Review

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Fibroblast growth factor 23: translating analytical improvement into clinical effectiveness for tertiary prevention in chronic kidney disease

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Abstract

Objectives: Fibroblast growth factor 23 (FGF23) plays a key role in the pathophysiology of chronic kidney disease (CKD) and of the associated cardiovascular diseases, ranking on the crossroads of several evolving areas with a relevant impact on the health-care system (ageing, treatment of CKD and prevention from cardiovascular and renal events). In this review, we will critically appraise the overall issues concerning the clinical usefulness of FGF23 determination in CKD, focusing on the analytical performances of the methods, aiming to assess whether and how the clinical introduction of FGF23 may promote cost-effective health care policies in these patients.

Content: Our comprehensive critical appraisal of the literature revealed that we are currently unable to establish the clinical usefulness of FGF23 measured by ELISA in CKD, as stability issues and suboptimal analytical performances are the major responsible for the release of misleading results. The meta-analytical approach has failed to report unambiguous evidence in face of the wide heterogeneity of the results from single studies.

Summary and Outlook: Our review has largely demonstrated that the clinical usefulness depends on a thorough analytical validation of the assay. The recent introduction of chemiluminescent intact-FGF23 (iFGF23) assays licensed for clinical use, after passing a robust analytical validation, has allowed the actual assessment of preliminary risk thresholds for cardiovascular and renal events and is promising to capture the iFGF23 clinically relevant changes as a result of a therapeutic modulation. In this perspective, the analytical optimization of FGF23 determination may allow a marriage between physiology and epidemiology and a merging towards clinical outcomes.

Keywords: harmonization; healthcare; immunoassay; outcome; stability.

Introduction

Chronic kidney disease (CKD) affects more than 50% of individuals older than 75 years, it is rather uncommon but equally harmful in paediatric population since associated with increased cardiovascular disease (CVD) morbidity and mortality (i.e., CVD is responsible for approximately 50 and 25% of deaths in stage 5 CKD in adult and paediatric populations, respectively) [1–3]. With the ageing of the general population, morbidity and mortality trends are likely to increase and further acceleration of ageing has been causally associated to CKD through two distinct mechanisms: (a) increase in phosphate and (b) alteration of the associated endocrine axis [2]. In particular, the latter appears to play a crucial role in ageing progression and to this end recent studies have focused on evaluating the effects of increased fibroblast growth factor 23 (FGF23) biochemically and

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biologically characterized in Table 1 and Klotho co-receptor deficiency, both of which are pathognomonic of CKD [4–11]. Being strictly associated with aging, CKD represents a major public health problem due to poor health outcomes and very high health care costs [12, 13]. The main objectives of CKD management are: (a) to delay the rate of progression to stage 5 CKD, (b) to limit CVD morbidity, (c) to preserve health related quality of life (HRQoL), which is in itself compromised in these patients, (d) to restrain healthcare costs [12, 13]. Due to the heterogeneity of patients and the aetiology of the underlying disease, risk scores need to be refined to identify patients most likely to benefit from an intervention (dialysis vs. conservative management, use of phosphate binders), considering the gain in life expectancy and HRQoL [13]. Economic models, developed to simulate long-term outcomes of CKD to inform cost-effectiveness evaluations of treatments, could benefit from prognostic markers, whose changes in the pattern are associated with changes in risk and the effects of pharmacotherapy [13].

To these aims, the Kidney Disease Improving Global Outcomes (KDIGO) working group approved of investing in more research that contributes to understanding the clinical usefulness of the aforementioned FGF23, which increases in very early stages of CKD, even before the onset of appreciable hyperparathyroidism and hyperphosphataemia [12]. FGF23 was initially identified as a phosphatonin, and its effect on

Table 1: FGF23 characterization and biological loop in CKD.

FGF23 structure and molecular features	Native peptide: 251 amino-acid (aa) Secretory protein: 227 aa, 32 kDa	
FGF23 release	Mainly by osteocytes and osteoblasts	
Tissue expression	Heart, liver, thyroid, parathyroid, small intestine, testis and skeletal muscle	
Main causes of FGF23 elevation	Physiological condition	– Increased serum phosphorus concentrations[a] – Increased calcium concentrations (calcioprotein particles) – Increased 1,25 $[OH]_2D$ (released by kidney)[a] – PTH increase
	Nephron loss[b]	– Predominant effect of 1,25 $[OH]_2D$ increase (released by osteoblast/osteocyte) – Increased serum phosphorus (lowest effect) – PTH mediation excluded
Additional factors	Conditions: diabetes type 1 and 2, inflammation, insulin resistance	**Positive regulator of transcription**: iron deficiency, pro-inflammatory cytokines (IL1B, IL6, IL10), NF-kB activation, erythropoietin, leptin, aldosterone. **Negative regulator of transcription:** insulin, adiponectin
FGF23 resistance	Lowest klotho expression in parathyroid gland and kidneys	Physiological effects – No phosphaturic effects[c] – No counter-regulatory effect on PTH secretion Clinical effects – Secondary hyperparathyroidism, – Vascular calcification – Left ventricular hypertrophy
Endocrine function	Receptor	Klotho
	Main targets	– Renal proximal tubule – Parathyroid glands – Intestine
	Additional targets	Heart (cardiac fibrosis, left ventricular hypertrophy, arrhythmia), Liver (inflammatory cytokines synthesis) Immune system (inhibition) Skeleton (demineralization) Bone Marrow (decrease erytrophoiesis)
	Main actions of FGF23 increased levels	– Phosphatonin (enhancement of renal excretion/decrease intestinal absorption of phosphate) – Decrease the synthesis of 1,25 $[OH]_2D$ (inhibition of renal 1-α-hydroxylase) – Decrease the secretion of PTH – Increase of blood pressure

