

Research Article

Assessment of Potential Role of Fibroblast Growth Factor 23 and Klotho Gene Polymorphism in Cardiovascular Calcification Associated with Chronic and End Stage Renal Diseases

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ABSTRACT

Background: The high cardiovascular mortality in patients with chronic kidney disease (CKD) is closely associated with vascular calcification (VC). Defects in endogenous anti-calcification factors such as fibroblast growth factor 23 (FGF23), and Klotho may play an important role in these complications of CKD.

FGF23 inhibits secretion of parathyroid hormone (PTH). This effect is dependent on the presence of klotho, which is highly expressed in the kidney and the parathyroid glands and acts as a co-receptor for FGF23 by markedly increasing the affinity of FGF23 for ubiquitous fibroblast growth factor receptors FGFR. Defects in either FGF23 or Klotho cause a combination of metabolic disturbances, including hyperphosphatemia, hypercalcemia, and hypervitaminosis.

Aim of the work: The aim of this study was to determine the possible effects of FGF23 and klotho gene polymorphisms (KL-VS and C1818T) on the cardiovascular calcification in relation to mineral metabolism in chronic kidney diseases (CKD) and end stage renal diseases (ESRD).

Subject and Methods: The present study was conducted on sixty subjects. Forty patients, recruited from the outpatient clinic of the Nephrology Department Medical Research Institute, Alexandria University, were classified in to two groups **Group 1:** Included twenty Chronic Kidney Disease patients' stages 3-5. Their mean age was 48.3 ± 12.5 . **Group2:** Included twenty hemodialysis patients under maintenance hemodialysis for one year (3 times/week and 4hours/session) on polysulphon membrane. Their mean age was 49.1 ± 12.5 . **Control subjects:** Included twenty healthy volunteers of comparable age and sex to the patients groups. Written consents will be obtained from all participants before being involved in the study. **The following was done to all the enrolled subjects:**

- To all participants general health parameters assessed included screening history, physical examination, blood tests (FGF23, sKlotho, Ca, Pi, Creatinine, Urea, Uric acid, Total cholesterol, Triglyceride, Albumin and Total protein).
- Ultrasound examination of common carotid artery (CCA) to determine Carotid Intima media thickness (CIMT).
- Molecular study [genotyping of KL-VS and C1818T] polymorphisms of Klotho by polymerase chain reaction /restriction fragment length polymorphism (PCR/RFLP)].

Results: In the present study, serum levels of FGF23 showed significant increase in the mean values ($p \leq 0.05$) in the CKD and ESRD groups as compared to normal control group 1. There was an inverse relationship between FGF23 and eGFR of dialysis patients when compared to healthy controls. Levels of Klotho showed a decrease in the mean values in CKD and dialysis groups as compared to normal control group ($p \leq 0.05$). In this study, patient groups with lower levels of serum Klotho exhibited significantly lower ($p \leq 0.05$) eGFR levels. The present study revealed significant increases in the parathyroid hormone levels, the phosphorus levels, triglyceride levels and cholesterol levels in the stud groups as compared to the normal control group ($p \leq 0.05$). This study showed a significant increase in CIMT in CKD and dialysis compared with the values reported for normal populations ($p \leq 0.05$). In the present study no correlation was found between CIMT and serum levels of calcium, phosphorus and PTH in HD patients. Negative correlation was found between CIMT and serum levels of Klotho and eGFR in HD patients. The study also showed a significant relationship between CIMT and age in CKD. Negative correlation was found between CIMT and serum levels of FGF23 and calcium in CKD patients. In the molecular study of Klotho gene polymorphism, we did not detect kl-vs and C1818T polymorphism in patients who underwent CKD and dialysis which requires further investigations to elucidate the revolutionary origin of this variant or any other mechanism involved

Conclusion:

- High serum FGF-23 concentrations predict more rapid disease progression in CKD patients who were not on dialysis and increased mortality in patients on maintenance hemodialysis. FGF-23 may therefore prove to be an important therapeutic target for the management of CKD and cardiovascular disease.
- Reduced Klotho protein levels with progressive renal failure may be a modifiable factor involved in the pathogenesis of cardiovascular and renal disease in at-risk populations.
- Although we were unable to specifically identify the causal variant in the klotho gene, this work provides important information in understanding how renal disease progresses to end-stage kidney failure.

KEYWORDS: CRONIC KIDNEY DISEASE, FIBROBLST GROWTH FACTOR-23, KLOTHO, Carotid Intima media thickness, parathyroid hormone, hemodialysis patients, Klotho gene kl-vs and C1818T polymorphism

1.0 INTRODUCTION

The high cardiovascular mortality in patients with chronic kidney disease (CKD) is closely associated with vascular calcification (VC). Defects in endogenous anti-calcification factors such as fibroblast growth factor 23 (FGF23), and Klotho may play an important role in these complications of CKD.^(1,2)

FGF23 is primarily secreted by osteocytes and has several endocrine effects on mineral metabolism. FGF23 induces phosphaturia by decreasing phosphate reabsorption in the proximal tubule through down-regulation of luminal sodium-phosphate co-transporters.^(3,4,5)

FGF23 inhibits secretion of parathyroid hormone (PTH). This effect is dependent on the presence of klotho, which is highly expressed in the kidney and the parathyroid glands and acts as a co-receptor for FGF23 by markedly increasing the affinity of FGF23 for ubiquitous fibroblast growth factor receptors (FGFR).^(6,7,8)

FGF23 levels increase progressively as glomerular filtration rate (GFR) declines in early CKD, with some investigators observing significant increases already by stages 2 to 3 disease. The etiology of increased FGF23 levels in renal failure is likely a compensatory mechanism to the hyperphosphataemia.^(9,10,11)

