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ORIGINAL ARTICLE

Stability and degradation of fibroblast growth factor 23 (FGF23): the effect of time and temperature and assay type

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Abstract
Summary There is growing need for a reliable assay for measuring fibroblast growth factor 23 (FGF23), a regulator of phosphorus and vitamin D. In this work, we analyze and compare the performance of three available assays, including the effect of temperature and time. This knowledge will allow for better understanding of FGF23 in the future.
Introduction Intact and C-terminal FGF23 (iFGF23 and cFGF23) concentrations are important in the diagnosis of hypo- and hyperphosphatemic diseases. The effects of temperature, storage, and specimen handling on FGF23 levels are not well known. We investigated the effects of various factors on plasma and serum measurement of FGF23 using three different assays.
Methods Serum and plasma FGF23 were measured using three commercially available ELISA assays—two measuring iFGF23 and one measuring cFGF23. Samples from subjects with known FGF23 disorders were stored at 4, 22, and 37 °C and analyzed at different intervals up to 48 hours (h). A subset of samples underwent repeated freeze-thaw cycles, and samples frozen at −80 °C for up to 60 months were reanalyzed. The effect of adding a furin convertase inhibitor on FGF23 degradation was investigated using samples stored at 37 °C for 48 h. Intact FGF23 levels were measured from plasma samples of four different groups to test the correlation of the two assays.
Results Plasma FGF23 levels were stable when stored at 4 and 22 °C for 48 h. Both plasma and serum FGF23 levels demonstrated relative stability after five freeze-thaw cycles. Long-term storage at −80 °C for 40 months induced some variability in FGF23 levels. The addition of a furin inhibitor did not affect FGF23 degradation. Intact FGF23 levels showed good correlation only at the upper limit of the assay range when comparing the two assays.
Conclusions Sample type, handling, and choice of assay are factors that affect FGF23 levels and should be considered when measuring this hormone.

Keywords Assay · FGF23 · Immutopics · Kainos · Stability

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Introduction

Fibroblast growth factor 23 (FGF23) is a hormone produced by bone that was identified as a critical regulator of phosphate and vitamin D homeostasis in 2000 [1, 2]. Deranged levels of circulating FGF23 have been found in several diseases of phosphate metabolism, such as tumor-induced osteomalacia (TIO) [3], X-linked hypophosphatemic rickets (XLH) [3], autosomal dominant hypophosphatemic rickets (ADHR) [1], autosomal recessive hypophosphatemic rickets (ARHR) [4], fibrous dysplasia (FD) [5], hyperphosphatemic familial tumoral

calcinosis (FTC) [6], and the recently described cutaneous-skeletal hypophosphatemia syndrome (CSHS) [7]. FGF23 levels are also markedly elevated in patients with end-stage renal disease [8]. In addition to circulating as a full-length, biologically active hormone, intact FGF23 (iFGF23) may be cleaved into presumably inactive amino and carboxyl-terminal fragments by a subtilisin-type proprotein convertase, possibly furin [9–11]. Some disorders like FTC are characterized by primarily elevated cFGF23 [6], whereas monitoring post-operative response to tumor resection in TIO relies mainly on iFGF23 levels [12].

The two most frequently used, commercially available assays to measure FGF23 concentrations are the intact FGF23 (Kainos Laboratories International, Tokyo, Japan) and the C-terminal FGF23 (Immutopics, San Clemente, CA) ELISA kits. Recently, another assay for measurement of iFGF23 (Immutopics second generation, San Clemente, CA) using ELISA has been released. Kainos and Immutopics iFGF23 assays detect only the intact molecule while the Immutopics cFGF23 assay detects both intact and C-terminal fragments of FGF23.

Manufacturer recommendations for the sample type (serum versus plasma) used in the cFGF23 assay have changed over time, and the reliability of using serum in an assay designed for plasma has not been reported. Although Kainos recommends the use of serum, both serum and plasma have been widely used in published studies. Sample stability over time and at various temperatures has not been reported, nor has the effect of long-term storage and repeated cycles of freezing and thawing, on FGF23 levels.

The objectives of this study were to compare these three FGF23 assays as well as evaluate the effects of handling and storage conditions (i.e., temperature and time) on FGF23 levels, repeated freeze-thaw cycles and long-term storage, and serum versus plasma samples. Additionally, we explored a possible mechanism of FGF23 degradation ex vivo by examining the effect of adding the furin convertase inhibitor CMK to samples.

