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C-Terminal, but Not Intact, FGF23 and EPO Are Strongly Correlatively Elevated in Patients With Gain-of-Function Mutations in HIF2A: Clinical Evidence for EPO Regulating FGF23

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Abstract

Fibroblast growth factor 23 (FGF23) is a key phosphate- and vitamin D-regulating hormone. FGF23 circulates as an intact 251 amino acid protein or N- and C-terminal degradation products. Hormone activity resides in the intact molecule, but it has been suggested that high levels of the C-terminal protein can interfere with intact FGF23 (iFGF23) activity. New evidence points to involvement of the hypoxia-inducible factor (HIF)/erythropoietin (EPO)/iron pathway as important in FGF23 physiology. Exactly how this pathway regulates FGF23 is not clear. Various in vitro, in vivo, and clinical studies involving perturbations in this pathway at various points have yielded conflicting results. Many of these studies are complicated by the confounding, independent effect of renal insufficiency on FGF23. To gain insight into FGF23 physiology, we studied 8 patients with a rare paraganglioma/somatostatinoma syndrome who had elevated blood EPO levels as a result of somatic gain-of-function mutations in HIF2A (*EPAS1*) that stimulate tumoral EPO production. All patients had normal renal function. EPO levels varied; most were very elevated and highly correlated with C-terminal FGF23 (cFGF23) levels that were also markedly elevated. Blood phosphate and intact FGF23 levels were normal. These data from patients with normal renal function in whom HIF activation was the inciting event suggest a direct role of the HIF/EPO pathway in FGF23 transcription and translation. They also demonstrate that posttranslational regulation was finely tuned to maintain normal blood phosphate levels. Additionally, normal

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Disclosures

All authors state that they have no conflicts of interest.

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phosphate and intact FGF23 levels in the setting of markedly increased C-terminal FGF23 levels suggest intact FGF23 action is not attenuated by C-terminal FGF23.

Keywords

PTH/VITD/FGF23; DISORDERS OF CALCIUM/PHOSPHATE METABOLISM; ENDOCRINE PATHWAYS; MOLECULAR PATHWAYS

Introduction

Fibroblast growth factor 23 (FGF23) is a hormone central in the regulation of phosphate and 1,25-dihydroxyvitamin D that acts by decreasing renal sodium/phosphate cotransporters and 25-hydroxyvitamin D 1-alpha hydroxylase activity, leading to decreased blood phosphate and 1,25-dihydroxyvitamin D levels, respectively.[1,2] FGF23 excess leads to hypophosphatemia, causing rickets and osteomalacia.[3] FGF23 deficiency causes hyperphosphatemic tumoral calcinosis.[4] FGF23 is a 251-amino acid protein that undergoes O-linked glycosylation in the Golgi by the enzyme polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3).[5,6] Glycosylation of FGF23 at a putative furin protease cleavage site stabilizes the intact protein, preventing its degradation into biologically inactive C- and N-terminal fragments. Conversely, phosphorylation of FGF23 by the kinase FAM20C prevents glycosylation by GALNT3 and promotes breakdown of the FGF23 protein.[7]

It is has been established that FGF23 transcription and translation are regulated by phosphate,[8] 1,25-dihydroxyvitamin D,[9] and calcium,[10] consistent with its role in regulating phosphate and 1,25-dihydroxyvitamin D via classic endocrine feedback physiology. Observations from patients with autosomal dominant hypophosphatemic rickets (ADHR) suggested iron may play a role in FGF23-regulated blood phosphate levels.[11] These patients, who have altered posttranslational processing due to mutations altering glycosylation, have increased intact FGF23 (iFGF23) and can become hypophosphatemic when anemic and erythropoietin (EPO) levels are elevated. Control patients can augment FGF23 posttranslational processing, resulting in higher C-terminal FGF23 (cFGF23) but normal intact FGF23 and blood phosphate levels.[12] Recently, it was shown that treatment of ADHR patients with oral iron had the potential to normalize the elevated intact FGF23 levels and correct hypophosphatemia.[13] This is the opposite of what is found in non-ADHR patients, in whom iron infusions, especially ferric carboxymaltose, has been shown to increase intact FGF23, resulting in hypophosphatemia. It is thought that this phenomenon is a feature attributable to the carbohydrate moiety in ferric carboxymaltose.[14]

