Egyptian Journal of Cell and Tissue Research
Print ISSN: 2812-5436 / Online ISSN: 2812-5444

Molecular signaling pathways in an experimental model of Cardiorenal Syndrome

Arwa Mokhtar Ahmed [a], Ghada Mahmoud Abd Elaziz [a] , Hanan Hassan Foad [b] and Hoda Ramadan Riad [a]

[a] *Medical Biochemistry and Molecular Biology department, Faculty of Medicine, Beni-Suef University, Egypt*

[b] *Medical Biochemistry and Molecular Biology department kasralainy Faculty of Medicine Cairo University*

Abstract:

The goal of this study is to investigate the role of the fibroblast growth factor 23 (FGF23)/Klotho (Klotho) molecular signaling axis in an experimental model of cardiorenal syndrome type 3 resulting from renal artery ligation leading to renal reperfusion ischemia. The study used 12 male Wistar rats (6-8 months old, weighing 230-300g) split into 2 groups of 6 rats each (Group I, controls) and Group II, diseases, where Cardiac Hypertrophy was created by ligating the right renal artery. Gene expression of FGF23 and Klotho in cardiac and renal tissue was measured using quantitative real-time PCR, and blood urea and creatinine levels were measured clinically.All afflicted rats had increased amounts of creatinine in their blood, and histological analysis revealed renal ischemia and heart hypertrophy. According to real time PCR, FGF23 expression has dramatically increased in cardiac and renal tissues whereas alpha klotho expression has decreased.

Keywords: cardiorenal; klotho; FGF23.

1. Introduction:

Renal insults are a hallmark of cardiorenal syndrome types 3 and 4, which cause cardiac dysfunctions. When acute kidney injury (AKI) contributes to the development of acute heart failure, a condition known as cardio-renal syndrome, type 3 (CRSIII) is triggered. Direct or indirect effects of acute kidney damage on the heart include activation of inflammatory signaling pathways, production of reactive oxygen species (ROS), and release of neurohormones [1]. Volume overload,

electrolyte imbalances (such as hyperkalemia, hypocalcemia, and metabolic acidosis), and other causes of cardiac failure are often linked to kidney damage [2]. Alterations in the pharmacokinetics and pharmacodynamics of therapeutic medications are another way in which acute kidney damage affects heart functioning [3].

Modulation of the FGF23/Klotho signalling axis may be one modification brought on by renal injury. Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone secreted by osteoclasts that, together with its cofactor Klotho, inhibits renal 1-hydroxylase, preventing the body from producing 1,25(OH)2 vitamin D. [4]. The increased levels of FGF23 in individuals with acute or chronic renal disease may be attributed to a number of different reasons [5]. Causes of elevated FGF23 in patients with acute or chronic renal disease include impaired filtration and/or breakdown by the sick kidney, retained phosphate, and deficiency of the enzyme Klotho [5].

Recent in vivo and in vitro-experiments reveal that the 'off-target' effects of FGF23 on the myocardium are mediated, at least in part, via a direct, Klotho-independent mechanism [6]. Impaired vasoreactivity, increased arterial stiffness, and cardiovascular morbidity and mortality have all been linked to higher levels of fibroblast growth factor (FGF)-23, whereas KLOTHO has been shown to have a protective effect against endothelial dysfunction. There is yet little evidence that FGF23 and the KLOTHO signalling pathway are expressed in the human cardiovascular system, and the respective contributions of FGF23 expression and accompanying Klotho deficit in type 3 cardiorenal syndrome have not been studied [7].

