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Original Research Article

Fibroblast Growth Factor (FGF-23) in Pre-Dialytic Chronic Kidney Disease Patients

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Abstract
Objective: Fibroblast Growth Factor (FGF-23); a Phosphaturic peptide hormone, has recently been identified as a regulator of calcium-Phosphorus metabolism. Aim of the present study was to analyse FGF-23 in addition to the conventional indicators of bone metabolism in pre-dialytic CKD patients.
Methods: Pre-dialytic CKD Stage 1 & 2 (N=10) and Stage 3 & 4 (N=14) patients were observed for different indicators of bone metabolism including Total, intact (active) and C-terminal FGF-23 (inactive) levels at recruitment as compared to age matched controls (N=14) and as compared to their profile at six month.
Results: In CKD stage (1-4) patients, phosphorus levels were within normal range but showed negative correlation with eGFR (Linear correlation (r) = -0.467, p=0.021) and positive correlation with C-terminal FGF-23 levels (spearman correlation (rs)=0.464, p=0.022). As compared to the controls, in stage 1 and 2 Total, intact and C-terminal FGF-23 increased significantly (p=0.001) by 16.4, 2.1 and 19.2 fold and in stage 3 and 4 by 28.6, 2.4 and 32.3 fold respectively. Thus, increase in the C-terminal by intact-FGF-23 in control, CKD stage 1 & 2 and CKD stage 3 & 4 were 4.5, 41.8 and 60.6 fold respectively. These patients were on regular calcium and vitamin D treatment. Follow-up study at six month did not show any change in FGF-23 levels.
Conclusion: In the absence of hyperphosphatemia at early stages of CKD, FGF-23 levels increased with declining renal function. Increase in C-terminal FGF-23 over intact-FGF23 levels might greatly advance understanding of mineral bone disease in CKD.
Keywords: Hyperphosphatemia, Mineral bone Disease, Secondary Hyperparathyroidism.

1.Introduction
Chronic kidney disease (CKD) has become one of the leading global health problems which eventually lead to kidney failure creating a major economic strain on the health care system [1]. Mineral bone disorder (MBD) is a major complication of CKD wherein patients show impaired renal excretion of phosphate that leads to significant hyperphosphatemia [2]. Disturbed calcium - phosphate metabolism is known to affect cardiovascular morbidity and mortality in patients with CKD, particularly in patients with End Stage Renal Disease (ESRD) [3,4]. However, there is limited data on the pattern of MBD in the pre-dialytic CKD population.

In healthy individuals, kidney excretes approximately two third of the daily dietary phosphate load. However it has been observed that even with deteriorating kidney function in CKD, this excretion rate remains relatively well maintained and is seen to alter only when it reaches its end stage [5-8]. Hence, this might limit the use of serum phosphorus as an early indicator of its imbalance in CKD. The assessment of the deranged phosphate homeostasis and other parameters involved in its regulation, thus needs to be studied in pre-dialytic CKD stages. In CKD-MBD, phosphorus imbalance, low serum calcium and 1, 25-dihydroxyvitamin D (1, 25$(OH)_2$D/

Active Vitamin D) levels represent a classical triad that leads to secondary hyperparathyroidism [9]. Apart from parathyroid hormone (PTH) and 1, 25 $(OH)_2$ D, a phosphaturic hormone, Fibroblast Growth Factor 23 (FGF-23) has recently been identified as a regulator of phosphorus metabolism [10].

FGF-23 is a 251 amino acid protein with a molecular weight of 32 kDa, member of the FGF-19 subfamily of FGFs. It is produced by the osteocytes in bones. From this, a signal peptide of 24 amino acids is cleaved to give the active FGF-23 protein of 227 amino acids [10, 11]. FGF-23 maintains serum phosphate levels in the normal range by inhibiting NaPi-2a and NaPi-2c-co-transport on the brush border membrane of proximal tubules thereby promoting renal phosphate excretion [11-14]. FGF-23 requires a transmembrane protein Klotho for its signaling [15]. In order to transmit its signal FGF-23 forms a heterotrimer complex with receptor FGF1Rc and co-receptor Klotho. Klotho binds to the C-terminal of FGF-23. Between the N-terminal and C-terminal domains there is a cleavage site at 179 amino acid (amino acid number counting from signal peptide) that contains an RXXR motif which is cleavage site for enzymes of the proconvertase-type of subtilisin or kexine type of the serine protease family. In its dynamics, active intact-FGF-23 (iFGF-23) is proteolytically cleaved at its C-terminal to produce inactive C-terminal and N- terminal fragments [15,16].

