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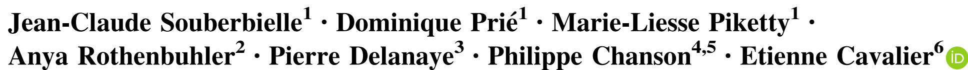

Evaluation of a New Fully Automated Assay for Plasma Intact FGF23

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Abstract Several FGF23 immunoassays are available. However, they are reserved for research purposes as none have been approved for clinical use. We evaluated the performances of a new automated assay for intact FGF23 on the DiaSorin Liaison platform which is approved for clinical use. We established reference values in 908 healthy French subjects aged 18–89 years, and measured iFGF23 in patients with disorders of phosphate metabolism and in patients with chronic kidney disease (CKD). Intra-assay CV was 1.04–2.86% and inter-assay CV was 4.01–6.3%. The limit of quantification was <10 ng/L. Serum iFGF23 concentrations were considerably lower than EDTA values highlighting the importance of using exclusively EDTA plasma. Liaison iFGF23 values were approximately 25% higher than Immutopics values. In the 908 healthy subjects, distribution of the Liaison iFGF23 values was Gaussian with a mean ± 2SD interval of 22.7–93.1 ng/L. Men had a slightly higher level than women (60.3 ± 17.6 and 55.2 ± 17.2 ng/L, respectively). Plasma iFGF23 concentration in 11 patients with tumour-induced osteomalacia, 8 patients with X-linked hypophosphatemic rickets, 43 stage 3a, 43 stage 3b, 43 stage 4, 44 stage 5 CKD patients, and 44 dialysis patients were 217.2 ± 144.0, 150.9 ± 28.6, 98.5 ± 42.0, 130.8 ± 88.6, 130.8 ± 88.6, 331.7 ± 468.2, 788.8 ± 1306.6 and 6103.9 ± 11,178.8 ng/L, respectively. This new iFGF23 assay available on a platform that already allows the measurement of other important parameters of the mineral metabolism is a real improvement for the laboratories and clinicians/researchers involved in this field.

Keywords FGF23 · Reference values · Vitamin D · Chronic kidney disease · Hypophosphatemia · Hyperphosphatemia

Introduction

Fibroblast growth factor 23 (FGF23) is a 251 amino-acid peptide that is mostly secreted by osteocytes and osteoblasts [1]. FGF23 decreases the reabsorption of phosphate by down-regulating the luminal expression of the sodium–phosphate co-transporters NPT2a and NPT2c in the proximal renal tubule and thus increases urinary phosphate excretion and decreases serum phosphate concentration. It also decreases the synthesis of 1,25 dihydroxyvitamin D ($1,25OH_2D$) and increases the inactivation of vitamin D into 24-hydroxylated compounds [1]. FGF23 induces these

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hyperphosphaturic and anti-vitamin D effects through the binding to a co-receptor complex made of one of the four FGF receptors (FGFR), mainly FGFR1, and a trans-membrane protein named Klotho [2]. Several FGF23-related diseases with disordered phosphate metabolism have been identified. When in excess, FGF23 induces hypophosphatemia with consequent hyperphosphaturia, with a low or low-normal 1,25OH$_2$D concentration and hypocalciuria and a clinical presentation of rickets/osteomalacia with demineralization of bone and diffuse pain. These conditions may be acquired, as in tumour-induced osteomalacia (TIO) due to (mostly benign) tumours secreting FGF23 [3], or inherited when different genes are mutated, the most frequent being a mutation in the *PHEX* gene causing X-linked hypophosphatemic rickets (XLH) [4]. On the opposite, the absence of FGF23 active form due to inactivating mutation in the FGF23 [5] or *GALNT3* gene (see below), or lack of FGF23 effect due to a mutation in the Klotho gene [6] are characterized by hyperphosphatemia with hyperostosis and/or tumoral calcinosis.

