01 were projected in all statistical analyses.

RESULTS

Subject data:

Table (5) presents the results for both healthy subjects and CKD patients, including age, sex, family history, hypertension, and body mass index. The data indicate that 34% of participants reported good health, whereas 20% were diagnosed with CKD. This was particularly true for those under the age of 40, in the 40 to 50 age group, 50% of individuals were reportedly in good health, while 38% were diagnosed with CKD. Additionally, 42% of individuals diagnosed with CKD were over 50 years old, while 16% appeared to be in good health. Furthermore, the investigation determined 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 percentage of individuals with a family history was 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. The body mass index indicated that 30% and 24% of healthy individuals and patients with CKD, respectively, were within the 18.5-25 range, while 70% and 76% of healthy individuals and patients with CKD, respectively, were within more than >30 range.

Table (5): Criteria obtained from the Questionnaire form for all subjects and CKD Patients in the present study.n (%)

Criteria		Control	CKD
Age (year)	<40	17 (34%)	30 (20%)
	40-50	25 (50%)	57 (38%)
	>50	8 (16%)	63(42%)
Sex	Male	23(46%)	72 (48%)
	Female	27 (54)	78 (52%)
Family history	Yes	8 (16%)	135 (90%)
	No	42 (84%)	15 (10%)
Hypertension	No	43 (86%)	60 (40%)
	Yes	7 (14%)	90 (60%)
Body mass index	18.5 – 25	15 (30%)	36 (24%)
	>30	35 (70%)	114 (76%)

Morphological and microscopical characterization

Approximately 200 urine samples were collected and cultured in Nutrient broth and on MacConkey agar. The results showed that, following microbiological examination, 150 patients with urinary tract infection-related pain were diagnosed with Gram-negative bacterial infection. They grew various colonies on MacConkey agar. Some of these colonies exhibited a profound purple hue on MacConkey agar due to lactose fermentation. The colonies observed were flat and round, with a moist texture and a partially defined edge on the surface, indicating the growth of lactose-fermenting species such as *Escherichia coli*, *Enterobacter, and Klebsiella*. While white colonies indicate the absence of lactose-fermenting species such as *Salmonella, Proteus, Yersinia,* and *Pseudomonas*

To confirm the diagnosis, colonies that fermented lactose were cultured on EMB agar plates. The majority of *Escherichia coli* strains on EMB agar displayed a distinct green sheen in their colonies. Microscopic analysis of this isolate revealed Gram-negative bacteria that appeared oval-shaped. They were pink rods, short to medium length, straight or slightly curved, slender, and non-sporulating. These bacteria were observed appearing as pairs or alone. The biochemical results for different isolates of Gram-negative bacteria are shown in Table (6). The *Escherichia coli* isolates were positive for the indole production test, indicating their capacity to hydrolyze tryptophan to indole via the tryptophanase enzyme. Additionally, they also yielded positive results in the methyl red test. Although the simmon citrate utilisation test and Voges-Proskauer test yielded negative results.

Table (6): Results of biochemical tests for Gram-negative bacteria isolated from UTIs

Biochemical Test	Indole	Methyl Red	Voges-Proskauer	Citrate	Oxidase	Catalase
Uropathogenic Escherichia coli	+	+	-	-	-	+
Klebsiella	-	-	+	+	-	+
Enterococci	-	-	+	-	-	-
Proteus	-	+	-	+	-	+

Klebsiella was negative for indole, methyl red, and oxidase, but positive for Voges-Proskauer, citrate, and catalase. *Enterococci*, on the other hand, showed a negative reaction for indole, methyl-red, citrate, oxidase, and catalase, while Voges-Proskauer indicated a positive reaction. Finally, *Proteus* showed indole, Voges-Proskauer, and oxidase-negative reactions, whereas methyl red, citrate, and catalase were positive.

