



Effect of essential amino acid ketoanalogues and protein restriction diet on morphogenetic proteins (FGF-23 and Klotho) in 3b–4 stages chronic kidney disease patients: a randomized pilot study

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Abstract

Background A low protein diet (LPD) with essential amino acid ketoanalogue supplementation (KA) may contribute in improving of chronic kidney disease (CKD), while the exact mechanisms of KA's effect are not established yet. We have conducted a prospective, randomized, controlled comparative study of LPD + KA and LPD alone in relation to serum Klotho, FGF-23 levels in CKD patients.

Methods 79 non-diabetic CKD 3b–4 stage patients, compliant with LPD diet (0.6 g/kg of body weight/day), had been selected. The patients were randomized into two groups. The first group (42 patients) received LPD + KA. The second group (37 patients) continued the LPD alone. In addition to routine tests, serum Klotho, FGF-23 levels, as well as bioimpedance analysis, sphygmography (stiffness (augmentation) indices (AI), central (aortal) blood pressure) with a «SphygmaCor» device; echocardiography (valvular calcification score (VCS) and LVMMI), were performed.

Results There were body mass indices' decrease ($p=0.046$), including muscle body mass in men ($p=0.027$) and woman ($p=0.044$) in the LPD group to the end of study (14th month). In addition, lower FGF-23 ($p=0.029$), and higher sKlotho ($p=0.037$) were detected in the LPD + KA group compared to the LPD one. The increase in AI ($p=0.034$), VCS ($p=0.048$), and LVMMI ($p=0.023$) was detected more often in the LPD group at the end of study.

Conclusion LPD + KA provides support for nutrition status and contributes to more efficient correction of FGF-23 and Klotho abnormalities that may result in cardiovascular calcification and cardiac remodeling decreasing in CKD. At the same time, a prolonged LPD alone may lead to malnutrition.

Keywords Chronic kidney disease · Essential amino acid ketoanalogues · Fibroblast growth factor-23 (FGF-23) · Serum alpha-Klotho (sKlotho) · Cardiovascular calcification · Cardiac remodeling

Introduction

The high risk of cardiovascular events (CVE) in patients with advanced chronic kidney disease (CKD) remains one of the major challenges for nephrologists, given an increasing

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number of patients with end-stage renal disease and a limited efficacy of conventional cardioprotective medications [1–3]. The accumulating evidence suggests that low protein diet (LPD) may retard progression of CKD, reduce the risk of cardiovascular complications and improve survival in pre-dialysis CKD patients [4–6]. The possible favorable effects of LPD include amelioration of metabolic acidosis, oxidative stress, chronic inflammation, and glomerular hyperfiltration that contribute to the deterioration of kidney function [7].

Fibroblast growth factor 23 (FGF-23) and Klotho are the key regulators of mineral metabolism that can predict cardiovascular risk and progression of kidney dysfunction in patients with CKD [8–10]. The available data suggest the changes in the FGF-23-Klotho system in patients with advanced CKD are associated with bone mineral disorders, metabolic acidosis, oxidative stress, chronic inflammation, protein–energy wasting, MIA syndrome (malnutrition, inflammation, atherosclerosis) and are closely linked with CVE [11–13]. Recent studies showed that increased FGF-23 production by osteocytes in advanced CKD correlated with endothelium dysfunction, left ventricular hypertrophy (LVH), vascular calcification, and atherosclerosis [14, 15]. Currently, FGF-23, such as parathyroid hormone (PTH), is considered to be a novel uremic toxin [16]. At early stages of CKD, serum FGF-23 increases as a compensatory response to phosphate retention [17]. However, an increased FGF-23 level may occur as a result of decreased kidney production of transmembrane Klotho protein that is a co-receptor for FGF-23 [18]. Ischemia, oxidative stress, metabolic acidosis, angiotensin II increase, inflammation, and protein–energy malnutrition can be important factors contributing to impaired Klotho expression [12, 18, 19]. In experimental mice, reduced Klotho levels induced vascular calcification, pathological bone fractures, and accelerated aging [19, 20], while adequate Klotho production provided antiapoptotic and antioxidative effects and prevented accelerated aging [21]. Notably, progressive deterioration of kidney function is associated with different disorders that are similar to those in general aging. However, in CKD they develop more rapidly, particularly in cardiovascular system [22]. Experimental models suggest that increased Klotho levels can have nephroprotective and cardioprotective effects [21, 23]. Therefore, different strategies aiming at increase of Klotho production and decrease of FGF-23 levels (i.e., administration of angiotensin II blockers, erythropoiesis stimulating agents, vitamin D analogs) could potentially prevent CKD and cardiovascular complications progression, assuming that may improve Klotho serum levels [24, 25]. At the same time, the role of the diet in contributing to support of serum Klotho and decrease of FGF-23 overproduction in CKD is unknown yet. However, the application of all possibilities for correction of the advanced CKD abnormalities could have an impact on improving the prognosis of CKD patients in

a greater degree. On the other hand, there is some caution against prolonged using of KA that they may increase cardiovascular calcification (CVC) due to calcium-containing salts.