Recent evidence suggests the effects of iron on FGF23 regulation may be part of a broader effect due to alterations in the hypoxia-inducible factor (HIF)/erythropoietin (EPO) pathway.[15–18] Clinkenbeard and colleagues injected EPO into wild-type mice, resulting in increased whole bone FGF23 mRNA and serum levels of both C-terminal and intact FGF23, along with an expected decrease in serum phosphate.[19] Notable in this setting, administration of EPO was sufficient to cause hypophosphatemia.[19] Interestingly, Daryadel and colleages administered EPO to mice and found increases in both C-terminal and intact FGF23 with

no change in phosphate levels.[20] Toro and colleagues demonstrated that EPO increased FGF23 by a mechanism partially dependent on the EPO receptor.[17] Mice with high endogenous EPO had elevations of C-terminal FGF23 to a greater degree than intact FGF23.[16] Furthermore, it appears that EPO and FGF23 may function in a feedback loop. In FGF23 knockout mice, EPO mRNA was elevated in the bone marrow, liver, and kidney.[21] Injection of FGF23 into wild-type mice decreased circulating EPO and erythropoiesis.[21] In a mouse model of renal failure, inhibiting FGF23 signaling increased hematopoietic stem cells committed to the erythroid lineage, stimulated erythropoiesis, and improved anemia.[22] (Animal data on the relationship between EPO and FGF23 are summarized in Supplemental Table S1.)

Several studies have suggested a link between EPO and FGF23 in humans (Table 1); {TBL 1} however, most were conducted in patients with renal failure or other illnesses, and effects on phosphate have been conflicting or not reported. Studies in four human cohorts with chronic kidney disease revealed correlations between EPO and total FGF23 levels but not intact FGF23 levels.[16] Phosphate was reported to be unchanged in one of these cohorts.[16] Patients with sepsis who developed acute kidney injury had increases in both intact and C-terminal FGF23, which correlated to increases in EPO.[17] In another study of very ill patients in an intensive care unit, the number of units of red blood cells transfused and C-terminal FGF23 levels were positively correlated; however, this study did not report phosphate levels, which could have been impacted by blood transfusions or sepsis.[15]

Two studies in patients with normal renal function examined the effect of EPO administration on intact and C-terminal FGF23 levels. In healthy control subjects, EPO administration (60,000 U) increased C-terminal but not intact FGF23 levels at 24 hours. Phosphate levels were not reported.[20] When EPO (20,000 to 40,000 U) was administered to 4 patients with chronic anemia, both intact and C-terminal FGF23 levels increased over 24 hours without a change in blood phosphate.[19]

To better understand the effects of chronic activation of the HIF/EPO pathway on FGF23 in humans without renal failure or anemia, we compared C-terminal and intact FGF23 levels and blood phosphate in normal control subjects to patients with EPO-producing tumors caused by gain-of-function mutations in HIF2A that resulted in activation of the HIF/EPO pathway, elevated EPO, and secondary polycythemia.[23] These mutations were somatic and present in a mosaic distribution throughout the body. This represents an ideal group to assess the primary effects of chronic HIF/EPO pathway activation on FGF23/phosphate homeostasis not confounded by altered renal function.

Materials and Methods

Study oversight

This study was approved by the institutional review board of the National Institute of Dental and Craniofacial Research and the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Patients or their parents provided written informed consent or assent.

Patients

Nine patients with paragangliomas and somatostatinomas due to somatic gain-of-function mutations in *HIF2A* were studied at the National Institutes of Health (NIH) Clinical Center. Patients ranged in age from 9 to 58 years. All had elevated metanephrine levels at presentation that normalized with tumor removal. For 8 of the 9 patients who had contemporaneous EPO, iron, phosphate, hematocrit, creatinine, and 1,25-dihyroxyvitamin D values, there was plasma available for FGF23 determinations (Table 2). {TBL 2} The interval between when the patients had resection of tumors and/or therapeutic phlebotomy varied. FGF23 was measured on stored plasma specimens that had been immediately processed at the time of collection and stored for up to 6 years at −80°C. All other laboratory values were otherwise determined at the time of collection. In addition, there were 51 stored samples with contemporaneous phosphate and EPO determinations available for analysis of the relationship between phosphate and FGF23.

