ISSN 1607-419X
УДК 616.61-085

# Circulating form of Klotho protein — a novel inhibitor of vascular calcification in chronic kidney disease

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*Received 10 October 2014; accepted 03 November 2014.*

**Abstract**

**Objective.** To study associations between serum soluble Klotho level and vascular calcification in patients with chronic kidney disease (CKD) 1–5 stages. **Design and methods.** We examined 70 patients with different stages of CKD, including 41 patients with chronic glomerulonephritis, among them 10 with systemic diseases, 22 with tubulo-interstitial nephritis (bacterial, gout and drug-induced) and 7 with hypertensive nephrosclerosis. All 70 patients with 1–5 stages of CRD underwent clinical examination and blood tests for serum Klotho levels by enzyme-linked immunosorbent assay. Vascular stiffness was assessed in 57 patients by applanation tonometry method (SphygmoCor, AtCor Medical, Australia). **Results.** Serum Klotho levels differed depending on the stage of CKD. When patients with different stages of CKD were compared, a reverse correlation between serum Klotho level and serum phosphorus level and intact parathormone was found in CKD progression. There is a correlation between serum Klotho level and an increase in left ventricular posterior wall thickness in patients with CKD and hypertension (n = 49). Also reduction in serum Klotho level is associated with a greater frequency of calcifications in heart and major arteries, increased vascular stiffness and reduced blood flow in the tibial arteries (ankle-brachial index). **Conclusions.** Thus, circulating Klotho plays an important role in mineral metabolism in CKD and demonstrates pleiotropic effects that might modify cardiovascular risk through the impact on vascular calcification and cardiovascular remodeling.

**Key words:** chronic kidney disease, hypertension, circulating Klotho protein, ectopic calcification, cardiovascular remodeling

*For citation: Milovanov YuS, Milovanova LYu, Sablina MM, Markina MM, Kryukova DV. Circulating form of Klotho protein — a novel inhibitor of vascular calcification in chronic kidney disease. Arterial'naya Gipertenziya = Arterial Hypertension. 2014;20(6):531–537.*

# Циркулирующая форма белка Klotho — новый ингибитор сосудистой кальцификации при хронической болезни почек

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*Статья поступила в редакцию 10.10.14 и принята к печати 03.11.14.*

**Резюме**

**Цель исследования** — комплексное изучение взаимосвязи сывороточного уровня белка Klotho с кальцификацией сердца и сосудов у больных хронической болезнью почек (ХБП) 1–5D стадий. **Материалы и методы.** Исследовано 70 пациентов с разными стадиями ХБП, включающими 41 случай хронического гломерулонефрита (ХГН), в том числе 10 при системных заболеваниях, 22 больных тубулоинтерстициальным нефритом (бактериальной, подагрической, лекарственной этиологии) и 7 пациентов с гипертензивным нефросклерозом. Наряду с общеклиническими исследованиями у всех больных с 1–5D стадиями ХБП изучен сывороточный уровень Klotho (иммуноферментный метод ELISA с набором анти-Klotho поликлональных антител). У 57 больных на момент взятия проб крови проводили анализ ригидности сосудов («SphygmoCor», «AtCor Medical», Австралия). **Результаты.** Сывороточная концентрация Klotho различалась среди изученных больных в зависимости от стадии ХБП. При сравнении больных с разными стадиями ХБП оказалось, что снижение концентрации в сыворотке крови больных Klotho при прогрессировании ХБП происходило в обратной корреляционной зависимости от содержания в сыворотке крови неорганического фосфора и интактного паратгормона. У больных ХБП с артериальной гипертензией (n = 49) выявлена связь между снижением уровня Klotho в сыворотке крови и увеличением толщины задней стенки левого желудочка. У этих же больных сниженная концентрация Klotho в сыворотке крови была ассоциирована с большей частотой выявления кальцификатов в сердце и магистральных артериях, увеличением ригидности сосудов и снижением кровотока в артериях голени (лодыжечно-плечевой индекс). **Выводы.** Таким образом, помимо участия в минеральном обмене, при ХБП циркулирующая форма белка Klotho может иметь значение в развитии кардиоваскулярных осложнений — артериальной кальцификации, ремоделирования сердца и сосудов.

**Ключевые слова:** хроническая болезнь почек, артериальная гипертензия, циркулирующая форма белка Klotho, эктопическая кальцификация, ремоделирование сердца и сосудов

*Для цитирования: Милованов Ю.С., Милованова Л.Ю., Саблина М.М., Маркина М.М., Крюкова Д.В. Циркулирующая форма белка Klotho — новый ингибитор сосудистой кальцификации при хронической болезни почек. Артериальная гипертензия. 2014;20(6):531–537.*

## Introduction

In recent decades, the role of cardiovascular disease (CVD) in the development of chronic kidney disease (CKD) has been widely recognized, which is due to the general aging of the population, increasing incidence of diabetes mellitus, hypertension and atherosclerosis [1].