Patients and methods

Subjects and samples

Samples were obtained from patients with TIO (7), XLH (5), FD (6), normal volunteers (117), and end-stage renal disease on dialysis (10), between 2005 and 2013. The study was approved by the National Institute of Dental and Craniofacial Research Institutional Review Board, and all subjects provided written informed consent, in accordance with NIH regulations. All blood samples were obtained after an overnight fast. Blood samples were immediately processed after they were drawn. Samples were placed on wet ice and then centrifuged at 4 °C within 30 minutes (min) of collection to allow for removal of serum or plasma. The aliquoted samples were then frozen on dry ice and stored at −80 °C, at each time point as described below, until batched for analysis. Blood for plasma was collected in purple-top tubes containing EDTA and red-top tubes for serum. The tubes did not contain other preservatives or protease inhibitors. All the samples were measured in duplicate. In the samples from subjects with primary disorders of FGF23, the samples had FGF23 levels that were well within the higher limit of detection of all assays used; as such they were not diluted for the experiments described.

FGF23 serum and plasma assays

The results of the Kainos and Immutopics iFGF23 assays will be referred to as iFGF23 while the results of the Immutopics C-terminal FGF23 assay will be referred to as cFGF23, even though the latter assay detects both the intact molecule and the C-terminal fragment. FGF23 concentrations were determined using either Kainos (iFGF23) [13] or Immutopics (cFGF23 or iFGF23) [14] second-generation assay ELISA kits according to the manufacturer's protocol. The manufacturer-reported detection limits are 2 pg/ml for iFGF23 using Kainos, 1.5 pg/ml for iFGF23 using Immutopics, and 1.5 RU/ml for cFGF23 (Immutopics). The intra- and inter-assay coefficients of variation are reported as 2–3 and 2.1–3.8 % for Kainos, 2.6–4.4 and 6.1–6.5 % for Immutopics (intact), and 1.4–2.4 and 2.4–4.7 % for Immutopics (C-terminal). For the iFGF23 assay, Kainos recommends using serum and Immutopics recommends measurement in plasma. For the Immutopics cFGF23 assay, prior to 2005, either plasma or serum was used, but since 2005, the manufacturer has recommended measurement in plasma only.

Time and temperature effect on plasma and serum FGF23

Blood from 2 patients with TIO was drawn concurrently in both EDTA and serum separator tubes. An aliquot was reserved as whole blood, and the rest was separated into serum and plasma. Serum and plasma samples were further aliquoted and stored at 4, 22, or 37 °C for 0, 4, 8, 24, and 48 h and then stored at −80 °C until batched analysis for FGF23.

Freeze/thaw stability

Aliquots of serum and plasma were frozen in dry ice and thawed for five freeze-thaw cycles. A single aliquot from one patient with TIO was used for measuring iFGF23 using Kainos and cFGF23. An aliquot from a different patient with TIO was used to determine freeze-thaw stability of iFGF23 using Immutopics.

FGF23 stability in frozen samples

Plasma samples from 5 TIO, 5 XLH, and 6 FD subjects, in which FGF23 concentrations had been previously measured, were selected for reanalysis. These samples had been stored at −80 °C for up to 60 months. For Kainos iFGF23, samples from 5 subjects with TIO and 5 subjects with XLH were assayed. For cFGF23, samples from 5 subjects with TIO and 6 subjects with FD were assayed. Comparing stability up to 60 months using Immutopics iFGF23 was not possible, as this newer assay only became available 12 months prior to this experiment.

Effect of furin inhibition on FGF23 degradation ex vivo

As furin is reported to be one of the possible enzymes responsible for FGF23 processing [1], we explored the effect of furin inhibition by adding the furin convertase inhibitor CMK (final concentration 1 mM) to fresh serum and plasma samples. Samples were then held at 37 °C for 0, 4, 8, 24, and 48 h and levels of iFGF23 (Kainos) and cFGF23 were measured. CMK was also added to the standards to assess effects on the standard curve. Linear regression analysis was done to compare the slopes of each of the two groups of data (with and without the furin inhibitor).

Correlation of iFGF23 levels using Kainos and Immutopics assays

Samples from 117 healthy volunteers, 10 patients on dialysis, 6 patients with FD, and 7 patients with TIO were obtained, and iFGF23 was measured using both Kainos and Immutopics.

Statistical analysis

To analyze the measures in each assay and in keeping with manufacturer recommendation, linear regression analysis was