We conducted a prospective, randomized, active-controlled study to compare the possibilities of LPD + KA and isolated LPD in their impact on morphogenetic proteins FGF-23 and Klotho, as well as the safety of these options in relation to both the risk of CVC and LVH in patients with CKD stage 3b to 4.

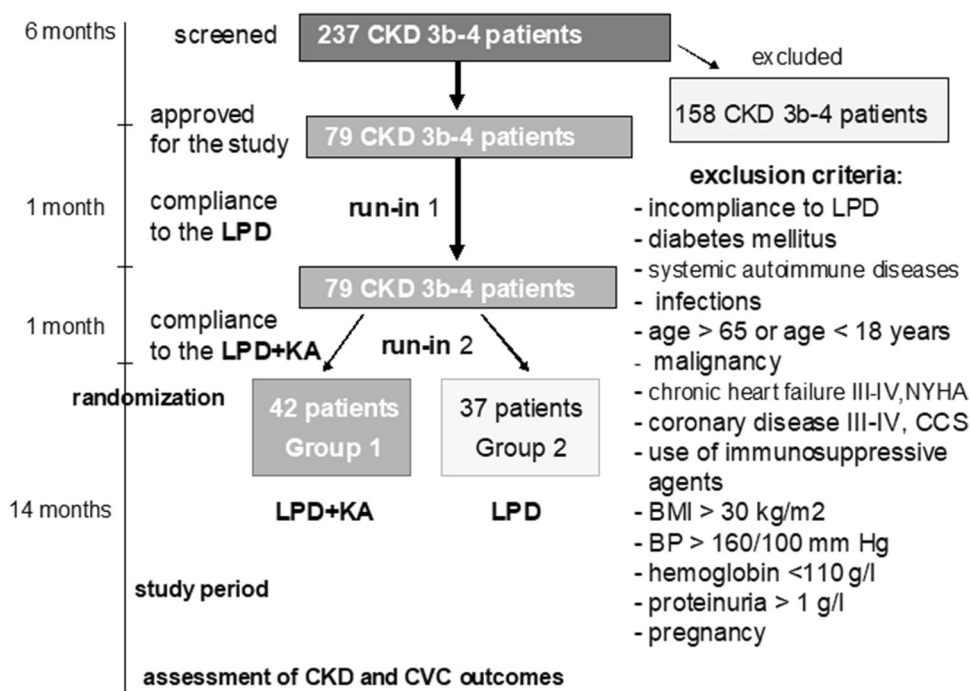
Materials and methods

A total of 79 non-diabetic CKD 3b–4 stage patients (from 237 CKD 3b–4 stage screened), 41 males and 38 females, mean age of 40.9 ± 9.5 years, compliant with LPD diet (0.6 g/kg of body weight/day), were approved for the study. All the patients were recruited from outpatients department of the university clinic. The patients were eligible for the study if they were diagnosed «CKD» stage 3b–4, according to KDIGO 2012 guidelines [1]. Estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation. Exclusion criteria were the following: diabetes mellitus, age less than 18 years or more than 65 years, severe chronic heart failure (III–IV class NYHA), refractory stable angina (III–IV class CCS) or acute coronary syndrome, malignancy, systemic autoimmune diseases, infections, body mass index (BMI) $> 30 \text{ kg/m}^2$, arterial hypertension ($> 160/100 \text{ mm Hg}$), serum hemoglobin $< 110 \text{ g/l}$, proteinuria $> 1 \text{ g/day}$, pregnancy, history of kidney transplantation, use of immunosuppressive agents, incomppliance to LPD.

All the eligible patients were entered in run-in phase on complacence to the LPD (0.6 g/kg of body weight/day, comprising 0.3 g of vegetable protein and 0.3 g of animal protein, with total phosphorus content $\leq 800 \text{ mg/day}$ and daily energy intake 34–35 kcal/kg/day). One month later, all the patients have been ketoanalogues (KA) of the essential amino acids (0.1 g/kg of body weight/day or 1 pill/ 5 kg of body weight/day «Ketosteril», Fresenius Kabi) added to the LPD. After the next 1 month, all the patients were randomized into two groups: the LPD + KA group (42 patients) and adjusted group, who continued the same dose of the LPD alone (37 patients). The duration of follow-up was 14 months (Fig. 1).