Additional confirmatory identification of the negative isolates to the species level was carried out using VITEK2 dense automated system tests. The results shown in Table (7) indicate that about 110 isolates (54.9%) were *E. coli*, 50 isolates (25.3%) were *Klebsiella*, 28 isolates (12.7%) were *Shigella*, and 12 isolates (7%) were *Proteus*.

Table (7): Classification and occurrence of microorganisms identified in urine cultures

Bacterium	Numbers
Escherichia coli	110(54.9%)
Klebsiella	50(25.3%)
Shigella	28(12.7%)
Proteus	12(7%)

The Hematology Analysis

The results showed that there was a significant increase (p-value ≤ 0.0001) in white blood cell, lymphocyte, and neutrophil counts, with values of 11.12, 2.8, and 6.77, respectively. AT the same time, there was a non-significant increase (p-value ≥ 0.001) in platelet count that was 264.9. The hematological test results of all patients with CKD are shown in Table (8).

Table (8): Hematological parameter tests for Patients with CKD and the control group

Group Parameter	Normal Range for Healthy Cases	Healthy Control Group	Patient Group	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	0.0702

*: significant, **: highly significant, p-Value ≤0.05

Biochemical analysis

The results of this study showed significant increases in urea and creatinine values, which were 62.71 mg/dL and 1.38 mg/dL, respectively, as shown in Table (9). Various biochemical indicators are present in both blood and urine to evaluate renal function.

Table (9): Biochemical parameters (Urea and Creatinine tests) for Patients with CKD and the control group

Group Parameter	Normal Range for Healthy Cases	Healthy Control Group	Patient Group	*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, *p*-Value ≤0.05

Gene expression of miRNA-126

Real-time PCR amplification program was done by using melting curve analysis for all evaluated gene expression according to the manufacturer's protocols, using relative gene expression. Total RNA extracted from blood samples was reverse-transcribed into cDNA for analysis of miRNA-126 gene expression by real-time PCR (qPCR). Expression of these genes was normalized to the housekeeping gene's level (U6) and quantified using the Ct value, as shown in Figure 1. Each colour in the curve displayed in these figures represents the Ct value.