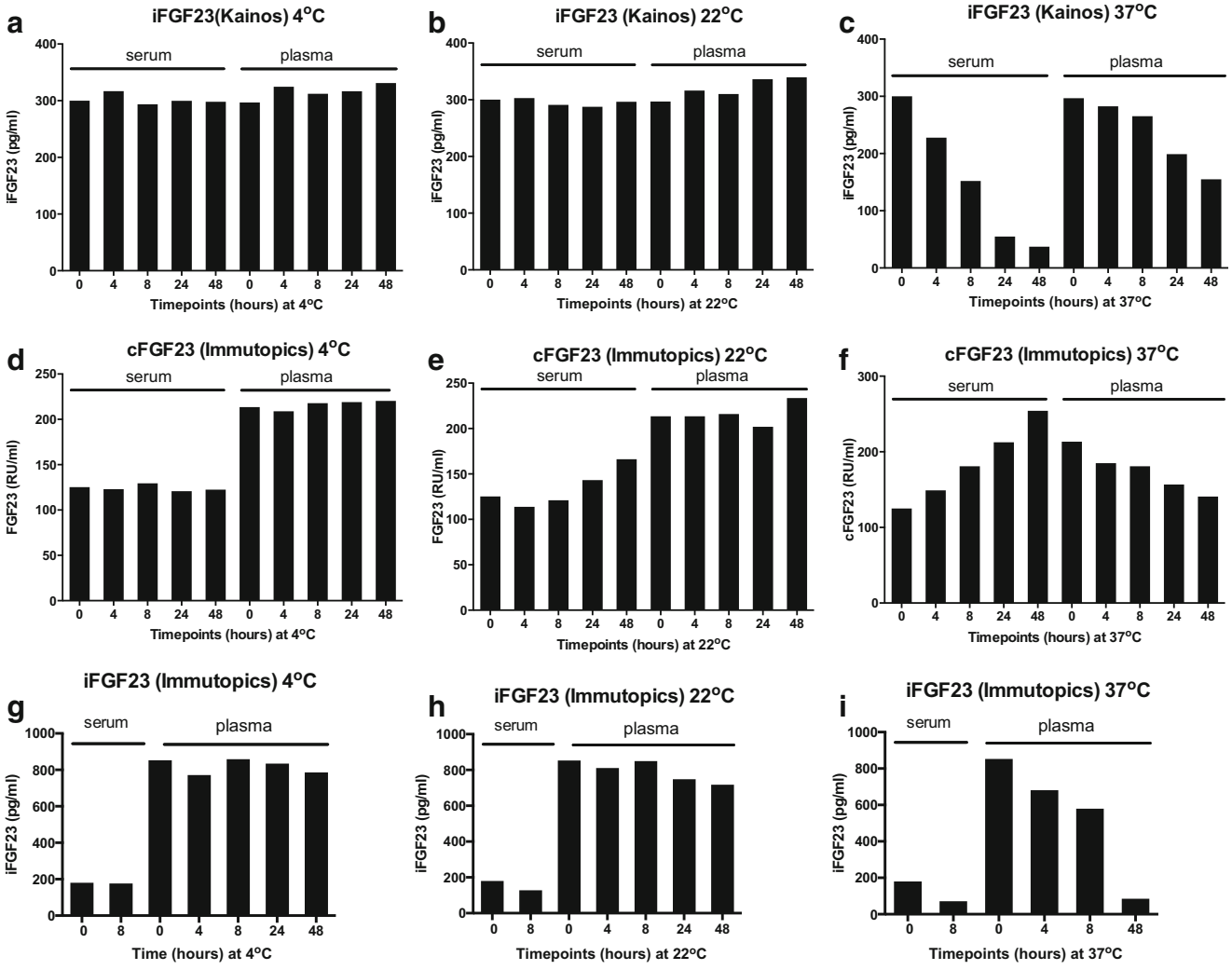

Fig. 1 Effect of temperature and time on FGF23 levels: iFGF23 (Kainos) and cFGF23 (Immutopics) were measured from a single patient in plasma and serum from samples frozen at 4, 22, and 37 °C and analyzed at different intervals (0, 4, 8, 24, and 48 h) (**a**–**f**). iFGF23 (Immutopics) using serum and plasma samples from a different patient were similarly handled and analyzed at different intervals (**g**–**i**)

used to generate the equation for the line and solve for unknown x using Microsoft Excel. To eliminate the hook effect, the highest standard was eliminated when appropriate. The detection threshold for each assay was calculated using a linear calibration curve as the limit of detection, since the standard curve is a linear curve; as such, LOD = 3Sa/b, where Sa is the standard deviation of the response and b is the slope of the calibration curve [15]. For correlation studies between Kainos and Immutopics in iFGF23 measurement, Passing-Bablok regression analysis was used to determine the correlation between the two assays, whereas Bland-Altman analysis was used to compare the two measurement techniques. All figures and calculations, including the half-life ($t_{1/2}$) for decay in serum, were generated using Prism 6, GraphPad Software, Inc.

Results

Intra- and inter-assay CV and detection threshold

Our intra-assay and inter-assay CV for intact FGF23 by Kainos were 1.98 and 3.5 %, respectively; for intact FGF23 using Immutopics 4.4 and 5.4 %, respectively; and for cFGF23 using Immutopics 2.3 and 1.5 %, respectively. Thus, all were within the manufacturer-provided ranges. The limit of detection (LOD) was 0.036 for iFGF23 by Immutopics, 0.040 for iFGF23 by Kainos, and 0.750 for cFGF23 (Immutopics).