Klotho was originally identified as an aging suppressor. Its gene product is a single-pass transmembrane protein that functions as a co-receptor for FGF23⁽¹²⁾. Klotho is expressed widely, but its level is highest in the kidney. Klotho is also secreted into the cerebrospinal fluid, blood, and urine.⁽¹³⁾

Klotho deficiency in rodents leads to a syndrome of premature aging where ectopic soft tissue calcification is a notable feature. Over-expression of Klotho rescues the Klotho-deficient phenotype including ectopic calcification⁽¹⁴⁾ suggesting that Klotho may be an inhibitor of ectopic calcification. Klotho-FGF23 signaling promotes renal phosphate excretion through sodium phosphate co-transporter 2a (NaPi2a) channels, thereby lowering blood phosphate levels, and down-regulates the expression of 1 α -hydroxylase to suppress production of active 1,25-dihydroxyvitamin D. Defects in either FGF23 or Klotho cause a combination of metabolic disturbances, including hyperphosphatemia, hypercalcemia, and hypervitaminosis D.⁽¹⁵⁾

Several studies have identified polymorphisms in klotho and association with a variety of phenotypes. Two studies have reported an association between a functional variant of klotho, known as KL-VS, and longevity in Caucasians and African Americans.⁽¹⁶⁾ KL-VS is also associated with cardiovascular disease in Caucasians and African Americans.⁽¹⁷⁾ Two other variants, G-395A and C1818T, are associated with reduced cardiovascular disease rates in Korean women.⁽¹⁸⁾

2.0 STUDY OBJECTIVE

The objective of this study was to determine the possible effects of FGF23 and klotho gene polymorphisms (KL-VS and C1818T) on the cardiovascular calcification in relation to mineral metabolism in chronic kidney diseases (CKD) and end stage renal diseases (ESRD).

3.0 SUBJECT AND METHODS

The present study was conducted on sixty subjects categorized as follows.

3.1 Case subjects:

Including forty patients recruited from the outpatient clinic of the Nephrology Department Medical Research Institute, Alexandria University. Those were classified into two groups.

Group 1: Included twenty Chronic Kidney Disease patients' stages 3-5. Their mean age was 48.3 ± 12.5 .

Group2: Included twenty hemodialysis patients under maintenance hemodialysis for one year (3 times/week and 4hours/session) on polysulphon membrane. Their mean age was 49.1 ± 12.5 .

3.2 Control subjects: Included twenty healthy volunteers of comparable age and sex to the patients groups written consents will be obtained from all participants before being involved in the study.

Exclusion criteria:

Diabetes Mellitus and autoimmune disease patients were excluded from the present study.

The following was done to all the enrolled subjects:

- To all participants general health parameters assessed included screening history, physical examination, blood tests (FGF23, Klotho, Ca, Pi, Creatinine, Urea, Uric acid, Total cholesterol, Triglyceride, Albumin and Total protein).
- Ultrasound examination of the common carotid artery (CCA) to determine Carotid Intima media thickness (CIMT).
- Molecular study [genotyping of KL-VS and C1818T polymorphisms of Klotho by polymerase chain reaction /restriction fragment length polymorphism (PCR/RFLP)].

Sample collection:

Morning 5ml venous blood samples were taken and divided as follows:

2ml blood in sterile vacuum tubes containing EDTA for DNA extraction and studying Klotho-2 gene polymorphisms

- 3ml blood was centrifuged (15,000 rpm for 15 minutes) and serum was separated. The resulted serum pipetted into aliquots and stored at -20°C till the time of analysis.
- At the time of assay, samples were thawed and vortexed gently.

3.3 Biochemical studies:**3.3.1 Estimation of Serum Fibroblast Growth Factor 23 (FGF23) :**⁽¹⁹⁾

Serum samples were assayed for FGF23 using a commercially available two-step sandwich enzyme-linked immunosorbent assay (ELISA) kit (Glory Science Co. China)

The samples was added to a well which is precoated with FGF23 monoclonal antibody, followed by incubation. FGF23 antibodies labeled with biotin were added, and combined with Streptavidin-HRP to form immune complexes. Then Chromogen Solution A, B was added, the color of the liquid changed into the blue. At the effect of acid, the color finally became yellow. The chroma of color and the concentration of the Human Substance FGF23 of sample were positively correlated. The absorbency of the plate was detected at 450 nm within 10 minutes after adding the stop solution.

The standard curve linear regression equation was calculated according to standards concentration and the corresponding Optical density (OD) values. From the curve it was possible to calculate the samples concentration.

3.3.2 Determination of serum klotho:⁽²⁰⁾ Serum samples were assayed for **klotho** using a commercially available two-step sandwich enzyme-linked immunosorbent assay (ELISA) kit (Glory Science Co. China).

The microtiter plate has been pre-coated with an antibody specific to KL. Standards or samples are added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for KL. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain KL, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of KL in the samples is then determined by comparing the O.D. of the samples to the standard curve.

3.3.3 Estimation of Serum human parathyroid hormone (hPTH)⁽²¹⁾

Human parathyroid hormone hPTH was assayed using a commercially available DIAsource hPTH-EASIA Kit.

3.3.4 Determination of serum Creatinine: - (22)

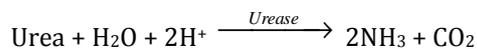
The method of Bowers and Wang was used in kinetic determination of creatinine without deproteinization. The creatinine concentration in the serum sample was calculated from the following equation:

$$\text{Creatinine concentration (mg/dL)} = \frac{A_{\text{sample}}}{2 \times A_{\text{standard}}}$$

Where 2 is the concentration of standard.