2. Materials and Methods:

2.1 Animals:

Twelve male Wistar rats (230–300g; 6-8 months old at the start of the trial) were utilised in this investigation. The animals came from Egypt's Kasr Al Ainy Faculty of Medicine's animal facility. Before beginning the experiment, animals were monitored for about 10 days to make sure no contagious diseases were spreading amongst them. The animals were kept at room temperature (22–24°C) with a typical daily illumination cycle (10–12h/day) in the animal house at the Kasr Al Ainy Faculty of Medicine in Egypt, where they also received a balanced standard feed and water ad libitum. The institutional animal care and use committee has approved all animal treatments (Approval No.021-126). Every attempt was made to minimise animal deaths and suffering. Rats were segregated into two groups; 6 rats in each group: **Group I (Control):** consists of 6 rats that received no medication and no surgical procedures were operated. **Group II (diseased) :** Rats were used to generate Cardiac Hypertrophy (in

vivo), by ligating their right renal artery as illustrated later.

2.2 Induction of cardiorenal syndrome type III :

To induce cardiac hypertrophy (in vivo), we ligated the right renal artery of rats. Xylazine (10 mg/kg) and ketamine (80 mg/kg) were administered intramuscularly to the animals to induce anaesthesia. After the abdomen was opened and the right renal artery was revealed, a plastic tube (a section of cannula tube, 22G) (outside diameter, 0.6 mm) was placed over the artery and secured in place with silk stitch. The catheter was removed from under the knot after 30 minutes, narrowing the artery to the size of the plastic catheter. Coated VICRYL®, an absorbable suture material, was used to seal the skin and abdominal wall. The operating room was sterile and the surgeons were meticulous. The animal's vital indicators, including its heart rate and breathing rate, have been stable since it became awake, indicating that the postoperative care provided was successful. Sprays containing antibiotics, analgesics, and antiseptics were used topically following surgery. After undergoing surgery, the rats were housed in ideal conditions for two days before being slaughtered.

After the duration of the experiment was complete, rat venous blood samples were obtained while the animals were under the effects of isoflurane anaesthesia so that biochemical parameters could be evaluated. Rats were used for histological analysis of their kidneys and hearts after they were killed.

2.3 Determination of serum creatinine and urea levels :

Serum creatinine and urea levels were determined using a colorimetric technique using commercial diagnostic kits (Biodiagnostic, Egypt), as per the manufacturer's instructions. We used a semi-automated technique to measure absorbance, with a spectrophotometer set at 340 and 580 nm and a water bath at 37 degrees Celsius. Each and every quality control measure was taken.

2.4 Histopathological Examination:

Kidneys and hearts were collected, then dissected, rinsed in physiological saline solution, frozen in tiny pieces using 10% neutral buffered formalin. To prepare the fixed tissues for embedding in Paraplast, they were dehydrated in a series of increasingly strong alcohols, clarified in xylol, impregnated, and finally dehydrated again. The overall histological structure of Paraplast blocks was analysed by staining them with hematoxylin and eosin (H&E), the neutral mucopolysaccharides with periodic acid - schiff (PAS), and the total protein content with bromophenol blue. A Leica binocular research microscope with a Canon digital camera was used to view and take images of the tissue sections.

2.4 FGF23 & Klotho Gene Expression analysis by real-time quantitative PCR (RT-qPCR):

Kidney and heart tissue's RNA was extracted using a NucleoSpin RNA nucleic acids extraction kit (Macherey-Nagel, Germany). cDNAs were synthesised by reverse transcription of mRNA, as per the manufacturer's instructions.

Quantitative real-time PCR was performed using the ViPrimePLUS One Step Taq RT-qPCR Green Master Mix I with ROX (SYBR Green Dye) and the Step One real-time PCR Systromal (Applied Biosystromals, USA) was used for data analysis (no. QLMM14-R). A total of 20 L was obtained by combining 1 L of primers, 5 L of template cDNA, 4 L of nuclease-free water, and 10 L of 1x master mix. To do this, we used primers that target particular regions of the fibroblast growth factor 23 (Fgf23) and Klotho genes **.(Table 1).**

