

# Expression profile of miR-15 and miR-16 in peripheral blood mononuclear cells of patients with steroid-resistant nephrotic syndrome

Sepideh Zununi Vahed<sup>1</sup> , Shahram Ghiyasvand<sup>1,2</sup>, Seyyedeh Mina Hejazian<sup>1,3</sup> , Mohammadali M. Shoja<sup>4</sup> , Mohammadreza Ardalan<sup>1\*</sup>

<sup>1</sup>Kidney Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

<sup>2</sup>Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

<sup>3</sup>Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran

<sup>4</sup>Clinical Academy of Teaching and Learning, Ross University School of Medicine, Miramar, FL, USA

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## ABSTRACT

**Introduction:** Steroids are considered first-line therapeutic opportunities in cases with idiopathic nephrotic syndrome (INS). The trustworthy biomarkers for steroid-resistant NS (SRNS) would permit more precise decisions in the treatment of INS.

**Objectives:** This study aimed to evaluate the miR-15 and miR-16 levels in NS cases.

**Patients and Methods:** Adult cases with primary NS (n=60) including 30 FSGS (Focal segmental glomerulosclerosis) and 30 membranous glomerulonephritis (MGN) patients and 24 healthy individuals were included. The evaluation of miR-15 and miR-16 expression in blood cells was performed using real-time polymerase chain reaction (qPCR). Moreover, gene ontology analysis and prediction of the miRNA targets were completed to recognize the biological procedures and signaling pathways involved in the pathophysiology of NS.

**Results:** A significant increase was observed in miR-15a-5p expression in cases with primary MGN and FSGS compared with healthy subjects. Conversely, the miR-16-5p expression was significantly decreased in both conditions compared with healthy controls. In the clinical subdivision of FSGS group to steroid-resistant-FSGS and steroid-responsive-FSGS, significant elevated levels of miR-15a-5p and diminished levels of miR-16-5p were observed in both groups compared to normal controls. Gene annotation demonstrated that these miRNAs contribute to cell cycle, ion transport, biological adhesion, cation transport, cellular response to endogenous stimulus, and regulation of small GTPase-related signal transduction.

**Conclusion:** Dysregulated levels of miR-15 and miR-16 may be involved in the pathogenesis and response to steroid therapy in patients with INS.

## Implication for health policy/practice/research/medical education:

Dysregulated levels of microRNAs were observed in clinical samples of adult patients with steroid resistant nephrotic syndrome. This microRNAs may be good biomarker in isolation of steroid resistant nephrotic syndrome from steroid sensitive nephrotic syndrome.

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## Introduction

Idiopathic nephrotic syndrome (INS) defines a group of renal glomerulus pathologies described by heavy proteinuria, edema, and hypoalbuminemia. Based on the underlying molecular mechanisms, NS can be classified into genetic-mediated, immune-mediated, and those with

circulating factor. This new classification with disease biology insights rather than histological description helps INS subgroups to receive a targeted therapy (1).

Steroids are considered the first-line therapeutic opportunities in patients with INS (2, 3). Recurrent relapses and drug-related toxicities are the main clinical obstacles

in steroid-sensitive NS (SSNS) (4), while steroid-resistant NS (SRNS) without any improvement in their proteinuria are facing with gradual decline of kidney function and are destined to end-stage renal diseases (ESRD) (5, 6). Constant proteinuria after 8-12 weeks of steroid regimens is a simple definition for SRNS. Glomerular podocytes foot processes effacement is the main ultrastructural morphological feature in both SSNS and SRNS cases (7, 8). In patients with SRNS, even prolonged steroid therapy is ineffective and facing them with harmful side effects. In these patients, calcineurin inhibitors, plasmapheresis and rituximab were associated with remission in some cases. Hence, the identification of trustworthy biomarkers for SRNS would let more precise decision in the treatment of INS.