The FGF23 molecule is the sole member of the FGF family to possess a proteolytic cleavage site (^{176}RXXR^{179}). Intact FGF23 (iFGF23) is considered as the physiological active form of FGF23. iFGF23 can be cleaved at the RXXR site by a still unidentified enzyme. The cleavage results in a N- and a C-terminal fragments (nFGF23, cFGF23) [7]. Besides the regulation of the secretion of FGF23 by the bone cells which is stimulated by high phosphate intake, 1,25OH$_2$D, parathyroid hormone (PTH), and hyperphosphatemia [8], a second level of regulation of FGF23 secretion linked to this mechanism of cleavage has been described. Indeed, in some clinical conditions such as iron deficiency, the synthesis of FGF23 by bone cells is greatly increased but an increase in the intracellular proteolytic cleavage also occurs, so that an increased quantity of inactive fragments is released by the cells while iFGF23 concentration is normal with no modification of serum phosphate or calcitriol concentrations [9]. Also, it must be noted that FGF23 is a glycosylated protein, and that this glycosylation is important for the stability of the iFGF23 molecule. Inactivating mutations in the gene-encoding *N*-acetyl-galactosaminyl transferase 3 (*GALNT3*), the enzyme which glycosylates FGF23, result in high plasma concentration of inactive fragments whereas iFGF23 concentration is low, leading to hyperphosphatemia and severe tumoral calcinosis [10].

Finally, it is now well known that plasma FGF23 concentration increases at the early steps of chronic kidney disease (CKD) [11] and markedly augments as GFR decreases to reach very high levels in patients treated by chronic dialysis [12].

Available reagents for measuring iFGF23 or C-terminal FGF23 are supplied as manual Elisa kits by various manufacturers [13]. An automated iFGF23 that is not currently available in Europe has also been developed [14]. The iFGF23 assays are thought to measure only iFGF23 while the C-terminal assays measure the sum of iFGF23 and C-terminal fragment. Unfortunately, most FGF23 assays are not approved for clinical use and should thus be reserved for research purposes. For this reason, the manufacturers do not provide reference values established in healthy populations. In the present study, we evaluated a new fully automated assay for iFGF23 in plasma that is now approved for clinical use by the European Community authorities. We evaluated the analytical performances of this assay and provided adult reference values obtained from a well-defined cohort of French healthy subjects. We also used this assay to measure iFGF23 in patients with CKD and in patients with disorders of phosphate metabolism.

Subjects and Methods

Healthy Subjects and Patients

We enrolled healthy volunteers who participated in the VARIETE study, a population-based cross-sectional study designed to recruit a reference population for serum IGF-I values and other hormones in adults (ClinicalTrials.gov identifier: NCT01831648) [15]. They were recruited between January 2011 and February 2012 by the clinical research units of 10 university hospitals distributed throughout France. Inclusion criteria were a normal physical work-up (weight, height, blood pressure, nutritional status and gonadal/sexual status), normal laboratory values determined after an overnight fast (plasma sodium, potassium, calcium, phosphate, creatinine, glycemia, total cholesterol, liver enzymes, TSH, blood cell counts, albuminemia, prothrombin time, and HIV and HCV serology), age 18–89 years and BMI between 19 and 28 kg/m^2, and a written informed consent to participate in the study. The exclusion criteria were a medical history of thyroid, renal, hepatic, cardiovascular, pulmonary, intestinal or psychiatric disorders, cancer, epilepsy, intercurrent illness occurring during the week preceding inclusion, current consumption of tobacco or other toxics, and treatment potentially modifying IGF-I or calcium/phosphorus metabolism (antiandrogens or antiestrogens, loop diuretics, hydrochlorothiazide, CYP-inducing drugs). In addition to the blood samples necessary for the screening biological evaluation, 50 mL of whole blood and 30 mL of EDTA blood were obtained from each subject. Blood was promptly centrifuged (3000*g* at 4 °C), and serum or plasma was aliquoted in polypropylene tubes that were immediately stored at −80 °C. This study was funded by

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We also obtained EDTA plasma samples from eight hypophosphatemic adults with XLH after a 7- to 10-day wash-out of phosphorus and active vitamin D treatment, 11 hypophosphatemic adults with TIO sampled at the time of diagnosis (i.e. not yet receiving phosphorus and/or active vitamin D treatment), and one hyperphosphatemic young woman with a mutation of *GALNT3*. Finally, we collected plasmas from 173 patients with CKD monitored in the same nephrology department by one of us (PD). Forty-four of them were treated by chronic dialysis. Among the other patients with CKD, 44, 43, 43 and 43 had a CKD stage 5, 4, 3b and 3a, respectively.