Time and temperature effect on iFGF23

iFGF23 levels showed negligible changes when serum or plasma was stored at either 4 or 22 °C up to 48 h using Kainos (Fig. 1a, b) or Immutopics (plasma) (Fig. 1g, h). Serum samples stored at 4 or 22 °C were significantly lower than plasma levels when iFGF23 was measured by Immutopics, supporting the manufacturer's recommendation of using only plasma for this assay (Fig. 1g, h). When stored at 37 °C, iFGF23 levels were unstable using both assays, showing a steady decline in levels over 48 h (Fig. 1c, i). At 37 °C, values decreased in a time-dependent fashion with a decrease of 87 % for serum and 47 % for plasma at 48 h when measured using the Kainos assay (Fig. 1c) and a 90 % decline in plasma at 48 h when measured by Immutopics (Fig. 1i). At 37 °C, by one-phase decay analysis, the $t_{1/2}$ for decay in serum was 7.1 h ($R^2 = 0.99$) whereas in plasma it was 24.2 h ($R^2 = 0.99$) using Kainos and 13.8 h ($R^2 = 0.99$) using Immutopics assay (data now shown).

Time and temperature effect on cFGF23

cFGF23 values in serum specimens that had been stored at 4 °C were significantly lower than values in plasma; however, levels were stable for up to 48 h (Fig. 1d). cFGF23 levels from serum stored at 22 and 37 °C were also lower than values in plasma, but tended to increase with time (Fig. 1e, f). For plasma samples, cFGF23 levels were stable when stored at 4 and 22 °C for up to 48 h (Fig. 1d, e). When plasma samples were stored at 37 °C, there was a time-dependent decrease in values over 48 h of 34 % (Fig. 1f). By one-phase decay analysis, the $t_{1/2}$ for plasma samples at 37 °C was 8.8 h ($R^2 = 0.97$).

Freeze/thaw stability

There was no apparent effect of repeated freeze-thaw cycles on either iFGF23 (Kainos and Immutopics) or cFGF23 (Fig. 2). Mean ± SD plasma and serum intact FGF23 levels (Kainos) were 228.2 ± 9.9 and 223.3 ± 14.9 pg/ml,

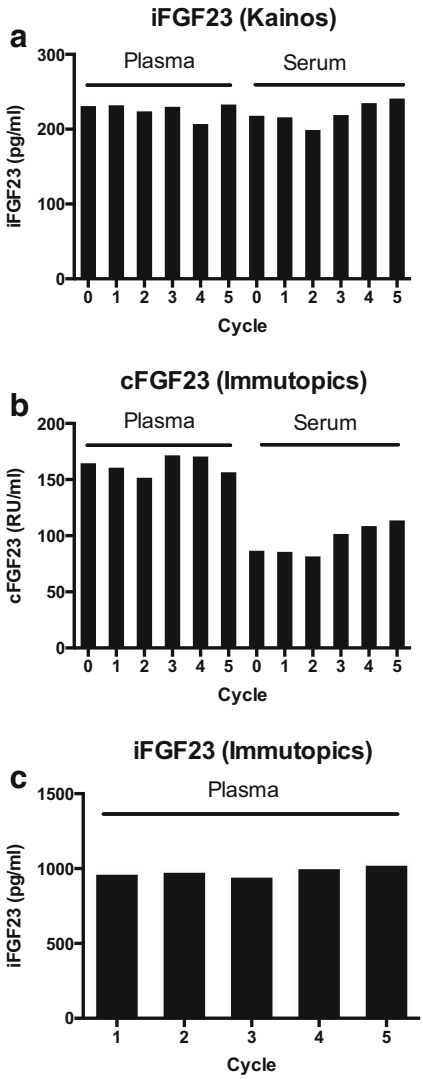

Fig. 2 Effect of freeze/thaw on FGF23 levels. A sample (of plasma and serum each) from one patient was aliquoted, frozen, and thawed for five cycles. iFGF by Kainos (**a**) and cFGF by Immutopics (**b**) were measured in plasma and serum. A plasma sample was aliquoted from a different patient and similarly handled for iFGF23 measurement by Immutopics (**c**)

respectively. Plasma and serum C-terminus levels were 164.0 ± 7.8 and 97.7 ± 13.4 RU/ml, respectively, again demonstrating lower measurements when serum is used. Mean plasma iFGF23 (Immutopics) was 988.2 ± 31.1 pg/ml.

Long-term storage effect on FGF23

The effect of long-term storage on FGF23 stability was assessed by comparing FGF23 values in plasma samples that had been measured before and after storage at −80 °C up to 40 months (iFGF23, Kainos) and up to 60 months (cFGF23) (Fig. 3). For both iFGF23 and cFGF23, levels decreased with time, with the exception of two samples, which showed a significant increase in levels (samples 5 and 6; Fig. 3b, d).

