Letter to the Editor

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FGF23 measurement in burosumab-treated patients: an emerging treatment may induce a new analytical interference

<https://doi.org/10.1515/cclm-2020-0460>

Received April 6, 2020; accepted June 8, 2020; published online July 12, 2020

Keywords: burosumab; FGF23; interference; immunoassay; X-linked hypophosphataemia (XLH).

To the Editor,

Fibroblast growth factor 23 (FGF23), a peptide mostly secreted by osteocytes and osteoblasts is a key regulatory factor for phosphate homeostasis and vitamin D metabolism: it decreases the reabsorption of phosphate in the proximal renal tubule and the secretion of 1,25(OH)₂ vitamin D (1,25(OH)₂D). Several FGF23-related diseases have been described. When in excess, FGF23 induces hypophosphatemia/hyperphosphaturia, with a low-normal concentration of 1,25(OH)₂D and a clinical presentation of rickets/osteomalacia, with demineralization and bone pain, and growth retardation in children. These conditions may be acquired, as in tumor-induced osteomalacia due to tumors secreting FGF23, or inherited, the most frequent heritable disease being X-linked hypophosphataemia (XLH) due to a mutation in the *PHEX* gene, leading to upregulation of FGF23 production from the bone [1].

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Recently, burosumab, a fully human IgG1 monoclonal antibody that binds intact FGF23 at the N terminal domain, has emerged as a breakthrough treatment for XLH [2, 3].

Different types of two-sites “Sandwich” FGF23 immunoassays exist. They detect either the intact form (iFGF23), or the C-terminal fragments (C-FGF23) [4]. There is no standardization among the different assays. Moreover, some assays are expressed in ng/L, some others in pmol/L, or in relative unit (RU/mL) [5]. Accordingly, the reference ranges are different [4, 5]. iFGF23 is the physiological active form of FGF23.

After an alert by a colleague about extremely high, unexpected C-FGF23 results in burosumab-treated patients, we tested both intact and C-FGF23 in 57 XLH children currently following burosumab therapy in Bicêtre Hospital. We evidenced opposite results when comparing intact (Liaison™, Diasorin, Saluggia, Italy) and C-FGF23 (Biomedica ELISA, Wien, Austria) in XLH children treated with burosumab: C-FGF23 concentrations were extremely high (above the upper calibrant of 20 pmol/L), whereas Liaison™ iFGF23 concentrations were dramatically lower in net samples.

The discrepancy was identified in every patient, whatever the moment of the blood sampling after last burosumab dose.

These discrepancies between the two methods led us to perform dilution tests with both the intact and the C-terminal assay, using the diluents recommended by the manufacturers. Typical results obtained in two patients in whom we had the opportunity to use another intact assay in addition to the two above-mentioned assays, are presented in Table 1. These two patients were diagnosed as XLH from clinical, radiological and biochemical evaluation (hypophosphataemia, hyperphosphaturia, low 1,25(OH)₂D and high alkaline phosphatase concentrations). XLH was confirmed on genetic basis (*PHEX* gene mutated).

The dilution ratio recommended by the manufacturers is 1/11 for both Biomedica assays and 1/10 for the Liaison™ assay. The recommended diluents were assayed and found below the limit of quantification of their respective assay

(no matrix effect observed). Therefore, we tested higher dilutions.

Even a 1/500e dilution did not produce a reliable result in Liaison™ intact assay as apparent concentration continued to increase at higher dilution rates, suggesting a still-present inhibitory influence in patients' plasma. The Biomedica iFGF23 also exhibited a negative interference in undiluted samples, and inconsistent progressive dilutions (Table 1).

No information concerning the epitopes recognized were obtained from both manufacturers, these data being considered as proprietary. Possibly, burosumab causes a steric hindrance, preventing the binding of at least one of the reagent antibodies in the intact assays. Besides, the iFGF23 measurement on Liaison™ is reported to

present a hook effect above 10,000 ng/L [6]. For all assays, heterophilic binding tubes (HBT) pretreatment and PEG precipitation did not eliminate burosumab Interference (data not shown).

Most likely two negative interferences are superimposed in intact assays (Hook effect plus inhibition of antibody reagents binding by burosumab). Matrix effect of assay diluent is another possible confounding factor if extreme dilution rates are tested.

Although the Liaison™ intact assay is analytically more performant, the use of C-terminal assay will avoid a wrong result on net sample in burosumab-treated patients. The Biomedica iFGF23 assay gives moderately elevated concentrations, whereas the Liaison™ intact assay gives apparently normal results in the two patients presented.

