Background: Fibroblast growth factor-23 (FGF-23) and Klotho constitute the main regulatory system of phosphorus homeostasis. Beyond this physiological role, there is growing evidence suggesting that this system has relevant pathophysiological implications in different clinical processes.

Content: In this review we discuss the pathophysiological implications of the FGF-23/Klotho system and the potential utility that measurements of its components may have as clinical biomarkers in different clinical settings, such as progression of chronic kidney disease, acute renal failure, and secondary hyperparathyroidism, as well as vascular dysfunction, atherosclerosis, and cardiovascular morbidity and mortality. We outline and discuss the current commercially available assays for determination of FGF-23 and Klotho and the assay limitations that must be overcome to translate these biomarkers into reliable indicators in clinical practice.

Summary: In addition to its physiological role, the FGF-23/Klotho system appears to provide important information regarding the pathophysiology of several clinical conditions. Although there has been increasing study of the components of this new biological system and their potential use as clinical biomarkers, the ultimate value of this system in clinical practice will not be known until remaining assay limitations can be overcome and adequately designed studies have been conducted to demonstrate its clinical utility.

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In this manuscript we review the most relevant pathophysiological implications of the FGF-23/Klotho system and the characteristics and limitations of the assays for FGF-23 and Klotho measurements from the perspective of the potential use of these molecules as clinical biomarkers.

Pathophysiological Implications of FGF-23 and Klotho: Potential Utility as Biomarkers

Alterations of phosphorus homeostasis are known to be involved in multiple pathophysiological mechanisms and clinical processes. Variations not only in phosphate concentrations but also in phosphate-regulatory factors have been linked with morbidity and mortality. The recent discovery of the FGF-23/Klotho regulatory system has opened the possibility that measurements of its subcomponents may have potential clinical utility as biomarkers.

CHRONIC KIDNEY DISEASE

Current markers of chronic kidney disease (CKD) (mainly increases in serum creatinine and albuminuria) have limitations as clinical predictors owing to their lack of sensitivity and specificity and the relatively late appearance of alterations of these markers in the course of the disease. Therefore, new reliable and early markers of renal disease progression are needed.

The serum concentrations of FGF-23 progressively increase as renal function declines. These increases may represent a compensatory response to maintain normal phosphatemia or reflect an end-organ resistance to the phosphaturic stimulus due to a renal Klotho deficiency. Importantly, the increase of FGF-23 begins very early in the course of kidney dysfunction, even before serum phosphate concentrations reach values that are outside the reference intervals. This early increase has suggested that the measurement of FGF-23 is a more accurate biomarker of body phosphate accumulation and mineral disorders in CKD. In addition, an increasing number of clinical studies have indicated the utility of FGF-23 as a predictor of renal disease progression.

Table 1. Clinical trials linking FGF-23 and Klotho with clinical variables and their potential use as clinical biomarkers.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Clinical use</th>
<th>Population</th>
<th>Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased FGF-23 concentrations</td>
<td>Prognosis</td>
<td>Elderly men</td>
<td>Decreased renal function</td>
<td>Marsell et al. (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Children</td>
<td>Decreased renal function</td>
<td>Bacchetta et al. (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CKD patients</td>
<td>Decreased renal function, initiation of dialysis, left ventricular hypertrophy, coronary artery disease, cardiovascular events, mortality</td>
<td>Fliser et al. (9), Kendrick et al. (10), Akimoto et al. (13), Faul et al. (24), Kanbay et al. (25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Community</td>
<td>Incident CKD, vascular dysfunction, atherosclerosis, mortality</td>
<td>Semba et al. (11), Mirza et al. (27), Arnlov et al. (28), Semba et al. (29)</td>
</tr>
<tr>
<td></td>
<td>Predicting response to therapy</td>
<td>Dialysis patients</td>
<td>Resistance to vitamin D therapy</td>
<td>Kazama et al. (22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Refractory secondary hyperparathyroidism</td>
<td>Nakanishi et al. (23)</td>
</tr>
<tr>
<td>Decreased sKlotho concentrations</td>
<td>Diagnostic</td>
<td>Patients with AKI</td>
<td>AKI</td>
<td>Kazama et al. (22)</td>
</tr>
</tbody>
</table>

Finally, an interesting question is whether circulating FGF-23 can identify individuals at risk of developing CKD in the general population. In a recent study that included a group of older, disabled community-dwelling women without CKD, increased serum FGF-23 was an independent risk factor for incident CKD after adjustment for other potential confounders, including age, race, basal renal function, phosphate, calcitriol, and PTH.