Normal controls

Normal control blood samples were obtained from healthy controls on an IRB-approved NIH protocol (99-CC-0168, Clinical Trials number NCT00001846). All donors provided written informed consent and samples were de-identified before distribution. Controls were selected prospectively. The first 8 healthy control patients who presented to the Blood Bank and who were matched by age, within 10 years, were selected. For the 9-year-old patient, blood from the youngest control available (22 years) was used. All other control subjects were able to be age-matched within 6 years. For controls, EPO, phosphate, hematocrit, iron, and creatinine were measured at the NIH Clinical Center laboratory. Plasma was obtained and stored at −80°C for later determination of FGF23 by ELISA (Table 2).

FGF23 assays

Intact and C-terminal FGF23 levels were measured from plasma using human FGF23 intact and C-terminal ELISA kits (Immutopics, Quidel, San Diego, CA, USA). Each sample was assayed in duplicate.

Statistics

Data were analyzed with GraphPad Prism Version 7.0c (La Jolla, CA, USA). Given small groups, which for some parameters did not have a normal distribution, nonparametric testing was performed using the Wilcoxon–Mann–Whitney test.

Results

In the 8 patients with stored plasma samples available for FGF23 determination, the contemporaneous EPO levels were significantly elevated compared with controls, 112.8 ± 80.5 versus 10.9 ± 9.2 mIU/mL, $p = 0.0006$ (mean ± SD) (Fig. 1A). {FIG1} Both blood phosphate and intact FGF23 levels were not different: 3.8 ± 0.6 versus 3.5 ± 0.3 mg/mL, $p = 0.320$, and 66.3 ± 28.8 versus 57.0 ± 18.1 pg/mL, $p = 0.595$, between patients and controls, respectively (Fig. 1B, C). C-terminal FGF23 levels were significantly elevated in patients compared with controls, 832 ± 694 versus 85.5 ± 45.8 RU/mL, $p = 0.003$ (Fig. 1D). Of note, C-terminal FGF23 levels were very tightly correlated with EPO levels (R = 0.969, p

< 0.0001, Fig. 1E). There was no correlation between C-terminal FGF23 levels and blood phosphate (not shown).

One control patient (C-7) had a slightly elevated EPO (33.1, normal 2.6 to 31.5 mIU/mL) that was associated with a slightly elevated C-terminal FGF23 (182, normal <180 RU/mL). The cause of the elevated EPO level is not known, but we postulate it may be due to a history of repeated blood donations, even though at the time of blood collection that patient's hematocrit was normal. Linear regression analysis of the relationship between EPO and C-terminal FGF23 in the control group, as in the patients, revealed a significant positive correlation, albeit less so than in the patients (R = 0.862, $p = 0.006$ versus R = 0.969, $p < 0.0001$, data not shown).

In addition, there was a significantly higher hematocrit in the patients versus controls ($p = 0.002$). There was also a significantly lower iron in the patients than the controls ($p = 0.0007$). This would be expected given the elevated EPO in the patient population, which is known to cause a secondary polycythemia and could contribute to lower iron levels. There was no significant difference in age or creatinine between groups ($p = 0.558$ and $p = 0.941$, respectively).

From the 8 patients, there were 51 samples with contemporaneous EPO and phosphate measurements. EPO and hematocrit levels varied in relationship to either tumor excision and/or therapeutic phlebotomy, which is performed for the polycythemia that results from elevated blood EPO levels. There was no relationship between blood EPO and phosphate levels (Supplemental Fig. S1).