Blood for all healthy subjects and patients was collected in the morning after an overnight fast, except for the dialysis patients for whom blood was obtained just before a dialysis session.

Description and Analytical Validation of the New Automated iFGF23 Assay

We evaluated a new automated iFGF23 immuno-chemiluminescent sandwich assay developed by DiaSorin (Saluggia, Italy) on the Liaison XL platform. This assay uses three monoclonal antibodies, one coated on microparticles and directed against the N-terminal portion of the iFGF23 molecule, another, labelled with fluoresceine, directed against the C-terminal portion, and the 3rd one, bound with isoluminol, directed against fluoresceine. According to DiaSorin, this assay is reserved for the measurement of iFGF23 in EDTA plasma as this manufacturer found that serum values were greatly lower than EDTA plasma values. The assay range is 5–5000 ng/L. We performed our own analytical evaluation of the Liaison iFGF23 assay and tested the intra- and inter-assay coefficient of variation (CV), the linearity of serial dilutions, and the limit of quantification (LOQ) of the assay. We verified the difference between serum and EDTA plasma values by measuring iFGF23 in 33 pairs of samples obtained in patients undergoing an extensive exploration of calcium/phosphorus metabolism in our unit. We also tested the stability of iFGF23 in serum and EDTA plasma in samples from healthy subjects ($n = 5$) and dialysis patients ($n = 5$) that were aliquoted. One aliquot was immediately analysed for iFGF23 concentration (T0) and served as a reference, while other aliquots were let standing at room temperature (RT) and were tested after 1, 4, 7 and 24 h. Another aliquot was placed at −20 °C and tested after 24 h at this temperature. Finally, we compared the new Liaison iFGF23 assay with another "intact" assay, the Immutopics iFGF23 assay (San Clemente, CA) in 87 EDTA plasmas from our daily routine practice.

Other Laboratory Methods

The biological parameters of the healthy volunteer screening evaluation were determined locally by the laboratories attached to the clinical research units, using standard chemistry methods. The CKD-epi formula was used to estimate glomerular filtration rate (eGFR). PTH, 25OHD and 1,25OH$_2$D measurements in the healthy subjects were centralized and done in batches by means of immunochemiluminometric assays on the LIAISON XL, using serum samples that had never been thawed. Analytical characteristics of these assays have been described elsewhere [16].

In our hands, the Immutopics iFGF23 assay presented inter-assay CVs of 25.5 and 6.2% in two EDTA plasma pools with a mean concentration of 31.6 and 155.6 ng/L respectively, and a LOQ of 21 ng/L.

Statistical Analysis

Quantitative variables are reported as mean ± SD and median [interquartile range (IQR)]. Normality of the distribution of iFGF23 concentration in the healthy subjects was assessed with the Kolmogorov–Smirnoff test. Between-group comparison of continuous variable was assessed by ANOVA. Repeated data were compared with the non-parametric Wilcoxon test. Simple regression was used to test the relationship between iFGF23 and continuous variables in the VARIETE population as well as between iFGF23 and eGFR in the non-dialyzed CKD patients. Multiple regression analysis was performed in the VARIETE population with iFGF23 as the dependent variable, and the variables which showed a statistically significant correlation with iFGF23 in simple regression analysis as independent variables. Bland–Altman representation was used to compare Liaison iFGF23 values with Immutopics values. A p value ≤ 0.05 was considered as significant.

Results

Analytical Evaluation of the Liaison iFGF23 Assay

We found an intra-assay CV of 1.20 and 1.04% in 20 replicates of the two quality controls (QC) provided by DiaSorin in the iFGF23 kit with a concentration of 262.9 and 886.9 ng/L, respectively. In 30 replicates of two EDTA plasma pools prepared from our routine practice, intra-assay CV was 2.86 and 2.33% at 50.5 and 242.3 ng/L,