Table 1: Primers sequence for RT - qPCR

NM_031336.2 Rattus norvegicus Klotho (Kl),	Forward 5'– GGCTCTTGCTGCTCCGTTTG –3' Reverse 5'– GAAGCAGAGCTCGGCGTAAT –3'
NM_130754.1 Rattus norvegicus fibroblast growth factor 23 (Fgf23)	Forward 5'– ACAGCTACAGCCAGGAACAG -3' Reverse 5'– CCGGGCTGAAGTGATACGAT -3'

Starting with a 10-minute cycle at 55 °C, we moved on to an 8-minute cycle at 95 °C, and finally finished with 40 cycles of 10-seconds at 95 °C and 60-seconds at 60 °C. The information was shown as a cycle-threshold plot (Ct). Relative abundance of each target gene was calculated using delta-delta Ct. (2-ΔΔCt) .

Statistical methodology

SPSS 25 for Windows was used to analyse the data. Numeric scale variables in the study were normally distributed, so they were expressed as mean and standard deviation.

Independent student's T test was used to compare the two groups. P-value was considered significant at ≤0.05 (two sided).

3. Results:

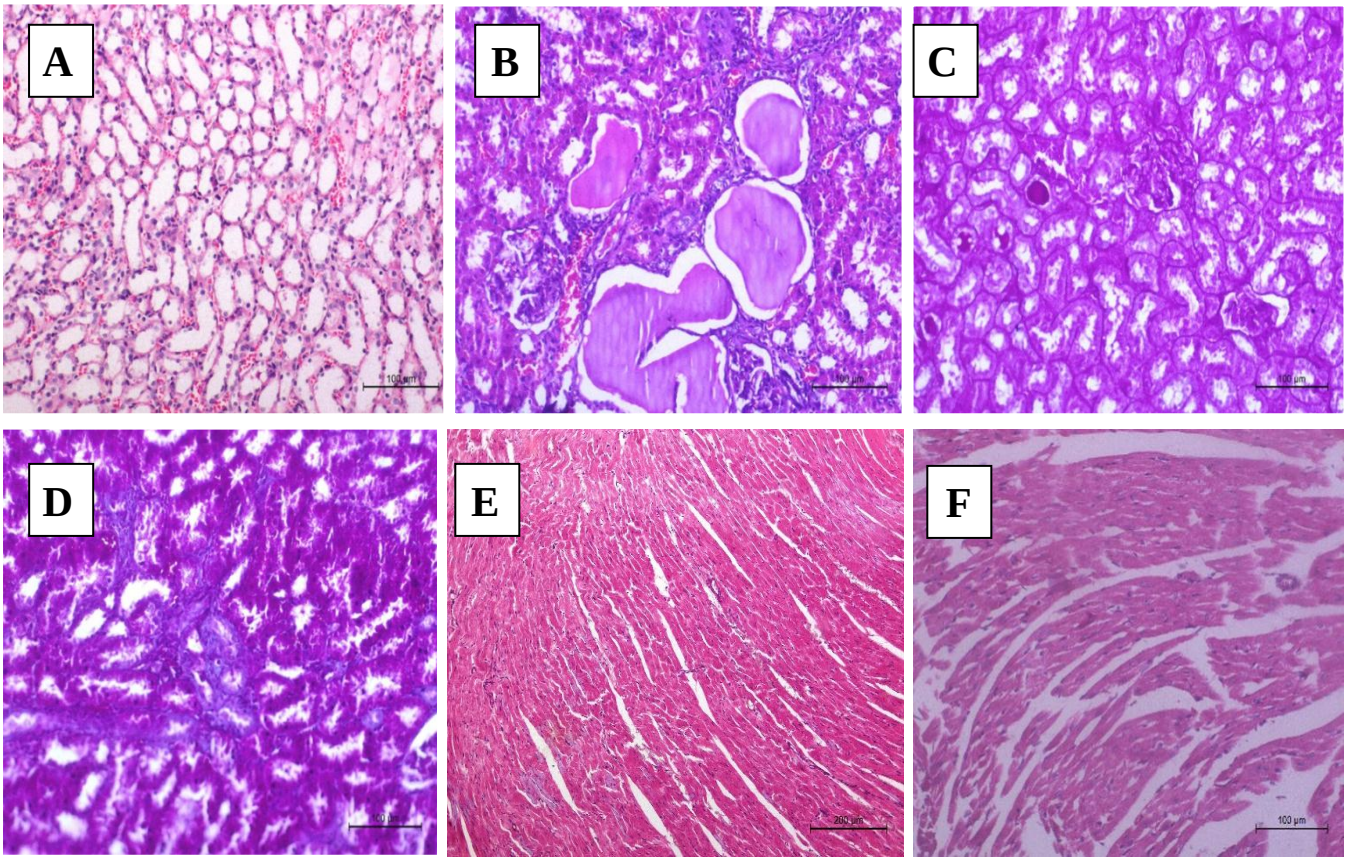
3.1 Histopathological results

3.1.1 Kidney sections:

The microscopic examination of the control rats stained with H&E revealed normal histological structure of renal tubules and renal glumeruli, renal medulla showing normal histological structure of renal tubules(Fig. A) While the examined renal sections of diseased rats with H&E showed signs of renal ischemia in the form of interstisial nephritis in the form of mononuclear infiltration and mild fibrosis and congestion of intertubular blood vessels. Some renal tubules showed cystic dilataltion and filled with hyaline casts. (Fig.B). PAS stained kidney sections from diseased rats (X 200) and (X400), showed thickening of the basement membrane of tubules and glomerulonephritis (Fig. C). MT stained kidney sections from diseased rats (X 200) showed interstisial nephritis in the form of mononuclear filtration and mild fibrosis (Fig. D).