Discussion

In this cohort of patients with gain-of-function mutations in HIF2A that caused increased EPO secretion, EPO levels were tightly correlated with marked elevations in C-terminal FGF23, consistent with EPO regulating FGF23 transcription and translation. Intact FGF23 and blood phosphate were normal, consistent with increased posttranslational processing of FGF23 to maintain euphosphatemia. Whether posttranslational processing is also HIF2A-regulated or due to a finely tuned, phosphate-sensing (or other) mechanism remains to be demonstrated. Importantly, normal phosphate levels in the setting of normal intact FGF23 and markedly elevated C-terminal FGF23 levels suggest that excess C-terminal FGF23 does not interfere with intact FGF23 action.

There is an accumulating body of evidence from mouse models and clinical studies that iron, EPO, and activation of the HIF pathway play a role in the FGF23 regulation (Table 1 and Supplemental Table S1). Almost all of the relevant studies support that regulation takes place at the transcriptional and translational level with increases in both FGF23 mRNA and protein. Since intact but not C-terminal FGF23 appears to regulate phosphate and vitamin D levels, posttranslational control of FGF23 processing to biologically inactive C-terminal FGF23 is especially important in conditions like anemia, in which transcription and translation are increased. This highlights the importance of posttranslational regulation of FGF23. Most human studies and many of the mouse studies to date have been confounded

by the presence of renal insufficiency, which has profound and complicated effects on FGF23 physiology, and/or intercurrent disease such as sepsis, which also likely affects FGF23 regulation. For these reasons, studying patients with primary activation of the HIF pathway by somatic gain-of-function mutations in HIF2A provides a unique, powerful, and less confounded model to study FGF23 physiology.

It is important to note that in 6 of the 8 human studies cited in Table 1, the pattern of elevated C-terminal FGF23 but normal intact FGF23, as is found in this study, was also observed. But in the rodent models reviewed in Supplemental Table S1, there were elevations in both intact and C-terminal FGF23 in 11 of the 17 studies, with only 6 of the 17 studies showing the pattern found in this study and the majority of published human studies. The differences in findings between the human and mouse literature may be due to differences in the effects of the physiological driver (in this case HIF) on FGF23 regulation and/or species differences. In diseases of primary FGF23 elevation, such as tumor-induced osteomalacia (TIO) and X-linked hypophosphatemic rickets (XLH), posttranslational regulation is absent or deficient and patients are hypophosphatemic due to increased levels of intact FGF23. Differences between HIF-mediated FGF23 excess and primary excess such as in TIO and XLH may be due to loss of phosphate sensing. In TIO, it has been shown that both HIF1[24] and FGFR1 activation may be involved in FGF23 production,[25] yet virtually all of the FGF23 made by the tumors is intact.[26] If both HIF and FGFR1 activation are in play in TIO, but only intact FGF23 is made by these tumors, it suggests that pathologic FGFR1 signaling may be downstream and supersede the effect of HIF, phosphate, or other factors in affecting FGF23 posttranslational regulation.

It has been shown that EPO is made by the paragangliomas/somatostatinomas;[23] however, the source of the FGF23 is not clear. It is accepted that FGF23 is produced in bone cells, specifically osteoblasts/osteocytes, but new evidence points to production of FGF23 by erythroid progenitor cells of the bone marrow.[16,17,20] Furthermore, EPO injection was found to induce FGF23 transcription in erythroid precursors but not bone cells.[20] The production of FGF23 in the bone marrow was fully suppressed by blockade of the EPO receptor; however, EPO receptor blockade only partially suppressed the increase in circulating FGF23,[17] suggesting that both bone cells and bone marrow cells may contribute to FGF23 levels and that these processes are differentially and perhaps independently regulated. The correlation between EPO and C-terminal FGF23 shown here (Fig. 1) supports a role of EPO-mediated FGF23 production by erythroid precursor cells but does not exclude a contribution by bone cells.

It has been suggested that C-terminal FGF23 fragments may compete and interfere with intact FGF23 action, even causing hyperphosphatemia in one model.[27] However, in spite of markedly elevated levels of C-terminal FGF23, blood phosphate and intact FGF23 levels were normal in the study patients, suggesting C-terminal FGF23 does not have a significant effect in modulating intact FGF23 action in humans.