In addition to routine tests, serum Klotho Human soluble α -Klotho ELISA Assay (IBL-Takara 27998-96Well), FGF-23 (Human Fibroblast Growth Factor-23 ELISA (Merk Millipore MILLENZFGF-23-32K) assay, using monoclonal antibodies to the native human FGF-23 molecule), phosphate, parathyroid hormone (PTH), total calcium, albumin and transferrin levels were measured.

Fig. 1 A flowchart of patients' selection for the study. We screened 237 patients with CKD stage 3b–4. 79 from them were enrolled in the study and were randomly distributed into group 1 ($n=42$) and group 2 ($n=37$) at the end of run-in period



The protein–energy malnutrition (PEM) examination (BMI, fat body mass, muscle body mass) was performed using bioimpedance analysis («OMRON» device, USA). The stiffness (augmentation) indices (AI) and central (aortal) BP were measured using «SphygmoCor» device (Australia). Cardiac valvular calcification score by semiquantitative scale and left ventricular myocardium mass index (LVMMI) were measured using bi-dimensional echocardiography. The increase in LVMMI during the study period was assessed using the difference between medians of the absolute LVMMI value at the end of the study and baseline. LVH was diagnosed if LVMMI was > 95 g/m² in females and > 115 g/m² in males, according to the European Society of Cardiology, 2013 guidelines [26]. Cardiac (valvular) calcification score was evaluated by a semiquantitative scale, according to KDIGO 2012 guidelines and the National Recommendations on CKD-MBD [2, 27] (Table 1, Supplementary data).

Sampling of the whole blood and instrumental measurements was performed in the randomization period and 14 months after the beginning of the study. The whole blood was centrifuged for 15 min at 3000 rpm (rotation per minute), and the obtained serum was frozen and stored at −80 °C.

Normal serum Klotho and FGF-23 levels were studied in 40 healthy volunteers of comparable age and gender (Table 2, supplementary data).

The protocol of the study was approved by the Ethics committee of the Sechenov First Moscow State Medical University (№ 06–14, 16.06.2014). All the patients provided written informed consent. All the described procedures were

performed in accordance with the Helsinki Declaration. The study was supported by Russian Science Foundation (Grant № 14-15-00947 2014).

Statistical analysis

Standard descriptive statistics with calculated median (inter-quartile range), mean ± standard deviation, or frequencies n (%) were used to assess the baseline characteristics of the study groups and the same characteristics at the end of the study. To assess the statistical significances between two groups, χ^2 test (for qualitative variables) and Mann–Whitney U test (for quantitative variables) were used. The quantitative data were presented as medians and quartiles, qualitative data were presented in percents. Spearman's rank correlation analysis was used to identify and assess the tightness of the relationship between two sets of quantitative variables. Two-tailed P values were considered statistically significant at < 0.05 level. All statistical analyses were performed using SPSS software version 21.0 (Chicago, Illinois, USA).

Results

We screened 237 patients with CKD 3b to 4 stages (Fig. 1). Seventy nine of them were enrolled in our study and were randomly distributed into group 1 ($n=42$) and group 2 ($n=37$) at the end of run-in period. Two groups were comparable by baseline demographic and clinical characteristic (Table 1). There were no significant baseline

Table 1 Main metabolic determinants, renal function (eGFR), central blood pressure (BP), and medications in the study groups, at baseline and at the end of the study