Thus PTH and FGF-23 induce phosphorus excretion. However, FGF-23 by suppressing 1-alpha hydroxylase acts as a counter regulatory hormone for vitamin D and decreases its synthesis and accelerates degradation of 1, 25$(OH)_2$ D [11-14]. Sieler *et al* [17] have reported the association of high serum concentration of FGF-23 with rapid progression of CKD in predialysed patients while Gutierrez *et al* [18] have demonstrated increased FGF-23 levels to be associated with increased mortality of patients on hemodialysis. Till date health programs for prevention of CKD in India have mainly focused on hypertension, diabetes mellitus and cardiovascular disease (CVD). Recently from India a study has been reported by Prasad *et al*, which elaborates the role of FGF-23 in post renal transplant period [19]. However, in spite of high prevalence of CKD related bone diseases there is paucity of data on CKD-MBD and the regulation of FGF-23among the early stages of CKD. The present study aimed to evaluate FGF-23 levels in addition to the conventional indicators of bone metabolism in pre-dialytic CKD patients among the Indian population.

2. Materials and Methods

2.1 Study Population

Subjects included were controls (n=14) and pre-dialytic CKD patients (n=24). Pre-dialytic CKD patients comprised of CKD stage 1 & 2 combined into one group (N=10) & CKD Stage 3 & 4 into another group (N=14). These pre-dialytic CKD patients were then followed up every month for a period of six months. Here data of these 24 pre-dialytic CKD patients at recruitment as compared to controls and as compared to their profile at six month is presented. This study was approved by the Institutional Ethics Committee (Letter dated by 2nd May, 2011) which follows the ethical standards laid down by the ICMR's ethical guidelines for biomedical research on human participants. Patient Information Sheet (PIS) was explained to each patient in the language understood by them in the presence of the house doctor and patient's relatives. On agreeing to participate in the study, signature on PIS and Informed consent (IC) was obtained from all patients before blood and urine sample collection.

Study population was selected as per the inclusion and exclusion criteria set for the study. On the basis of the renal profile carried out during enrollment, patients were classified according to CKD stages on the basis of estimated glomerular filtration rate (eGFR) calculated using "Modification of Diet in Renal Disease" (MDRD) method and presence of microalbumin creatinine ratio (ACR) more than 30mg/gm creatinine in urine. [20] CKD Stage-1 patients were with eGFR≥ 90 ml/min/1.73 m^2, CKD Stage-2 were with 60–89 ml/min/1.73 m^2, CKD Stage-3 were with 30–59 ml/min/1.73 $m^2$and CKD Stage-4 were with 15–29 ml/min/1.73 m^2. Healthy individuals recruited for the age match comparison with the CKD patients were volunteers in the study with no CKD or any other organic disease and normal eGFR (eGFR ≥ 90 ml/min/1.73 m^2) and ACR less than 30mg/gm creatinine. They were confirmed healthy on the basis of their clinical history and routine biochemical investigations.

After enrolment medication of vitamin D and calcium were stopped for fifteen days of CKD patients. After this washout period, on 16th day which was considered as the first day of the recruitment, blood and urine samples were again collected and renal profile was repeated. Intact PTH (iPTH), 25 (OH)D (Total Vitamin D) and 1, 25 $(OH)_2$ D, Total FGF-23, iFGF-23 and bone alkaline phosphatase (BALP) levels were also analysed. On the basis of the renal profile subsequent to the wash-out period of the above said medications, these patients were again prescribed calcium and vitamin D treatment. After recruitment, renal profile was repeated every month while iPTH, 25 (OH) D and 1, 25 $(OH)_2$ D were repeated at 3rd and 6th month and accordingly the doses of calcium and vitamin D were adjusted. Calcium tablets (SHELCAL 500MG Tablet) OD was given if serum Ca was less than 8.0 mg/dl and stopped if serum Ca was more than 11 mg/dl. Calcitriol (Rocaltrol, 0.25 mg Capsule) OD/BD was prescribed if serum 1, 25$(OH)_2$ D was low and