respectively. Inter-assay CV calculated from 33 replicates of the two DiaSorin QCs was 4.01 and 4.72% at 251.6 and 886.7 ng/L, respectively. In 17 replicates of two EDTA plasma pools, inter-assay CV was 6.3 and 2.1% at 34.6 and 123.7 ng/L, respectively. Using a diluent provided by DiaSorin in the iFGF23 kit, serial dilutions (up to 1/20) of a plasma with a very high FGF23 level yielded recovery values of 92–99%. To evaluate LOQ of the assay, we serially diluted a plasma with an iFGF23 concentration of 67.8 ng/mL and calculated the CV of 10 replicates of each of the dilutions with low FGF23 values. The findings were a CV of 5.2, 4.2 and 5.7% at a concentration of 15.1, 13.0 and 11.0 ng/mL, respectively in favour of a LOQ <10 ng/L. Although serum and EDTA iFGF23 concentrations were significantly correlated, serum iFGF23 was significantly lower than EDTA iFGF23 (serum iFGF23 = 0.51 EDTA iFGF23-6.6). The serum iFGF23/EDTA iFGF23 ratio was 0.433 ± 0.101 (min: 0.233; max: 0.609). There was a significant positive correlation ($R = 0.38$; $p = 0.04$) between this ratio and the Liaison iFGF23 values indicating that the relative difference between serum and EDTA iFGF23 was higher at low iFGF23 concentrations. We found that EDTA iFGF23 was fairly stable at RT and at −20 °C for 24 h (Table 1). This was also the case for serum iFGF23, albeit to a lesser extent, as a slight but significant loss of concentration was observed after 4 h (−4%), 7 h (−5%) and 24 h (−10.3%) at RT. Figure 1 shows the relationship between the Liaison iFGF23 and the Immutopics iFGF23 values. The concentrations measured with these two assays were significantly correlated ($p < 0.0001$) with lower Immutopics values than Liaison values except in a few cases.

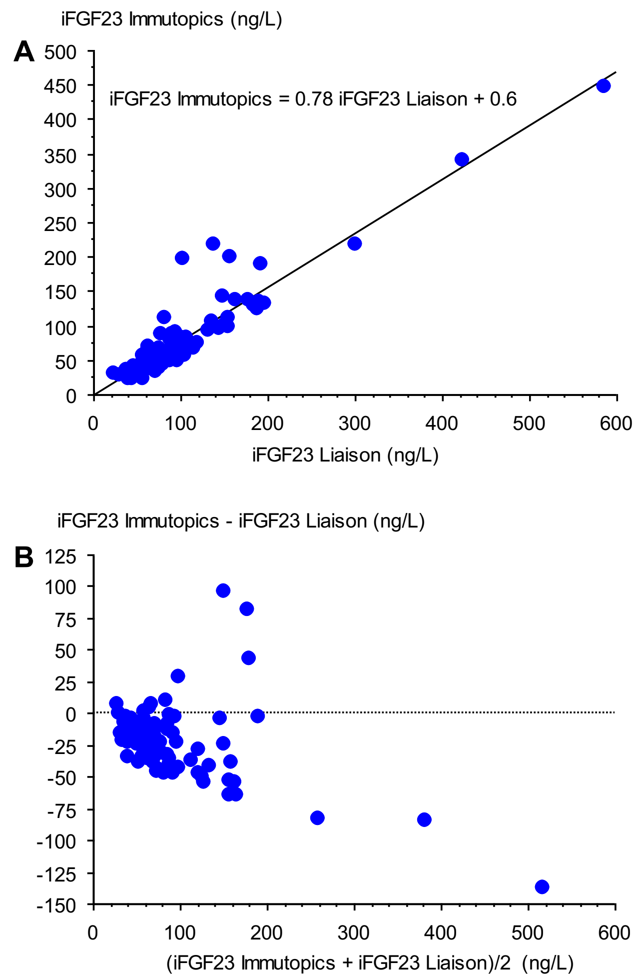

Fig. 1 Regression (**a**) and Bland–Altman plot (**b**) of the plasma iFGF23 concentrations measured with the Liaison automated assay and the Immutopics assay ($n = 87$)