3.1.2 Heart Sections :

The microscopic examination of the control rats heart (group I) showed normal histological structure of heart, showing normal myocardial muscle (Fig. E). While the examination of the diseased rat sections showed congestion of subendocardial blood vessels, congestion and perivascular edema of coronaries Hemorrhages were present within myocardial tissue (Fig. F).



3.2 RT- qPCR results:

Table (2) Comparison between the studied groups regarding the renal FGF23 level:

items	Control group	Diseased group	P-value
FGF23	1.03±0.02	6.00±0.62	<0.001*

**P-value is significant*

Table 2 showed that there was a significant difference between the two groups regarding the renal FGF23 level. The higher level was in diseased group.

Table (3) Comparison between the studied groups regarding the renal KLOTHO level:

items	**Control group**	**Diseased group**	**P-value**
KLOTHO	1.01±0.01	0.25±0.04	<0.001*

**P-value is significant*

Table 3 showed that there was a significant difference between the two groups regarding the renal KLOTHO level. The Lower level was in diseased group.

Table (4) Comparison between the studied groups regarding the cardiac FGF23 level:

items	**Control group**	**Diseased group**	**P-value**
FGF23	1.03±0.01	4.67±0.31	<0.001*

**P-value is significant*

This table showed that there was a significant difference between the two groups regarding the cardiac FGF23 level. The higher level was in the diseased group.

Table (5) Comparison between the studied groups regarding the cardiac KLOTHO level:

items	**Control group**	**Diseased group**	**P-value**
KLOTHO	1.02±0.01	0.41±0.08	<0.001*

**P-value is significant*

This table showed that there was a significant difference between the two groups regarding the cardiac KLOTHO level. The lower level was in the diseased group.