Effect of furin inhibition on FGF23 degradation ex vivo

To examine the effect of furin on FGF23 degradation, the furin convertase inhibitor CMK was added to serum and plasma samples. The addition of the CMK inhibitor to the standards did not change the standard curve. While iFGF23 (Kainos) and cFGF23 levels were lower in the CMK-treated samples at all time points, the addition of CMK had little effect on the observed changes in iFGF23 and cFGF23 when stored at 37 °C for up to 48 h (Fig. 4a–d). When comparing slopes, no difference was seen in the decline in levels with or without the furin inhibitor for plasma iFGF23 ($P = 0.06$), serum iFGF23 ($P = 0.48$), plasma cFGF23 ($P = 0.49$), or serum cFGF23 ($P = 0.12$). FGF23 levels declined as expected when stored at 37 °C, with or without the furin inhibitor, except for serum cFGF23.

Correlation of iFGF23 levels using Kainos and Immutopics assays

The mean ± SD of iFGF23 in 171 healthy volunteers was 41.5 (18.3) pg/ml using Kainos and 42.9 (20.8) pg/ml using Immutopics. Intact FGF23 levels showed variability at the lower limit of the normal range (healthy volunteers) ($R^2 = 0.38$) but high correlation at the upper limit of the assay range (R^2 range 0.95–0.98) as seen in patients with FD and TIO and in patients on dialysis (Fig. 5a, b). This was also confirmed by Bland-Altman analysis plotting the differences in both assays against the Kainos assay (Fig. 5b).

Discussion

We have shown that plasma and serum FGF23 levels were not significantly affected by storage at 4 or 22 °C for up to 48 h prior to analysis, following manufacturer recommendation for measuring plasma or serum. Plasma was preferred over serum for both intact and C-terminal FGF23 measurement when using Immutopics assays. Although Kainos assay instructions recommend measurement in serum, our study demonstrated

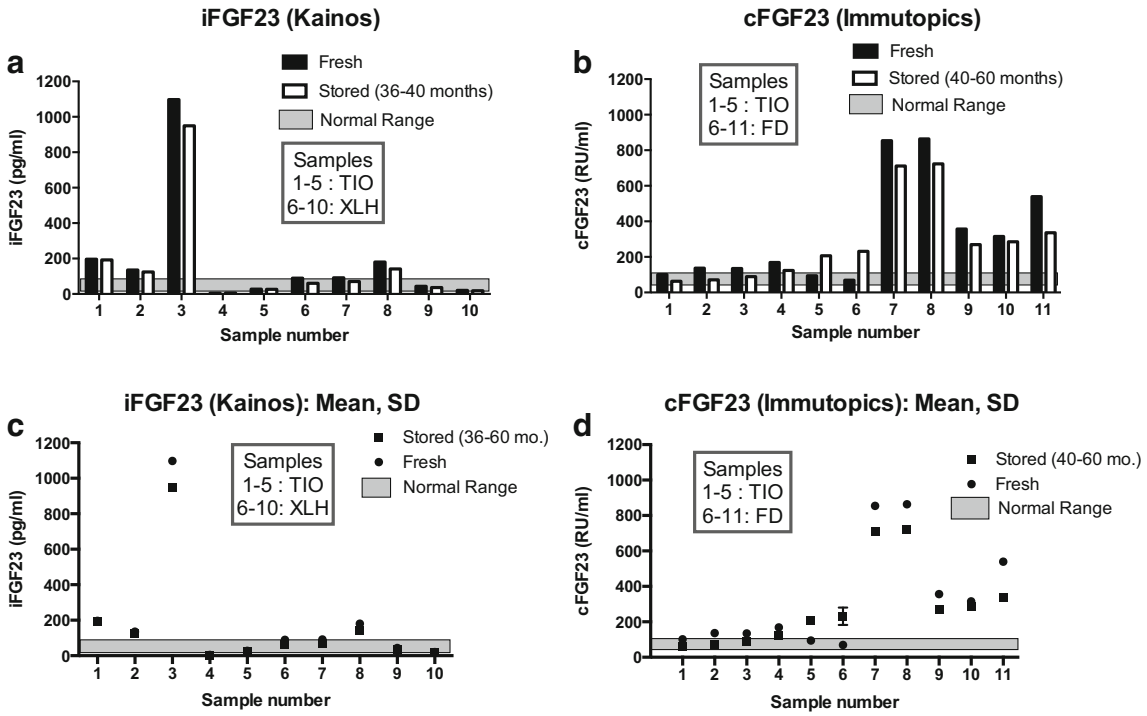

Fig. 3 Effect of storage on FGF23 levels. Samples from patients with TIO, XLH, and FD, which had been previously measured (*Fresh*), were thawed and reassayed (*Stored*) for iFGF23 (Kainos) (**a**) and cFGF23 (Immutopics) (**b**). The *mean and SD bars* of sample duplicates are plotted in **c** and **d** to reveal variability of some measures when comparing low and high FGF23 concentrations. The normal range for iFGF23 was calculated as the 95 % range using mean + 2SD (8–78 pg/ml). The normal range for cFGF23 is established as <180 RU/ml