Genetically-based podocyte structural derangement, epigenetic and pharmacogenetics elements all are involved in the pathogenesis of SRNS (9). However, factors outside the kidney could be also responsible for SRNS. The proposed mechanism of circulating factors in the pathogenesis of some forms of focal segmental glomerulosclerosis (FSGS) has been verified by its recurrence after kidney transplantation as this factor is waiting to damage the new kidney again. Moreover, the favorable effects of plasmapheresis in most of these recurrent patients highlights the accuracy of this theory much more (10-12). Inflammatory cytokines are other proposed factors connected to NS pathophysiology (13,14). Despite the effectiveness of immunosuppressive drugs in some SRNS patients, a major group remain unresponsive. The current knowledge does not provide us optimal therapeutic options or predict the responsiveness or recurrences (11,15). Thus, an inclusive outlook based on the molecular mechanism -directed therapeutic options is required.

microRNAs (miRNAs) are non-coding RNAs regulating gene expression negatively through repression of protein translation or induction of messenger RNA degradation. They have significant roles in different biological pathways including cell apoptosis, proliferation, metabolism, immunity, and oncogenesis. Dysregulated levels of miRNAs are reported to be involved in the physiopathology of NS.

### Objectives

The involvement of microRNAs is also reported in the regulation of steroids responsiveness in different pathological conditions such as acute lymphoblastic leukemia and asthma. It is likely that miRNAs act as steroid responsiveness markers and could be applied as a screening test to stratify the therapeutic direction. Recently, it is demonstrated that glucocorticoid (GC) receptors (GCRs) and miRNAs can interact with each other in a bidirectional way and miRNA regulation is an important component of GC signaling (16-18); consequently, it is important to study their profile among SRNS. In so doing,

the present study evaluated the expression of miR-15 and miR-16 in blood cells of NS patients. These immune cells can be useful clinical sources in NS since NS is highly associated with the immune system (19, 20).

## Patients and Methods

### Study population

The target group in our study included patients with primary NS (Urinary protein excretion higher than 3 g/24 h) patients that hospitalized in Imam Reza Hospital, Tabriz, Iran (November 2017 to March 2019). In the present cross-sectional study, primary NS cases (n=60) with age range between 20-60 years were included. Secondary NS patients (such as diabetic nephropathy, amyloidosis, systemic lupus erythematosus, viral infections and drug-induced NS) were excluded. Other exclusion criteria were positive HIV test, active bacterial infections, urinary tract obstruction, patients with a cancer background, autoimmune systemic disease and acute renal failure. Serum examination for antiphospholipase A2 receptor antibody (Anti-PLA<sub>2</sub>-R antibody) and renal biopsy findings were utilized to obtain additional information regarding the etiological and histopathological details. In FSGS patients based on their proteinuria response to glucocorticoid therapy, they were divided to responsive and resistant once. Patient with primary membranous glomerulonephritis (MGN) was included as another podocytopathy group with steroid-resistant nature proteinuria. Healthy volunteers (n=24) were also included as normal control group.

### PBMC preparation and RNA extraction

EDTA pre-coated vacutainer tubes were utilized to collect freshly obtained blood samples prior to peripheral blood mononuclear cell (PBMC) isolation. To do this, phosphate buffered saline (PBS) was used to dilute peripheral blood (4 mL) which was then added into equal volume of Ficoll-Paque solution. The centrifugation processes (500 ×g for 10 minutes) was repeated in a new RNase-free falcon after washing the cells. Afterwards, the supernatant was removed and the previous centrifugation process was performed. Ultimately, Trizol (1mL) was utilized to solve the cell pellet which provided storable samples at -70°C for future application. Trizol protocol (RiboEx™, South Korea) was applied to extract the total RNA and the properties of RNA (quality, quantity and integrity) were estimated by calculation of Optical Density (OD) (NanoDrop™ One<sup>C</sup> Thermo Scientific, USA).

### Quantification of the transcripts

Synthesis of first strand cDNA and the amplification of the synthesized cDNA in PBMCs specimens were performed by PCR and real-time PCR methods, respectively. In this way, 15 µL volume of reverse transcriptase (RT) reaction were included of 1 µg of RNA, 3 µL RT buffer, 0.8 µL RT enzyme, 1.5 µL dNTP, 0.375 µL Ribolock, 3 µL primer mix (miR-15, and miR-16 specified primers, Table 1) and 1.325

**Table 1.** Sequences of designated primers in the study

| Primers    | Type | Sequence (5'-3')                                    |
|------------|------|-----------------------------------------------------|
| miR-15a-5p | RT   | GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACCACAAA  |
|            | F    | CCGTAGCAGCACATAATGGTTTGT                            |
| miR-16-5p  | RT   | GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACCGCCAAT |
|            | F    | GCTAGCACGTAAATATTGGCG                               |
| SNORD-47   | RT   | GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACAACCTCA |
|            | F    | GCGATATCACTGTAAACCGTTCC                             |
|            | R    | GTGCAGGGTCCGAGGT                                    |

μL DEPC water that were incubated in a thermocycler at 42°C for 60 minutes, 10 minutes at 72°C and then incubated at 4°C. After that, quantitative PCR was done at 95°C for 15 minutes, 95°C for 10 seconds, and at 60°C for 30 seconds (×40 cycles) via SYBR Master Mix. The internal control for PBMCs was the SNORD-47 primer.

