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Pre-analytical stability of FGF23 with the contemporary immunoassays

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ABSTRACT

Background: Several publications have reported on the pre-analytical stability of fibroblast growth factor 23 (FGF23) and some recommend coating blood collecting tubes with protease inhibitors, in order to prevent degradation. These recommendations are based on observations for a first generation assay for the measurement of intact FGF23. However, if this also applies for the contemporary immunoassays, and at what stage of pre-analysis, is unknown.

Methods: We reviewed data from these previous reports on the issue of FGF23 stability and complemented these findings with data from novel experiments.

Results: We concluded that the contemporary intact FGF23 assays by Immutopics, Kainos, Millipore and DiaSorin do not suffer from immediate loss of FGF23 signal and do not require blood withdrawal in protease inhibitor-coated collecting tubes. Nevertheless, FGF23 concentrations do decline when centrifugation is delayed up to 8 h and prompt centrifugation is therefore advised.

1. Introduction

The bone-derived hormone fibroblast growth factor 23 (FGF23) regulates phosphate and vitamin D homeostasis and is widely used as a marker in chronic kidney disease research. It is rarely measured for clinical purposes, but of great value in cases of tumour-induced osteomalacia (TIO), unexplained hypophosphatemic rickets, and in some very rare hyperphosphatemic disorders [1]. For measurement of FGF23, currently four immunoassays from four manufacturers are commercially available. Immutopics (1st and 2nd generation, San Clemente, CA, USA), Kainos (Tokyo, Japan), Millipore (Billerica, MA, USA) and DiaSorin (Saluggia, Italy) all have marketed assays for the measurement of the intact 251 amino-acid protein. Only the assay from DiaSorin is marked for use in diagnostics. Additionally, Immutopics has developed a C-terminal assay which measures both the intact protein and the 72 amino-acid C-terminal fragment of FGF23, formed after cleavage by proprotein convertases, possibly furin [2,3]. Unfortunately, the assays are neither standardized nor harmonized and differ substantially in their reported values and even in their units [4–10]. This impairs suitability in research and particularly in clinical diagnostics, as comparing results from different assays is impossible. One factor that might contribute to their lack of comparability is the supposed instability of intact FGF23 post-venepuncture. Concerns about the stability of this protein after blood collection have led some to coat the collection tubes with protein stabilizers as the instability is ascribed to protein cleavage within the blood collecting tube itself. This may occur in whole blood directly after venepuncture (pre-centrifugation, before removal of blood cells and clotting factors) or in serum/plasma after centrifugation. For clarity and practicality, we here distinguish immediate pre-centrifugation stability (centrifugation < 1 h), delayed centrifugation stability (centrifugation after 8 h) and delayed storage stability (after centrifugation) of FGF23.

In 2011, Smith et al. first assessed the stability of FGF23 at room temperature both in whole blood and in promptly centrifuged plasma using the two 1st generation assays from Immutopics (the intact and C-terminal FGF23

Abbreviations: FGF23, fibroblast growth factor 23; TIO, tumour-induced osteomalacia

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assays) [6]. They reported higher baseline concentrations of intact FGF23 at 30 min when collection tubes were pre-coated with a protease inhibitor cocktail or aprotinin, suggestive of immediate protein proteolysis in tubes that did not contain protease inhibitors. Assumed breakdown of FGF23 in uncoated tubes endured when centrifugation was further delayed between 30 min and 4 h, resulting in a gradual loss of intact FGF23 and a surprising increase of measured FGF23 concentrations over time using the C-terminal FGF23 assay. They additionally investigated post-centrifugation stability and showed the same gradual loss of intact and increase of C-terminal FGF23 concentrations occurs in separated EDTA plasma. Two years later, this same group performed similar experiments with the updated (2nd generation) intact and C-terminal FGF23 Immutopics assays, alongside the assays by Millipore and Kainos, to evaluate pre-centrifugation instability when centrifugation was delayed for up to 8 h. The 2nd generation intact FGF23 assay from Immutopics again showed a significant decrease (mean 23%) in signal when centrifuged 8 h after collection as compared to prompt centrifugation after venepuncture [7]. As in their previous study, protease inhibitors negated this decline. Strikingly, the Kainos and Millipore intact FGF23 assays showed negligible differences (5–7%) between the promptly centrifuged samples and those that were centrifuged 8 h after collection. Thereupon, more groups started to investigate the stability of FGF23. A post-centrifugation stability study in only two promptly separated plasma and serum patient samples by El-Maouche et al. in 2016 showed no decline of intact FGF23 after a delay of measurement of up to 48 h at room temperature using the Kainos and 2nd generation intact Immutopics assays [5]. One year later, Cui et al. demonstrated that intact FGF23 is indeed unstable pre-centrifugation with the Immutopics assay, albeit their observed differences were not significant, possibly due to a small sample size ($n = 4$) [11]. That same year, Souberbielle et al. evaluated the DiaSorin intact FGF23 assay on the automated Liaison platform [8]. When EDTA samples are promptly centrifuged after collection, intact FGF23 was shown to be stable for up to 24 h at room temperature. In 2018, van Helden and Weiskirchen studied the delayed centrifugation stability without protease inhibitors with the DiaSorin assay and observed no change in intact FGF23 concentrations up to 8 h [12]. Unfortunately, neither of the latter studies obtained information on the possible immediate pre-centrifugation protein instability with the assays used.