Normal Subjects from the VARIETE Study

Nine-hundred and seventy-two subjects were initially recruited. Sixty-four were excluded (two excluded because their informed consent was not available, 60 excluded because abnormal biological values were found in the screening evaluation, two excluded because no EDTA plasma sample was available for iFGF23 testing). The study population thus consisted of 908 subjects (482 men, 426 women) whose main characteristics are summarized in Table 2. All had an eGFR above 60 mL/mn, and normal calcemia, phosphatemia, albuminemia. In these 908 healthy subjects, the distribution of plasma iFGF23 was Gaussian (Fig. 2) with a mean ± SD of 57.9 ± 17.6 ng/L and thus a calculated normal range (mean ± 2SD) of

Table 1 Stability at room temperature (RT) and at −20 °C of iFGF23 concentration in serum and EDTA plasma samples of five healthy controls and five dialysis patients

Conditions of conservation	Serum iFGF23 (ng/L)	EDTA iFGF23 (ng/L)
Tested immediately (T0)	476.2 ± 531.2	954.0 ± 1021.3
Tested after 1 h at RT	474.5 ± 530.0	947.2 ± 1021.2
Tested after 4 h at RT	458.8 ± 512.7*	950.2 ± 1018.2
Tested after 7 h at RT	453.5 ± 508.4*	942.9 ± 1015.6
Tested after 24 h at RT	426.9 ± 477.8*	957.2 ± 1024.8
Tested after 24 h at −20 °C	477.7 ± 535.7	957.1 ± 1032.6

Data are mean ± SD

*$p < 0.01$ versus T0 (Wilcoxon test)

Table 2 Main characteristics of the healthy subjects participating in the VARIETE study

n	908
Sex ratio: men/women	482/426
Age (years)	39.7 ± 18.6
BMI (kg/m^2)	23.0 ± 2.4
Serum 25OHD (ng/mL)	23.8 ± 8.1
Serum PTH (ng/L)	20.6 ± 8.0
Serum 1,25(OH)$_2$D (ng/L)	52.9 ± 14.5
Serum calcium (mmol/L)	2.30 ± 0.10
Serum phosphate (mmol/L)	1.09 ± 0.18
Serum albumin (g/L)	43.0 ± 3.9
eGFR (CKD-epi) (mL/min)	100 ± 7

Continuous variables are presented as mean ± SD

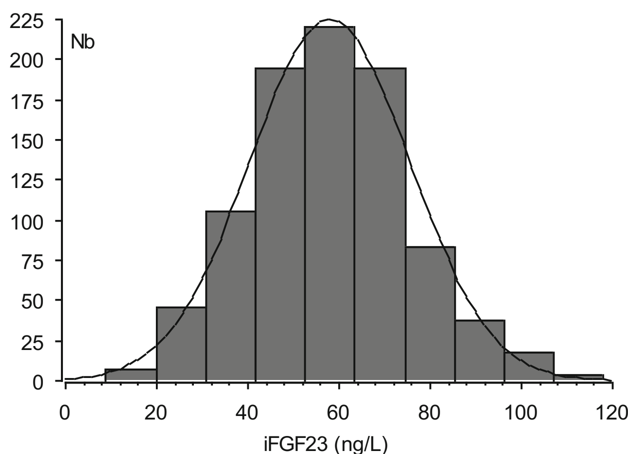

Fig. 2 Distribution of the iFGF23 concentration in the 908 adult healthy subjects

22.7–93.1 ng/L. After stratification of the 908 healthy subjects into three groups of age, we found that subjects 60 years old and older had a higher mean iFGF23 (63.0 ± 19.4 ng/L) than those aged 30–59 years (56.1 ± 17.3 ng/L) and than those aged 18–29 years (56.9 ± 17.3 ng/L). It must be noted however that, as expected, these older subjects had a lower eGFR ($p < 0.0001$) than those aged 30–59 years and those aged 18–29 years (79.8 ± 11.3, 97.8 ± 13.3, and 111.1 ± 13.1 mL/mn, respectively). We thus tested whether excluding subjects with an eGFR <75 mL/mn ($n = 74$) had any influence on the iFGF23 reference range. This was not the case as mean iFGF23 concentration was 57.4 ± 17.3 ng/mL in the 834 remaining subjects. We also found that iFGF23 was slightly but significantly ($p = 0.003$) higher in men (60.3 ± 17.6 ng/L) than in women (55.2 ± 17.2 ng/L).