### *In-silico analysis*

Gene ontology (GO) analysis were applied for shared gene sets enrichment (<http://software.broadinstitute.org/gsea/msigdb/annotate.jsp>) and miRwalk online tool (<http://mirwalk.umm.uniheidelberg.de/>) were performed to predict the miRNA targets.

### *Statistical analysis*

Percentages and numbers were used to represent categorical variables. Mean ±SD (standard deviation) or median (Max-Min) were used to present normally or Non-normally distributed variables, respectively. To compare quantitative and categorical variables, Mann-Whitney *U*/Student's *t* test and chi-squared tests were performed, respectively. One-way ANOVA analysis followed by Tukey's HSD post hoc test was used to compare normal variables whereas or Kruskal-Wallis compared non-normally distributed variables.

## **Results**

### *Subjects*

Sixty patients with INS were enrolled in this study with mean age  $45.76 \pm 13$  years. Thirty patients were diagnosed as FSGS, most of which (N=21) were unresponsive to steroid, they were named steroid-resistant-FSGS. Thirty cases were diagnosed as primary MGN. To underscore the general effect of proteinuria, at first, we compared NS as a whole group with controls and then the FSGS group results were compared with MGN and healthy individuals. No significant differences were observed in GFR (mL/min/1.73 m<sup>2</sup>) [Median: 80 (18-137) and 79 (10-130),  $P=0.324$ ], serum urea (mg/dL) [Median:  $38.2 \pm 16.5$  and  $44.2 \pm 20$ ,  $P=0.18$ ], uric acid (mg/dL) [Median:  $6.61 \pm 1.2$  and  $6.3 \pm 1.1$ ,  $P=0.94$ ], serum creatinine (mg/dL) [Median:  $2.21 \pm 0.55$  and  $1.2 \pm 0.5$ ,  $P=0.17$ ] and 24-hour urine protein excretion [Median: 3013 (85-15000) and 1360 (97-15900),  $P=0.26$ ] between the MGN and FSGS groups, respectively.

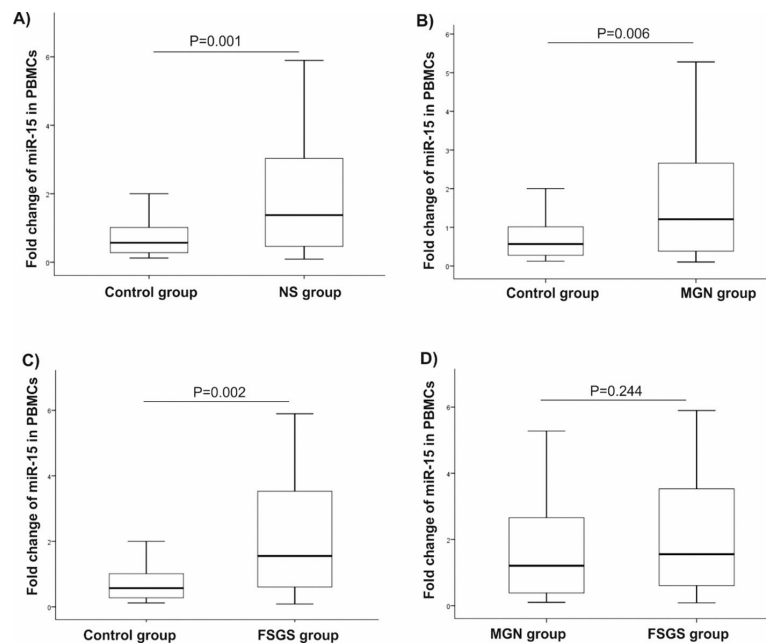
On the whole, we compared the miRNAs levels in patients based on the histopathology and response to steroids. A RT-qPCR assay was performed in the studied groups (FSGS and MGN patients and controls) to evaluate the expression levels of miR-15 and miR-16 in PBMCs samples. There were a significant increase in miR-15 level in NS ( $P=0.001$ ), FSGS ( $P=0.002$ ), and MGN ( $P=0.006$ ) groups comparing to controls (Figure 1A-C). Moreover, an elevation in miR-15 expression level detected in FSGS group compared to MGN group that was not significant statistically ( $P=0.244$ , Figure 1D). There was a decrease in PBMCs levels of miR-16 in NS cases ( $P=0.022$ ). The reduced levels of miR-16 was significant in FSGS subgroup when compared to controls,  $P=0.13$  (Figure 2A-C). No significant differences was observed between subtypes of NS ( $P=0.209$ , Figure 2D).