Further studies are needed to
evaluate the role of FGF-23 as a useful and reliable predictor of CKD in the general population.

FGF-23 requires Klotho to exert its actions. Renal Klotho gene expression is markedly decreased very early in CKD (12). However, the protein concentrations of the membrane form and the soluble Klotho (sKlotho) concentrations have been measured in only a few studies, which show conflicting results. Plasma sKlotho concentrations in end-stage renal disease (13) showed a mild-to-moderate decrease, whereas concentrations in early-stage CKD have been reported to be below, within, or even above the reference interval (14–15). In 2 studies performed with urine, sKlotho concentrations have shown significant and sustained reductions with the progression of CKD (12, 16), for which the amount of urinary sKlotho was inversely related to the estimated glomerular filtration rate (eGFR) (16). Similarly, a significant inverse relationship has been observed between eGFR and plasma sKlotho in CKD (14). Importantly, the decline of sKlotho starts earlier than the alteration in the serum concentrations of other key molecules in CKD, including vitamin D, FGF-23, PTH, and phosphate. Moreover, although concentrations are variable, serum sKlotho has been found to be inversely related to serum creatinine in healthy individuals (17), which could be useful for identifying individuals at risk of developing CKD.

ACUTE KIDNEY INJURY
Traditionally, diagnosis of acute kidney injury (AKI) is based on an increase of serum creatinine or a decrease in urine output. However, both of these manifestations appear late after the insult, when kidney injury has already been established and has progressed. Several novel biomarkers have been suggested to improve the early diagnosis of AKI, including interleukin-18, neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule-1. NGAL and interleukin-18 are more sensitive biomarkers of early kidney injury, but less specific than kidney injury molecule-1.

Animal models of AKI show reduced concentrations of Klotho in kidneys, urine, and blood, which return to reference intervals upon recovery (18). Importantly, the decrease of sKlotho in plasma and urine is detectable as early as 3 h after injury, long before serum creatinine and NGAL increase. No studies have been performed to investigate renal Klotho expression in AKI in humans, but reduced expression of this protein as seen in CKD might be a common phenomenon in patients with kidney damage. On the contrary, clinical studies have shown that urine sKlotho concentrations are lower in patients with AKI than in healthy individuals (18). Thus, sKlotho might be a novel biomarker for AKI.

SECONDARY HYPERPARATHYROIDISM
The progressive increase in circulating FGF-23 may have adverse end-organ effects, including a net stimulatory effect on parathyroid glands leading to secondary hyperparathyroidism (SHPT). In the evolution of CKD, FGF-23 concentrations increase with reduced renal function long before calcitriol concentrations decline or PTH rises (19). The decrease in serum calcitriol is critical in the development of SHPT, and thus the inhibitory effect on renal calcitriol synthesis by increased FGF-23 may be a key factor in the pathogenesis of SHPT.

The interaction between FGF-23 and PTH is complex, especially in the context of CKD. Although FGF-23 decreases PTH mRNA and secretion, PTH is increased in CKD despite increased serum FGF-23 concentrations. This is explained by the very low expression of the FGF receptor 1 and the coreceptor Klotho in uremic parathyroid glands, which results in resistance to the inhibitory stimuli of FGF-23 (20).