In simple regression analysis, Liaison iFGF23 was negatively and significantly correlated with 1,25OH$_2$D ($r = -0.26$; $p < 10^{-4}$) and eGFR ($r = -0.14$; $p < 10^{-4}$), and positively and significantly, albeit weakly, correlated with PTH ($r = 0.08$; $p = 0.02$), calcemia ($r = 0.10$; $p = 0.004$) and BMI ($r = 0.11$; $p = 0.0008$). iFGF23 was not significantly correlated with age, serum phosphate and 25OHD concentrations. In multiple regression analysis, only 1,25OH$_2$D remained correlated with iFGF23.

Patients with Various Disorders of Phosphate Metabolism and Patients with CKD

The 11 patients with TIO all had an iFGF23 above the upper limit of normal (ULN) of 93.1 ng/L found in the VARIETE population with a mean value of 217.2 ± 144.0 ng/L. This was also the case for all but one patient with XLH (150.9 ± 26.6 ng/L). The only XLH patient with a normal iFGF23 had a concentration of 88.6 ng/L that is close to our ULN. The patient with a *GALNT3* mutation had an iFGF23 concentration of 36 ng/L with a C-terminal FGF23 value of 1640 RU/mL measured with the Immutopics C-terminal FGF23 kit (normal values in our lab: <120).

In the CKD patients, we observed a progressive significant increase in the mean iFGF23 concentration as renal function declined (p for trend $<10^{-4}$) with, as expected, very high levels in dialysis patients. In the 130 non-dialyzed CKD patients, iFGF23 was significantly and negatively correlated with eGFR ($r = -0.35$; $p < 10^{-4}$). The iFGF23 concentrations of these various groups of patients are summarized in Table 3.

Discussion

In this work, we evaluated a new fully automated immunoassay for iFGF23 that is now approved for clinical use by the European Community authorities. We demonstrated excellent analytical characteristics for this new assay with low intra- and inter-assay CVs and a low LOQ corresponding to much better performances in our hands than the ones of the Immutopics iFGF23 assay, most likely due to automation.

The results we obtained with the DiaSorin Liaison FGF23 assay are quite similar to those observed with the other only automated method (unavailable in Europe) described in [14]. These authors obtained a slope of 0.85 when they compared their results with the Kainos ELISA assay for iFGF23, whereas we found a slope of 0.77 with the Immutopics ELISA iFGF23 kit. This highlights the necessity of standardizing the results of the different iFGF23 assays using a common international standard as already recommended by colleagues who compared three "research" iFGF23 assays [17].

Table 3 Summary of the Liaison iFGF23 concentrations of the healthy subjects and various groups of patients

Group	*n*	Liaison iFGF23 (ng/L) mean ± SD median [IQR]
Healthy subjects	908	57.9 ± 17.6 57.5 [45.5–68.9]
TIO	11	217.2 ± 144.0 170.2 [129.0–235.9]
XLH	8	150.9 ± 28.6 162.9 [139.8–169.4]
CKD stage 3a	43	98.5 ± 42.0 103.6 [59.2–129.0]
CKD stage 3b	43	130.8 ± 88.6 119.2 [73.6–159.4]
CKD stage 4	43	331.7 ± 468.2 216.3 [130.2–307.8]
CKD stage 5	44	788.8 ± 1306.6 350.0 [181.3–602.6]
Dialysis	44	6103.9 ± 11,178.8 1057 [389.5–4789.0]

Note the large difference between mean and median in the different CKD groups highlighting the non-Gaussian distribution of the iFGF23 distribution in these patients with some of them presenting very high levels. All groups differed significantly from each other ($p < 0.001$ at least)

We found that iFGF23 concentration was stable in EDTA plasmas stored at RT for up to 24 h, a result which is consistent with published results obtained with two other iFGF23 assays [18]. On the contrary, we found a slight but significant loss of concentration when serum iFGF23 was measured after 4–24 h at RT. It was shown in [18] that five freeze/thaw cycles did not affect the iFGF23 concentration, a result also reported by DiaSorin in the package insert of their iFGF23 kit. We confirmed that the type of sample is of great importance with the finding of huge differences between serum and EDTA plasma values. In addition to a slightly better stability of iFGF23 in EDTA than in serum, we found that the relative difference between serum and EDTA values varied with iFGF23 concentration with greater differences for the lowest values making that some normal subjects have a serum iFGF23 level close or below the LOQ. We thus recommend the use of EDTA plasmas only to measure iFGF23 with this assay.