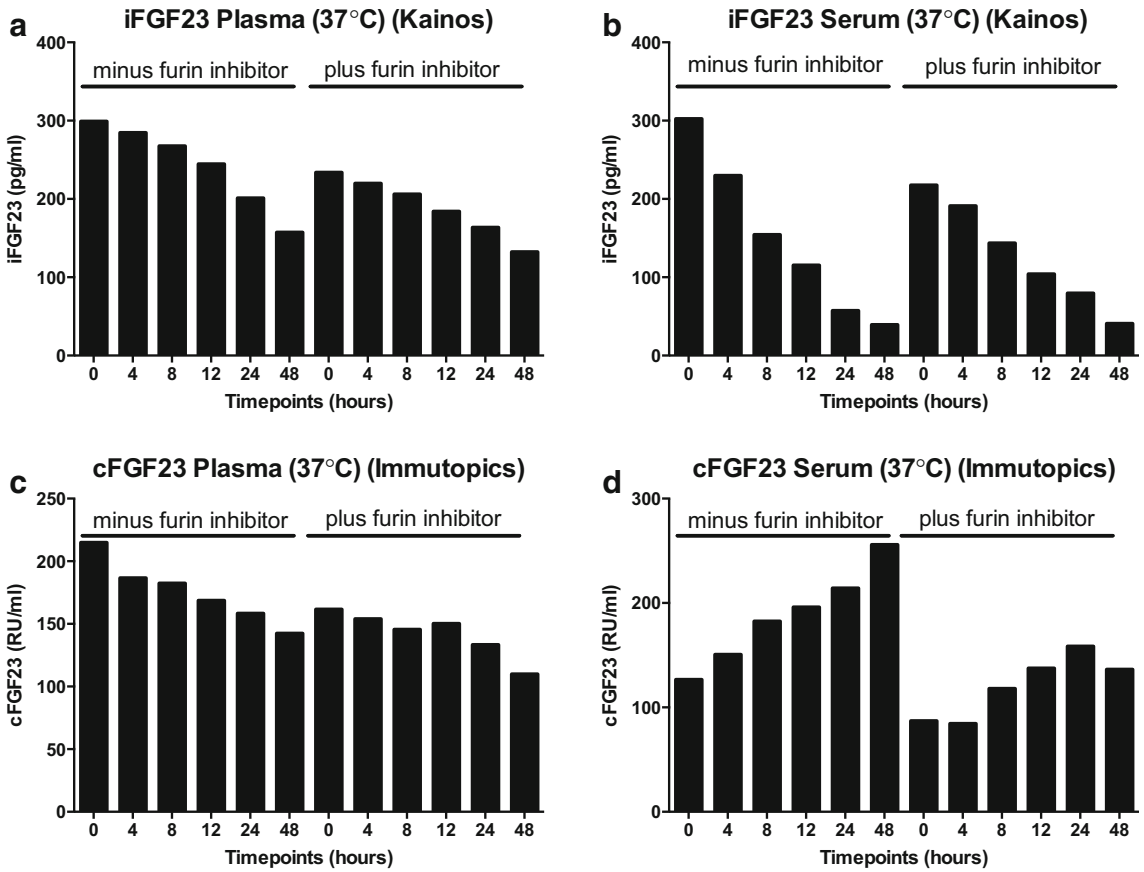

Fig. 4 Effect of furin inhibition on FGF23 degradation. The addition of the furin convertase inhibitor CMK had no significant effect on the degradation of plasma iFGF23 ($P = 0.06$) (**a**), serum iFGF23 ($P = 0.483$) (**b**), plasma cFGF23 ($P = 0.49$) (**c**), or serum cFGF23 ($P = 0.12$) (**d**)

that both serum and plasma were stable up to 48 h. As expected, at body temperature (37 °C), FGF23 levels were not stable in any form or assay used. Both forms of FGF23 levels were not significantly changed by repetitive cycles of freezing and thawing up to five cycles. Both iFGF23 and cFGF23 values showed slight changes when stored at −80 °C for up to 40 months or more. The two assays used to measure iFGF23 show significant correlation at the higher range only.

By performing serial measurements of serum and plasma levels, we have found that iFGF23 levels showed changes ≤15 % at 4 and 22 °C up to 48 h using either Immutopics or Kainos. Smith et al. previously conducted similar experiments using Immutopics assays that showed a significant decline in iFGF23 and a rise in cFGF23 plasma levels when kept at room temperature for 2 and 4 h [16]. In contrast to Smith's findings, our experiment using the same assays showed stable iFGF23 and cFGF23 plasma levels at 4 h when stored at 22 °C. However, at 48 h, our experiment showed a decline in plasma iFGF23 by 15 % whereas cFGF23 increased by 9 %. Per the Kainos instruction manual, the activity of a given sample declines to 86.6 % after 13 days in a refrigerator [13]. A possible explanation of why cFGF23 levels appeared more stable than iFGF23 is the greater biological variability in iFGF23 levels as compared to cFGF23 levels, as shown in healthy subjects and subjects with chronic kidney disease [17]. An important difference between our study and the one conducted by Smith [16] is the high levels of FGF23 in our samples, as compared to healthy volunteers in the study by Smith et al. It is unclear why serum cFGF23 rose with time when stored at 22 and 37 °C; this may reflect an interfering substance that binds to the assay antibody, giving false elevation with time, or denaturation of the protein resulting in the generation of additional molecular epitopes, generating falsely positive elevations.