In human studies, serum FGF-23 concentrations correlated with PTH in early and predialysis CKD patients (21), as well as in individuals with healthy kidneys or those with mildly impaired renal function (7). Importantly, serum FGF-23 is a predictor of resistance to active vitamin D therapy and progression to refractory hyperparathyroidism in CKD (22, 23) (Table 1). These findings suggest that increased FGF-23 concentrations could reflect long-term effects of hyperphosphatemia on parathyroid glands and the development of severe parathyroid hyperplasia in these patients. Collectively, these studies point to FGF-23 as a potential biomarker of parathyroid hyperplasia and resistance to vitamin D therapy for SHPT.

CARDIOVASCULAR DISEASE AND MORTALITY
Diverse studies have shown that FGF-23 is an independent predictor of survival and cardiovascular morbidity. In CKD patients, FGF-23 has been related to left ventricular hypertrophy (24), coronary artery stenosis (25), and mortality (13), whereas in community studies, FGF-23 has been associated with vascular dysfunction (26, 27), total body atherosclerosis (28), and mortality (29) (Table 1).

In addition to the potential utility of FGF-23 as a biomarker of cardiovascular dysfunction, a pathogenic role for this molecule has been suggested. This would be explained by direct Klotho-independent effects of FGF-23 mediated by low-affinity bindings under conditions of increased FGF-23 concentrations, such as advanced CKD. This has been recently confirmed by experimental studies demonstrating that intravenous or intramyocardial FGF-23 administration resulted in the induction of hypertrophy of rat cardiomyocytes and in the development of left ventricular hypertrophy.
in the animal model (24). These findings strongly suggest that a component of cardiovascular risk could be directly attributable to FGF-23.

With respect to vascular damage, only a few studies have investigated the effects of FGF-23 on vessel integrity. The recent demonstration of the expression of the FGF receptors and Klotho in the human vascular wall reinforce the possibility that they may influence vessel integrity (4, 5). Interestingly, recent studies (5, 30) point to the absence of Klotho protein and gene expression in mouse vascular tissue. These data indicate species-specific differences in the FGF-23/Klotho system, and suggest that FGF-23 does not signal through a Klotho-dependent pathway in mouse vessels, which could explain why overexpression of FGF-23 in mice is not associated with adverse cardiovascular effects (31). Additional research is needed to confirm the existence of this new model of action in the vascular tissue and to examine its substantial clinical implications. Strategies to decrease high FGF-23 concentrations in humans could emerge as a potential novel therapeutic approach.

Experimental studies have shown Klotho to have diverse independent beneficial effects on the vascular system, including stimulation of calcitriol and nitric oxide synthesis and suppression of Wnt signaling, oxidative stress, and vascular calcifications (2). In contrast to studies of FGF-23, studies that explore the relationships between human sKlotho concentrations and clinical cardiovascular phenotypes are scarce, owing, until very recently, to the lack of a reliable assay for measuring this protein (17). Among those investigations, a large longitudinal population-based study concluded that in older community-dwelling adults, plasma sKlotho is an independent predictor of all-cause mortality (29). In addition, genetic studies have demonstrated that Klotho gene (KL) polymorphisms are associated with the incidence of coronary artery disease (32). The recently described expression of Klotho in human vascular smooth muscle cells may partially contribute to explain these effects over the cardiovascular system (4, 5).

**Clinical Use of Commercial Assays for Determination of FGF-23 and sKlotho**

**DETERMINATION OF FGF-23**

FGF-23 is a bone-derived 32-kDa protein consisting of 251 amino acids, including a leader sequence of 24 amino acids which encodes for a signal peptide. Circulating FGF-23 can be found in both active and inactive forms. The inactive forms result from a proteolytic cleavage between Arg179 and Ser180 by protein convertases during secretion, generating N-terminal and C-terminal fragments.