3.3.5 Determination of serum urea: (23)

The method depends on the enzymatic determination of urea according to (urease-Berthelot reaction). The method depends on the enzymatic determination of urea according to the following reaction (urease-Berthelot reaction).



The urea concentration in the serum sample was calculated according to the following equation:

$$\text{Urea mg /dl} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Concentration of standard.}$$

3.3.6 Determination of serum uric acid: (24)

It was determined enzymatically using uricase according to the following reactions



The rose color produced (oxidized chromogen), which was proportional to uric acid concentration in the sample was measured spectrophotometrically at λ 520 nm and compared to a known concentration of standard similarly treated.

$$\text{Uric acid mg /dl} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Concentration of standard.}$$

3.3.7 Evaluation of glomerular filtration rate (GFR) :- (25)

Estimated Glomerular Filtration Rate (eGFR) calculated by Cockcroft-Gault

Cockcroft-Gault formula in mg/dl:

$$\text{GFR}_{\text{Cockcroft}} = \frac{(140 - \text{age}) \times \text{mass (kg)} [\times 0.85 \text{ if female}]}{72 \times \text{serum creatinine (mg/dl)}}$$

3.3.8 Determination of serum Calcium: (26)

Calcium concentration (mg/dL)= 10 ×

Calcium ion forms a violet complex with O-Cresolphthalein complex one in an alkaline medium.

The calcium concentration in the serum sample was calculated from the following equation:

$$\text{Calcium concentration (mg/dL)} = 10 \times \frac{A_{\text{sample}}}{A_{\text{standard}}}$$

Where 10 is the concentration of standard.

3.3.9 Determination of serum Phosphorus: ⁽²⁷⁾-

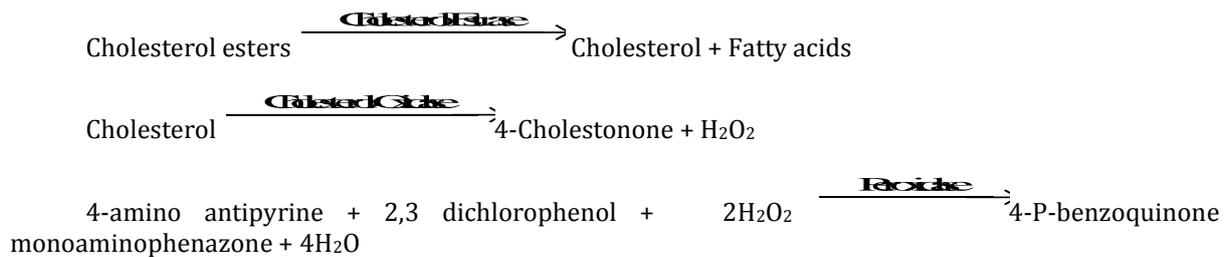
Inorganic phosphate reacts in acid medium with ammonium molybdate to form a phosphomolybdate complex with yellow color. The intensity of the color formed is proportional to the inorganic phosphorus concentration in the sample. The phosphorous concentration in the serum sample was calculated from the following equation:

$$\text{Phosphorous concentration (mg/dL)} = 5 \times \frac{A_{\text{sample}}}{A_{\text{standard}}}$$

Where 5 is the concentration of standard.

3.3.10 Determination of Total Cholesterol :⁽²⁸⁾

Cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed by the reaction of hydrogen peroxide and 4- aminoantipyrine in the presence of phenol and peroxidase

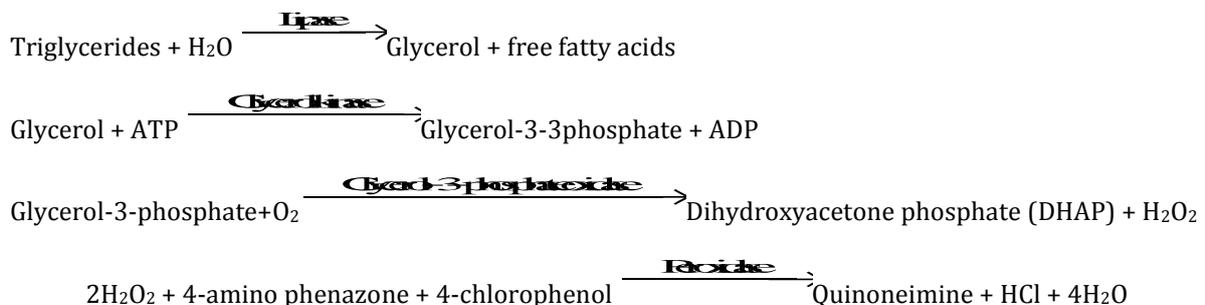


The intensity of the red dye Quinonimine formed is proportional to the cholesterol concentration in the sample.

$$\text{Cholesterol concentration (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times (\text{Standard Concentration})$$

3.3.11 Determination of Triglycerides: ⁽²⁹⁾

Triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is quinoneimine which is formed from hydrogen peroxide, 4- amino phenazone and 4- chlorophenol under the catalytic influence of peroxidase.



$$\text{Concentration of triglycerides in Sample (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times (\text{Standard Concentration})$$

3.4 Ultrasound examination:

3.4.1 Determination of Carotid Intima-Media Thickness (CIMT):⁽³⁰⁾

Siemens Acuson X 300 Premium edition diagnostic ultrasound system, used in par with the protocol outlined by the American Society of Echocardiography (ASE). A correct image showed double line for both the near and far wall of the carotid artery; these lines are the lumen-intima interface and media-adventitia interface. Once the images were taken, border detection programs were used that trace the far wall interface of the leading edge of the lumen-intima to the leading edge of the media-adventitia and calculate the CIMT.