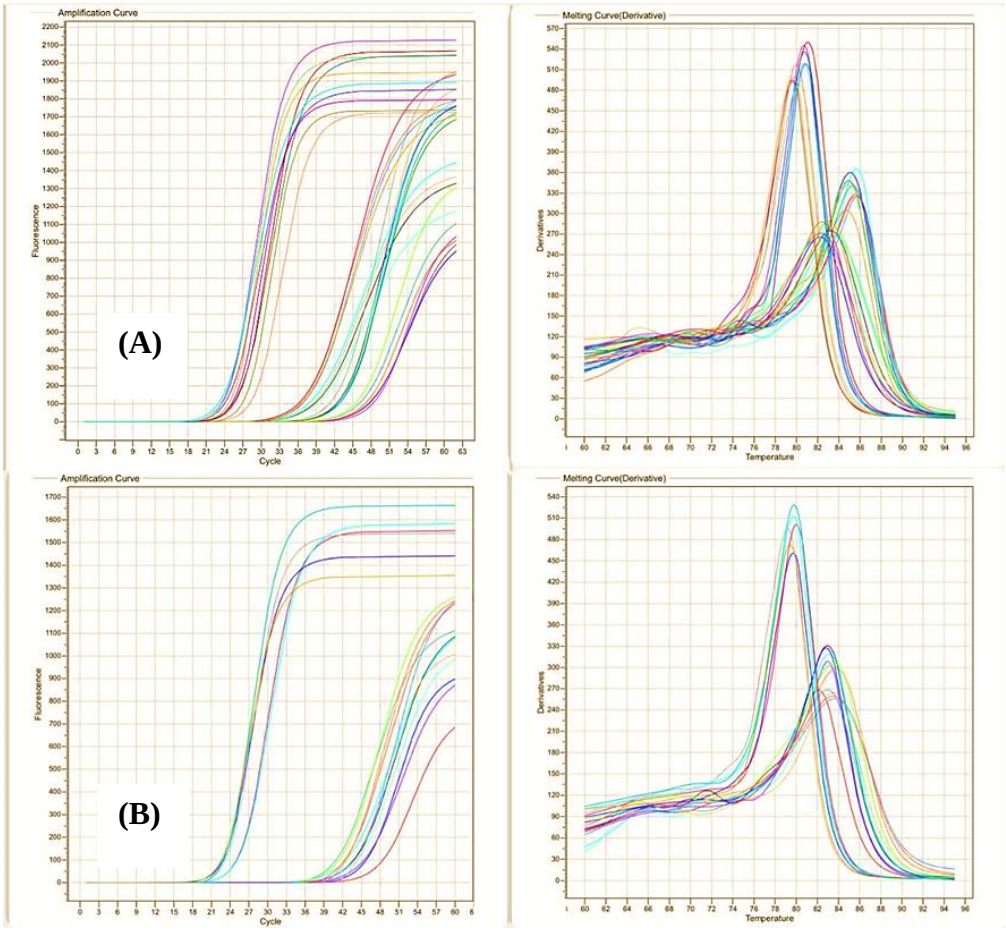

Figure (1): (A) amplification plots for miRNA 126 and U6 obtained by RT-qPCR, (B) melting curve analysed for miRNA-126 and U6 expression.

The gene expression of miRNA-126 was analyzed by qRT-PCR, using the reference gene U6 for quantification, and comparing the Ct values between the studied groups (patients and healthy controls) in different stages of gene expression level, Ct, ΔCt, ΔΔand fold change, as shown in Table (10).

Table (10):Comparison between the studied groups (patients and healthy controls) in different stages of gene expression level Ct, ΔCt, ΔΔCt, and fold.

Groups	Mean Ct	Ct U6	ΔCt =Mean Ct of miRNA126 - Mean Ct of U6)	ΔΔCt=ΔCt patient – ΔCt control	Fold = $2^{-\Delta\Delta Ct}$
Patients	48.17	24.80	23.37	2.17	0.227
healthy controls	46.00	24.70	21.2	0	1

The Ct value correlates with the level of gene expression; the lower Ct value indicates the presence of higher copies of the target gene.

The results showed that the Ct value for miRNA-126 was 48.17 in the patient group and 46.00 in the healthy control, and the Ct value for U6 was 24.80 in both groups.

The ΔCt value was calculated as the difference between the patient's miRNA-126 Ct and the control's miRNA-126 Ct, normalized to the U6 Ct. These results showed that the ΔCt of miRNA-126 was 23.37 in the patient group and 21.2 in the control group. Using the 2^-ΔΔCt and Livak equations, the fold change analysis showed downregulation of miRNA-126 in patients with CKD.

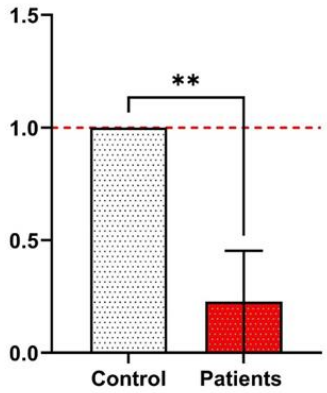

Figure (2): fold of change ($2^{-\Delta\Delta ct}$) miRNA126 expression of the two study groups.