The findings reported by these various studies may appear confusing or sometimes even contradictory, and certainly some information was still lacking. We have therefore performed additional experiments to elucidate some of the inconsistencies and to fill remaining knowledge gaps in the available literature.

2. Methods

Samples obtained at the Amsterdam UMC were from the Longitudinal Aging Study Amsterdam (LASA) [13]. Samples obtained at the Royal Melbourne Hospital have been described previously [7]. Bland-Altman analyses (MedCalc, Ostend, Belgium) were used to assess differences.

3. Results

To examine whether the 2nd generation intact FGF23 assay from Immutopics, similar to the 1st generation assay, suffers from immediate pre-centrifugation signal loss of intact FGF23 and requires collection tubes with protease inhibitor, we compared intact FGF23 results in healthy volunteers using the Immutopics assay of samples collected in EDTA Aprotinin tubes (50 KIU/mL aprotonin, BD, Franklin Lakes, NJ, USA) with those collected in regular EDTA tubes (BD) at the Amsterdam UMC ($n = 28$ each). Measured FGF23 concentrations were on average 6% higher in EDTA tubes versus EDTA tubes with added aprotonin (95% CI 104%–108%, $p \leq .0001$). We concluded that test results are not affected in a clinically relevant way when a protease inhibitor is not used. Hence, immediate pre-centrifugation protein instability is not a relevant issue any longer after introduction of the 2nd generation intact assay by Immutopics. We performed the same experiment using the DiaSorin assay ($n = 30$ each) and found no difference between the two groups

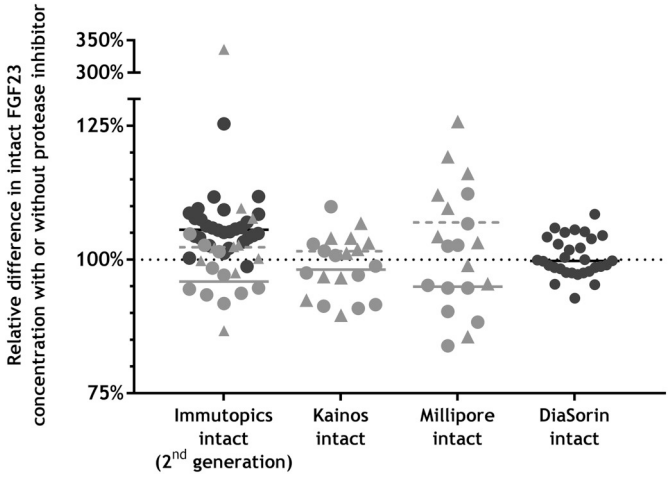

Fig. 1. Pre-analytical stability of intact FGF23 at room temperature in EDTA plasma with or without protease inhibitor(s) with the contemporary immunoassays. FGF23 concentrations expressed as percentage without protease inhibitor(s) as compared to with protease inhibitor(s) (dashed line at 100%). Healthy volunteers (circles, solid lines) or patients on dialysis (triangles, dashed lines) in the Amsterdam UMC (black) or Royal Melbourne Hospital (grey). None of the assays showed any significant relative difference. Horizontal lines represent median.

($p = .723$). At the Royal Melbourne Hospital, we additionally investigated the impact of delayed centrifugation (8 h) on stability of intact FGF23 in healthy volunteers ($n = 10$), the immediate instability and the impact of delayed storage of intact FGF23 in healthy volunteers and patients on dialysis (n = 10 each) with the 2nd generation Immutopics, Kainos and Millipore assays. None of the assays showed any signs of immediate instability of intact FGF23, comparing P100 tubes (containing a cocktail of protease inhibitors, BD) to regular EDTA tubes (BD) (all $p > .05$, Fig. 1). After an 8 h delay of centrifugation, the Kainos (−5%, 95% CI 3%–8%, $p = .002$), Millipore (−7%, 95% CI 5%–10%, $p = .0001$) and Immutopics (−12%, 95% CI 5%–18%, $p = .004$) assays all showed a modest decrease in intact FGF23 concentration in healthy volunteers. To asses, delayed storage stability, we compared samples stored at -80 °C immediately after processing (including centrifugation), with those left at room temperature after processing for 8 h before storage (all $p > .05$). None of the assays showed any signs of signal loss if appropriate storage was delayed. A summary of the current literature regarding this topic, as well as our data, is compiled in Table 1.