3.5 Molecular studies:

3.5.1 Genomic DNA isolation:⁽³¹⁾

Genomic DNA was extracted from whole blood using the GeneJET™ Genomic DNA Purification Kit (Fermentas).

3.5.2 Genotyping of KL-VS and C1818T polymorphisms of Klotho by PCR-RFLP:⁽³²⁾

Genotyping of KL-VS and C1818T polymorphisms of Klotho were analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for detection of substitution of Phenylamine by Valine at the position 352 for KL-VS polymorphism and Cytosine→Thymine (C→T) nucleotide substitution for C1818T polymorphism.

A segment of the Klotho gene encompassing the KL-VS and C1818T polymorphic sites were amplified by polymerase chain reaction (PCR).

3.6 Statistical analysis of the data.⁽³³⁾

Data were analyzed using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). Test of normality was applied on the data by using Kolmogorov-Smirnov test, Shapiro-Wilk test also D'Agostino Quantitative data were expressed using ange, mean, standard deviation and median. Quantitative data were analyzed using F-test (ANOVA to compare the three categories of outcome. No-normally distributed quantitative data was analyzed using Mann Whitney test for comparing two groups while for more than two groups Kruskal Wallis test was applied. Pearson coefficient was used to analyze correlation between any two variables. P value was assumed to be significant at 0.05.

4.0 RESULTS

4.1 Biochemical studies:

In the present study, serum levels of FGF23, klotho, urea, creatinine, uric acid, parathyroid hormone, phosphorus, cholesterol, triglyceride and eGFR in CKD and dialysis groups and normal control group are illustrated in table 1.

The results showed significant increase in the mean values of FGF23 ($p \leq 0.05$) in CKD and dialysis groups as compared to normal control group while levels of Klotho showed significant decrease in the mean values in CKD and dialysis groups as compared to normal control group ($p \leq 0.05$).

There were significant increases in the mean values of urea, creatinine, uric acid, parathyroid hormone, and phosphorous levels in CKD and dialysis groups as compared to normal control group. Levels of triglyceride and cholesterol were significantly increased in CKD group as compared to normal control group ($p \leq 0.05$). The study showed a significant increase in CIMT in CKD and dialysis compared with the values reported for normal populations,⁽⁶⁵⁾ and significantly higher than the value of control group. ($p \leq 0.05$).

Significant decrease was noticed in the mean values of eGFR Protein and Albumin levels in CKD and dialysis groups as compared to normal control group ($p \leq 0.05$). Statistical analysis showed no significant

change in the mean values of calcium in CKD and dialysis groups as compared to normal control group ($p \leq 0.05$).

In the present study no correlation was found between CIMT and serum levels of calcium, phosphorus and PTH in HD patient's. Negative correlation was found between CIMT and serum levels of Klotho and eGFR in HD patients and between CIMT and serum levels of FGF23 and calcium in CKD patients.

Table (1): Clinical data and blood biochemical analysis in normal control and patient groups (values expressed as mean \pm SD)

	CONTROL n=20	Patient group n=40		Test of significant	
		CKD n=20	Dialysis n=20	F	P
FGF (pg/ml)	162.3 \pm 30.1	230.6 \pm 155.8	1222.7*# \pm 868.4	27.117	0,000*
Klotho (pg/ml)	670.9 \pm 79.3	388.2* \pm 58.4	326.0*# \pm 63.3	66.473	0,000*
PTH (pg/ml)	25.9 \pm 2.7	46.7* \pm 22.5	489.1*# \pm 458.9	19.454	0.000*
eGFR (ml/min/1.73 m ²)	169 \pm 50	33.3* \pm 13.9	7.2*# \pm 3.2	165.421	0.000*
Urea (mg/dl)	25.7 \pm 5.7	116.6* \pm 39.4	210.0*# \pm 30.5	205.55	0.000
Creatinine (mg/dl)	0.6 \pm 0.2	3.0* \pm 1.5	10.7*# \pm 2.0	276.173	0.000
Total protein (g/dL)	7.3 \pm 0.5	6.8* \pm 0.5	7.1 \pm 0.4	5.319	0.008*
Albumin (g/dl)	4.0 \pm 0.2	3.2* \pm 0.4	3.7 \pm 0.3	28.042	0.000*
Uric acid (mg/dl)	5.0 \pm 1.0	6.7* \pm 0.8	6.2* \pm 1.3	13.481	0.000*
Ph (mg/dl)	4.5 \pm 0.7	5.0 \pm 1.5	7.9*# \pm 3.3	15.391	0.000*
Calcium (mg/dl)	7.7 \pm 0.7	7.9 \pm 1.3	8.3 \pm 1.9	0.788	0.460
TG (mg/dl)	110.8 \pm 42	180.3* \pm 37	194.0* \pm 27	30.969	0.000*
Cholesterol (mg/dl)	208.3 \pm 38.5	259.3* \pm 52.9	228.0*# \pm 34.8	7.230	0.002*
CIMT (mm)	0.4 \pm 0.1	1.0* \pm 0.2	1.2*# \pm 0.3	74.051	0.000

F for ANOVA test

*: compares control versus patient groups. #: compares CKD versus Dialysis patient