The limitations of this study include the small sample size and that the analyses were retrospective and confined to stored samples. Additionally, in this model, it is not possible to differentiate the effect of EPO alone versus that of increased HIF2A activity or low iron

levels, which could have direct effects on FGF23 independent of EPO. Arguing against increased HIF2A activity, however, is that two of the patients with *EPAS1* mutations (encoding HIF2A) who had normal EPO levels also had normal C-terminal and intact FGF23 levels, consistent with the hypothesis that the observed findings are EPO mediated. The variable EPO levels in patients is attributed to varying degrees of tumor burden, either due to previous tumor resection and/or the nature of disease burden in patients with somatic mosaic conditions.

These data support the hypothesis that EPO upregulates FGF23 production; however, the physiologic reasons for this relationship are unclear and alternative hypotheses are also plausible. For example, it is possible that catecholamines and somatostatin regulate both C-terminal FGF23 and erythropoietin independently. If this were the case, a significant correlation between catecholamines and/or somatostatin and C-terminal FGF23 and/or EPO would be expected, but this was not the case. There were no significant correlations between C-terminal FGF23 and metanephrines, catecholamines, somatostatin, or hematocrit, nor were there correlations between erythropoietin and metanephrines, catecholamines, somatostatin, or hematocrit (Supplemental Figs. S2 and S3 and Supplemental Table S2). If iron deficiency, due to exuberant erythropoiesis, stimulates C-terminal FGF23 independently of HIF2a and erythropoietin, it would be expected there would be a correlation between C-terminal FGF23 and hematocrit, which was not the case. The possibility that HIF2A directly stimulates C-terminal FGF23 independent of EPO and that the highly correlated EPO and C-terminal FGF23 levels observed are coincidental cannot be excluded, but under normal physiological conditions, there is an obligate increase in EPO in response to HIF2A activation, making it impossible to tease this out. Finally, it is possible that HIF2A stimulates cleavage of intact FGF23, resulting in upregulation of C-terminal FGF23, but this is also unlikely and ignores the elevations in EPO, the correlation of C-terminal FGF23 with EPO, and the preclinical models supporting the direct role of EPO.

It is possible that EPO could have an effect on other components of the posttranslational regulatory pathway and FGF23 is a bystander. For instance, the kinase FAM20C has been shown to phosphorylate FGF23 at Ser 180 adjacent to the 176–179 HTHR glycosylation/furin degradation domain. It has been shown in vitro that when Ser 180 is phosphorylated, glycosylation by GALNT3 is inhibited and furin degradation ensues.[7] If EPO upregulated FAM20C, cleavage of FGF23 might be increased, creating the need for upregulation of FGF23 to maintain enough intact protein to preserve serum phosphate in the normal range. Thus, future studies are needed to better understand the physiological relationship between EPO and FGF23.