DISCUSSION

Chronic kidney disease (CKD) is characterised by a long-term and permanent decline in kidney function. It has a global impact on millions of people and raises the likelihood of developing cardiovascular disease and mortality. CKD disrupts the control of important metabolic pathways (19). *Escherichia coli is* the primary cause of urinary tract infections (UTIs), followed by *Klebsiella*. Other significant species in these infections include *Salmonella*, *Enterobacter*, and *Proteus* (20,21). The diagnosis of UTI was based on clinical history and urinalysis, which was confirmed by a urine culture (22). *Escherichia coli* was identified as the predominant bacterium responsible for urinary tract infections (UTIs) in various investigations, with reported percentages of 41% and 56% (35,36). *K. pneumoniae* (18.5%) and *Enterococcus faecalis* (7.7%) were other possibilities. Updated urine pathogen prevalence data by sex, age, and location must be considered when selecting an antibiotic (23,24). The results showed a significant increase (p-value ≤ 0.0001) in white blood cell, lymphocyte, and neutrophil counts, with values of 11.12, 2.8, and 6.77, respectively, while there was a non-significant increase (p-value ≥ 0.001) in platelet count, with a value of 264.9. Chronic kidney disease (CKD) affects around 10% of the population and has major social and economic effects (25). In the elderly, chronic renal disease was common (26). Urinalysis results include white blood cells, a positive nitrite test, bacteria, and red blood count. The minimal, median, and maximal white blood cell counts were defined. Their total blood count showed a median WBC value within the reference range (27).

Escherichia coli and non-*Escherichia coli* UTI patients had similar rates of aberrant laboratory test results, according to another study by (28). The most common abnormal urine analysis results were cloudy urine, proteinuria, and leukocyturia, which were the most prevalent abnormal urine outcomes. *Escherichia coli* and *non-Escherichia coli* UTIs must be diagnosed early to ensure appropriate empirical antibiotic treatment. Preventing irreversible kidney damage, comorbidities, lengthy treatment, recidivism, and chronicity is crucial (28).

White blood cells, also known as WBCs, are immune cells that play crucial roles in serious medical conditions such as malignancies, infections, and inflammatory illnesses. The total count of WBCs observed was (11.12 $*10^3/\mu L$) for CDK patients, while the control group was (6.76 $*10^3/\mu L$). The results showed a highly significant difference between patients who had CKD, and the healthy control group (p-value ≤ 0.001)(29). Studies by (30,31,32,33) results were in line with this study.

Lymphocytes are a type of agranulocyte that make up 20 to 40% of the total leukocyte count. They are an integral part of the adaptive immune system. Lymphocytes are mobile immune cells that possess the ability to recognise and respond to antigens. They constantly move among different lymphatic tissues. Changes in neutrophil and lymphocyte cell counts were linked to sickness severity in many conditions, including infections (34), which is a significant increase (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).

Lymphocytes play a role in CKD, where their diversity and ability to change shape contribute to their several functions in causing kidney damage and scarring. Innate and adaptive lymphocytes work together in the process and exhibit a phenomenon of sequential responses, highlighting the significance of separate therapies that target specific subsets of lymphocytes. The study of lymphocyte interference in living organisms and the transfer of these cells has opened up possibilities for therapeutic techniques connected to lymphocytes. Regulation of the gut microbiota and metabolites to modulate lymphocyte immunological responses associated with chronic kidney disease (CKD) demonstrates promising therapeutic potential for the development of future drugs targeting kidney illnesses (35).

T-lymphocyte subsets serve as indicators of a patient's immunological state and have been linked to negative outcomes in many diseases. Nevertheless, the correlation between T-lymphocyte subsets and primary infection, as well as renal prognosis in patients with chronic kidney disease, has not been fully examined (36).

The results of this study showed that the neutrophile count was highly significant, with a value of (6.77 $*10^3/\mu L$). Immature phagocytes, known as neutrophils (NET), have a very short half-life. They can release oxygen free radicals and proteolytic enzymes, which are known to exacerbate inflammation and its associated damage (37). release a variety of cytotoxic chemicals, including reactive oxygen intermediates, enzymes, and microbicidal polypeptides; these products play a crucial role in inflammation and tissue destruction, especially in acute renal failure. 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 (38).