Assay kits for measuring circulating FGF-23 detect intact FGF-23 (iFGF-23) alone or both iFGF-23 and C-terminal FGF-23 (cFGF-23) fragments. Most assays are based on a double antibody sandwich ELISA with colorimetric reading. Antibodies of iFGF-23 tests detect epitopes within the amino-terminal and the carboxyl-terminal portions. In the cFGF-23 assay, antibodies detect 2 different epitopes in the carboxyl terminal portion. Therefore, the cFGF-23 assay measures both the intact molecule and the large carboxyl terminal fragment of human FGF-23. Fig. 1 shows the basis of the most widely referenced assays detecting iFGF-23 (Kainos® FGF-23 ELISA kit and Immutopics® Human Intact FGF-23 ELISA kit) and cFGF-23 [Immutopics Human FGF-23 (C-term) ELISA kit].

It is unclear if the cFGF-23 assay provides comparable sensitivity to that for iFGF-23 in patients with different stages of renal function. Some studies suggest that measurements of iFGF-23 rather than cFGF-23 may be more physiologically relevant, whereas other investigations show significant associations with only cFGF-23. It is possible that the use of only one assay may result in a significant underestimation of important biological signals. However, a recent clinical study has demonstrated that virtually all detectable FGF-23 is in the active form, and thus, measurements obtained with iFGF-23 and cFGF-23 assays would reflect the
same circulating moiety (33). Additional studies are needed to determine whether this applies regardless of kidney function.

The performance characteristics of the assays, as reported by the manufacturers, vary widely in precision (intraassay CV between 3% and 11%), measurement ranges, detection limits, and even sample types (cell culture media, serum, plasma, urine, tissue homogenates, and other biological fluids) (Table 2). Importantly, the assays are designed only for research use and do not appear ready for clinical use because they are not harmonized. So, in the absence of certified reference materials, the measurements obtained with the different assays have a marked lack of analytical agreement among results. Furthermore, the units of measure reported are different (pg/mL for iFGF-23 vs RU/mL for cFGF-23). Moreover, although it has been shown that iFGF-23 is highly susceptible to in vitro proteolytic degradation, the instructions provided with the kits lack recommendations regarding use of protease inhibitors to minimize intact hormone loss after collection.

Most of the assay protocols report interferences by hemolysis, lipemia, or both, but they do not describe their intensity, and there are few published studies on interfering substances. Similarly, only a few publications consider crucial preanalytical variables. Thus, it has been reported that plasma iFGF-23 concentrations undergo substantially greater diurnal variation (measured in the early morning up to a mean increase of 30%) than the concentrations determined using the cFGF-23 assay (34). By contrast, other authors have reported no significant diurnal variations in circulating iFGF-23 concentrations (35). Fasting and nonfasting mean concentrations do not differ significantly with either assay, and no association with sex or age has been observed (35).

The potential use of this protein as a biomarker has stimulated great interest in developing automated methods to improve the performance of the FGF-23 immunoassays by overcoming the limitations of the ELISA kits. A new automated chemiluminiscence immunoassay has been recently developed to measure intact and cFGF-23 concentrations (36). This assay is fully automated in a random access chemiluminiscent immunoanalyzer and shows very good performance: sensitivity of 1 pg/mL, a wide analytical measurement range (up to 15 000 pg/mL), and intrassay CV/\% 5% over a wide range of FGF-23 concentrations. The assay requires smaller amounts of sample, gives the first result within 20 min, and has a defined cutoff for chronic hypophosphatemia of 25 pg/mL, with very good analytical sensitivity and specificity. In addition, the effects of interfering substances are reported, with <5% variability in the FGF-23 estimated values (at 500 mg/dL hemoglobin, 50 mg/dL bilirubin, and 300 mg/dL ascorbic acid).