FGF23, fibroblast growth factor 23; 1,25 [OH] D_2, 1,25-(OH_2) vitamin D; PTH, parathyroid hormone. [a]Both in equal contribution and under PTH mediation. [b]In this condition FGF23 early increases with respect to PTH, D vitamin and phosphate alteration. [c]Experimentally observed in absence of a PTH effect and in presence of increased PTH levels. The information reported in Table 1 was extracted from Supplementary References [5–11].

lowering serum 1,25-(OH_2) vitamin D_3 [1,25(OH_2)D_3], although prominent, has not been consistently observed, and thus its central role in vitamin D metabolism has been substantially overlooked [4, 14, 15]. Several clinical and basic research studies have contributed to the construction of the complex feedback loop involving the regulation and secretion of FGF23 in CKD [12, 14]. Further data have characterized how increased FGF23 levels can promote the suppression of 1,25(OH_2)D_3 and trigger the development of secondary hyperparathyroidism in the early stages of CKD and left cardiac ventricle hypertrophy through a cardiac receptor that is not Klotho-dependent [4]. A large body of evidence on FGF23 suggests that in CKD circulating increased levels: (a) correlate with decline in glomerular filtration rate, (b) are associated with disease severity (i.e., highest concentrations in stage 5 CKD), (c) are independent predictors of deterioration in renal function (even after adjustment for key indices of mineral metabolism), (d) indicate the need for replacement therapy/dialysis in diabetic and non-diabetic patients [15–20]. Despite some controversial data, in patients with CKD elevated FGF23 levels appear to be associated to increased all-cause and cardiovascular mortality rates and poor cardiovascular outcomes [17, 18, 20]. However, there are no clear indications on the absolute levels and the percentage increases of FGF23 that can be considered predictive for poor outcomes, and several authors have pointed to the lack of harmonization of the FGF23 assays as major limitation in estimating risk thresholds [21].

Here, we critically appraise all these issues with a particular focus on the analytical performances of the methods, with the aim of assessing whether and how the determination of FGF23 may promote cost-effective health care policies in patients with CKD.

FGF23 in CKD: analytical issues and clinical usefulness

To define the analytical issues and the clinical usefulness of FGF23 a systematic review of the literature published since 1965 up to May 2022 was performed by searching the PubMed and Embase databases, using “Fibroblast growth factor 23 and chronic kidney disease” as key words and setting “humans” as filter. The search resulted in 169 clinical trials and randomized controlled trials and 592 narrative and systematic reviews (SRs), documents and metanalyses. The original list of clinical studies was compared to those quoted in the reviews and metanalysis. Among all retrieved articles, we finally considered 52 original papers reporting on the clinical value of FGF23 determination in CKD and on analytical issues, as eligible for our further evaluation and analysis, to reinforce and contribute additional evidence to the basic one retrieved from updated SRs and metanalyses. The following selection criteria were applied to the original articles:

(a) Determination of FGF23 in plasma/serum using immunoassays licensed for research and clinical use; (b) estimation of the analytical performances of FGF23 assays; (c) estimation of the clinical performances of FGF23 testing: (1) in risk stratification of CKD, (2) in prediction of renal failure progression, of adverse outcomes (CVD and mortality), and (3) as therapeutic target; (d) the papers selected at point (c) were retained if FGF23 average and/or median levels in the investigated case series were shown.