Additionally, platelet count results showed no significant difference between the patient group and the healthy control group, which were (264.9* 10^3/µL). Platelets, which originate from bone marrow megakaryocyte cells, are the smallest blood cells and play a crucial role in hemostasis and coagulation (39).

A study investigated the role of platelet parameters in various disorders, including UTIs (40). Platelets can promote the inflammatory process by recruiting leukocytes and preventing the death of monocytes and neutrophils. This leads to increased levels of inflammatory mediators such as chemokines and cytokines (41). During the inflammatory process, there is an increase in platelet count as a component of the acute phase reaction (42).

Biochemical markers are crucial for accurately diagnosing conditions, evaluating risk, and implementing treatment strategies to enhance clinical outcomes. Rather than using the invasive methods of urine analysis, serum measurements of renal function markers, including urea, creatinine, electrolytes, uric acid, and blood urea nitrogen, are regularly employed. The kidneys excrete urea, a major nitrogenous waste product resulting from the breakdown of proteins and amino acids, and creatinine, a byproduct of the breakdown of creatine phosphate in muscle. Urea and creatinine are reliable markers of normal kidney function, whereas elevated levels in the bloodstream indicate renal failure. Creatinine is a generally established and commonly used indicator for measuring renal function (43).

In this study, a significant increase in urea level was observed in all groups for patients who had CKD and a healthy control group (*p*-value 0.0267); (62.71±59.51) and (32.33±5.9), respectively.

On the other hand, there were highly-significant differences in the creatinine level between the CKD patient and healthy control group (*p*-value 0.0019), which were(1.38) and (0.90), respectively.

Blood creatinine and urea are crucial parameters for assessing renal function and play a significant role in determining the severity of the condition (44). Glomerulus filtered creatinine, and the quantity of creatinine in the serum was used as an indirect indicator of glomerular filtration. The rise in serum creatinine levels indicates the progression of renal disease and is a more reliable indicator than urea for predicting adverse outcomes (45). Approximately 85% of urea was excreted through the renal system, whereas the remaining portion was removed via the gastrointestinal (GI) tract. Conditions characterised by decreased renal clearance, such as acute and chronic renal failure, lead to elevated serum urea levels. This study suggests that urea levels may also increase in conditions unrelated to renal disorders, including upper gastrointestinal haemorrhage, catabolic states, high-protein meals, and dehydration. Urea levels may fall in cases of hunger, severe liver illness, and a low-protein diet. Urea was less reliable than serum creatinine for renal function. In renal disease, urea is elevated early, consistent with this study (46).

Several microRNAs (miRNAs) are associated with CKD mortality and progression (47). miRNAs have recently attracted interest as potential biomarkers for assessing the severity and/or causes of kidney disease (48). An important benefit of miRNAs is their inherent steric stability, which renders them well-suited to serve as non-invasive biomarkers (49). Therefore, these short RNAs may be sufficiently reliable for potential therapeutic applications (50). MicroRNAs (miRNAs) are a recent advance in gene expression regulation. They are skilled musicians recognised for their ability to regulate gene expression post-transcription. miRNAs are small, unpaired RNA molecules that bind to specific areas of target mRNAs, primarily inside the 3′ untranslated region. It is now understood that miRNAs play a role in the pathophysiology of the kidneys. miRNAs play a significant role not only in cellular processes in kidney disease but also in patients with chronic kidney disease and acute renal injury (51).

miRNAs have the potential to be valuable biomarkers in nephrology. However, before they can be utilised in routine clinical practice, their expression must be evaluated in large cohorts. Additionally, efforts must be made to establish standardised measuring techniques and determine the most appropriate tissues and biofluids for analysis. Furthermore, manipulating the expression of miRNAs, either increasing or decreasing their levels, represents a new and promising therapeutic strategy for treating renal illnesses. Additionally, miRNAs have the potential to serve as biomarkers for these conditions (52).