*Statistically significant at $p \leq 0.05$

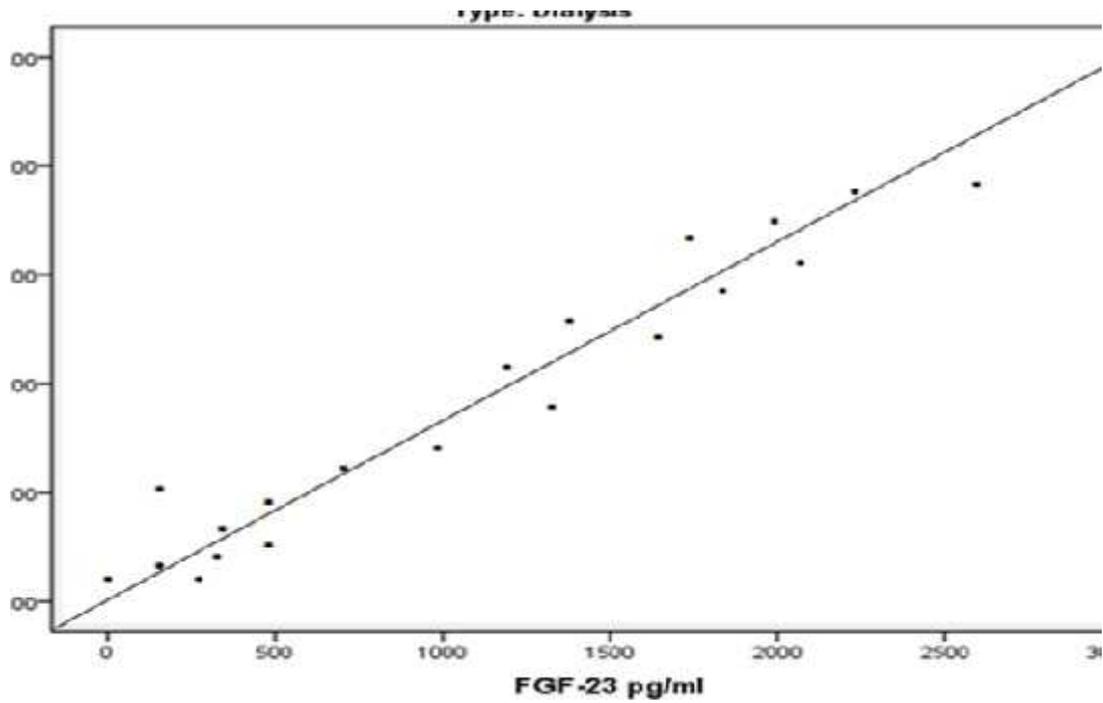


Figure (1): Correlation between FGF-23 and Creatinine in Dialysis patients.

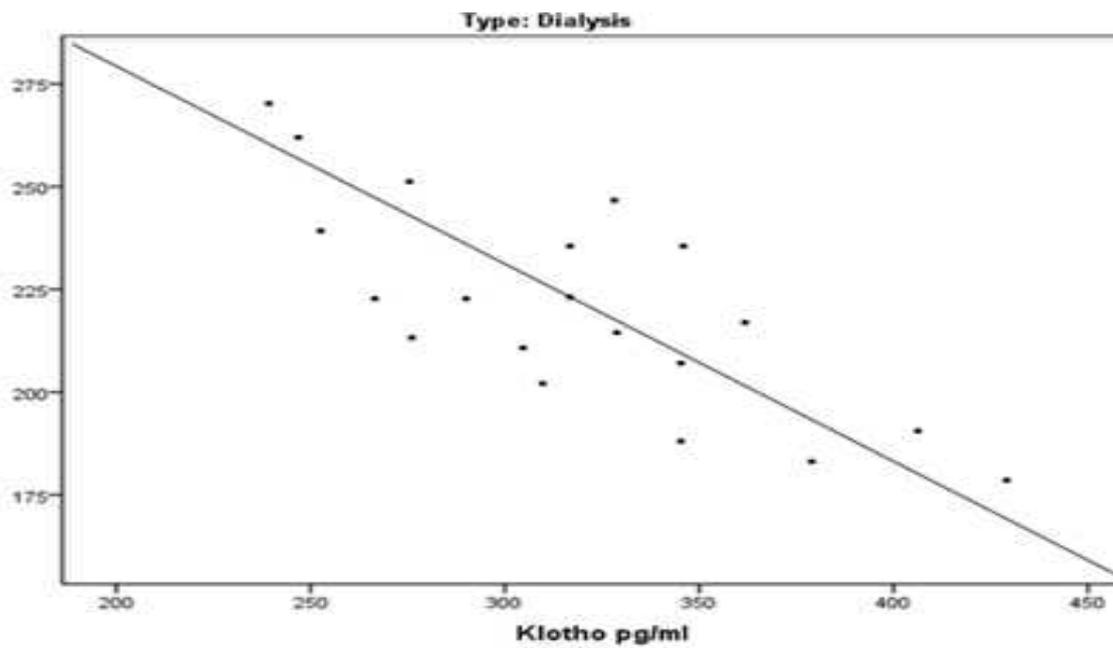


Figure (2): Correlation between Klotho and Urea in Dialysis patients

4.2 Genetic analysis:

None of the individuals were carrying the Klotho gene polymorphisms.

The amplicon from wild type homozygotes (352FF) was digested into two bands (319 bp and 186 bp), whereas the heterozygotes (352FV) had three bands (319 bp, 265 bp and 186 bp).