Both forms of FGF23 levels were not significantly changed by repetitive cycles of freezing and thawing up to five cycles, regardless of the assay used. Both forms showed a minimal change (generally a decline) in levels when stored at −80 °C for up to 36–60 months, except for two samples that showed an increase in cFGF23 levels with long-term storage (Fig. 3b, d). Although all samples were handled in a standard manner, the variance on these two samples (8 and 49 %—displayed in Fig. 3d depicting the mean and SD for each duplicate) was high, and this could be explained by handling error. Alternatively, long-term storage leads to denaturation and/or degradation of the proteins, which in turn generates more fragments containing available epitopes that are recognized by polyclonal antibodies (such as Immutopics), and generates a higher signal. This phenomenon of increased signal when

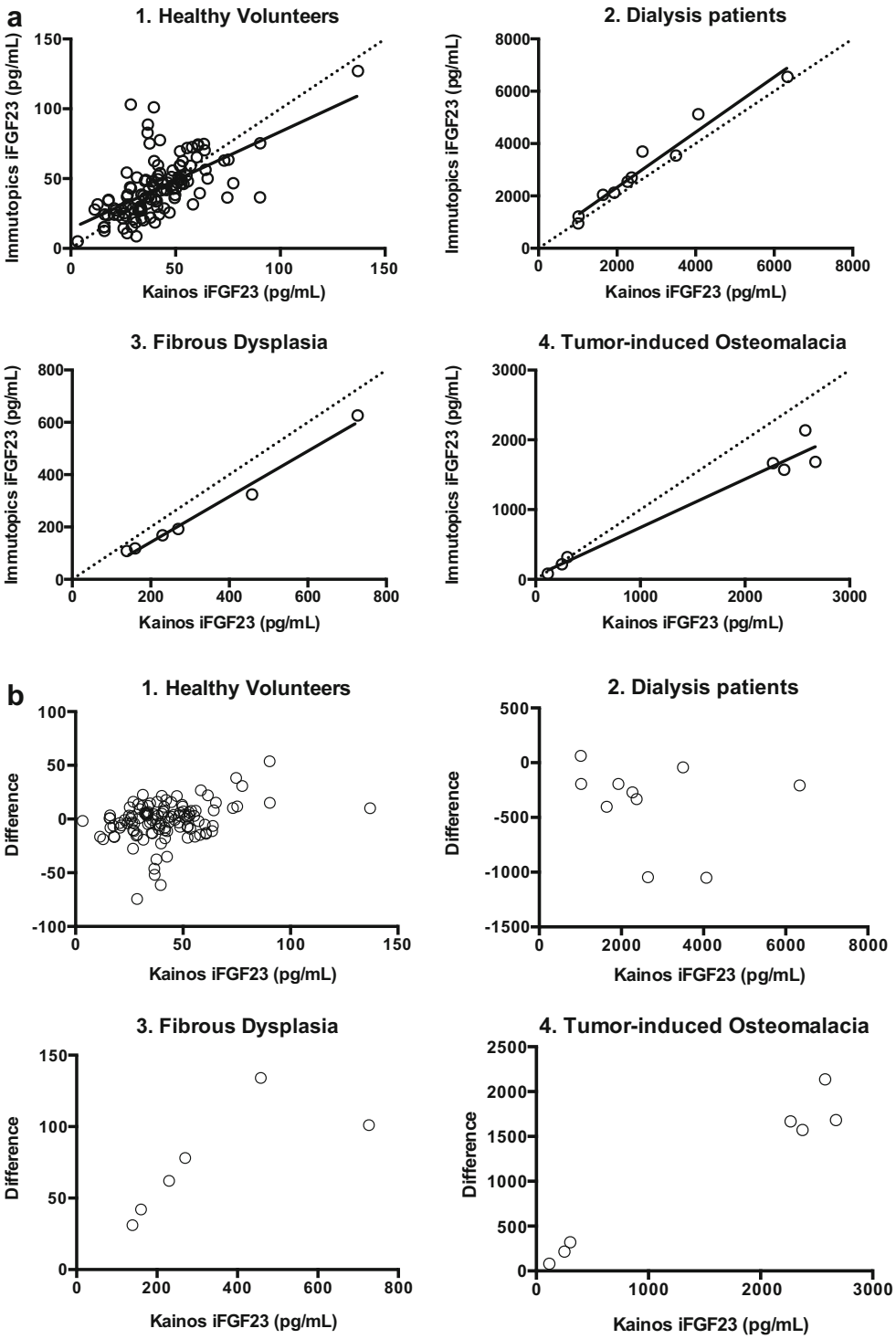

Fig. 5 **a** Correlation of iFGF23 levels as measured by Immutopics (*Y*-axis) and Kainos (*X*-axis) assays in the sample of (*1*) 117 healthy volunteers ($R^2 = 0.38$); (*2*) 10 patients on dialysis ($R^2 = 0.95$); (*3*) 6 patients with fibrous dysplasia ($R^2 = 0.98$); and (*4*) 7 patients with tumor-induced osteomalacia ($R^2 = 0.96$). **b** Bland-Altman analysis was performed to plot the difference between the two assays (*Y*-axis) against the Kainos assay (*X*-axis) (*1–4*)

using polyclonal antibodies was demonstrated by Jain et al., where a rabbit polyclonal antibody recognized fragments of the protein in question whereas the monoclonal antibody did not [18]. Other factors that can affect levels during sample storage include prolonged contact of serum with erythrocytes leading to exchange of substances and either dilution or increase of concentration, hemolysis (again causing either dilution or increase concentration), and interference of hemoglobin in the measurement (specifically in the photometric quantification of constituents) [19]. Overall, these findings are consistent with Kainos' instructions of stability after six thaw/freeze cycles and stability at −80 °C for 3 years [13]. This knowledge allows for a cost-effective approach for long-term sample storage and performing analysis on samples of FGF23 in batches.

The ex vivo degradation of both FGF23 forms showed longer $t_{1/2}$ as compared to published in vivo degradation time

(ex vivo plasma $t_{1/2}$ of 8.8 h compared to in vivo serum $t_{1/2}$ of 46 min for cFGF23 and ex vivo serum $t_{1/2}$ of 7.1 h as compared to in vivo serum $t_{1/2}$ of 58 min for iFGF23) [20]. Although furin is a convertase enzyme possibly involved in FGF23 processing [10], addition of a furin inhibitor had no effect on either serum or plasma sample degradation of FGF23 levels in our experiment. In contrast to our finding, addition of a broad-spectrum protease inhibitor by Smith et al. resulted in stabilization of FGF23 levels over 4 h [16]. It is therefore likely that more proteases are involved in FGF23 regulation occurring at different levels. Future studies should evaluate the role of various proteases on FGF23 stability and processing.