The clinical usefulness of FGF23 measurement in CKD patients as a predictor of adverse outcome has been investigated by a large body of clinical research studies employing manual double antibody sandwich enzyme-linked immunosorbent assays (ELISA) with colorimetric readout approved for research use only [21, 22]. Several SRs have thoroughly described for all immunoassays the analytical performances (i.e., design antibody specificity, calibration) together with the pre-analytical pitfalls (i.e., proteolysis of the forms, interfering substances), which did not follow a pre-marketing rigorous evaluation and were investigated after the publication of the epidemiological studies [21–27]. Expert opinions emphasized the lack of harmonization of the FGF23 immunoassays used as the main cause of heterogeneity and controversy in the published trials, neglecting the critical issues concerning the stability of the measurand and therefore the impact on the reliability of the results [21, 28–31]. Stability issues should be accounted when discussing whether measurement of the intact form (iFGF23) or both iFGF23 and C-terminal fragments (cFGF23, only detectable by Immutopics assay) could be more robustly associated with hard endpoints in the CKD population. There is a consensus on the evidence that in dialysis patients, iFGF23 appears to be the predominant circulating form with a biological and physiological relevance greater than cFGF23 [20, 32, 33]. Conversely, some evidence report that cFGF23 outperforms iFGF23 (both by Immutopics assay) in predicting decline in renal function [15, 34]. Sound literature reports that iFGF23 levels (Millipore assay) decrease significantly after successful renal transplantation and remain within normal limits with stable graft function. The role of FGF23 in inorganic phosphorous homeostasis is more prominent in the early period after transplantation [35]. Abnormalities in phosphate homeostasis are common in renal transplant recipients, ranging from hypophosphataemia within

3 months after transplantation to hyperphosphataemia, hyperparathyroidism, and high FGF23 levels in the late post-transplantation stage [35]. Persistent high levels of FGF23 and intact parathyroid hormone (iPTH) during the first months post-transplantation, and the influence of immunosuppressive drugs, ischaemia–reperfusion injury, and metabolic acidosis, can result in post-transplantation hypophosphataemia [35]. Long-term exposure to high levels of phosphate and FGF23 likely have distinct yet associated adverse effects on the cardiovascular system, kidney, and bone in renal transplant recipients and although data are limited, renal transplant recipients might benefit from dietary and pharmacologic interventions to improve phosphate metabolism [36].

Only iFGF-23 levels measured by the Kainos assay compared to cFGF23 appear to be modulated by dietary phosphate intervention, and sensitive to binder type [37, 38]. The lack of comparative data using Kainos and Immutopics iFGF23 in the same studies, and some evidence of significant association shown only for the former strengthened the hypothesis that significant effects may depend on the assay used and not only on the form tested [28, 34, 39]. The Kainos and Immutopics iFGF23 assays still differ in epitope stability, although a greater improvement was obtained for the latter with the second-generation release [27]. Suspected loss of immunoreactive iFGF23 due to degradation was the most likely explanation for undetectable concentrations with the first-generation Immutopics assay reported in some clinical studies, and this was largely attenuated by the use of protease inhibitors [28, 30, 31]. Pre-analytical sample handling conditions undoubtedly played a crucial role in the stability of the detected form. Delayed centrifugation of samples for 6–8 h implies a 45, 23, 7 and 5% reduction of iFGF-23 concentrations in 1st, 2nd generation Immutopics, Millipore and Kainos assays, respectively and no significant decrease for the chemiluminescent immunoassay (CLIA) DiaSorin [27, 40]. Delayed retention affected only Immutopics 1st generation assay (40% decrease) [27]. Considering that most of evidence on FGF23 relates to sub-studies of clinical trials it is important to consider that 3 repeated freeze–thaw cycles, implied an average loss of iFGF23 immunoreactivity of 37 and 11% for Immutopics and Kainos respectively [28, 41]. The aforementioned pre-analytical issues, in addition to differences in assay design and epitope recognition have strongly influenced the agreement between results. The widely emphasized "lack of a reference material" to build a metrological traceability chain allowing for standardization and harmonization of the assays is undoubtedly a great drawback to the inference of the results of epidemiological studies, and may undermine the clinical usefulness of the marker [21]. However in the context of CKD, it is well reported that also the harmonization of iPTH immunoassays is challenging, making it difficult to assess true PTH [1–84] concentrations over a broad range of estimated glomerular filtration rates. Anyway, the disagreement between the results of different assays does not prevent the clinical use of iPTH immunoassays [42]. Data on the comparison between iFGF23 methods are very scarce and of low methodological quality (i.e., unclear use of 1st or 2nd generation Immutopics assays, no compliance to Measurement Procedure Comparison and Bias Estimation, missing/unreliable estimation of the inter-methods agreement). In most cases, the simple visual inspection of the correlations between iFGF23 results obtained by Kainos and Immutopics 1st and 2nd generation assays on the same clinical case series has fuelled speculation about the poor ELISA agreement [30, 31, 33]. Under-recovery and negative proportional bias were reported for the iFGF23 Immutopics assay compared to Kainos (–22%) on highly-purified FGF23 standards and compared to DiaSorin (–25%) on CKD patient samples [22, 40, 43]. Kainos assay appeared to exhibit an over-recovery at high iFGF23 concentrations of standard preparations vs. Millipore, not confirmed by spiking FGF23 in human sera (recovery 56 vs. 80% respectivssely) [22, 41]. Paradoxically, visual inspection of the correlation (r^2=0.97) between iFGF-23 results obtained by Millipore and Kainos assays was considered enough for the validation of the former method to be applied to CKD patients as it has a 3-fold higher upper calibration level [41].