Determinants	Group 1 LPD + KA (n = 42)	Group 2 LPD (n = 37)	P value
eGFR (ml/min/1.73 m ²)			
Baseline	35.1 (19.8–43.4)	34.9 (20.1–44.1)	0.571
End of study	29.1 (16.6–40.1)	26.6 (14.7–39.9)	0.047
Central systolic BP (mm/Hg)			
Baseline	115.0 (109.5–130.0)	113.0 (107.0–129.5)	0.632
End of study	118.0 (115.0–133.3)	131.0 (117.5–146.5)	0.039
Body mass index (kg/m ²)			
Baseline	24.4 (23.2–29.1)	25.0 (23.9–30.1)	0.765
End of study	24.2 (23.0–30.4)	19.6 (19.1–22.9)	0.046
Muscle body mass%			
Baseline	<i>m</i> 35.1 (33.4–38.9) <i>f</i> 27.2 (25.1–29.4)	<i>m</i> 35.4 (33.5–39.1) <i>f</i> 26.9 (24.9–29.7)	0.821
End of study	<i>m</i> 35.4 (33.4–39.0) <i>f</i> 26.9 (24.8–29.2)	<i>m</i> 28.33 (22.05–37.75) <i>f</i> 20.1 (19.6–24.6)	0.027 0.044
Medications (n, %)			
Antihypertensive drugs			
Baseline	35 (83.3)	31 (84.1)	0.571
End of study	37 (88.0)	37 (100)	0.051
Phosphate binders			
Baseline	7 (16.2)	6 (16.4)	0.944
End of study	9 (21.4)	16 (43.2)	0.024
Vitamin D therapy			
Baseline	10 (23.8)	9 (24.3)	0.920
End of study	13 (30.9)	16 (43.2)	0.070
Laboratory values			
Serum phosphate (mmol/l)			
Baseline	1.41 (1.21–1.55)	1.39 (1.2–1.58)	0.824
End of study	1.24 (1.22–1.57)	1.55 (1.39–1.87)	0.037
Calcium total (mmol/l)			
Baseline	2.27 (2.19–2.37)	2.29 (2.21–2.36)	0.713
End of study	2.42 (2.28–2.51)	2.12 (1.9–2.42)	0.041
Albumin (g/l)			
Baseline	38.48 (36.7–44.3)	39.43 (36.9–45.2)	0.811
End of study	37.97 (36.4–40.8)	36.10 (35.9–39.2)	0.482
Total protein (g/l)			
Baseline	76.6 (74.9–83.4)	77.3 (75.6–84.1)	0.893
End of study	76.2 (73.0–83.1)	66.8 (63.8–77.8)	0.039
Triglycerides (mmol/l)			
Baseline	1.11 (1.00–2.10)	1.2 (1.02–2.00)	0.146
End of study	1.15 (1.14–2.00)	1.0 (1.0–1.67)	0.060
Transferrin (g/l)			
Baseline	2.91 (2.10–3.61)	3.00 (2.7–3.6)	0.881
End of study	2.7 (2.4–3.40)	2.1 (1.9–2.5)	0.048
Parathyroid hormone (pg/ml)			
Baseline	67.0 (45.0–115.6)	69.5 (55.5–127.6)	0.631
End of study	75.0 (59.6–132.4)	119.0 (72.0–295.0)	0.032

Table 1 (continued)

Determinants	Group 1 LPD + KA (n = 42)	Group 2 LPD (n = 37)	P value
Serum urea (mmol/l)			
Baseline	7.8 (7.2–8.6)	8.1 (7.4–8.8)	0.893
End of study	8.3 (7.6–9.1)	12.6 (9.6–14.4)	0.027
Serum uric acid (μmol)			
Baseline	435 (397–481)	450 (399–479)	0.784
End of study	433 (375–451)	462 (420–489)	0.074
Serum standard bicarbonate (SB) (mmol/l)			
Baseline	23.1 (19.0–26.0)	24.0 (20.0–25.8)	0.713
End of study	24.4 (20.1–25.9)	19.5 (18.5–23.4)	0.029
C-reactive protein (mg/l)			
Baseline	4.1 (3.6–5.0)	4.0 (3.8–5.1)	0.484
End of study	4.3 (3.8–5.3)	6.2 (5.8–7.1)	0.018
FGF-23 (pg/ml)			
Baseline	97.0 (67.0–360.5)	85.9 (59.0–337.5)	0.648
End of study	110.3 (78.4–478.5)	353.0 (110.0–792.9)	0.029
Klotho (pg/ml)			
Baseline	429.9 (368.5–517.1)	433.2 (373.2–534.2)	0.814
End of study	418.1 (351.3–498.2)	221.7 (198.4–316.7)	0.037

Results are expressed as medians (interquartile range) or frequencies *n* (%), as appropriate. *P* value reflects the significance between the groups by Mann–Whitney *U* test or χ^2 test, as appropriate

eGFR estimated glomerular filtration rate, *BP* blood pressure, *LPD* low protein diet, *KA* ketoanalogues of essential amino acids, *m* male patients, *f* female patients

P values < 0.05 were identified as statistically significant are in bold

differences between LPD + KA and LPD groups in the age [41.1 (18.0–64.3) vs 42.0 (18.2–63.8)]; sex [female gender 20 (47.6%) vs 18 (48.6%)], primary renal disease [chronic glomerulonephritis in 22 (52.4%) vs 20 (54.05%) and tubulointerstitial nephropathies in 20 (47.6%) vs 17 (45.9%)]; proteinuria levels [0.81 (0.1–0.9) vs 0.69 (0.0–0.9) g/day]; hemoglobin levels [114.0 (110.0–122.0) vs 115.0 (110.5–123.5) g/l]; mean arterial systolic BP [126.0 (110.0–143.0) vs 123.0 (109.0–145.0) mm Hg]; patients with stable coronary artery disease (angina pectoris) class I–II, CCS [4 (9.5%) vs 3 (8.1%)]; stable patients with chronic heart failure class I–II, NYHA [1 (2.4%) vs 0 (0.0%)]; patients with optimal BP control [38 (90.5%) vs 33 (89.2%)]; patients receiving angiotensin converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers [4 (9.5%) vs 3 (8.1%)] and some others (Table 1).