A comparison analysis was also performed in FSGS patients based on their responses to steroid therapy and their proteinuria levels. As shown in Figure 3A, there was a statistically significant increase in miR-15 level in steroid-responder-FSGS ( $P=0.008$ ) and steroid-resistant-FSGS ( $P=0.011$ ) compared to healthy controls. Furthermore, there was a meaningful decrease in miR-16 expression level ( $P=0.009$ ) of resistant FSGS group compared with healthy controls (Figure 3B).

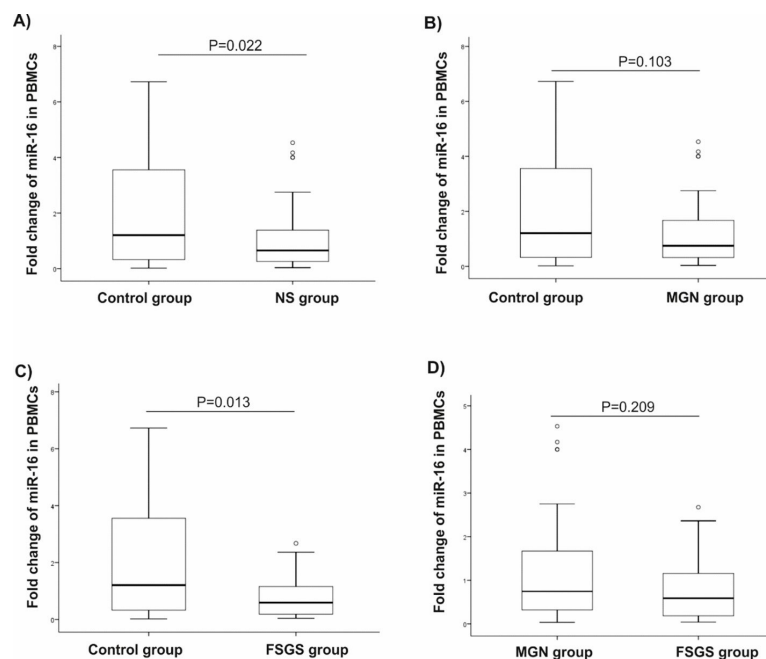
### *GEO data sets and gene ontology enrichment (GO) analysis*

Gene ontology (GO) annotation illustrated that in MGN, miR-15 mostly contributes to cytoskeleton organization, cellular component morphogenesis, reply to endogenous stimulus, positive control of molecular function and organelle organization control. GO for miR-16, positive regulation of molecular function, organelle organization, and protein modification process, as well as cytoskeleton organization and response to endogenous stimulus (Figure 4A, B).

GO annotation demonstrated that in FSGS, the miR-15 mostly contributes to ion transport, biological adhesion, cation transport, regulation of GTPase-related signal transduction, and cellular reply to endogenous stimulus. GO also demonstrated that miR-16 may be involved in biological adhesion, epithelial tube morphogenesis, epithelium development, regulation of GTPase activity, and protein phosphorylation in FSGS (Figure 4C, D).



**Figure 1.** miR-15a-5p expression level in PBMCs samples of patients with NS and healthy controls. Expression level of miR-15a-5p in (A) nephrotic syndrome, (B) MGN and (C) FSGS groups compared to healthy controls and (D) MGN group in compared to FSGS group. NS: Nephrotic syndrome, MGN: Membranous glomerulonephritis, FSGS: Focal segmental glomerulosclerosis.