One of the main improvement to promote cost-effective applicability of the test in overall stages of CKD was the extension of calibration range of CLIA (up to 5,000 ng/L) with respect to ELISAs (800 ng/L of Kainos, and 2,200 ng/L Immutopics, 2,400 ng/L Millipore). Preliminary data suggested that only ~25% of dialysis patients may require sample dilution which has no effect on measurement reliability, while this implied a significant deviation from linearity for the ELISA manual assays [22, 40].

Getting value to FGF23 among cardiovascular markers in CKD

Several authoritative SRs and opinion papers support the "clinical utility" of FGF23 in whole CKD population to improve risk stratification for renal failure progression, CVD and mortality, while no data yet endorse its use as therapeutic target [5, 12, 21, 26, 37]. However, most evidence is simply based on statistically significant associations between increased level of the marker and adverse outcomes, thus suggesting that FGF23 might be a candidate prognostic

marker in these patients, being probably involved in the causal pathway to cardiovascular pathology (left ventricular hypertrophy, arterial stiffness) as effect of its altered endocrine feedback loop and increased concentrations [14]. Further evaluations focused on the added contribution of FGF23 (i.e., C statistics, net reclassification index (NRI)) to other risk factors have provided contrasting data, and the clinical usefulness appears to be biased towards prediction of cardiovascular events in overall CKD patients and this in particular in those on haemodialysis showing a wide dispersion of FGF23 concentrations (Table2) [5, 15, 26, 37, 40, 43–58].

For clinically validated cardiovascular biomarkers (i.e., high sensitive troponins, natriuretic peptides [NP]) significant associations emerged between elevated levels and adverse outcomes across overall CKD stages, being stronger in dialysis-dependent than in non–dialysis-dependent patients and in those with stage 4–5 CKD than in stages 1–3 (i.e., simple comparison of areas under the ROC) [2]. Contrasting evidence emerges from two meta-analysis of data on the prognostic value of FGF23. The wide heterogeneity of FGF23 results from the various assays (and forms) within the same CKD stages and their highly skewed distributions (Table 2) reflects the

Table 2: IFGF23 concentrations reported by clinical studies according to the various assays used in the clinical investigations and the CKD stage.

CKD	Sample size, n	iFGF23 concentrations, pg/mL	iFGF23 assay	Study reference
Stage 3a	43	Median (IQR) 103.6 (59.2, 129.0)	DiaSorin	43
Stage 3	38	Median (upper-lower range) 58 (24, 201)	Kainos	44
	30	Median (IQR) 24.5 (23.2, 30.3)	Millipore	45
	63	Median (IQR) 30 (19, 44)	Immutopics	15
	1,664	Median (IQR) 42 (33–53)	Kainos	46
	109	Median (IQR) 68.8 (51.2, 85.2)	Kainos	47
	525[b]	Median (IQR) 135.5 (104.4, 187.2)	DiaSorin	48
	205	123.8 (94.1, 164.0)		
	29	Median (5th, 95th percentile) 176 (144–211)	DiaSorin	40
Stage 3b	14[a]	Mean (SD) 90.7 (28.4)	Kainos	49
		Mean (SD) 110.2 (73.7)		
	43	Mean (SD) 130.8 (88.6)	DiaSorin	43
		Median (IQR) 119.2 (73.6–159.4)		
Stage 3-4	40	Median (IQR) 97 (64–142)	Kainos	50
	200	Median (IQR) 61 (46, 76)	Immutopics	51
Stage 3b-4	88	Median (IQR) 120 (88–176)	Immutopics	37
	57	119 (95–169)		
	205	Median (10th, 90th percentiles) 99 (59, 205)	Kainos	52
Stage 4	43	Median (IQR) 216.3 (130.2, 307.8)	DiaSorin	43
	265[b]	192.9 (142.3, 292.9)	DiaSorin	48
	31	Median (5th, 95th percentile) 239 (199, 411)	DiaSorin	40
Stage 4/5	43	Mean (SD) 77 (83)	Immutopics	15
	173[b]	Median (IQR) 318.6 (179.4, 545.4)	DiaSorin	48
	176	278.7 (178.9, 450.5)		
	32	Mean (SD) 226 (11)	Kainos	53
Stage 5	44	Median (IQR) 350.0 (181.3, 602.6)	DiaSorin	43
	75	Median (5th, 95th percentile) 855 (379, 24, 925)	DiaSorin	40
Haemodialysis	1,340	Median (IQR) 3,118 (726, 12, 928)	Kainos	54
	44	Median (IQR) 1,057 (389.5–4, 789.0)	DiaSorin	43
		Mean (SD) 6,103.9 (11, 178.8)		
	2,985	Median (10th, 90th percentile), 5,555 (580, 19, 540)	Millipore	41
	229	Median (10th, 90th percentile), 2,526, (431, 19, 495)	Kainos	55
	92	Mean (SD) 1,336 (2, 164.7)	Kainos	56
	549	Mean (SD) 100,654.1 (198, 581)	Immutopics	57
		Median (IQR) 39,600 (11, 300; 90, 300)		
	165	Median (IQR), 382 (145, 2977).	Millipore	58