In conclusion, in patients with normal renal function and gain-of-function mutations in HIF2A that led to elevations in EPO, there was a significant elevation in C-terminal FGF23 that was highly correlated with elevated EPO levels but normal intact FGF23 and phosphate levels. These data support the notion that HIF/EPO pathway activation leads to coordinated control of FGF23 transcription and translation and that high levels of C-terminal FGF23 do not interfere with intact FGF23 function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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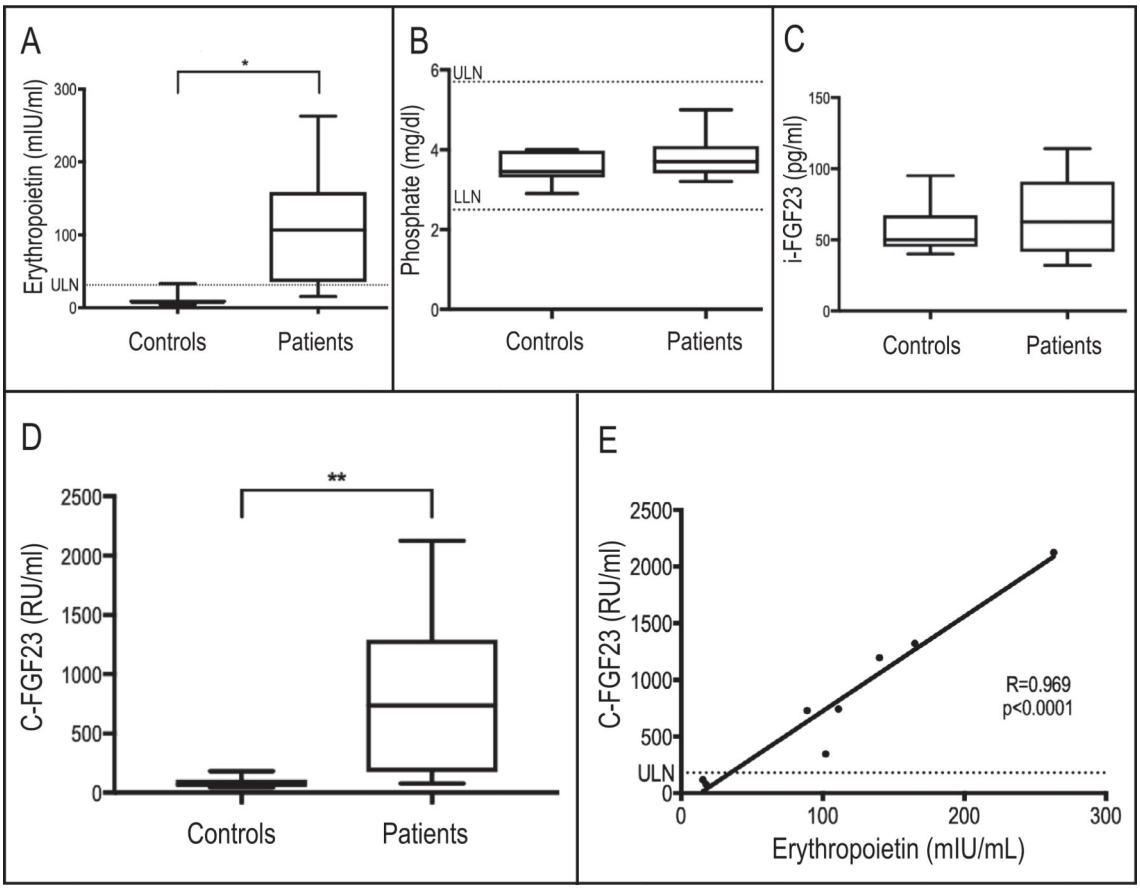

Fig 1.

Erythropoietin, phosphate, and FGF23 levels in patients with gain-of-function mutations in *HIF2A* and controls. (*A*) Erythropoietin levels from 8 patients with HIF2A mutations were significantly higher than controls (mean ± SD: patients 112.8 ± 80.48; controls 10.9 ± 9.16 mIU/mL, *Wilcoxon–Mann–Whitney test, $p = 0.0006$). Normal range for erythropoietin levels: 2.6 to 31.5 mIU/mL. (*B*) The patient blood phosphate levels were not different from controls (mean ± SD: 3.8 ± 0.6 versus 3.5 ± 0.34 mg/mL, respectively, $p = 0.320$). Normal range for phosphate levels: 2.5 to 5.7 mg/dL. Of note, the normal phosphate range for children is age-dependent and higher than adults. To account for the 9-year-old in the group, the upper limit was set at 5.8 mg/dL. (*C*) Intact FGF23 levels were not different between the patients and controls (mean ± SD: 66.3 ± 28.8 versus 57.0 ± 18.1 pg/mL, $p = 0.595$). (*D*) C-terminal FGF23 was significantly elevated in the patients compared with controls (mean ± SD: 832 ± 694 versus 85.5 ± 45.8 RU/mL, **$p = 0.003$). Normal range for C-terminal FGF23: <180 RU/mL. (*E*) There was a significant linear correlation between EPO and C-terminal FGF23 levels (R = 0.969, $p < 0.0001$). ULN = upper limit of normal; LLN = lower limit of normal.

Table 1.