Current assays should be evaluated and standardized more rigorously and there is need for further studies to establish clearly the influence of preanalytical fac-

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**Table 2. Characteristics of ELISA assays for FGF-23 and soluble Klotho determinations.**

<table>
<thead>
<tr>
<th>Assay Provider</th>
<th>Sample Type</th>
<th>Units</th>
<th>Sample Range</th>
<th>Sensitivity</th>
<th>Intraassay CV, %</th>
<th>Interassay CV, %</th>
<th>Interferences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immutopics</td>
<td>C-term</td>
<td>RU/mL</td>
<td>15–1500</td>
<td>1.5</td>
<td>1.4–2.4</td>
<td>2.4–4.7</td>
<td>Lipemia</td>
</tr>
<tr>
<td>ALPCO</td>
<td>P, CCM</td>
<td>30–2300</td>
<td>3</td>
<td>5</td>
<td>5–7.3</td>
<td>Lipemia</td>
<td></td>
</tr>
<tr>
<td>Immutopics, ALPCO</td>
<td>Intact pg/mL</td>
<td>P, CCM</td>
<td>6–650</td>
<td>1</td>
<td>2.6–4.4</td>
<td>6.1–6.5</td>
<td>Lipemia</td>
</tr>
<tr>
<td>Kainos</td>
<td>S</td>
<td>3–800</td>
<td>3</td>
<td>2.0–3.0</td>
<td>2.1–3.8</td>
<td>Lipemia</td>
<td></td>
</tr>
<tr>
<td>Millipore</td>
<td>S, P, CCM, AE</td>
<td>9.9–2400</td>
<td>3.5</td>
<td>7.8–11.2</td>
<td>2.4–11.3</td>
<td>Hemolysis, lipemia</td>
<td></td>
</tr>
<tr>
<td>Uscn Life Science</td>
<td>S, P, BF</td>
<td>15.6–1000</td>
<td>5.4</td>
<td>&lt;10</td>
<td>&lt;12</td>
<td>Hemolysis</td>
<td></td>
</tr>
<tr>
<td>Mybiosource</td>
<td>S, U, CCM, TH</td>
<td>3.1–200</td>
<td>0.78</td>
<td>&lt;8</td>
<td>&lt;10</td>
<td>Hemolysis</td>
<td></td>
</tr>
<tr>
<td>Klotho</td>
<td>S, P, TH</td>
<td>0.156–10</td>
<td>0.39</td>
<td>&lt;8</td>
<td>&lt;10</td>
<td>Hemolysis</td>
<td></td>
</tr>
<tr>
<td>Cusabio</td>
<td>S, P, U</td>
<td>93.7–6000</td>
<td>6.15</td>
<td>2.7–3.5</td>
<td>2.9–11.4</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>EliAab</td>
<td>S, P, U, CCM, BF, TH</td>
<td>7.8–500</td>
<td>2.4</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>My Biosource</td>
<td>S, P, CCM, BF, TH</td>
<td>0.5–10</td>
<td>0.1</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Hemolysis, lipemia</td>
<td></td>
</tr>
<tr>
<td>My Biosource</td>
<td>S, P, U, TH, SAL</td>
<td>0.05–20</td>
<td>0.01</td>
<td>&lt;10</td>
<td>&lt;12</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Uscn Life Science</td>
<td>S, P, BF</td>
<td>0.156–10</td>
<td>0.058</td>
<td>&lt;10</td>
<td>&lt;12</td>
<td>Hemolysis</td>
<td></td>
</tr>
</tbody>
</table>

*P, plasma; S, serum; CCM, cell culture media; AE, adipocyte extracts; BF, biological fluids; U, urine; TH, tissue homogenates; NR, not reported; SAL, saliva.
Moreover, population-based studies focused on defining clear cutoff values for clinical risk assessment are scarce. Furthermore, some cutoff values are referred to specific assays and overlook variables which could alter the measures, so the utility is limited. Before introduction of these assays into clinical practice, more robust reference values are needed, including validated recommended cutoff values for treatment targets. Such studies should consider the biological variability of FGF-23 in assessing the usefulness of this biomarker. In this regard, a recent study reported that the plasma concentrations of cFGF-23 in healthy individuals showed lower intraindividual variation (8.3%) than iFGF-23 (18.3%), suggesting that the desirable assay imprecisions should be <4.15% and 9.15%, respectively. However, the interindividual variability was higher for cFGF-23, resulting in a lower index of individuality, suggesting that conventional population-based reference intervals may be not the most sensitive method for detecting abnormalities in serial monitoring results. Finally, the use of cFGF-23 gave a lower threshold for clinically significant change in consecutive results in an individual than iFGF-23 (25% vs 54%). Additional studies are needed to provide supporting evidence of the biological variation of various forms of FGF-23 and to improve the knowledge and clinical use of this hormone.