1. Nutrition status determinants.

To the end of 14-month follow-up, patients from the group 2 (LPD alone) have achieved a statistically significant reduction in BMI ($p = 0.046$), including muscle body mass in men ($p = 0.027$) and in women ($p = 0.044$), according

to bioimpedance analysis. At the same time, there were no signs of PEM in patients from group 1 (LPD + KA) [Table 1]. In addition, to the end of the study, we found out the statistically significant decrease in serum total protein ($p=0.039$) and transferrin ($p=0.048$) levels also in the group 2, while average values of serum albumin levels remained in normal range in both groups. Serum urea levels were lower at the end of the study in the patients from the LPD + KA ($p=0.027$). Serum bicarbonates levels were statistically significant higher ($p=0.029$) in the group 1 compared to group 2 at the end of the study (Table 1).

2. Glomerular filtration rate (eGFR) levels

At the end of the study, the average eGFR was significantly higher ($p=0.047$) in the patients from group 1 (Fig. 2; Table 1).

3. Calcium–phosphate and PTH levels

Long-term administration of the LPD supplemented with KA had favorable effects on serum phosphorus, calcium and PTH levels compared with those of LPD alone (Table 1). In patients from group 1, average serum calcium level has increased during follow-up, however, it did not exceed the reference values. At the same time, serum PTH was lower in the group 1 compared to group 2 ($p=0.032$) to the end of the study. In addition, serum phosphate level was higher in the group 2 at the end of the study ($p=0.037$), which increased the need in phosphate binder use ($p=0.024$), Table 1.

Moreover, in patients from group 1, serum phosphate (0.81–1.45 mmol/l) and total calcium (2.10–2.55 mmol/l) target levels were achieved and maintained by lower doses of phosphate binders. Median daily doses of sevelamer hydrochloride were 1600 and 2000 mg, and median daily doses of calcium carbonate were 1000 and 1750 mg (for groups 1 and 2, respectively), though these differences did not reach statistical significance ($p=0.130$ and $p=0.094$, respectively).

4. FGF-23, Klotho and a diet type.

In patients from group 1, serum FGF-23 level at the end of follow-up was lower ($p=0.029$) than in patients from group 2, while serum Klotho level was higher ($p=0.037$) (Table 1; Fig. 3).

5. Central BP, valvular calcification score and LVH

To the end of the study, we have also observed the statistically significant differences between the studied groups in the central systolic BP levels ($p=0.039$). In the group 1, they were lower than in patients of the group 2 (Table 2). Taking into account that KA may increase serum calcium levels, but at the same time with obtained unexpected lower central systolic BP levels in the LPD + KA group, we performed the analysis of cardiovascular stiffness/calcification. The data for this analysis were also obtained at the randomization and at the end of the study. The main characteristics are shown in Table 2.

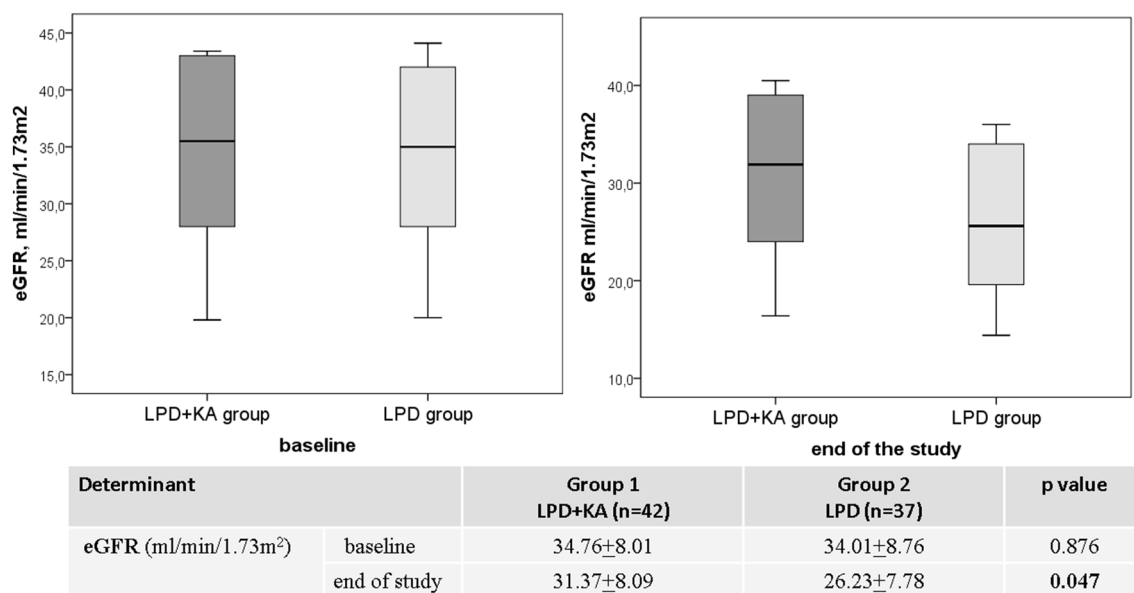


Fig. 2 eGFR average values as mean ± standard deviations (SD) in the study groups at the start of the study were comparable. To the end of study, the extent of eGFR falling was lower in the group