The role of miRNAs in the pathophysiology of human diseases has generated significant interest in their potential for diagnosis and treatment. Modifications to miRNAs can concurrently impact many elements of a signalling pathway. Various methods for inhibiting miRNA activity have been developed, including antisense tactics, antagomirs, and Decoy or Sponge technologies (53). A potential therapeutic strategy may involve suppressing miRNAs implicated in albuminuria, extracellular matrix accumulation, EMT, and podocyte dysfunction, or restoring miRNA activity in kidney disorders in which miRNAs are downregulated (54). There is a suggestion that by modulating dysregulated miRNAs in living organisms, it may be possible

to reduce the occurrence of certain disorders (55). miRNAs play a crucial role in regulating gene responses to diverse pathophysiological stressors and therefore have the potential to be used in therapy (56). To achieve an effective treatment response in CKD, it is necessary to administer miRNA precursors for overexpression and miRNA inhibitors for inhibition in a precisely timed, tissue-specific manner (57). Quantitative PCR is a widely employed method for precise measurement of miRNA levels in blood samples and can be implemented in clinical laboratories. When performing quantitative PCR, it is crucial to select a suitable control gene, particularly because miRNA levels are often low in the bloodstream. Certain teams have opted to utilise short endogenous RNAs, such as U6, to standardise circulating miRNA levels. However, outcomes have been inconsistent, reflecting significant fluctuations in RNA expression across clinical contexts (58).

microRNA-126 The study's findings indicate that miRNA-126 was downregulated. miRNA-126 is specific to endothelial cells and plays a role in maintaining blood vessel integrity and promoting angiogenesis by regulating vascular endothelial growth factor (VEGF) signalling. Consequently, it also reduces inflammation in blood vessels (59). miRNA-126, which was downregulated in CKD in the general population (50, 60,61), and that is in line with this study.

Upon quantifying miRNA-126 levels in serum and whole blood samples, a negative correlation with CKD was observed (62). An observational study showed that lower miRNA-126 levels were associated with a higher likelihood of CKD and a rapid deterioration in renal function over a 5-year period (63). Additionally, miRNA-126 has been shown to protect against atherosclerosis by promoting turnover of vascular smooth muscle cells (VSMCs) and regulating their contractile phenotype (64). Collectively, decreased miRNA-126 expression can lead to vascular dysfunction, a common feature of CKD. miRNA-126 could serve as a promising biomarker for early detection of CKD and as a potential target for preventing or treating vascular complications associated with CKD. Conversely, few studies have demonstrated increased miRNA-126 expression in persons with diabetic kidney disease (DKD) (65,66,67,68) when measured in urine, plasma, and serum. This may be a compensatory mechanism that leads to increased miRNA-126 release when microvascular endothelial cells are subjected to stressful conditions (69,70). miRNA-126 is secreted by glomerular endothelial cells in response to cytokines associated with diabetic kidney disease (DKD) (60,64). miRNA-126 is highly expressed in endothelial cells (ECs) and has been extensively explored in the field of vascular biology and disorders (70,71,72). The endogenous miRNA-126 is potentially linked to different vascular functions (angiogenesis, leukocyte adhesion, and inflammation) in atherosclerotic lesions. It achieves this by reducing the expression of proteins involved in signalling pathways, such as Sprouty-related enabled/ VASP homology domain-containing protein 1 and phosphatidyl inositol 3-kinase regulatory beta (61,62). Reductions in these molecules trigger rapid activation of fibrosarcoma via the mitogen-activated protein kinase signalling pathway, thereby enhancing vascular endothelial growth factor expression and stimulating angiogenesis. Harris et al. found that elevating miRNA-126 levels in endothelial cells (ECs) not only promotes angiogenesis but also suppresses vascular cell adhesion molecule 1 expression. As a result, leukocyte adherence to ECs is reduced (73,74). Furthermore, miRNA-126 upregulates sirtuin1 and superoxide dismutase-2 expression, thereby reducing oxidative stress in endothelial cells (ECs). Higher circulating miRNA-126 levels may be associated with preserved vascular function and a reduced risk of chronic kidney disease (CKD) (75). The univariate analysis revealed significant correlations between circulating miRNA-126 levels and age, haemoglobin and cholesterol levels, and platelet count. Our multivariate analysis revealed that miRNA-126 levels were independently associated with eGFR, platelet count, haemoglobin level, and age (76,77,78,79).