3.2 Biochemical results:

Figure 1: Comparison between the studied groups regarding the creatinine level at different times

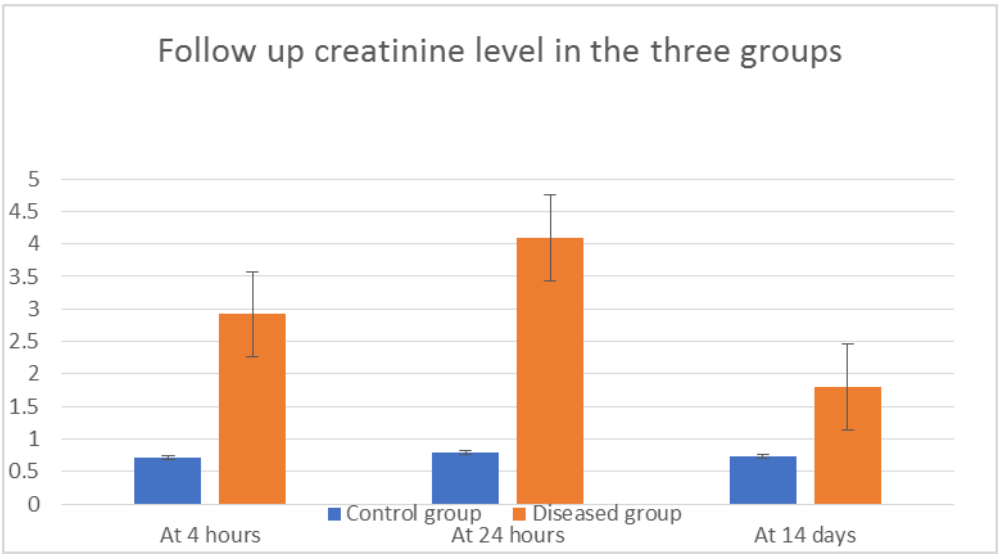
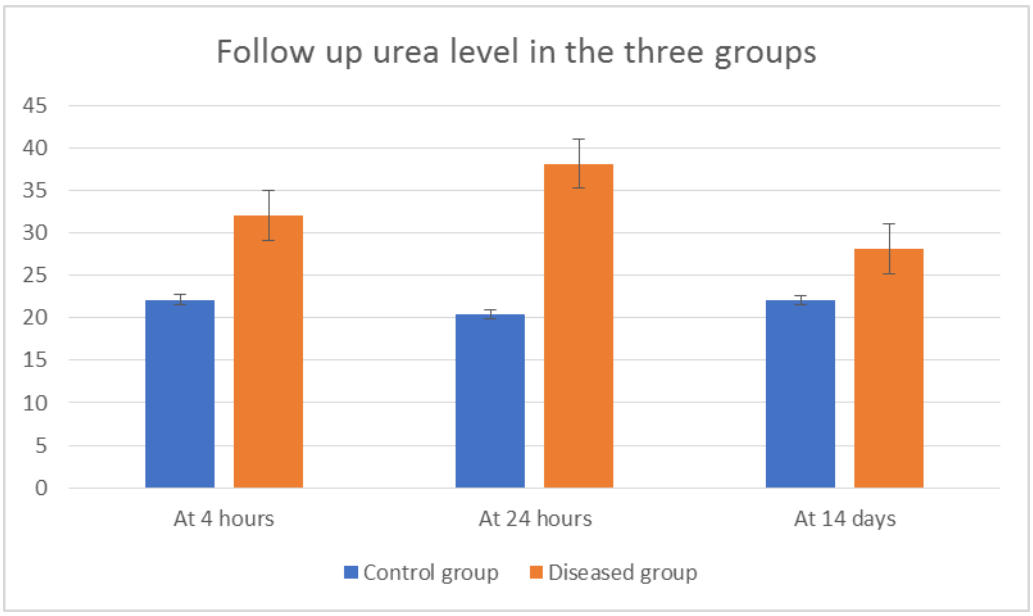


Figure 2: Comparison between the studied groups regarding the urea level at different times



4. Discussion:

Our study was conducted to evaluate the characterization of the molecular signaling axis FGF23/Klotho in an experimental model of cardiorenal syndrome type 3 induced by acute renal ischemia through ligating right renal artery for 30 minutes followed by reperfusion.