We established reference values for the Liaison iFGF23 assay from a well-defined cohort of healthy French adults. It must be underlined that these reference values may not be applicable to other populations, in particular in less industrialized countries. Indeed, it has been shown that FGF23 levels differ across populations by degree of industrialization with lower values in less industrialized countries [19]. This is probably due to higher phosphate intake in more industrialized countries with higher consumption of meat and processed food preserved with phosphate additive. In our healthy subjects, we found that the distribution of the iFGF23 concentrations was Gaussian unlike Smith et al. who found a right-skewed distribution with the Immutopics iFGF23 kit in 170 healthy adults with a mean age of 56 years and an eGFR >60 mL/mn/1.73 m^2 [20]. This may be due to the use of different iFGF23 assays in [20] and in the present study and/or to the inclusion of outliers in [20]. Indeed, as indicated above, the recruitment of the VARIETE participants was drastic and we excluded 60 apparently healthy subjects because of abnormal biological values in their screening evaluation. It is thus possible that some of the apparently healthy subjects of the Smith's study would have been excluded from the VARIETE population. Men had a significantly, albeit slightly, higher iFGF23 level than women while Yuen et al. found the contrary in black adults from different countries [19]. As these authors studied subjects aged 25–45 years, they hypothesized that this difference might have been due to the effects of estradiol which has been shown to stimulate FGF23 production [21]. In our population however, women aged 18–29 years had a lower iFGF23 (54.3 ± 16.9 ng/L) than women aged 60 and more (59.1 ± 17.7 ng/L) on the one hand, and men aged 18–29 years (59.2 ± 16.9 ng/L) on the other hand, a result not in favour of this hypothesis. As Yuen et al. used a C-terminal FGF23 assay in their study, a more likely explanation for the higher FGF23 level found by these authors in women may be that young women have generally lower iron stores than men due to menstrual bleeding, and that iron deficiency increases the intracellular cleavage of FGF23 and increases the release of C-terminal fragments. The reason for the higher iFGF23 in men than in women in the present study may be due to higher phosphate intake in men than in women. Unfortunately this can only be considered as an hypothesis as phosphate intake was not recorded in the VARIETE study. We also found that subjects aged 60 years and more had higher iFGF23 levels than younger subjects. We hypothesized that this might be due to lower GFR in the older subjects. However, excluding those with an eGFR <75 mL/mn had no effect on the iFGF23 values, a result consistent with the lack of correlation between eGFR and iFGF23 in multiple regression analysis. It must be underlined that we estimated GFR with the CKD-epi formula which does not take into account the loss of lean mass in the elderly so that the frequency of impaired renal function may have been underestimated compared to a direct measurement of GFR with a reference method such as the iohexol clearance. In our group of healthy subjects considered as a whole, iFGF23 concentration was negatively and significantly correlated with $1,25OH_2D$ as found by

others [22]. This can be explained by the fact that FGF23 inhibits $1{,}25OH_2D$ synthesis on the one hand, and as $1{,}25OH_2D$ stimulates FGF23 secretion on the other hand [23].

FGF23 testing is essential in the differential diagnosis of hypophosphatemia. Here, we asked our XLH patients to stop their phosphorus and active vitamin D therapy for 7–10 days as this treatment is known to increase FGF23 circulating levels [24]. All our patients with TIO and all but one patient with XLH had an iFGF23 level above the ULN found in our healthy subjects. This is consistent with the previously published series of TIO or XLH where most patients had an elevated FGF23 concentration [25–27]. The only XLH patient with a normal iFGF23 value had a concentration close to the ULN which may be considered as inappropriate with regard to its hypophosphatemia.

We tested one hyperphosphatemic patient with a *GALNT3* mutation. Like in some patients described in the previous series of *GALNT3* mutations [10], its iFGF23 concentration was in the low-normal range at a level that is associated with a normal phosphatemia in the general population. This can be surprising as an undetectable iFGF23 level could have been expected because, in this pathology, hyperphosphatemia and calcinosis are due to an instability of the FGF23 molecule inducing an absence of FGF23 effects. A probable explanation relates to the very high concentration of C-terminal FGF23 fragment which has been shown, in a rat model, to compete with iFGF23 for binding to the FGFR–Klotho complex, antagonizing thus the action of any residual iFGF23 in these patients [28].