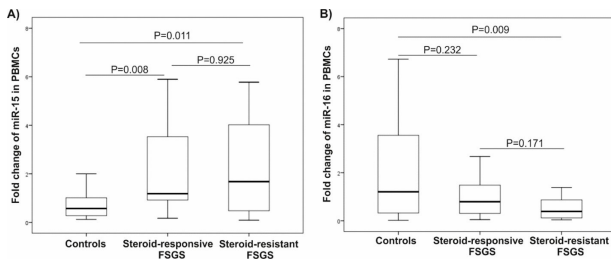
**Figure 2.** miR-16-5p expression level in PBMCs. Expression level of miR-16-5p in (A) nephrotic syndrome, (B) MGN and (C) FSGS groups compared to healthy controls and (D) MGN group in compared to FSGS group. NS: Nephrotic syndrome, MGN: Membranous glomerulonephritis, FSGS: Focal segmental glomerulosclerosis

## Discussion

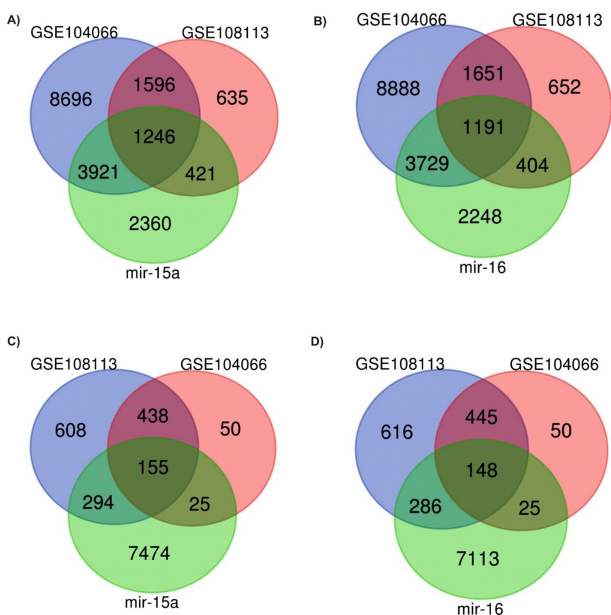
In this report, a significant elevation in miR-15a-5p level was seen in cases with primary MGN and FSGS compared with healthy subjects. Conversely the miR-16-5p expression was diminished significantly in both conditions compared with healthy controls. In clinical subdivision of FSGS group to steroid resistant- FSGS

and steroid responsive- FSGS, significant elevated levels of miR-15a-5p and diminished level of miR-16-5p were observed in both groups compared to normal controls. In future intergroup comparison, the same pattern of differences existed; however, it was not statistically significant.

In a Mexican study on pediatric NS, a reduced expression



**Figure 3. Comparison of miR-15a-5p and miR-16-5p expression levels in patients with FSGS.** Expression levels of miR-15a-5p (A) and miR-16-5p (B) in steroid-resistant FSGS in compared to steroid-responsive FSGS and healthy controls in PBMCs samples.



**Figure 4. GO annotation analysis for miR-15a-5p and miR-16-5p in MGN and FSGS pathogenesis.** GSE104066 and GSE108113 GO annotation for miR-15a-5p (A) and miR-16-5p (B) in pathology of MGN and miR-15a-5p (C) and miR-16-5p (D) in pathophysiology of FSGS. MGN: Membranous glomerulonephritis, FSGS: Focal segmental glomerulosclerosis.

was reported in miR-16-1 and miR-15a compared to normal controls and the miR-16 reduction was statistically significant (21). In another report, a reduced circulating levels of miR-16 was detected in NS cases in comparison with normal controls (22). In concordance with the exciting knowledge, in our study, a diminished levels of miR-16 was detected in FSGS cases compared to MGN and healthy subjects. The diminished level of miR-16 was more prominent in steroid resistant-FSGS than steroid-responsive-FSGS patients.

In a bioinformatic analysis, ten hub genes including VCL, KIF2C, SMAD4, CCND, VEGFA, FBXW7, KIF23, MAP2K1, CHEK1 and BTRC were obtained as targets of miR-15 and miR-16. These miRNAs function as tumor-suppressors and modulate cell cycle G1/S transition by

silencing Cyclin E1/E2, Cyclin D1-3 and CDK4,6 (23). By targeting Bcl2, they also cause cell apoptosis (24) and in tumor cells they arrest cell cycle at the G0/G1 phase (25). In a study, miR-15 has the highest expression in PBLCs (peripheral blood lymphocyte cells) of MGN cases compared to healthy controls (26). Given a mark increase of miR-15a-5p expression in MGN and FSGS in our study; it is postulated that miR-15 can induce a Bcl2 decline, podocyte's cycle arrest and apoptosis, leading to glomerular barrier impairment and proteinuria.

miR-16 inhibits vascular endothelial growth factor (VEGF) production (27, 28) in transgenic animals, an increased VEGF expression is associated with proteinuria and collapsing glomerulopathy (29, 30). In children with SRNS, a significantly increased podocytes VEGF expression has been reported in immune-histochemical study of kidney biopsy samples (31). Transmission of overexpressed miR-16-5p-containing exosomes, extracted from human urinary stem cells, could reduce VEGF expression and prevent podocytes apoptosis. It provides hopeful insights for treatment of diabetic nephropathy (28).