4. Discussion

According to the 2011 article by Smith et al. the intact FGF23 protein suffered from immediate pre-centrifugation proteolysis using the 1st generation Immutopics assay [6]. Our data suggest that this is not an issue when using the contemporary Immutopics, Kainos, Millipore or Diasorin assays for intact FGF23 (Fig. 1). Therefore, the addition of protease inhibitors, like aprotinin, or the use of P100 tubes, containing protease inhibitors, is not necessary. Samples will need to be centrifuged promptly after venepuncture, as the contemporary assay all show signal loss upon delayed centrifugation (8 h). In their 2013 article, Smith et al. reported a mean reduction of intact FGF23 concentration of 23%, when centrifugation is delayed for 8 h in patients on dialysis measured despite using the 2nd generation Immutopics assay, a finding corroborated by another study in CKD patients, while the Kainos and Millipore assays showed only small (5–7%) reductions (Table 1) [7,11]. Here, we confirm analogous results in healthy volunteers (Table 1). However, these small reductions seem of less clinical importance as they are invalidated by a greater intra-individual variation of intact FGF23 concentrations [14]. Nevertheless, rapid processing, in particular centrifuging, after sample

Table 1
Pre-analytical stability of FGF23 at room temperature in EDTA tubes with the contemporary intact FGF23 immunoassays.

Assay	Immediate centrifugation (< 1 h)	Delayed centrifugation (> 1 h)[a]	Delayed plasma storage[a]
Immutopics iFGF23 (EDTA plasma) 1st generation	**59% decrease** without protease inhibitor cocktail or aprotinin[b] in healthy adults (n = 15)[e] [6].	**45% decrease** over 4 h in healthy adults (n = 15)[e] [6].	**40% decrease** between 30 min – 4 h in healthy adults (n = 15)[e] [6].
2nd generation	**6% increase** without aprotinin[b] in healthy adults (n = 28)[c,e] **No change** without P100 tubes in healthy adults and patients on dialysis (n = 10 each)[c].	**12% decrease** over 8 h in healthy adults (n = 10)[c,d] **23% decrease** over 8 h in patients (n = 10)[e] [7]. **23% decrease** over 6 h in CKD patients (n = 4)[f] [11].	**No change** over 48 h in a TIO patient (n = 1) [5]. **No change** over 8 h in healthy adults (n = 10) or patients on dialysis (n = 10)[c].
Kainos iFGF23 (EDTA plasma/serum)	**No change** without P100 tubes in healthy adults and patients on dialysis (n = 10 each)[c].	**5% decrease** over 8 h in healthy adults (n = 10)[c,d] **5% decrease** over 8 h in patients on dialysis (n = 10)[d] [7].	**No change** over 48 h in a TIO patient (n = 1) [5]. **No change** over 8 h in healthy adults and patients on dialysis (n = 10 each)[c].
Millipore iFGF23 (EDTA plasma)	**No change** without P100 tubes in healthy adults and patients on dialysis (n = 10 each)[c].	**7% decrease** over 8 h in healthy adults (n = 10)[c,e]. **7% decrease** over 8 h in healthy adults and patients on dialysis (n = 10)[d] [7].	**No change** over 8 h in healthy adults or patients on dialysis (n = 10 each)[c].
DiaSorin iFGF23 (EDTA plasma)	**No change** without aprotinin[b] in healthy adults (n = 30)[c].	**4% decrease** over 8 h in healthy adults (n = 5)[g] [12].	**No change** over 24 h (n = 10) [8].

[a] In tubes without proteases inhibitor(s).
[b] Protease inhibitor.
[c] Current study.
[d] $p < .05$.
[e] $p < .001$.
[f] NS.
[g] p unknown.

collection is advisable to limit total variation to the minimum. The processes accounting for the apparent delayed centrifugation instability have yet to be elucidated and it is challenging to reconcile why signal loss is greater using certain assays. In vivo, FGF23 can be cleaved intracellularly by proprotein convertases like furin into C-and N-terminal fragments [2]. It is currently unclear whether FGF23 secreted into the circulation is also susceptible to proteolysis. If intact FGF23 were to be cleaved ex-vivo by proteases prior to centrifugation, one might expect it to be reflected equally by all intact FGF23 assays. Moreover, arguing against the involvement of proprotein convertases, these enzymes require calcium for functioning [15], which, however, is chelated in EDTA plasma tubes, and should largely prevent enzymatic proteolysis. In turn, a variable ex vivo modification of the amino acids within the epitope regions recognized by the specific antibodies used in the different assays better explains the observed phenomena. This could explain why only the 1st generation of the intact FGF23 assay by Immutopics (using two polyclonal antibodies) suffered from an immediate decline in intact FGF23 signal, while intact FGF23 appears stable when using the 2nd generation assay by Immutopics (using two monoclonal antibodies) or the other contemporary assays, all using antibodies directed against different epitopes on the FGF23 protein. Similarly, the differences in delayed centrifugation stability using the contemporary assays can be attributed to the same phenomena. In short, immediate FGF23 signal loss does no longer seem to be an issue with any of the contemporary assays for intact FGF23. Consequently, coating the collection tubes with protease inhibitors is not required. Centrifugation should nonetheless not be delayed beyond one hour to prevent possible diminution of the antibodies' capacity to capture FGF23. After centrifugation, in serum or plasma, FGF23 is demonstrated to be stable.

Declaration of interest

None.

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