CONCLUSION

It was concluded that there is a relationship between infection with G-ve bacteria, the causative agents of CKD, and changes in miRNA expression. Serum levels of white blood cells (WBC), lymphocytes (LYM), neutrophils (NTR), platelets (PLT), urea, and creatinine are used as indicators for diagnosing chronic kidney disease. The strong positive association between miRNA-126 expression and CKD suggests that this biomarker plays a significant role in disease development and bacterial infection, particularly with Gram-negative bacteria. It could serve as a valuable marker for the diagnosis and treatment of these conditions.

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تقييم التعبير عن رنا 126 في مرضى أمراض الكلى المزمنة المصابين بالبكتيريا سالبة الجرام

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قسم التقنيات الحيوية الجزيئية والطبية، كلية التقنيات الأحيائية، جامعة النهرين، بغداد، العراق.

الخلاصة

الخلفية عن الموضوع: يعد مرض الكلى المزمن (CKD) من المشاكل الصحية الرئيسية والقاتلة في المنطقة وأصبح سببا للوفاة في جميع أنحاء العالم. وتسببه أنواع مختلفة من العوامل الميكروبية، من الجلد أو المستقيم، التي تدخل إلى مجرى البول وتصيب المسالك البولية.**الهدف من الدراسة:** صممت هذه الدراسة لمعرفة العلاقة بين التعبير الجيني لـ mi RNA-126 ومرض الكلى المزمن كمؤشرات حيوية في عينة من المرضى العراقيين. **المواد و طرق العمل:** تم جمع العينات التي خضعت للفحص الميكروبيولوجي من عينات البول لجميع المرضى المصابين بالتهاب المجاري البولية باستخدام الفحص المجهري والخصائص المظهرية حيث استخدمت نوعين من الوسائط الانتقائية MacConkey وEMB، وتم تشخيصهم بواسطة Vitek. أظهرت النتائج أن 110 عزلة أعطت الصفات المظهرية النموذجية والاختبارات الكيموحيوية التابعة لجنس الإشريكية القولونية والتي كانت (54,9%)، 50 عزلة (25,3%) كليبسيلا، 28 عزلة (12,7%) شيجلا و12 عزلات 7(%) بروتيوس. تم تحديد المؤشرات الحيوية السريرية لجميع عينات المرضى المصابين بمرض الكلى المزمن،**النتائج:** أظهرت النتائج أن الاختبارات الأكثر شيوعا في الإصابة بعدوى المسالك البولية هي خلايا الدم البيضاء، الخلايا الليمفاوية، العدلات، عدد الصفائح الدموية، اليوريا والكرياتينين مقارنة بمستوياتها في الأصحاء. أظهرت النتائج وجود زيادة معنوية (p-value $\geq$0.05) في خلايا الدم البيضاء، العدلات، الخلايا الليمفاوية، يوريا الدم والكرياتينين بينما كانت هناك زيادة غير معنوية (p-value $\geq$0.05) في عدد الصفائح الدموية. تم إجراء الدراسة الجزيئية من خلال التحليل بواسطة (RT-qPCR) لتحديد تعبير miRNA-126 كمؤشر حيوي للمرض. **الاستنتاجات:** بينت الدراسة انخفاض في مستوى التعبير الجيني لـ-mi RNA 126في المرضى مقارنةً بالمجموعة القياسية (0.227).

الكلمات المفتاحية : الإشريكية القولونية، كليبسيلا، شيجلا، بروتيوس.