IQR, interquartile range; SD, standard deviation. [a]Same group randomized 1:1 after washout. [b]Vitamin D supplemented.

aforementioned analytical difference in detection and further contribute variability to the heterogeneity of individual risk profiles at baseline [15, 37, 40, 43–58]. By considering the different fonts of variability and heterogeneity there is a high risk to obtain biased cumulative relative risk (RR) estimates from pooling data. Paradoxically Marthy et al. observed that RRs do not follow the trends of FGF23 increase across the pooled studies, and significant association of "elevated FGF23" (i.e., generally the third quartile of the distribution of FGF23 levels in each study) with mortality are observed in CKD patients not on dialysis despite exhibiting much lower absolute levels vs. those on dialysis [59]. In contrast, Gao et al. found in maintenance haemodialysis patients that "elevated FGF23" was associated with a 25 and 22% increased risks of all-cause mortality and cardiovascular events [60]. To translate clinical usefulness into effectiveness, clinical research would need to provide robust glomerular filtration rate-specific FGF23 predictive thresholds for risk stratification (i.e., higher gradations of thresholds for increasing CKD stages). These were provided only more recently by one study which used a clinically validated assay (CLIA DiaSorin) first, and demonstrated this added test improved the risk classification (i.e., NRI) for cardiovascular (≥177.7, 228.1, 528.5 pg/mL in CKD stage 3, 4 and 4–5 respectively) and renal events (≥141.2, 192.5, 311.1 pg/mL in CKD stage 3, 4 and 4–5 respectively) [48]. Assessing FGF23 thresholds is undoubtedly challenging considering the hormonal nature as NP and that likewise its increase is modulated by several factors heterogeneously distributed within CKD population in addition to renal clearance and vitamin D supplementation. Decreased renal clearance with solute retention, general chronic inflammation associated with CKD, and extensively overhydration in stage 5 CKD patients on haemodialysis, cause a spurious highest increase of FGF23 levels (and of other cardiovascular markers) independently of the cardiac history of the patients [61]. These need to be considered to avoid overestimates of FGF23 predictive value. Indeed, deranged fluid homeostasis and chronic fluid overload are *per se* associated to an increased risk of cardiovascular events and mortality which however is likely to be further enhanced by the statistically significant interaction between high concentrations of these markers (well described for NP) and overhydration [62]. Higher exposure to adverse events according to the combination of cardiovascular markers' increase and overhydration should receive more attention in this context to endorse their clinical introduction. In 1/5 of patients on dialysis a major cardiovascular event or death occurs [62, 63].

Managing variability issues

Most of the clinical interest relates to dialysis-dependent patients and clinical research of NP in this context has shown that it is crucial to account for the interplay between: (a) time/conditions of sample collection, (b) half-life and biological variability (BV) of the forms (NT-proBNP/BNP; iFGF23/cFGF23), (c) analytical performances of the assay used. In this context the first two issues are crucial as both NP and FGF23 can reflect differences in changes in volume status and dialyzer clearance [61]. Half-life should be long enough to avoid plasma concentrations changing significantly during the dialysis session, which is why NT-pro-BNP has been considered more stable and reliable than BNP (half-life ~70 vs. ~20 min) [62]. Similarly, iFGF23 (Kainos) seems to outperform to c-FGF23 (Immutopics 1st generation), having an average half-life of ~58 vs. ~46 min, although c-FGF23 (Immutopics 2nd generation) does not seem to change significantly during the single haemodialysis session [64]. Furthermore, to minimize the effect of volume status, the blood sample should be obtained when some marker stability is reached after the post mid-week dialysis session [62]. Most investigations consider samples obtained just prior the first dialysis session, when highest concentrations reflect overhydration, whereas a consistent but variable decrease occurs thereafter with its treatment [62].