Summary of Studies Investigating the FGF23/EPO Pathway in Flumans

Model	*n*	EPO	CFGF23	iFGF23	CFGF23 (C) relative to iFGF23 (i)	Phos	Ref.	Comment
Anemia	4	rhEPO 20,000–40,000 IU	↑ 12, 18, 24 hours	↑ 12,18 hours	C ~ i	↔	(19)	
Critically ill ICU	131		↑ (correlates with RBCs transfused)	NR	NR	NR	(15)	EPO not assessed Transfusions could ↑ phos
Healthy control	32	rhEPO 60,000 IU vs. placebo	↑	↔	C > i	NR	(20)	One time point, 24 hours Placebo/crossover design
ICU pts ± AKI 48 hr after septic shock	45	23 develop AKI → ↑ EPO	↑ (correlates with baseline EPO)	↑ (correlates with baseline EPO)	C ~ i	NR	(17)	All analyses on baseline numbers Acute → 48-hour time point
Non-dialysis CKD	42	No exogenous EPO given	↑ (associated with EPO)	↔ (not associated with EPO)	C > i	↔	(16)	
Dialysis	79	Mean 214 U/kg weekly	↑ (associated with rhEPO dose)	↔ (not associated with rhEPO dose)	C > i	↑	(16)	
Adult renal transplant	680	Grouped by tertiles of EPO	↑ (associated with EPO)	↔ (not associated with EPO)	C > i	↔	(16)	
Anemic CKD and CHF	56	EPO 50 IU/kg/wk; in two groups (Hb rise and Hb stable) vs. control	↑	↔	C > i	↓	(28)	↔ in iron

cFGF23 = C-terminal FGF23; iFGF23 = intact FGF23; phos = phosphate; ref = reference; ↔ = no change; NR = not reported; pts = patients; ICU = intensive care unit; pRBC = packed red blood cells; AKI = acute kidney injury; CKD = chronic kidney disease; CHF = congestive heart failure; Fib = hemoglobin.

This summary of eight studies of FGF23 and EPO in humans shows that C-terminal FGF23 is increased consistently in all eight studies. In six of the eight studies, intact FGF23 is unchanged or does not correlate with EPO levels; in two of the eight, intact and C-terminal FGF23 are increased. Inconsistencies also exist in effects on phosphate, which is not reported in five of the studies, unchanged in two, and decreased in one.

Table 2.

Selected Laboratory Values in Patients With Gain-of-Function Mutations in *HIF2A* and Control Subjects

Subject number	Age	Race	EPO (2.6–31.5 mIU/mL)	PHOS (2.5–5.7 mg/dL)	HCT (32.4–51.0%)	Iron (37.0–158.0 Mcg/dL)	Cre (0.30–1.17 Mg/dL)	CFGF23 (<180 RU/mL)	iFGF23 (pg/mL)[a]	1,25 OH_2-VitD (18–78 pg/mL)
1	44	White	165				0.68	1321	74	
2	9	White	111	5	45.9	39	0.38	743	95	
3	32	White	15.2	4.1	51.3	52	1.23	117	78	
4	32	White	263	3.4	46.4	7	0.67	2123.5	46	46
5	23	White	102	3.7	54.8		0.78	346	114	39
6	28	White	88.9	4.0	51.1	28	1.13	728	51	
7	39	White	17.4	3.5	43.8	38	0.76	79	40	61
8	58	White	140	3.2	38.5	17	0.72	1196	32	47
C-1	41	White	8.9	3.3	41.4	55	0.77	87	49	
C-2	22	White	5.2	4.0	40	73	0.88	71	95	
C-3	32	White	4.4	2.9	37.1	97	0.68	44	40	
C-4	32	White	8.8	3.5	37.9	67	0.98	77	62	
C-5	34	Asian	8.4	3.9	37.0	176	0.72	118	51	
C-6	29	White	9.3	4.0	35.7	113	0.61	49	45	
C-7	45	Flispanic	33.1	3.3	40.1	57	0.62	182	69	
C-8	60	Black	9.4	3.4	37.6	123	0.82	54	45	

Phos = phosphate; HCT = hematocrit; Cre = creatinine; cFGF23 = C-terminal FGF = 23; iFGF23 = intact FGF23.

Patients with gain-of-function mutations in HIF2A are numbered 1–8 and control subjects C-1-C-8. Patient 6 was male and all other subjects were female.

[a]The normal range for intact FGF23 is not well defined.