stopped if serum 1, 25 $(OH)_2$ D was above normal or serum calcium was more than 11.0mg/dl. Total FGF-23, iFGF-23 and BALP levels were repeated along with all other parameters at six month. All the parameters were also analysed in controls at recruitment.

2.2 Methodology

Aliquots of serum and plasma were immediately obtained from the blood sample and then stored at -80 degrees until further use. Another aliquot of separated serum was analysed for renal profile and urine sample was checked for the presence of microalbumin. Serum levels of iPTH, were measured using immune-radiometric assay kits from DIA Source (hPTH-120 min-ITMs [KIP149*1], Belgium) which is a two-step immune-radiometric assay based on coated tube separation. Samples and calibrators were captured by goat antibodies specific to 1-34 PTH fragment (N-terminal) and subsequently detected by 125I labeled monoclonal antibody specific to 44-60 hPTH fragments. Total Vitamin D (25 [OH] D) and active vitamin D (1, 25 $[OH]_2$ D) were analysed by Radioimmunoassay method (DIA Source, 25 OH-Vitamin D total-RIA-CT, KIP1971 and 1, 25 (OH)2-VIT.D-RIA-CT, KIP1929) wherein fixed amount of 125I labelled 25[OH] D and 1, 25 $[OH]_2$ D competes with respective vitamin present in serum for a fixed amount of antibody site immobilized on the wall of the polystyrene tube. Plasma iFGF-23 and Total FGF-23 was measured using a two-step iFGF-23 and C-terminal FGF-23 ELISA kit (Second generation Immunotopics Inc, San Clemente, USA) respectively according to the manufacturer's protocol. iFGF-23 ELISA assay had the antibodies that recognized the epitopes between N-terminal and C-terminal portion of the processing site of FGF-23, i.e. it recognized only the biologically active portion of FGF-23 and values expressed in pg/ml. In contrast, C-terminal assay detected both, the biological active portion i.e. iFGF-23 which was not cleaved and the C terminal inactive fragment of FGF-23 which was produced after cleavage of active iFGF-23. Hence the above C-terminal ELISA assay estimates Total FGF-23 levels present in the circulation and values expressed in relative units (RU)/mL.1 RU/mL roughly equates to 2 pg/mL according to the manufacturer [21]. The inactive C-terminal FGF-23 levels were then derived by subtracting the levels of active iFGF-23 (pg/ml) fragments from Total FGF-23 (pg/ml). The iFGF-23 assay showed an intra-assay coefficient of variation (CV) of 5.4% and inter-assay CV of 7.2% and that of C-terminal assay showed an intra-assay CV of 3.5% and inter-assay CV of 4.7%.

2.3 Statistical Analysis

Measured variables as Mean ± SD or median (25^{th}/75^{th} percentiles) were compared between patient groups and controls by unpaired student's t test or Mann–Whitney U test respectively and between two time intervals by paired t test or Wilcoxon signed-rank test respectively. Correlations were studied by either Pearson correlation (r) or Spearman Correlation (rs) test. $p<0.05$ was considered as statistically significant. Analyses were performed using statistical software SPSS (version 21.0, Chicago, IL).

3. Results

3.1 CKD Patient Data Analysis at Recruitment

Table 1 & 2 show the demographic and biochemical data respectively of pre-dialytic CKD patients at baseline i.e. at the time of the recruitment and of age and BMI matched controls. The mean values of biochemical parameters observed at baseline showed that,there was no significant increase in calcium (Ca), Phosphorus (P) or Ca-P product (Table 2) as compared to controls.