Table 1: Net results and progressive dilutions for two patients, with iFGF23 assays (Liaison™ and Biomedica ELISA), and C-FGF23 assay, as obtained on the same sample. Raw results (and results corrected by dilution factor).

	iFGF23, ng/L (× dilution factor) Liaison, Diasorin	iFGF23, ng/L (× dilution factor) Biomedica	C-FGF23, pmol/L (× dilution factor) Biomedica
Reference range	23–95	10–50	0.7–3.5
Measuring range	5–5000	10–1600	0.2–20
Patient 1			
FGF23, before burosumab (conventional therapy)	106	ND	ND
Net plasma FGF23, during burosumab treatment	36.7	142	>20
Dilution 1/2	102 (204)		>20
Dilution 1/5	295 (1,475)		>20
Dilution 1/10	520 (5,200)		>20
1/20	631 (12,620)		>20
1/50	690 (34,490)		>20
1/100	553 (55,300)		>20
1/500	246 (123,000)	332 (166,000)	>20
1/1,000	154 (154,000)	228 (228,000)	10.1 (10,090)
1/2,000	91 (182,000)	138 (276,000)	3.85 (7,700)
1/3,000	63 (189,000)	105 (315,000)	2.47 (7 410)
1/4,000		90 (360,000)	
1/5,000		77 (385,000)	
Patient 2			
FGF23, before burosumab (conventional therapy)	159	ND	ND
Net plasma FGF23, during burosumab treatment	92.6	556	>20
Dilution 1/2	221 (442)		>20
Dilution 1/5	508 (2,540)		>20
Dilution 1/10	677 (6,770)		>20
1/20	668 (13,370)		>20
1/50	513 (25,675)		>20
1/100	403 (40,300)		>20
1/500	124 (62,000)	229 (114,635)	8.56 (4,280)
1/1,000	72 (72,000)	144 (144,000)	3.63 (3,630)
1/2,000	36 (72,000)	78 (156,000)	1.68 (3,360)
1/3,000	25 (75,000)	59 (177,000)	1.08 (3,240)
1/4,000		42.5 (170,000)	
1/5,000		42.8 (214,000)	

Patient 1: 12 years old girl, burosumab dose 1.0 mg/kg. Patient 2: 13 years old girl, burosumab dose 0.4 mg/kg. ND, not done.

However, both intact assays are largely underestimating FGF23 concentrations. The reason why the two intact assays behave differently in net samples may be related to the different epitopes recognized in these two assays, leading to steric hindrance of different extent caused by burosumab.

The possibility of immunological interference in immunoassays is well-known for decades. Endogenous antibodies can bind the reagent antibodies (e.g. heterophilic antibodies), or the analyte to be measured (e.g. thyroglobulin and insulin assays, in the presence of anti-thyroglobulin and anti-insulin antibodies, respectively) [7]. Their impacts in immunoassays are diverse, either giving high, or falsely low results, depending on the assay design and epitopes recognition [7]. Dilution tests, HBT pretreatment and comparison with alternative assays, are common tools to evidence an erroneous result, provided that a clinical suspicion is raised [8].

Hence a warning note addressed by the laboratory to the prescribing physician is mandatory to be able to suspect and manage this interference. What clinical significance can have an exact result of either iFGF23 or C-FGF23 concentration in this context? Informing the physician of an extremely high concentration could be clinically sufficient. Taking into account the recommended dilution ratios, we suggest that reporting a very high level (e.g. >50,000 ng/L for intact Liaison™ and >220 pmol/L for C-terminal Biomedica assays) is a cautious approach. We also recommend that manufacturers introduce a warning in their instructions for use, and propose a detailed protocol for this clinical context.

Burosumab therapy is effective because it blocks FGF23 effect on target tissues, notably on proximal renal tubule [9]. Hence the very high FGF23 concentrations recorded are biochemically ineffective, and do not reflect the *in vivo* results on phosphate control. Most likely, circulating FGF23 is being complexed and neutralized by burosumab. *In vitro*, burosumab inhibits recognition of the iFGF23 molecule, as if it could not be “seen” anymore. In practice, the *in vivo* control of XLH by burosumab is monitored using serum phosphate, alkaline phosphatase and 1,25(OH)₂D measurements [1, 9].

In previous studies and clinical trials, FGF23 concentrations obtained during burosumab therapy are not described [1, 9]. Usually FGF23 concentrations at the diagnosis of XLH are moderately elevated, or inappropriately high-normal in the setting of hypophosphataemia [1]. So, these extremely high results are unexpected.

The mechanisms that regulate FGF23 secretion are only poorly understood, as well as the mechanism by which *PHEX* loss of function results in elevated circulating

FGF23. The reason why FGF23 increases tremendously with burosumab therapy is also obscure.

In conclusion, if FGF23 is to be measured in burosumab-treated patients, the Biomedica C-FGF23 assay is analytically reliable. Otherwise, a particular analytical protocol must be defined for iFGF23 measurement in this context. In any case, the management of this interference needs an alert note for the prescribing physician. The interest of measuring FGF23 in this context, if any, will have to cope with extremely high concentrations together with an antibody interference, both of which representing an analytical challenge. The reason why the FGF23 concentration increases dramatically during burosumab therapy deserves further studies which, maybe, will shed some light on the molecular mechanisms of phosphate control in *PHEX* patients.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Research funding: None.

Competing interests: Authors state no conflict of interest.

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