The present result on the gel electrophoresis showed wild type homozygotes for Kl-vs polymorphism

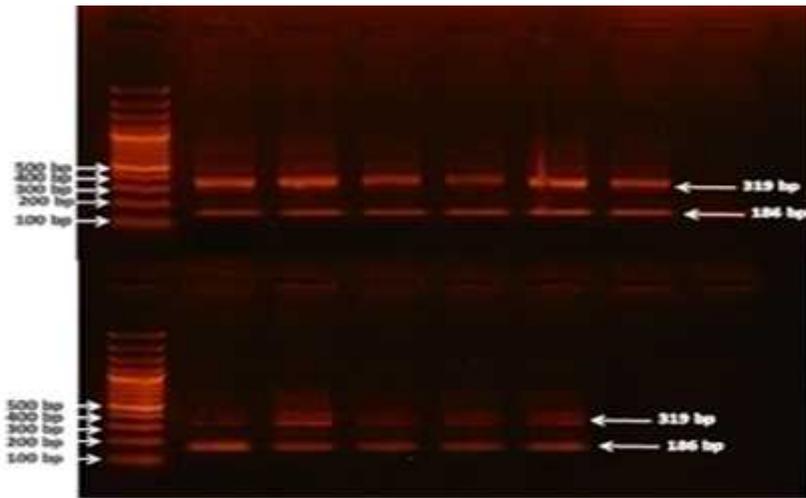


Figure (3): polymerase chain reaction /restriction fragment length polymorphism (PCR/RFLP)] of Klotho vs polymorphism of Klotho gene.

The DNA segment from TT homozygotes was digested into 272 and 180 bps. For the undigested CC wild homozygotes, a single band of 452 bp was observed. Our result on the gel electrophoresis indicate that our samples was CC wild homozygotes for C1818T polymorphism

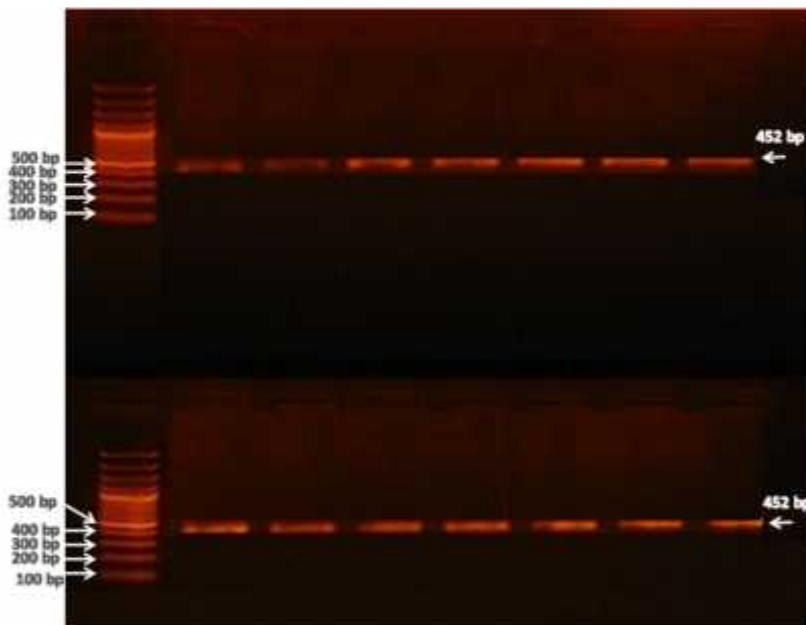


Figure (4): polymerase chain reaction restriction fragment length polymorphism (PCR/RFLP)] Of C18118T polymorphism of Klotho gene

5.0 DISCUSSION

FGF23 is an endocrine hormone that is secreted by osteocytes and osteoblasts. ⁽³⁴⁾The classical effects of FGF23 in the kidney and parathyroid glands are mediated by its binding to FGF receptors (FGFR) complexed to the co-receptor klotho, which increases the binding affinity of FGF23 for FGFR. ⁽³⁵⁾ The primary physiological actions of FGF23 are to stimulate phosphaturia, ⁽³⁶⁾ reduce systemic levels of 1,25-dihydroxyvitamin D ⁽³⁷⁾ and inhibit PTH secretion. ⁽³⁸⁾

Klotho is a single-pass transmembrane protein that exerts its biological functions through multiple modes. First mode through membrane-bound, Klotho acts as coreceptor for the major phosphatonin

FGF23, while the second mode through soluble Klotho functions as an endocrine substance. In addition to its function in the distal nephron where it is abundantly expressed, Klotho is present in the proximal tubule lumen where it inhibits renal Pi excretion by modulating Na-coupled Pi transporters via enzymatic glycan modification of the transporter proteins –an effect completely independent of its role as the FGF23 co-receptor.⁽³⁹⁾

Soft tissue calcification, and especially vascular calcification, is a dire complication in CKD, associated with high mortality. Klotho protects against soft tissue calcification via at least 3 mechanisms: phosphaturia, preservation of renal function and a direct effect on vascular smooth muscle cells by inhibiting phosphate uptake and de-differentiation.⁽³⁹⁾

The present study revealed significant increase in the levels of serum FGF23 in CKD and ESRD groups as compared to normal control group. These results were in agreement with Nakanishi *et al.*⁽⁴⁰⁾ who suggested that serum FGF23 levels were progressively increased as kidney function declines and markedly elevated once on dialysis. Also Husen *et al.*⁽⁴¹⁾ proved that, higher levels of FGF23 were found in stage 5 compared to stages 1 and 2 in CKD.