Table 1: Demographic data of CKD stage 1-4

Demographic	Control (N=14)	Recruitment CKD Stage 1&2 (N = 10)	RecruitmentCKD Stage 3 &4 (N = 14)
M/F	4/10	6/4	7/7
Age (yrs)	55.3±6.62	56.5 ± 11.3NS	63.5±6.68 P=0.004
Weight(kg)	59.8±18.2	60.8± 12.2NS	63.1±17.6NS
BMI (Kg/m^2)	23.8±6.2	24.5 ± 3.69NS	24.3±4.92NS
Smoking	-	2 (20%)	3 (21%)
Alcohol	-	3 (30%)	7 (50%)
Diabetes	-	1 (10%)	10 (71%)
Hypertension	-	8 (80%)	9 (64%)

NS: non-significant

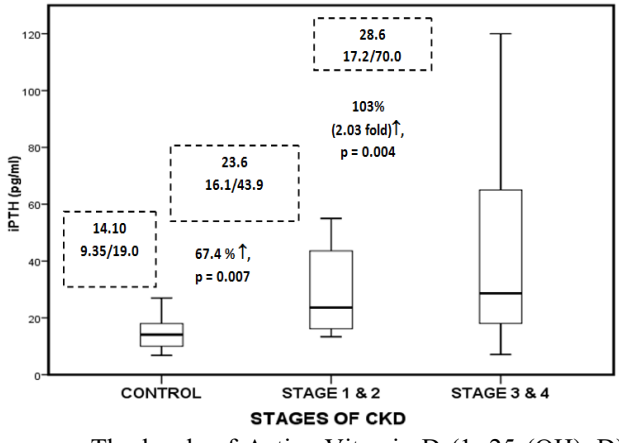
Table 2: Biochemical data of CKD Stage 1-4

Biochemistry Data	Control (N=14)	Recruitment CKD Stage 1&2(N = 10)		RecruitmentCKD Stage 3 &4 (N = 14)	
BUN (mg/dl)	8.0 (7.75/9.25)	11.5 (10.5/14.8)	43.8% ↑, p = 0.01	16 (13.8/24.5)	100.0% (2.0 fold)↑ p = 0.001
Creatinine (mg/dl)	0.85 (0.70/1.0)	1.0 (0.9/1.15)	17.6 % ↑, p = 0.04	1.60 (1.37/1.75)	88.2%↑ p = 0.001
GFR (ml/min/1.73m^2)	90.8±13.6	73.0±10.5	19.6% ↓, p = 0.001	45.0±12.8	50.4% ↓ p = 0.001
Total Protein (gm/dl)	7.02±0.4	7.2±0.45	NS	7.33±0.57	NS
Albumin (gm/dl)	4.07±0.9	4.2±0.42	NS	4.14±0.36	NS
Calcium (Ca) (mg/dl)	9.57±0.5	9.74±0.71	NS	9.70±0.61	NS
Corrected Calcium (Cr.Ca) (mg/dl)	9.49±0.7	9.56±0.74	NS	9.71±0.48	NS
Phosphorous (P) (mg/dl)	3.52±0.9	3.72±0.60	NS	3.98±0.81	NS
Corrected Ca x P (mg^2/dl^2)	33.1±7.7	35.4±4.72	NS	38.3±8.50	NS

NS: non-significant

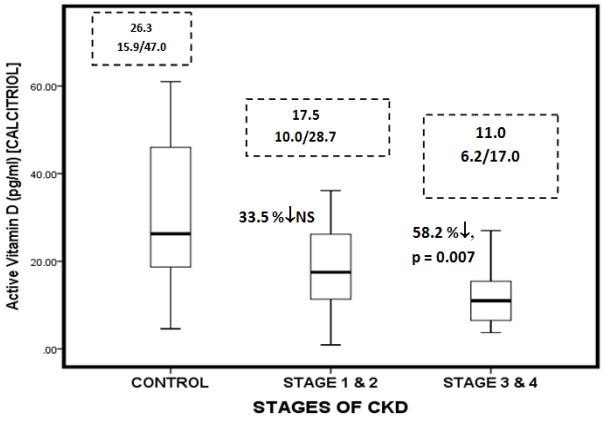
iPTH increased significantly in CKD stage 1 & 2 (67.4%, p=0.007) and in CKD stage 3 & 4 (103% [2.03 fold], p=0.004) patients as compared to the age match controls (Figure 1).