Vinculin (VCL), is a cytoplasmic adapter protein and regulator of podocyte actin filament that attaches to glomerular basement membrane (GBM). VCL is a target of miR-15/16 (32). VCL protein has an essential role in maintaining the glomerular barrier integrity (33).

miR-15 family (miR-497, miR-322, miR-195, miR-16 and miR-15a/b) limits the unrestrained TGF- $\beta$  activation, a critical pathway in fibrosis. miR-15 targets both activators (SMAD3) and repressors (SMAD7) limbs of TGF- $\beta$  signaling; however, net effect seems to be more in favor of repression (34). Moreover, the inflammatory role of miR-15 on enhancement of translocation of NF- $\kappa$ B p65 into nucleus and upregulation of pro-inflammatory cytokines IL-8 and INF-gamma has been reported in colonic epithelial cells (35).

Evidence shows differentially expressed genes between SSNS and SRNS. In contrast to SRNS patients, transcriptome sequencing of PBMC samples of pediatrics with SSNS course were enriched in genes related to the TGF- $\beta$ 1, p53 signaling, cytokines (IL-4 and IL-6), and targeted genes of miR-16 and miR-106 (36). The cluster of miR-15/16 can be upregulated by GCs and their overexpression increases GCs sensitivity (16). Additionally, GC therapy induces lymphoblast apoptosis that is mediated via miR-15b and miR-16 elevation (37). Because of the inhibitory function of miR-16 on VEGF production, it is a plausible explanation for miR-16 depletion-related podocyte injury and steroid resistant.

It seems that dysregulated expression miR-15/16 through blocking the cell cycle pathways and altering the inflammatory cytokines induces functional and structural impairments on podocytes. Despite major differences in the pathogenesis mechanisms of FSGS and MGN, quit some differences in our study, it looks that common

miRNA dysregulated pathways are involved in both FSGS and MGN patients. We knew that steroid responsiveness or resistance are old terms and we are going toward new outlooks to find the mechanistic-based therapies, but in order to be connected with existing clinical environment, we used those terminologies in our study.

### Conclusion

Dysregulated levels of miR-15 and miR-16 may be connected with podocyte damages, the pathogenesis of NS, and responses to steroid treatment. Future study is needed to increase our understanding of this area.

### Limitations of the study

Extracted results are in concordance with existing knowledge, however, we are aware about our limitations; small sample size and lack of electron microscopic results were examples.

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### Authors' contribution

**Conceptualization:** Mohammadreza Ardalan, Sepideh Zununi Vahed.

**Data curation:** Sepideh Zununi Vahed.

**Formal analysis:** Sepideh Zununi Vahed.

**Investigation:** Shahram Ghiyasvand.

**Methodology:** Seyyedeh Mina Hejazian, Shahram Ghiyasvand.

**Project administration:** Mohammadreza Ardalan, Sepideh Zununi Vahed.

**Resources:** Seyyedeh Mina Hejazian.

**Software:** Sepideh Zununi Vahed.

**Supervision:** Mohammadreza Ardalan, Mohammadali M Shoja.

**Validation:** Seyyedeh Mina Hejazian.

**Visualization:** Mohammadreza Ardalan.

**Writing—original draft:** Seyyedeh Mina Hejazian.

**Writing—review & editing:** All authors.

### Conflicts of interest

The authors declare that they have no competing interests.

### Ethical approval

The research conducted in this study adhered to the principles outlined in the Declaration of Helsinki. Tabriz University of Medical Sciences Ethics Committee approved this project (IR.TBZMED.REC.1397.907). All stages of the study were clearly described to all patients and healthy individuals and written informed consent was obtained from them. This research was funded by the Kidney Research Center of Tabriz University of Medical Sciences, Tabriz, Iran. This article was extracted from the subspecialty thesis of Shahram Ghiyasvand (Thesis#61888). The authors also completely have observed the ethical issues including data fabrication, falsification, plagiarism, double publication misconduct, or submission and redundancy.

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