The choice of FGF23 assay would depend on several factors, including sample type availability and, most importantly, the clinical setting. XLH, TIO, and ADHR are characterized by high iFGF23 whereas FTC and the hyperostosis-hyperphosphatemia syndrome are characterized by low iFGF23 but high cFGF23, explained by high cleavage of iFGF23 [9, 21, 22]. Only the intact full-length form FGF23 exerts biological effects [11], and measurements of iFGF23 and cFGF23 are therefore not interchangeable. Studies that have measured both forms in disorders of phosphorus and FGF23 impairment have found discrepant cFGF23 and iFGF23, especially when FGF23 levels were not high [23, 24]. Similarly, at the lower end of the range, we have found greater variability among the two assays we used to measure iFGF23. Smith et al. compared four commercial ELISA assays measuring FGF23 and found a poor correlation between the ELISA kits measuring intact FGF23, mainly due to calibration [24]. Furthermore, regardless of the assay used, it is important to interpret FGF23 levels in the clinical setting; for example, a normal FGF23 (rather than suppressed) level in the setting of low phosphorus may be abnormal, suggesting a primary FGF23 disorder.

The limitations of this study include a small sample size; for example, blood from two patients was used for experiments 1 and 2. Although the sample size is small, we had performed studies similar to this in the past, for shorter lengths of time and similar conditions, that produced similar results (data unpublished). We therefore believe that these samples are representative and generalizable. Although we did not use a positive control for the CMK inhibitor, adding the CMK inhibitor to the standards did not result in changes to the curve. Although there has been no change in the raw materials or components of the Kainos assay kit over the years, Immutopics introduced the second generation cFGF23 kit in early 2009 (which included a change in one antibody and a new source of peptide used for the standards, resulting in a more robust and sensitive assay). It is unclear if this technical change could influence the results.

In conclusion, FGF23 is a hormone that is central to a variety of disorders of phosphorus homeostasis. Proper measurement of FGF23 is important for both clinical and research purposes. Our findings provide clinicians and researchers with important knowledge on the effects of handling and storage on the reliability of FGF23 measurements, which can be used in future studies to improve our understanding of FGF23 in health and disease.

Compliance with ethical standards The study was approved by the National Institute of Dental and Craniofacial Research Institutional Review Board, and all subjects provided written informed consent, in accordance with NIH regulations.

Conflicts of interest None.

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