Figure 1: iPTH levels



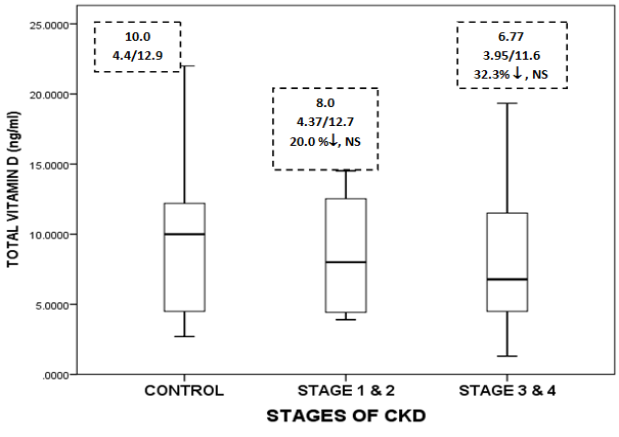
The levels of Active Vitamin D (1, 25 $(OH)_2$ D) decreased in CKD stage 1 & 2 (33.5% non-significant, [NS]) and further reduced significantly in CKD stage 3 & 4 (58.2%, p=0.007) (Figure 2).

Figure 2: 1, 25$(OH)_2$ D (Active Vitamin D) levels



Total Vitamin D (25 (OH) D) decreased in both CKD stage 1 & 2 (20%) and in CKD stage 3 & 4 (32.3 %), but was statistically not significant as compared to controls (Figure 3).

Figure 3:25 (OH) D (Total Vitamin D)



In CKD stage 1 and 2, Total FGF-23, iFGF-23 and C terminal FGF-23 increased by 16.4 fold (p=0.001), 2.1 fold (p=0.001), and 19.2 fold (p=0.001) respectively and in stage 3 and 4 by 28.6 fold (p=0.001), 2.4 fold (p=0.001), and 32.3 fold (p=0.001) respectively as compared to controls (Figure 4, 5, 6). The increase in the C-terminal FGF-23 by iFGF-23 in control, CKD stage 1 & 2 and CKD stage 3 & 4 were 4.5 fold, 41.8 fold and 60.6 fold respectively.