DETERMINATION OF sKLOTHO

The existence of KL transcripts for a putative form of secreted Klotho was discovered in 1998, but the presence of this protein in both human sera and cerebrospinal fluid was not confirmed until recently. KL comprises 5 exons and encodes a single-pass transmembrane protein of 1012 amino acids with a large amino-terminal extracellular domain, consisting of 2 internal repeat sequences (KL1 and KL2) and 2 short membrane-spanning (21 amino acids) and intracellular carboxyl (11 amino acids) domains (Fig. 2). sKlotho can be generated through 2 different pathways, by an alternative Klotho messenger RNA splicing putatively encoding only KL1 (5) and by a proteolytic cleavage by membrane-anchored A desintegrin and metalloproteinases-17 (ADAM-17) and ADAM-10, which release the full-length extracellular domain into the extracellular space (Fig. 2). Furthermore, the amino acid sequence between the repeat sequences KL1 and KL2 (Lys-Lys-Arg-Lys) forms a potential site for proteolytic cleavage. Although these fragments are not detected in human serum and cerebrospinal fluid, the existence of the alternatively spliced KL1 transcript in urine should not be excluded, and it would be interesting to determine whether these fragments are biologically active.

The full-length extracellular domain is detected by the Human soluble α-Klotho Assay Kit - IBL (Immuno-Biological Laboratories) (17), which is the ELISA assay more frequently used to measure sKlotho in human blood. This sensitive and specific assay has become available only recently and provides a reliable measurement of circulating Klotho, although other assays for sKlotho are available (Table 2).

There is a paucity of data validating the assays for this biomarker. Devaraj et al. (37) evaluated the human Klotho ELISA kit (CUSABIO BIOTECH) with some modifications, and demonstrated that it has intra- and interassay imprecision values of <8.3% and 13.3%, respectively. There were no significant effects on Klotho measurements with the addition of common interferents, such as ascorbate, triglycerides, or hemolysis; only bilirubin (250 mg/L) significantly reduced Klotho values. There was also a significant reduction of Klotho values in samples with glycohemoglobin concentrations >6.5%.

Unlike FGF-23, sKlotho is subject to diurnal variation decreasing to a circadian nadir at midnight and rising to a maximum in the early morning (35). Although the physiological significance of this pattern is unknown, it should be taken into account when collecting blood and urine samples. A negative relationship between Klotho concentrations and age has been reported in healthy volunteers (17), in patients with X-linked hypophosphatemia (35), and in children with CKD (38). There were no apparent differences regarding sex (35).

The reference interval for blood sKlotho has not been clearly established. The first human data on
plasma sKlotho have been recently reported (37, 38), with the differences observed among the studies most likely a result of the different assay antibodies directed to different epitopes. It would be expected that urinary Klotho, rather than the serum Klotho concentrations, should be linked to the magnitude of the functioning nephrons in CKD patients, but to date, few clinical studies have examined urinary Klotho. A recent investigation revealed that the Klotho/creatinine (Klotho/Cr) ratio in random urine samples does not serve as a reliable surrogate for the amount of Klotho excreted in urine over 24 h. Moreover, the finding that the random urine Klotho/Cr ratio trended toward lower values with advancing stages of CKD and was significantly associated with eGFR has spurred extensive evaluation regarding the clinical impact of measuring random urine Klotho/Cr ratios (39).

Much further research is needed to validate and standardize this biomarker in the translational arena, including studies to define reference values and measure its biological variability.

Conclusions

FGF-23 and Klotho are emerging as new biomarkers. Their potential utility seems to transcend the field of renal failure and include other areas such as cardiovascular disease. However, several important considerations must be overcome to establish FGF-23 and Klotho as reliable biomarkers in clinical practice.

References