In patients with CKD, we found the expected increase in iFGF23 levels paralleling the decrease in eGFR, with a mean concentration in dialysis patients that was more than 100 times higher than the mean value found in the healthy controls. This increase in iFGF23 levels occurs early during the course of CKD as reported by others [11] and as highlighted by the 70% higher mean concentration in our stage 3a CKD group than in healthy controls. This early increase in FGF23 may be an adaptative response to phosphate retention but other reasons have also been suggested, such as a decrease in Klotho that induces a resistance to FGF23 effects. It has been shown in several observational prospective cohort studies performed in patients with CKD, either dialyzed or not, including renal transplant recipients, that those with the highest elevations in FGF23 circulating levels have a significantly increased risk of cardiovascular and all-cause mortality as well as cardiovascular events and faster progression of CKD [29–35]. This may be the basis for recommending routine FGF23 measurement in patients with CKD [36]. However, whether this association between higher FGF23 and mortality is causal remains to be fully demonstrated, even if experimental data showing that FGF23 directly induces left ventricular hypertrophy in mice through Klotho-independent effects are consistent with this causality [37]. The definitive evidence in favour of routine FGF23 testing in CKD would come from placebo-controlled intervention studies showing that decreasing FGF23 secretion in CKD patients has beneficial effects. However, targeting only FGF23 in CKD patients with, for example, a monoclonal anti-FGF23 antibody is now considered with caution as such an intervention in a rat model of CKD–mineral and bone disorders caused severe hyperphosphatemia resulting in vascular calcifications and increased mortality [38]. Other medications such as non-calcium phosphate binders and calcimimetics are able to decrease FGF23. In a secondary analysis of the EVOLVE study, a large randomized clinical trial comparing cinacalcet and placebo in addition to conventional therapy in dialysis patients, FGF23 levels significantly decreased [39]. It was shown that a $\geq$30% reduction in FGF23 between baseline and week 20 was significantly associated with a reduction in the primary composite endpoint (time to death or a first non-fatal cardiovascular event), cardiovascular mortality, sudden cardiac death and heart failure. Although it was not possible to discern whether these associations were due to a direct or indirect effect of FGF23, it must be noted that the observation was independent of usual confounders. The "pros and cons" for the integration of FGF23 measurement in the management of CKD patients will certainly be actively discussed by nephrology experts during consensus conferences such as an updating of the KDIGO guidelines [40]. It must also be remembered that, until recently, no FGF23 assays were approved for clinical use and that this represented a brake to recommend routine FGF23 testing in CKD. This point at least is now solved with the recent availability of the Liaison iFGF23 assay evaluated in the present study.

In conclusion, we have evaluated a new fully automated immunoassay for iFGF23 that is now approved for clinical use by the European Community authorities. This assay presents excellent analytical characteristics. We established adult reference values and obtained the expected concentrations in patients with various disorders of phosphate metabolism and in patients with CKD. The availability of this new assay on an analytical platform that already allows the measurement of other important parameters of the mineral metabolism (PTH, 25OHD, $1{,}25OH_2D$ and several bone markers) is a real improvement for the laboratories and clinicians/researchers involved in this field.

Compliance with Ethical Standards

Conflict of interest JCS reports lecture fees and/or travel/hotel expenses from DiaSorin, Roche Diagnostics, Abbott, Amgen, Shire, MSD, Lilly and Rottapharm; EC is a consultant for IDS and DiaSorin

and has received lecture fees from IDS, DiaSorin, Roche, Abbott and Amgen; PD is a consultant for IDS and has received lecture fees and/or travel expenses from DiaSorin, Amgen, Shire, Fresenius, Menarini and Sanofi; DP, M-LP, AR and PC have nothing to disclose.

Human and Animal Rights and Informed Consent All procedures performed in our patients/healthy subjects were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its latter amendments or comparable ethical standards. All healthy subjects and patients gave informed consent to have their blood sample tested.

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