Figure 4: Total FGF-23 levels

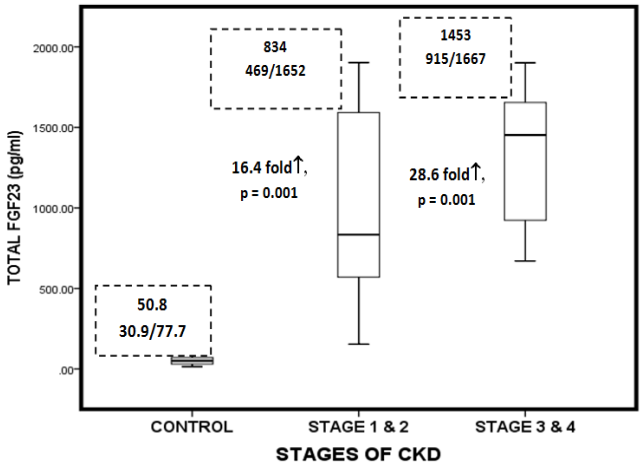


Figure 5: Intact FGF-23 levels

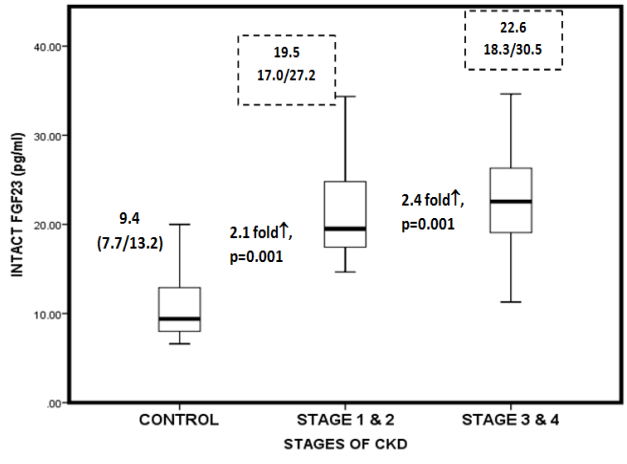
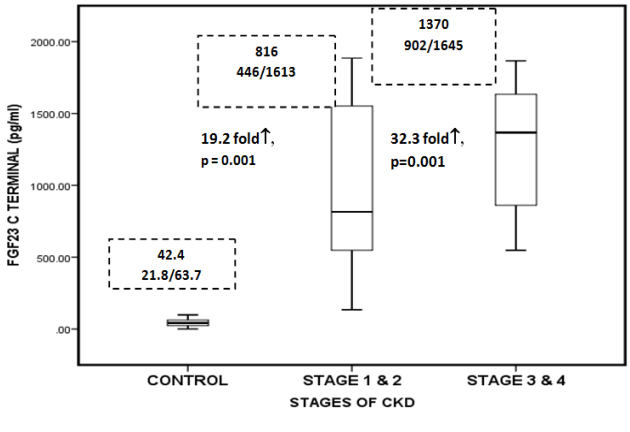
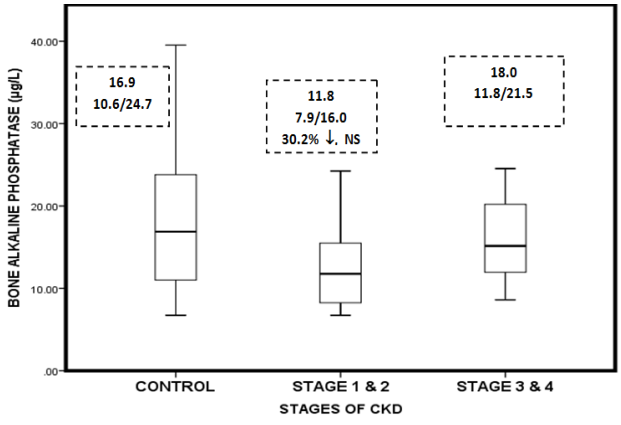


Figure 6: C terminal FGF-23 levels



As compared to the controls, there was decrease in BALP levels in CKD stage 1 & 2 (30.2%, NS) while in CKD stage 3 & 4 it was similar to that of controls (Figure 7).

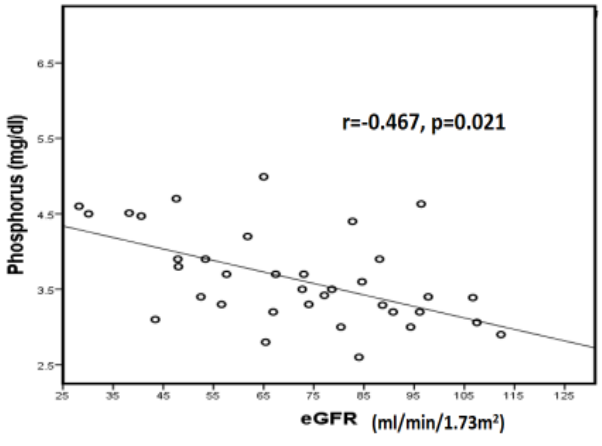
Figure 7: Bone Alkaline Phosphatase levels



3.2 Correlation Data

Bivariate correlation analysis done by combining all CKD stages, demonstrated a negative correlation of iPTH with serum calcium (Spearman (rs) =-0.478, p=0.018) and 25 (OH) D (rs= -0.559, p=0.005) (Figures not shown). P showed an inverse correlation with eGFR (r= − 0.467, p=0.021) (Figure 8) and direct correlation with C-terminal FGF-23 (rs=0.464, p=0.022) & Total FGF-23 (rs=0.466, p=0.022).