LPD + KA than in the group LPD alone. The difference was statistically significant ($p=0.047$), according to Mann–Whitney *U* test

Fig. 3 The dynamics of mean serum FGF-23, Klotho and PTH levels in the CKD patients according to the diet type during the study period (Mann–Whitney *U* test). The patients from the first group had statistically significant lower FGF-23 ($p=0.029$) and higher sKlotho ($p=0.037$) serum levels at the end of the study compared to the patients from the second group. Serum levels of PTH also were significantly lower ($p=0.032$) in LPD + KA group to the end of the study

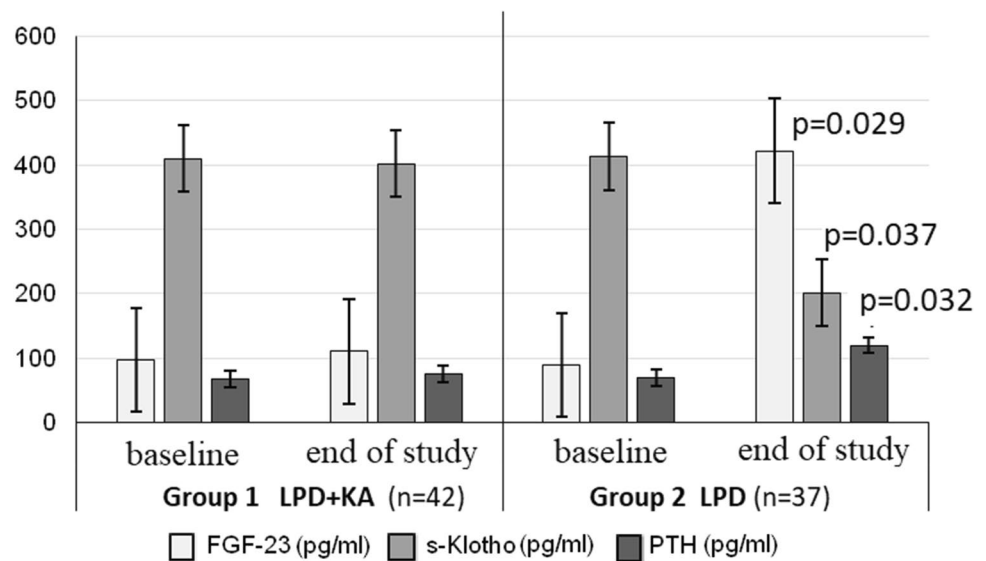


Table 2 Augmentation index (AI), cardiac (valvular) calcification score and left ventricular myocardium mass index (LVMMI) in the studied groups

Determinants	Group 1 LPD + KA (n=42)	Group 2 LPD (n=37)	P value
Augmentation index, %			
Baseline	19.7 (16.0–25.0)	19.4 (15.4–25.0)	0.734
End of study	21.2 (17.5–27.0)	29.9 (19.5–36.9)	0.034
Cardiac (valvular) calcification score, n (%)			
«0 points»			
Baseline	7 (16.6)	5 (13.5)	0.593
End of study	5 (11.9)	0 (0)	0.048
«0.5–1 points»			
Baseline	31 (73.8)	29 (78.3)	0.384
End of study	31 (73.8)	31 (83.7)	0.263
«> 1.5 points»			
Baseline	4 (9.5)	3 (8.1)	0.738
End of study	6 (14.3)	6 (16.2)	0.676
Cardiac remodeling			
LVMMI, kg/m ²			
Baseline	109 (100–116)	111 (95–119)	0.590
End of study	111 (102–117.5)	116 (100–124)	0.023
LVH, n (%)			
Baseline	23 (54.8)	21 (56.8)	0.582
End of study	26 (61.9)	30 (81.1)	0.051

Results are expressed as medians (interquartile range) or frequencies *n* (%) as appropriate. *P* value reflects the significance between the groups by Mann–Whitney *U* test or χ^2 test, as appropriate

LVMMI left ventricular myocardium mass index, LVH left ventricular hypertrophy

P values < 0.05 were identified as statistically significant are in bold

Augmentation index and cardiac (valvular) calcification score were significantly higher in group 2 compared to those in group 1 (Table 2). Furthermore, the percentage of patients with LVH to the end of the study also was higher in the group 2 (81.1 vs 61.9%). The difference in changes

of LVMMI during the study between two groups was also statistical significant ($p=0.023$).