Sliem *et al.*⁽⁴²⁾ explained the increase in FGF23 in two explanations; the first is the kidney, which is the principal target of FGF23, becomes no longer responsive to FGF23 in CKD. The second is that, in early stage CKD, serum FGF23 is elevated to maintain normal serum phosphate levels, by promoting urinary phosphate excretion. However, in patients at the advanced stage, overt phosphate loading may overcome such compensation for decrease glomerular filtration rate (GFR) despite markedly elevated FGF23 levels. Plasma FGF23 concentrations begin to increase early in CKD. Increasing FGF23 levels are independently associated with left ventricular hypertrophy, CKD progression, and mortality, possibly through off-target actions. Lowering serum phosphate levels through the use of phosphate binders may lower FGF23 levels.⁽⁴³⁾ During the past decade, clinical data showed the association between FGF23 and CVD have been accumulated and recent translational research has suggested a direct pathophysiological link between FGF23 and CVD in CKD. Improved understanding of the mechanisms by which FGF23 confers the cardiovascular risks is necessary to establish new therapeutic approaches to mitigate this risk.⁽⁴⁴⁾

This inverse relationship between FGF23 and eGFR has been demonstrated in many studies, both in the pediatric and adult population.^(45,46) The elevated FGF23 levels observed in CKD have been explained by both increasing production by altered osteocyte function and by accumulation secondary to decreased renal clearance, as FGF23 is a low molecular weight protein that is freely filtered across the glomeruli.^(47, 48)

The increased level of FGF23 was positively correlated to the elevation of serum creatinine and blood urea, as illustrated in the present study. Devaraj *et al.*⁽⁴⁹⁾ have shown that FGF23 levels were significantly increased in patients with creatinine levels of more than 2 mg/dL (177 μ mol/L).

In the present study, levels of Klotho showed decrease in the mean values in CKD and dialysis groups as compared to normal control group. It has been reported that In CKD 1-5, Klotho and 1,25D linearly decreased, whereas both FGF23 and PTH showed a baseline at early CKD stages and then a curvilinear increase.⁽⁵⁰⁾

In this study, patient groups with lower levels of serum Klotho exhibited significantly lower ($p \leq 0.05$) eGFR levels, as previously reported by Terada⁽⁵¹⁾ in CKD patients and by Yokoyama⁽⁵²⁾ in patients on hemodialysis. It has been reported that the mRNA and protein expression levels of Klotho were severely reduced in the kidneys of patients with chronic renal failure compared to control subjects⁽⁵³⁾. However, it seems that the serum Klotho levels were not completely depleted, even in patients with stage 5 CKD on hemodialysis. This finding suggested that a basal level of Klotho production from other organs than the kidneys, such as the brain and parathyroid glands, might exist in humans, as has been previously reported in mice.⁽⁵³⁻⁵⁵⁾

Some studies indicated that the transcriptional suppression of Klotho by a protein-bound uremic toxin, indoxyl sulfate, results from cytosine and guanine (CpG) hypermethylation of the Klotho gene⁽⁵⁶⁾. Since indoxyl sulfate may play a significant role in the vascular disease and higher mortality observed in CKD patients⁽⁵⁷⁾, epigenetic modification of the Klotho gene by a uremic toxin such as indoxyl sulfate might be a mechanism underlying the association between the decline of serum Klotho levels and arterial stiffness in CKD patients observed in the current study.

The decline in soluble Klotho levels represents a negative event occurring in the early stages of cardiovascular-renal disease, Klotho might be considered as a useful biomarker that predicts atherosclerosis and vascular calcification. Further long-term clinical studies are required to establish the role of this exciting new potential marker and predictor of cardiorenal disease.⁽⁵⁸⁾

A significant increase in the parathyroid hormone levels in studying groups as compared to normal control group were supported by Rodriguez *et al.*⁽⁵⁹⁾ who stated that, in CKD the incorrect control of PTH secretion was attributed to the reduced vitamin D receptor (VDR) and Ca receptor expression which occur in parallel to the parathyroid gland growth. Parathyroid gland hyperplasia and the consequent increase in PTH secretion are responsible for hyperparathyroidism observed in CKD. Komaba and Fukagawa⁽⁶⁰⁾ explained the failure of increased FGF23 levels to suppress PTH, by the parathyroid resistance that might be due to the decreased expression of the Klotho-FGF R1 complex in the hyperplastic parathyroid gland.

Gutierrez *et al.*⁽⁶¹⁾ documented that in CKD, serum FGF23 levels were increased together with secondary hyperparathyroidism, indicating resistance of the parathyroid to FGF23. Measurement of FGF23 seemed to have prognostic significance in the treatment of secondary hyperparathyroidism.

Parathyroid and Klotho levels affected with declining renal function in CKD patients. This may be related to an intrinsic glandular defect, although Krajisnik⁽⁶²⁾ data suggested biochemical changes related to CKD, such as hypercalcemia and high FGF23 levels, to be a probable cause. This may explain the co-occurrence of high circulatory FGF23 and PTH levels, and the failure of FGF23 to prevent PTH hyper-secretion in late CKD.

Canalejo *et al.*⁽⁶³⁾ results demonstrated that PTH is necessary for FGF23 secretion. Actually, high phosphate does not stimulate FGF23 when PTH is low, and hence in this context PTH is likely to be more important than phosphate in the regulation of FGF23 secretion.

In the current study, the phosphorus levels were significantly elevated in the studied dialysis patients as compared to normal control group. These findings were consistent with the study of Fourtounas *et al.*⁽⁶⁴⁾ who found that, in CKD, the kidneys fail to excrete the phosphorus, resulting in positive phosphorus balance. The skeleton through the disorders of the bone that accompany CKD, contributes to this hyperphosphatemia, as it fails to handle the exceeding phosphorus.