Figure 8: Correlation between Phosphorus and eGFR throughout the CKD stages 1-4



3.3 Follow up Data of CKD Patients at Six month

Biochemical data of CKD stage 1 and 2 and CKD stage 3 and 4 patients, at the end of six month follow up is depicted in the Table 3 and 4 respectively. In CKD stage 1 and 2 patients, iPTH levels decreased by 53.4% (p=0.02)(Table 3) and in CKD stage 3 and 4 patients 25 (OH) D levels increased significantly (65.4%,p=0.03) at the end of six months as compared to their recruitment levels (Table 4). Other parameters did not show any significant change.

Table 3: Six monthly follow up Biochemical and Clinical data of CKD stage 1 & 2

	Recruitment CKD STAGE 1 & 2 (N = 10)	**6TH Month Follow Up CKD STAGE 1 & 2 (N = 10)**	
BUN (mg/dl)	11.50(10.5/14.8)	11.50(9.3/18.0)	NS
Creatinine (mg/dl)	1.0(0.9/1.15)	1.0(0.80/1.23)	NS
eGFR (ml/min/1.73m^2)	73.0±10.5	77.0±21.8	NS
Albumin (gm/dl)	4.2±0.42	3.8±0.19	NS
Globulin (gm/dl)	2.98±0.44	3.2±0.4	NS
Total Protein (gm/dl)	7.2±0.45	7.07±0.45	NS
Calcium (Ca) (mg/dl)	9.74±0.71	9.72±0.51	NS
Corrected Calcium (Cr.Ca) (mg/dl)	9.56±0.74	9.87±0.31	NS
iPTH (pg/ml)	23.6(16.1/43.9)	11.0(8.1/30.5)	53.4% ↓ p = 0.02
25 (OH) D (ng/ml) (Calcidiol)	8.0(4.37/12.7)	10.8(9.6/14.82)	35.% ↑NS
1,25 $(OH)_2$ D(pg/ml) Calcitriol)	17.5(10.0/28.7)	22.70(16.1/35.2)	29.7%↑NS
Phosphorous (P) (mg/dl)	3.72±0.60	3.92±0.63	NS
Corrected Ca x P (mg^2/dl^2)	35.4±4.7	38.7±6.2	NS
Total FGF-23 (pg/ml)	834(469/1652)	658(454/1234)	21.1%↓NS
Intact FGF-23 (pg/ml)	19.5(17.0/27.2)	20.1(17.5/22.8)	NS
C Terminal FGF-23 (pg/ml)	816(446/613)	635(438/1216)	22.2%↓NS
Bone Alkaline Phosphatase (BALP) (µg/L)	11.8(7.9/16.0)	10.8(9.55/16.1)	NS

NS non-significant

Table 4: Six monthly follow up Biochemical and Clinical data of CKD stage 3 & 4

Biochemistry Data	**Recruitment CKD STAGE 3 & 4 (N = 14)**	**6th Month Follow Up CKD STAGE 3 & 4 (N = 14)**	
BUN (mg/dl)	16 (13.8/24.5)	22 (13.5/35.5)	37.5% ↑ p=0.03
Creatinine (mg/dl)	1.6 (0.37/1.75)	1.4 (1.2/1.7)	12.5 % ↓ P=0.01
eGFR (ml/min/1.73m^2)	45.0±12.8	45.2±17.1	NS
Albumin (gm/dl)	4.14±0.36	3.7±0.30	NS
Globulin (gm/dl)	3.35±0.67	3.42±0.33	NS
Total Protein (gm/dl)	7.33±0.57	7.19±0.46	NS
Calcium (Ca) (mg/dl)	9.7±0.61	9.64±0.38	NS
Corrected Calcium (Cr.Ca) (mg/dl)	9.71±0.48	9.88±0.33	NS
iPTH (pg/ml)	28.6(17.2/70.0)	28.4(16.1/63.4)	NS
25 (OH) D (ng/ml)	6.77(3.95/11.6)	11.2(5.50/15.9)	65.4% ↑ p = 0.03
1,25 $(OH)_2$D (pg/ml)	11.0(6.2/17.0)	11.3(6.1/19.9)	NS
Phosphorous (P) (mg/dl)	3.98±0.81	4.11±0.80	NS
Corrected Ca x P (mg^2/dl^2)	38.3±8.50	40.6±7.75	NS
Total FGF-23(pg/ml)	1453(915/1667)	1411(879/2374)	NS
Intact FGF-23 (pg/ml)	22.6(18.3/30.5)	25.7(16.2/141)	13.7%↑NS
C Terminal FGF-23(pg/ml)	1370(902/1645)	1385(860/2251)	NS
Bone Alkaline Phosphatase (BALP) (µg/L)	18(11.8/21.5)	13(10.9/19.1)	27.8% ↓NS