6. Evaluation of confounding factors concerning FGF-23 and Klotho in CKD.

Among the CKD-related factors potentially affecting Klotho levels, we had examined the effect of the following determinants: eGFR ($r=0.595$, $p<0.01$), central systolic blood pressure ($r=-0.536$, $p<0.01$), serum hemoglobin level ($r=0.612$, $p<0.01$), and erythrocyte sedimentation rate ($r=-0.454$, $p<0.01$) using Spearman's rank correlation analysis. The effect of the low protein diet and ketoanalogues (LPD + KA) was also statistically significant ($r=0.547$, $p<0.01$).

Among the factors potentially affecting FGF-23, the following parameters were considered: eGFR ($r=-0.516$, $p<0.05$), Klotho ($r=-0.713$, $p<0.01$), phosphorus ($r=0.697$, $p<0.01$), PTH ($r=0.475$, $p<0.05$), and LPD + KA use ($r=-0.496$, $p<0.05$).

Thus, despite the statistically significant correlations of FGF-23 and Klotho levels with the possible confounding factors, the effect of LPD + KA on serum FGF-23 and Klotho was also statistically significant, especially on the Klotho level. In addition, to the end of the study, all the confounding factors were significantly improved in LPD + KA group in comparison with the group of patients taking LPD only (Table 1).

Patients' compliance to the diet

The carefully selected patients had, as expected, a high compliance to the LPD regarding both protein and energy intake. The protein intake was close to prescription throughout the study period [0.60 (0.58–0.62) vs 0.61 (0.59–0.62)]. The daily energy intake was also close to KDIGO recommendations (34–35 kcal/kg per day) in both groups at all study moments. All patients completed a 14-month study.

Discussion

Recent studies suggested that LPD might be a safe option to retard the progression of CKD in pre-dialysis patients [3–7]. However, there is no consensus to which extent it is desirable to restrict protein intake in patients with advanced stages of CKD. Several studies showed that a very low protein diet (up to 0.3 g/kg/day) supplemented with KA of the essential amino acids may have advantages over moderate protein restriction (0.6–0.8 g/kg daily) in relation to the risk of CKD progression and cardiovascular outcomes [6, 28]. However, other studies raised concerns that very strict diet will be poorly tolerated in the long term and can increase the risk of malnutrition that is strongly associated with an unfavorable prognosis, including subsequent hemodialysis issues [7]. It seems that long-term moderate dietary protein restriction (up to 0.6 g/kg daily) supplemented with KA has a favorable safety/benefit ratio in patients with CKD stage 3b to 4, while a very low protein diet may be more appropriate

in patients with CKD stage 5 and can postpone initiation of renal replacement therapy [4, 7, 29]. To clarify the risks and benefits, we have attempted to assess the potential of moderate LPD (0.6 g/kg/body weight) in combination with ketoanalogues for a long-term prognosis in relation to nutritional status, CKD and cardiovascular complication (CVC) progression in CKD 3b–4 stage patients. As a comparison group, the patients that were comparable to the main group by basic CKD parameters, but did not include ketoanalogues (KA) in the LPD, were recruited. We have additionally studied the dynamics of the serum levels of FGF-23 and Klotho as key markers of CVC and CKD progression. Over 14 months, LPD supplemented with KA compared with LPD alone maintained adequate nutrition, prevented a further decrease of eGFR, and was associated with a lesser increase of vascular stiffness, valvular calcification and LVH. Moreover, serum FGF-23 and Klotho levels did not change significantly in patients from group 1, while in patients from group 2 there were 3.7-fold increase in serum FGF-23 level and 1.7-fold reduction in serum Klotho level.

Serum Klotho level is closely associated with vascular calcification that is considered to be a major contributor to cardiovascular mortality in patients with CKD [9, 10, 15, 19]. Membrane Klotho is a co-receptor for FGF-23, known as a uremic toxin and having the direct effects on heart and vessels [15]. Reduced Klotho levels were shown to increase FGF-23 production in patients with progressive CKD [18]. The other determinant of increased FGF-23 levels is phosphate retention occurring in patients with impaired kidney function [17, 18]. Therefore, correction of hyperphosphatemia is one of the priorities in the current management for CKD aiming at prevention of renal and cardiovascular outcomes [1–3]. The results of the recent studies suggest that correction of serum Klotho and FGF-23 levels should be a part of cardioprotection and nephroprotection [24, 25, 30, 31].