As CKD progresses, elevated FGF23 levels are no longer able to enhance urinary phosphate excretion, thus leading to the development of hyperphosphatemia. This may be partly related to declining Klotho expression and a reduction in functional nephrons.⁽⁴³⁾

The results was confirmed with that of Sakan H *et al.*⁽⁴⁶⁾ who demonstrate that FGF23 levels rise to compensate for renal failure-related phosphate retention in early and intermediate CKD. This enables FGF23-klotho signaling and a neutral phosphate balance to be maintained despite the reduction in klotho. In advanced CKD, however, renal klotho declines further. This disrupts FGF23 signaling, and serum phosphate levels significantly increase, stimulating greater FGF23 secretion. The results also suggest the serum sKL concentration may be a useful marker of renal klotho expression levels.

Furthermore, our findings were in agreement with Komaba and Fukagawa⁽⁶⁰⁾ who stated that reduced renal function directly affects phosphorus reabsorption. The kidney becomes incapable of filtering enough phosphorus and its high level in blood directly stimulates the parathyroid gland which in turn stimulates FGF23 synthesis and secretion by the osteocytes.

The study showed a significant increase in CIMT in CKD and dialysis compared with the values reported for normal populations,⁽⁶⁵⁾ and significantly higher than the value of control group.

Benedetto and colleagues⁽⁶⁶⁾ showed an increase in CIMT as an independent predictor of cardiovascular death, retaining an independent effect in a model that included left ventricular mass. End-stage renal disease is considered as a risk factor for arterial stiffness and increased arterial CIMT. Increased CIMT, arterial sclerosis, stiffness and calcification of the coronary arteries have been reported in hemodialysis patients. Intima-media thickness is linked with concentric left ventricular hypertrophy in dialysis patients and serves as an independent predictor of cardiovascular events and cardiovascular and all-cause mortality in these patients.^(56,68)

In the present study no correlation was found between CIMT and serum levels of calcium, phosphorus and PTH in HD patients which were compatible with previous studies.^(69,70)

Okhuma *et al* ⁽⁷¹⁾ found significant correlation between serum calcium level and CIMT. Kawagashi *et al.*⁽⁷²⁾ found correlation between CIMT and serum phosphorus level and PTH in HD patients. Impaired calcium-phosphorus metabolism may affect lipoprotein metabolism and may contribute to the acceleration of atherosclerosis.

This study also showed a significant relationship between CIMT and age in CKD, which indicated the natural progression of atherosclerotic progression with increasing age as reported with Hojs *et al* ⁽⁷³⁾ and others.^(74,75)

This study showed an increase in the mean values of triglyceride and cholesterol in CKD and dialysis groups as compared to normal control group. The associations between CIMT and lipid disorders have inconsistently been reported in patients on dialysis.⁽⁷⁸⁾ It has been demonstrated that an independent association exists between total cholesterol, LDL cholesterol, triglycerides and CIMT and plaque occurrence in HD patients ^(77,78) However, several studies reported no relationship between lipid profile and CIMT in HD patients. ^(71, 72, 79)

In the molecular study of Klotho gene polymorphism, we did not detect kl-vs and C1818T polymorphism in patients who underwent CKD and dialysis which requires further investigations to elucidate the revolutionary origin of this variant or any other mechanism involved. Various frequencies of kl-vs mutation have been reported in previous studies ⁽⁸⁰⁻⁸²⁾ in different populations including, African American, Caucasians, Italian and Japanese, with lower frequency in Korean. Therefore it seems that the prevalence of kl-vs polymorphism is considerable in some populations, but it is rarely observed in others. On the other hand, prevalence of kl-vs is strongly affected by the ethnic background. In a study by Imamura *et al.* ⁽⁸³⁾ it was shown that the human klotho gene polymorphism (-395A) may be a genetic risk factor for CAD but not for vasospastic angina in Japanese patients without significant fixed stenosis of the coronary arteries. Kim *et al.* ⁽⁸⁴⁾ reported the klotho gene polymorphism as a risk factor for ischemic stroke. However there were discrepancies for the results in different populations.

Kl-vs variant was absent in our studied group patients who had major cardiovascular risks. Our finding highlights the necessity of future studies to further clarify the role of klotho variants in various clinical conditions in different populations. The frequency of klotho kl-vs variant should be examined in more studies. These studies must be carried out on subjects recruited from different ethnic backgrounds in areas which population admixture is rare and their characteristics have been described. At least one ethnic group with known presence of kl-vs variant must serve as positive control. Also detection of serum level of klotho protein or its gene expression in tissues might be helpful in identification of klotho role in CAD in various populations.

6.0 CONCLUSION

- High serum FGF-23 concentrations predict more rapid disease progression in CKD patients who were not on dialysis and an increased mortality in patients on maintenance hemodialysis. FGF-23 may therefore prove to be an important therapeutic target for the management of CKD and cardiovascular disease.
- Reduced Klotho protein levels with progressive renal failure may be a modifiable factor involved in the pathogenesis of cardiovascular and renal disease in at-risk populations.
- Although we were unable to specifically identify the causal variant in the klotho gene, this work provides important information in understanding how renal disease progresses to end-stage kidney failure.

7.0 RECOMENDATION

-Integration of FGF-23 measurements into current clinical practice should be cautioned by the many questions that still remain unanswered. The exact role of FGF-23, the determination of its 'normal' range and the association of FGF-23 with dietary phosphate intake and mediators that affect its secretion all need to be further delineated.

Klotho may be an early clinical biomarker of acute and chronic renal injury CKD as its diminution precedes changes of other well-established markers/factors involved in the progression of renal failure. However, further long-term prospective studies are required to establish the utility/value of Klotho as an early marker of acute and chronic renal disease.

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