NS non-significant

4. Discussion

Present study demonstrated that although phosphorus levels across the pre-dialytic stages of CKD were found to be within the normal range, there was an inverse association of serum phosphorus with eGFR. This suggests that there was a rise in serum phosphorus levels with the declining eGFR levels. Similar findings of normophosphatemic CKD have also been reported [5-7]. This suggests that serum phosphorus cannot be used as an indicator of disturbed phosphorus metabolism in the early stages of CKD.

The present study also demonstrated that the levels of both active iFGF-23 and inactive C-terminal FGF-23 increased significantly in the early stages of CKD, even in the absence of hyperphosphatemia. Serum phosphorus was negatively correlated with eGFR suggesting that there was a rise in serum phosphorus which might have led to the increase in the FGF-23 levels as an appropriate response to minimize the phosphorus elevation. iPTH was also seen to have negative correlation with serum calcium and 25 (OH) D levels.

Earlier studies have also suggested that normal serum phosphate and calcium levels in the vast majority of patients with early and intermediate stages of CKD might be due to compensatory increase in FGF-23 and iPTH [22, 23]. PTH and 1, 25 $(OH)_2$ D are also involved in maintaining normal phosphate balance. Both of these actively contribute to the mineral ion transport process by influencing biological activities of the membrane structures and transport molecules [24]. PTH, by inducing renal expression of 1-alpha-hydroxylase, increases production of 1, 25 $(OH)_2$ D metabolite, which further enhances the intestinal phosphorus absorption [24]. However, contrary to iPTH, FGF-23 suppresses renal expression of 1-alpha-hydroxylase and thus synthesis of 1, 25 $(OH)_2$D [11-13]. This in turn increases iPTH levels contributing to secondary hyperparathyroidism [22, 23]. In concordance with the above studies, present study also demonstrated that FGF-23 and iPTH levels were elevated in CKD patients in its early stages, further increased as eGFR fell, and both increased before hyperphosphatemia first appeared. Thus stimulation of FGF-23 and iPTH in the early stages of CKD can be seen as a part of the homeostatic response at maintaining normal calcium and phosphorus levels [9, 25-27].

However, notable observation of the present study was that, the increase of C-terminal FGF-23 was many folds greater as compared to the iFGF-23 levels and serum P demonstrated a positive association with C-terminal and Total FGF-23 levels suggesting accumulation of inactive C-terminal FGF-23 with the increase in the severity of kidney damage. Thus although iFGF-23 may be able to control increased P associated with increase in eGFR, there was much greater increase of C-terminal FGF-23 occurring simultaneously. Goetz *et al.* [28] have provided *in vitro* and *in vivo* evidence that the isolated C-terminal FGF-23 domain antagonized the activity of the intact hormone by competing for the availability of the receptor complex and can be the portion that is present in higher concentration during the diseased condition.

We acknowledge small sample size of the present study. The follow up had to be restricted to six months due to the drop out of the patients. In conclusion, it can be suggested that in the absence of hyperphosphatemia at earlier stages of CKD, FGF-23 levels may be used as an indicator for phosphate imbalance. Maximum increase seen of C-terminal FGF-23 suggests that it might be strongly associated with the progression of CKD to its terminal kidney disease which in turn is associated with hyperphosphatemia. Thus, understanding the role of FGF-23 in CKD-MBD can help in the management of phosphate balance in CKD patients.

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