Additional sources of variability must be considered when assessing which form and assay is useful for: (a) predicting adverse outcomes; (b) being a surrogate index of treatment effects; (c) applied on a cluster of critical patients. Circadian rhythm, post-prandial modulation and BV (Table 3) are probably non-influential factors of variation when considering the greater intra- and inter variability of levels in haemodialysis patients [29, 32, 65]. The use of early morning fasting samples allows us to overcome the first two factors of variation, but we are unable to speculate on the effect of BV on i/c FGF23 concentrations in patients with mild to severe stages of CKD, who show a wide dispersions of FGF23 concentrations (Table 2), although under stationary conditions cFGF23 levels tends to remain stable over time [16]. Furthermore, the much lower BV CVs of iFGF23 DiaSorin vs. 2nd generation Immutopics assays likely reflects the difference in the epitope identification and not only an improved precision, and this can probably explain the dependence of the effect size on the assay used. Finally, the RCV is much lower for cFGF23 vs. iFGF23 and this reasonably suggests that the first marker could theoretically be considered as a more reliable index of marker change in response to treatment.

Table 3: Biological variability of iFGF23 and cFGF23 according to the population.

Form (assay)	CVi	CVg	RCV	Study reference	Population
cFGF23 (immutopics 2nd)	8.3	28.9%	25	32	Healthy individuals
iFGF23 (immutopics 2nd)	18.0	19.0	54	32	
iFGF23 (DiaSorin)	12.5%	13.4%	41	65	
cFGF23 (immutopics 2nd	36%	203.2%		29	Stable haemodialysis patients (predialysis samples)

iFGF23, intact fibroblast growth factor 23; cFGF23, C-terminal fragments FGF23; CVi, intra-individual coefficient of variability; CVg, inter-individual coefficient of variability; RCV, reference change value.

This issue, together with the dependence of BV data on the assay, should however be accounted when assessing (a) the response to phosphate intervention in stage 3–4 CKD and (b) the relationship of FGF23 change with other biomarkers [19, 39, 66, 67]. Accordingly, one might ask whether an average decrease in iFGF23 (Kainos) of 30–40% described in the literature can be considered a response to treatment (causal effect) or due to the BV of the markers (casual effect) [19, 66]. More likely, the 35% decreased concentrations of cFGF23 may be considered clinically relevant, but the actual effectiveness should be demonstrated by the impact on the reduction of CV morbidity/mortality rates [19].

The main challenge: FGF23 use in children with CKD

The epidemiology of CKD in children is showing an increasing trend of occurrence, mainly related to the long-term management of the disease and early diagnosis, thus proving the need of the promoting the use of biomarkers for tertiary prevention. Concerning the aetiology of CKD in children, the increasing incidence of low-birth weight, intrauterine growth retardation and small for gestational age newborns related to the growth in the number of premature children together with the exploding burden of paediatric obesity, are likely destined to significantly change the relative distribution of the causes of CKD [68].

Success with replacement therapy has actually improved morbidity and mortality trends in children with CKD. However, CVD remains the leading cause of death in both children with stage 5 CKD and in adults with childhood onset of CKD [3, 69, 70]. Death rates for CVD are similar in children on peritoneal dialysis and haemodialysis, whereas transplant recipients have a relatively lower risk of cardiovascular death [71].

Several studies in children with CKD have reported subclinical evidence of atherosclerosis with intimal plaque, medial vessel/soft tissue calcification, increased coronary calcium burden, coronary artery calcification, early significant left ventricular hypertrophy and dysfunction, carotid arterial wall and aorta stiffness [72, 73].

Twenty percent of hospitalizations in paediatric stage 5 CKD patients are reported to be due to arrhythmias, 10% to cardiomyopathy, and 3% to a cardiac arrest and this latter is the earliest (0–4 years) and the most common cause of cardiovascular death, followed by arrhythmia, cardiomyopathy, and cerebrovascular disease, with myocardial infarction rarely reported [70]. Cardiovascular morbidity and mortality in adulthood have roots in childhood, and recommendations are directed toward children with CKD stage 5, undergoing dialysis, and renal transplant recipients [12]. Accordingly, chronic cardiovascular risk factor reduction (mostly treatment of hypertension and of dyslipidaemias) is an essential part of clinical management of CKD in childhood. Furthermore, slowing the progression of CKD, avoiding long-term dialysis and, if possible, conducting preventive transplantation are reported to be the best strategies to decrease the risk of premature CVD in young CKD patients [73].

The traditional lipidic profile is recommended to be measured in childhood with CKD, being a good surrogate marker for predicting adult CVD (odds ratio 5.85 (95%CI 2.33–14.7) for triglyceride levels ≥110 mg/dL). The related PPV (7%) is however actually too low to consider the exclusive use of lipidic profile to promote preventive programs [74]. Therefore, current evidence support that additional markers, as FGF23, whose elevation carries a causal role in the progression of cardiovascular alterations, typical of these patients, should be introduced in the clinical management of CKD in children and adolescence [75, 76]. FGF23 was shown to induce hypertrophy of cardiomyocytes and left ventricular hypertrophy in experimental models of CKD via a direct interaction with the receptor FGFR4 [77, 78]. In myocardial autopsy specimens of the left ventricle from individuals with childhood-onset stage 5 CKD left ventricular hypertrophy was associated with upregulation of FGFR4 and activation of the calcineurin-nuclear factor of activated T cell signalling pathway [79].