The favorable effect of long-term LPD supplemented with KA on serum Klotho and FGF-23 concentrations may be related to adequate correction of nutritional status and a better control of oxidative stress, chronic inflammation and metabolic acidosis that develops as a result of protein–energy wasting. Experimental studies have shown that Klotho was very sensitive to the latter disorders that induced a reduction in the serum levels of this protein [12, 13, 15]. According to our data, Klotho's association with the use of LPD + KA ($r=0.547$, $p<0.01$) was stronger than FGF-23 ($r=-0.496$, $p<0.05$) ones, and it can be assumed that adequate correction and maintenance of nutritional status determinants in the first place can be associated with maintenance of Klotho levels, and FGF-23 decreases already for the second time. Retained Klotho level leads to a better phosphate excretion by FGF-23, a more adequate balance of calcium and PTH. Therefore, maintenance of Klotho and

FGF-23 levels, as early markers, can prevent subsequent shifts in phosphate–calcium metabolism in patients with advancing CKD and may protect against bone damage and cardiovascular complications.

In patients from group 1 who followed LPD supplemented with calcium-containing KA, the target levels of serum phosphate were achieved with the use of less frequency phosphate binder application and smaller its doses compared to patients from group 2. This difference can reflect a higher serum Klotho levels at the end of 14-month follow-up and improved Klotho-mediated phosphate excretion under the effect of FGF-23.

Paracrine effects of Klotho protein include activations of calcium channels receptors (TRPVs), particularly TRVP5 and TRVP6 [12, 19]. TRVP5 are located predominantly in the distal kidney tubules and participate in calcium reabsorption. TRVP6 are expressed in the heart and the intestinal epithelium cells, where they take part in the intestinal absorption of calcium [9, 12, 19, 32]. Therefore, serum Klotho can regulate calcium homeostasis through changes in its reabsorption in kidneys and intestine. In our opinion, calcium-containing KA can stimulate Klotho production to prevent transient episodes of hypercalcemia in patients with CKD. At the end of follow-up, the average calcium level tended to increase in patients from group 1. However, calcium levels did not exceed the reference values. In patients who followed LPD alone, the average serum calcium tended to decrease. This effect could contribute (in addition to elevated phosphate level) to an increased PTH level that was significantly higher in group 2 compared with group 1.

As possible effect of KA on FGF-23 serum levels may suggest the ability of calcium salts of ketoanalogues to bind phosphate in the intestine, forming phosphoric acid calcium, thus facilitating phosphate elimination through the gastrointestinal tract [2, 3]. In our study, serum phosphate and FGF-23 levels in patients followed the LPD + KA were lower than ones in patients group without KA.

Prolonged administration of calcium-containing KA can presumably lead to increasing calcification of the cardiac valves and vessels. However, in our study, 14-month LPD supplemented with KA was associated with a lower average valvular calcification score compared with that in patients from group 2. These data confirm that vascular calcification in patients with advanced CKD should not be regarded as just a passive deposition of calcium and phosphate from the circulation related to hypercalcemia. In patients with CKD, different trigger factors, including hyperphosphatemia and FGF-23, induce apoptosis of endothelial cells, increased production of reactive oxygen species, pro-inflammatory cytokines, pro-fibrotic growth factors, and pro-angiogenic growth factors, and impaired nitric oxide production. All these factors not only induce endothelial dysfunction but can stimulate proliferation of vascular smooth muscle cells

[9, 33, 34]. Moreover, a reduced secretion of annexin II by vascular smooth muscle cells and an activation of Wnt signal pathway lead to progressive vascular calcification, as vascular smooth muscle cells start to express proteins of osteoblastogenesis [34]. Therefore, Wnt pathway plays a key role both in bone metabolism and calcification of blood vessel media and cardiac valves in CKD. Klotho is able to inhibit Wnt signal pathway and to retard vascular calcification [35, 36]. In addition, Klotho protects against endothelial dysfunction, affecting the production of NO, oxidative stress and pro-inflammatory cytokines [9, 10, 12].

The preventive and therapeutic approaches aimed at control of Klotho and FGF-23 production in CKD patients, including LPD and its modifications, could significantly contribute to prevention of cardiovascular complications and CKD progression.

Conclusion

In summary, long-term administration of LPD supplemented with KA maintains the nutritional status, does not impair nitrogen balance, and can retard deterioration of kidney function, calcification of the cardiovascular system and progression of LVH in patients with the advanced stages of CKD. However, prolonged LPD alone, even with careful medical follow-up and intake of high-calorie carbohydrates, was associated with the signs of malnutrition (decreased BMI, muscle mass, total protein and transferrin in serum), development of negative nitrogen balance, aggravation of mineral-bone disorders (increase in serum FGF-23, PTH and phosphate levels and decrease in serum Klotho). Moreover, prolonged LPD without KA supplementation did not prevent the progression of CKD and cardiovascular calcification.

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Compliance with ethical standards

Ethical approval The study was approved by the Institutional Review Board of the Sechenov First Moscow State Medical University (Protocol No. 06–14, 16.06.2014). All the patients provided written informed consent. All the described procedures were performed in accordance with the Helsinki Declaration.

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