Acute renal injury in diseased group was induced by surgical ligation of the right kidney artery for 30 minutes then reperfusion was

allowed, and this goes along with the ischemic model conducted by [8], where they performed unilateral ischemia-reperfusion without contralateral nephrectomy. When comparing the changes in the kidney functions in the ischemic to the control group, we found significant increase in the levels of creatinine after 4 hours of renal artery ligation and more increase after 24 hours indicating impaired kidney functions and AKI. After 14 days we noticed significant decrease in serum creatinine levels due to the healing process. That confirmed the efficacy of the surgical procedure in inducing AKI. Regarding serum urea levels we found significant increase in the diseased group after 24 hours then significant decrease after 14 days but all levels were within normal ranges. The results of our work agreed with [9]. In their study Lipopolysaccharide (LPS) was used to induce septic cardiorenal syndrome type 5 animal model where sepsis leads to acute kidney and heart injuries. They assessed serum creatinine at 6 h, 24 h, 48h, and 72 h after LPS injection and they found that the serum creatinine significantly increased at 24 h ($p < 0\ 05$) and 48 h ($p < 0\ 05$) after LPS injection compared to the control group. In our study and for the purpose of confirming kidney injury, kidney sections stained with H&E, PAS and MT were examined. Tubulointerstitial lesions (inflammatory cell infiltration and tubular dilation), coagulative necrosis, and a moderate degree of fibroplasia were found, in contrary to control group where histopathological examination revealed normal renal glomerular structure and tubules with cuboidal epithelium. That was similar to histopathological results of [8] who showed prominent renal damage and severe loss of structure, atrophic renal cortex with disruption of tubular architecture, marked tubule necrosis and intratubular casts, and extensive interstitial inflammatory infiltration after unilateral ischemia-reperfusion without contralateral nephrectomy procedure. In our study to assess the possible cardiac damage, histopathological studies were conducted on heart sections from both diseased and control rats. Along with the results reported by [7], we found congestion and perivascular edema of coronaries; hemorrhages and mononuclear cell infiltration were also seen in cardiac tissue, which was related with fibrosis. While myocardial fibers with central nuclei and normal endocardial organization were seen in the control group's histopathological examinations. Data presented in the current study demonstrated that the expression of FGF23 gene in kidney tissue was significantly higher in the diseased group than the control group. That could be credited to a combination of factors including decreasing filtration and/or degradation by the diseased kidney, phosphate retention and Klotho deficiency. That goes along with the study [7] In their study, mice were subjected to surgical occlusion of left renal pedicle for 60 min followed by reperfusion in acute term (24, 48

and 72h), short term (for 8 and 15 days) and long term (4,6 and 8 weeks). They found a significant increase in expression of FGF23 gene in kidney tissue and it showed a peak in the first 24 hours, in the 8th day and 15th day. [10] conducted a similar study but on experimental model of CRS type II, where renal fibrosis is induced by acute myocardial infarction, and obtained evidence that not only circulating FGF23 but also the expression levels of FGF23 in kidney were increased, a finding that is consistent with our findings. Similarly, [11] have measured plasma level of intact and C-terminal FGF23 and its expression in kidney in normal individuals and patients with CKD and AKI, they found that patients with normal kidney function have low (physiologic) circulating levels of cFGF23 and iFGF23. Patients with AKI have very high circulating levels of cFGF23, but only moderately increased iFGF23, consistent with increases in both FGF23 production and cleavage. As CKD advances to ESRD, circulating levels of cFGF23 and iFGF23 progressively increase. According to [12], elevated FGF23 levels have been observed in multiple studies of human AKI. Plasma cFGF23 levels were 5.6-fold higher in patients with AKI versus age-matched patients without AKI. Increased FGF23 levels detected with the cFGF23 assay may reflect increased FGF23 production and/or impaired clearance but do not inform about FGF23 bioactivity. In the present study we observed that higher levels of serum urea and creatinine are not associated with more expression of FGF23 gene in both diseased and control group, that was against results reported in [12], who observed that higher levels of serum urea and creatinine are associated with more expression of FGF23 gene, while control group with normal BUN and serum creatinine showed a normal gene expression levels of FGF23. This controversy could be explained by the low number of the studied animals in each group. Regarding klotho level there was statistically significant decrease in level of expression in kidney tissue. The decrease in renal α-klotho has also been shown in several other animal models, such as LPS induced model, the unilateral ureteral ligation model and the cisplatin-induced AKI model, which indicates that the kidney may be the main source of α-klotho [9].