The aforementioned clinically used biomarkers of CVD risk (NP, troponins) are well validated as surrogate predictive tools of future cardiovascular death in adult CKD patients, but their clinical effectiveness needs to be demonstrated in paediatric patients and for FGF23 only few observational studies are currently available in childhood and adolescence.

The first studies reported in children with stage 3 CKD FGF23 far higher levels than those detected in early stages (average concentrations of 78 ± 83 ng/L and 111 ± 117 RU/mL for iFGF23 and cFGF23 both measured by Immutopics) [31]. Furthermore, FGF23 concentrations (Kainos) were found to significantly increase according to the severity of CKD in children (144 ± 91, 313 ± 275, 734 ± 397 ng/L for CKD stage 3, 4 and 5 respectively), and to predict hyperphosphatemia in stage 4 disease [80]. Furthermore, paediatric patients (age 13 ± 6 years) with high turnover renal osteodystrophy and treated with maintenance peritoneal dialysis were reported to be characterized by high median iFGF23 and cFGF concentrations (74, IQR: 57–115 pg/mL and 514, IQR: 373–893 RU/mL, respectively, both measured by Immutopics). In this cohort FGF23 increasing concentrations were associated with improved indices of skeletal mineralization and accordingly its use has been suggested as non-invasive tool for the diagnosis of skeletal mineralization defects in paediatric stage 5 CKD patients with secondary hyperparathyroidism [81]. In children and adolescents with pre-dialysis CKD (median age 12, IQR: 8–15 years) and with eGFR<45 mL/min per 1.73 m^2, cFGF23 (2nd generation Immutopics) concentrations ≥170 RU/mL were independently associated with a higher prevalence of left ventricular hypertrophy and the odds of left ventricular hypertrophy was three times higher than in those with <FGF23 levels 100 RU/mL [1]. Importantly, as a continuous variable, each doubling of FGF23 was associated with two-fold higher odds of left ventricular hypertrophy. The associations remained significant after adjusting for haemoglobin level and systolic blood pressure, this latter being reported as a strong independent predictor of left ventricular hypertrophy in children with CKD [1]. In advanced CKD, factors including hypertension and high BMI are relatively stronger determinants of left ventricular hypertrophy than FGF23 [1]. These findings are consistent with previous evidence on children and adolescents (sample size=26, aged 6–21 years) receiving haemodialysis and reporting that 1 SD increase in log-transformed cFGF23 (Immutopics) concentration was associated with a 17% greater left ventricular mass index, but no association was seen with iFGF23 (Millipore) [82]. The highest FGF23 levels were observed in children with concentric left ventricular hypertrophy, similar to findings in adults with CKD [83]. In contrast to previous evidence, some authors reported in CKD stages 2–5 (sample size=83, age 12.1 ± 3.2 years) no association between iFGF23 (Kainos) concentrations and left ventricular mass index [84].

In parallel to adult population, future research should investigate whether FGF23 can be a surrogate marker to guide strategies for the management of phosphorus balance in children with CKD [1].

Undoubtedly, a great challenge will be the assessment of CKD stage specific FGF23 predictive thresholds for risk stratification by CLIA, in this population, since the association of several cardiovascular risk factors with future CVD become stronger with increasing age. This means, first that the "time to progression" is a key element of the disease and a delay in the progression may be obtained by preventive early interventions using surrogate markers as target to tailor treatment, assess related effectiveness and avoid overtreatment. Second, the value of FGF23 increase as risk factor of CVD in children with CKD is potentially even greater than in adulthood, because unlike adults, most children with CKD do not exhibit multiple comorbidities such as preexisting advanced cardiac disease, peripheral vascular disease, or diabetes. Therefore, in the context of CKD in childhood and adolescence FGF23 measurement may gain a crucial role as stand-alone prognostic factor of CVD [1].