These results were in accordance with [10] who showed that the expression of Klotho was significantly down regulated in the kidneys. Immunohistochemistry showed that Klotho expressed in renal tubules rather than the glomeruli of sham mice, and its expression was significantly decreased in the renal tubules of CRS mice. In consistence with our study, Da Cruz Junho [7] investigated expression of klotho gene in renal tissue after acute renal ischemia using real-time PCR and reported that klotho gene expression was mostly reduced in renal tissue after 48h of reperfusion, evidencing even more the renal injury (once some authors consider Klotho is a

renal injury biomarker). Our results are reinforced by [9], who investigated the effects of α-klotho in acute cardiorenal injury in LPS-induced septic cardiorenal syndrome and have reported that after injection of LPS, the levels of α-klotho expression in the kidneys were significantly reduced as early as 6 h after injection. They also found that using recombinant α-Klotho protein as a pretreatment significantly alleviated acute cardiorenal injury, suggesting the protective role of klotho for kidney and heart tissues. In contrast to results reported in [12], we observed that higher levels of serum urea and creatinine are not associated with less expression of Klotho gene in renal tissue of both diseased and control group. Recent studies suggest that being secreted by cardiac myocytes, FGF23 can stimulate pro-fibrotic factors in myocytes to induce fibrosis-related pathways in fibroblasts and consequently cardiac fibrosis in a paracrine manner [13]. To evaluate this effect of FGF23 on cardiac tissue, we compared the level of FGF23 expression in diseased group, showing cardiac fibrosis and remodeling, and in control group with normal heart architecture, we found a significant increase in FGF23 expression in the heart tissue of the diseased group while it was normal in control group. These results have been previously reported by [14] who showed that baseline FGF23 levels were independently associated with higher LV mass, lower LV systolic function, and reduced left atrial function over long-term follow-up. Similarly, [15] have studied 6542 participants who were free of cardiovascular disease at baseline and reported that higher FGF23 levels are associated with incident HF and is also associated with left ventricular hypertrophy. On the other hand, [16] demonstrated that high circulating levels of FGF23 in mice did not induce cardiac hypertrophy, However, they used a mice model with X-linked hypophosphatemia, supporting a primary role for phosphate in inducing cardiac hypertrophy. Regarding effect of klotho on preserving cardiac function and histology, [17] investigated whether Klotho plays a role in cardiac aging and damage. Their results suggest that Klotho deficiency impairs heart function and causes cardiac hypertrophy mimicking accelerated cardiac aging. Our results concerning level of Klotho expression are compatible to [17] as we found a significant decrease in level of klotho expression in heart tissue of ischemic group while it showed normal levels in control healthy one. Interestingly, Interstitial fibrosis, a consequence of pathological cardiac hypertrophy and remodeling, was reduced in KL (−/−) old wistar mice after treatment with secreted Klotho protein, indicating attenuation of cardiac hypertrophy and this reinforce our results that suggest a significant decrease in klotho expression in diseased heart tissue [17].

5. Conclusion and Recommendations:

there is a significant relation between the molecular signaling pathway FGF23/Klotho and the cardiac and renal disfunctions related to cardiorenal syndrome type three

6. References:

1. Yap, C. & Lee, H. (2012): Acute kidney injury and extrarenal organ dysfunction: new concepts and experimental evidence. Anesthesiology; 116: 1139–1148.
2. Bagshaw, S., Hoste, E., Braam, B., et al. (2013): Cardiorenal Syndrome Type 3: Pathophysiologic and Epidemiologic Considerations. Contrib Nephrol.; 182: 137–157.
3. Di Lullo, L., Bellasi, A., Barbera, V., et al. (2017): Pathophysiology of the cardio-renal syndromes types 1–5: An uptodate. Indian Heart J.; 69: 255-265.
4. Navarro-García, J., González-Lafuente, L., Fernández-Velasco, M., et al. (2021): Fibroblast Growth Factor-23-Klotho Axis in Cardiorenal Syndrome: Mediators and Potential Therapeutic Targets. Front Physiol; 12: 2042.
5. Christov, M., Waikar, S., Pereira, R., et al. (2013): Plasma FGF23 levels increase rapidly after acute kidney injury. Kidney Int; 84: 639–641.
6. Isakova, T., Xie, H., Yang, W., et al. (2011): Fibroblast growth factor 23 and risks of mortality and end-stage renal disease in patients with chronic kidney disease. JAMA; 305: 2432–2439.
7. Da Cruz Junho, V., Caio-Silva, W., Ruiz-Hurtado, G. et al. (2019): Characterization of Klotho/FGF23 signaling in cardiorenal syndrome-induced cardiac hypertrophy. The FASEB Journal; 33: 831.1-831.1
8. Le Clef, N., Verhulst A, D'Haese PC, (2016): Unilateral Renal Ischemia-Reperfusion as a Robust Model for Acute to Chronic Kidney Injury in Mice. Plos One; 11: e0152153.
9. Liu, X., Niu, Y., Zhang, X., et al. (2019): Recombinant α-Klotho Protein Alleviated Acute Cardiorenal Injury in a Mouse Model of Lipopolysaccharide-Induced Septic Cardiorenal Syndrome Type 5. Anal Cell Pathol (Amst); 2019: 5853426.
10. Hao, H., Ma, S., Zheng, C., et al. (2021): Excessive fibroblast growth factor 23 promotes renal fibrosis in mice with type 2 cardiorenal syndrome. Aging (Albany NY); 13: 2982.
11. Christov, M., Neyra, J., Gupta, S., et al. (2019): Fibroblast growth factor 23 and klotho in AKI. Semin Nephrol; 39: 57–75.
12. Neyra J, Hu M, Moe O,(2020): Fibroblast Growth Factor 23 and αKlotho in Acute Kidney Injury: Current Status in Diagnostic and Therapeutic Applications. Nephron; 144: 665-672.
13. Leifheit-Nestler, M., Kirchhoff, F., Nespor, J., et al. (2018): Fibroblast growth factor 23 is induced by an activated renin–

angiotensin–aldosterone system in cardiac myocytes and promotes the pro-fibrotic crosstalk between cardiac myocytes and fibroblasts. Nephrol. Dial. Transplant; 33: 1722–1734.

14. Patel, R., Ning, H., de Boer, I., et al. (2020): Fibroblast Growth Factor 23 and Long-Term Cardiac Function: The Multi-Ethnic Study of Atherosclerosis. Circ Cardiovasc Imaging; 13: 11925.
15. Almahmoud, M., Soliman, E., Bertoni, A., et al. (2018): Fibroblast growth factor-23 and heart failure with reduced versus preserved ejection fraction: MESA. J Am Heart Assoc ; 7: e008334.
16. Liu, E., Thoonen, R., Petit, E., et al. (2018): Increased Circulating FGF23 Does Not Lead to Cardiac Hypertrophy in the Male Hyp Mouse Model of XLH. Endocrinology; 159: 2165–2172.
17. Chen, K., Wang, S., Sun, W., et al. (2021): Klotho Deficiency Causes Heart Aging via Impairing the Nrf2-GR Pathway. Circ Res.; 128: 492–507.