Concluding remarks

The interplay of risk factors is complex and risk profiles are heterogeneous among patients with CKD, and KDIGO recently recommended the clinical introduction FGF23 aiming to tailor tertiary prevention programme [12]. Our comprehensive critical appraisal of the literature revealed that the clinical utility of FGF23 in CKD is undoubtedly affected by several limitations of current clinical research including stability issues and suboptimal analytical performances of the first-generation ELISAs. Indeed, the meta-analytical approach has failed to report unambiguous evidence across the same CKD groups and the pooling of results obtained by the determination of cFGF23 and iFGF23 contributed further biological heterogeneity to the wide heterogeneity of the assays. However, it should be emphasized that the analytical evolution of FGF23 immunoassays has thoroughly contributed to disclose the pathophysiological role of FGF23 and may lead to novel approaches to treating inherited and acquired states of FGF23 [14]. Accordingly, several authoritative experts have recognized that "although FGF23 research has been performed by suboptimal methods and methodology, there is however a large body of evidence that testing FGF23 is

likely clinically useful to improve risk assessment in patients with CKD and enhance patients' management upon existing tests" [18]. We can agree that few markers such as FGF23 have a biological background and evidence from basic research linking increase of FGF23 with hard endpoints [14]. However, before introducing a biomarker into clinical research a number of pre-analytical and analytical issues need to be thoroughly examined, as they carry significant economic and practical implications for both the clinical laboratory and the health service alike [14, 85]. Our review showed that pre-analytical issues and analytical performances of ELISA assays were studied long after the publication of clinical research results, and this has contributed to misreporting and wasting research funds. The lack of a reference material and of standardized and harmonized assays, the limited stability of the measurand, the reliability of the results, the and finally the evidence that statistically significant relationships depend on the assay used, all together require caution in the critical evaluation of the evidence [86–88]. The strength of correlations and of associations between iFGF23/cFGF23 or other markers may unpredictably reflect the assay design (i.e., differences in epitope identification) as well as several source of bias (i.e., stability of the epitopes). Furthermore, the evidence that significant correlations were most often obtained with cFGF23 vs. iFGF23 (both Immutopics) or iFGF23 Kainos vs. Immutopics has undoubtedly conditioned the research towards the use of one assay and likely fuelled hypothesis on causality of FGF23 increase as risk factor or therapeutic target [19, 28, 34, 66]. It should be noted that the vast majority of the studies were performed by the use of cFGF23 and, among iFGF23 assays, Kainos was the one used to assess therapeutic effects.

One of the major limitations of clinical research studies on FGF23 was the poor reporting of laboratory methods and of pre-analytical sample management conditions, which likely affected the evidence as most samples were retrieved when performing randomized clinical trials. On the other hand, appropriate pre-analytical sample handling and the effects of interfering variables (haemolysis, lipaemia, bilirubin) have been poorly described in the ELISA assay protocols [21]. With the recent release of automated CLIA, the concentration of interfering substances has been correlated with % variability of iFGF23 concentrations. Several efforts have been made to: (a) optimize epitope stability and specificity (i.e., Immutopic and Millipore assays use 2 polyclonal antibodies, Kainos 2 monoclonal antibodies and DiaSorin 3 monoclonal antibodies), (b) define pre-analytical sample handling conditions, (c) assess the analytical performances (i.e., precision, repeatability, recovery), (d) extend the calibration range to achieve a cost-saving detection in overall CKD stages [21].

In conclusion, KDIGO clarifies the clinical needs that could be met through the clinical implementation of FGF23 determination, while recommending that more robust research results should be provided for clinical validation in CKD [12]. FGF23 has a key role in the pathophysiology of CKD mineral bone disorder and in the associated CVD, and it ranks on the crossroads of several evolving areas having a relevant impact on the health-care system (ageing, treatment and tertiary prevention of CKD) [19]. However the epidemiological impact of abnormalities associated with FGF23 now requires to be approached by robustly designed clinical trials, paying more attention to analytical methods and statistical issues. For instance, there is a high risk of overinterpreting research findings if the sample size is not adequately estimated accounting for the dispersion of FGF23 levels within CKD stages, and this latter is influenced by the analytical performances of the methods (i.e., precision, accuracy) [86].

Our review has largely demonstrated that clinical usefulness depends on the thorough analytical validation of the assay [89]. The recent introduction of CLIA licensed for clinical use has allowed the assessment of preliminary but robust risk thresholds for cardiovascular and renal events [48] and is promising to capture the FGF23 clinically relevant changes as a result of a therapeutic modulation. Both the estimate of FGF23 risk thresholds and of significant changes are mandatory to finally translate the clinical usefulness into effectiveness, maximizing the application of FGF23 towards its use as therapeutic target.

The assessment of the technical characteristics of the assay represents one of the main domains of Health Technology Assessment (HTA) model [90]. Furthermore, the complexity of FGF23 assay optimization together with the potential impact on healthcare are major determinants of the cost of the test, and the evaluation of both items may address a reimbursement comparable to that of NT-proBNP or 1,25 OH Vitamin D. Undoubtedly, FGF23 as a candidate to become an effective health care tool should benefit from the use of the HTA approach to allow quantification of patient health impact and to inform cost-effectiveness analysis in order to support regulatory decisions [13, 90].

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of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. S. Ferraro and G. Biganzoli, were involved in the provision of study material and conceptualization; E. Biganzoli and M. Plebani revised and corrected the final draft; G. Zuccotti and V. Calcaterra revised to approve the final draft. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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