On the other hand, epidemiological studies showed that CKD as an independent predictor of development and progression of cardiovascular complications [1–3].

Thus, the search of the factors inhibiting the progression of the CVD, including the cases in CKD, is highly relevant. The recent evidence shows that the circulating form of Klotho protein suppresses oxidative processes through the FoxO activation and increased expression of superoxide dismutase, as well as influences the processes of endothelial integration and function in CKD [4–5].

Recent experimental studies have confirmed that the circulating Klotho protein can act as a humoral factor with a protective cardiovascular effect [6–8]. Over-expression of Klotho provides with both renal and cardiovascular protection [8].

Increased production of Klotho prevents atherosclerosis occurrence and slows aging in experimental animals [9]. Moreover, clinical studies confirmed a protective role of Klotho in the pathogenesis of cardiorenal interactions in CKD [10–12].

The aim of our study was a comprehensive assessment of the relationship between serum level of Klotho protein and cardiac and vascular calcification in patients with CKD stages 1–5D.

## Design and methods

*Patients*

We enrolled 70 patients with different stages of CKD, including 41 patients with chronic glomerulonephritis (CGN), including 10 cases with systemic diseases (systemic lupus erythematosus, systemic vasculitis), 22 — with tubulointerstitial nephritis (bacterial, gout, medication-related) and 7 subjects with hypertensive nephrosclerosis (30 males and 40 females). The mean age at baseline was 50,0 ± 14,9 years (from 20 to 84 years).

CKD stages were determined by NKF K/DOKI criteria (2002), while the glomerular filtration rate (GFR) was calculated according to the equation CKD-EPI (2009) (Table).

At baseline, 21 (30%) out of 70 included patients had normal blood pressure (BP) level (110/70–140/80 mm Hg), 49 (70%) subjects had hypertension (HTN) of different severity. For BP control antihypertensive agents were prescribed alone or in combination therapy depending on the severity of hypertension, including angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, beta-blockers, diuretics.

Among 49 hypertensive patients, BP was controlled within the target level 130/80–140/80 mmHg in 27 (55.1%) subjects, the remaining 22 (44.9%) patients regularly received antihypertensive drugs, and at baseline they still had elevated BP (150/90–165/100 mm Hg).

In 22 (31.4%) patients with persistent hypercalcemia and elevated levels of intact parathyroid hormone (iPTH) iPTH paricalcitol was prescribed.

Patients with systemic diseases were included in case of absent activity manifestations

*Table*

**THE DISTRIBUTION OF PATIENTS BY STAGE OF CHRONIC KIDNEY DISEASE**

| The stage of chronic kidney disease | The range of estimated flomerular filtration rate (ml/min/1.73 m$^2$) | Mean | Number of patients, n (%) |
|---|---|---|---|
| 1 | 92–100 | 96.2 | 11 (15.7%) |
| 2 | 60–85 | 76.6 | 12 (17.1%) |
| 3 | 30–55 | 39.3 | 25 (35.7%) |
| 4 | 15–27 | 21.8 | 11 (15.7%) |
| 5 | 10–14 | 9.7 | 5 (7.1%) |
| 5D | 5–9 | 6.7 | 6 (8.6%) |
| Total | – | – | 70 (100%) |

(hypocomplementemia, high levels of antibodies to double-stranded deoxyribonucleic acid and anticytoplasmic antibodies — p-ANCA and c-ANCA).

*Methods*

Serum level of Klotho was assessed in all 70 patients with CKD stages 1–5D using immunoassay analysis ELISA and a set of anti-Klotho polyclonal antibodies (company "Millipore", USA). It was conducted according to a standard protocol in the diagnostic laboratory "LiTEH (Laboratory of intellectual and technical chemistry)". Blood was sampled in the vacuum centrifuge tubes, and then it was centrifuged at a speed of 2500 rev/min for 15–20 minutes, the serum was frozen and stored at –28 °C.

The examination included a detailed history, analysis of symptoms in order to clarify the etiology of CKD, CVD, duration of hypertension, the rate of CKD progression, residual renal function before hemodialysis (HD) was started. All patients underwent clinical blood and urine tests. Biochemical tests were performed with the use of biochemical analyzer "Technicon" (USA) and biochemical laboratory device "Spectrum" (Abbot, USA).

BP was measured by Korotkoff method in all patients. Hypertensive patients also underwent heart ultrasound ("ACUSON 128 HR10", USA) in accordance with a standard protocol [13, 14].

Moreover, arterial stiffness was assessed in 57 patients using the device "SphygmoCor" (Australia) that is based on the measurement of the pulse wave by a sensor installed at the site of radial and femoral arteries. To assess the degree of peripheral vascular calcification ankle-brachial index (Engl. ABI — ankle-brachial index) was evaluated in the same patient sample by the equation: ABI = ankle MAP/brachial MAP (lower reference value is 0.9, upper — 1.42).

*Statistical analysis*

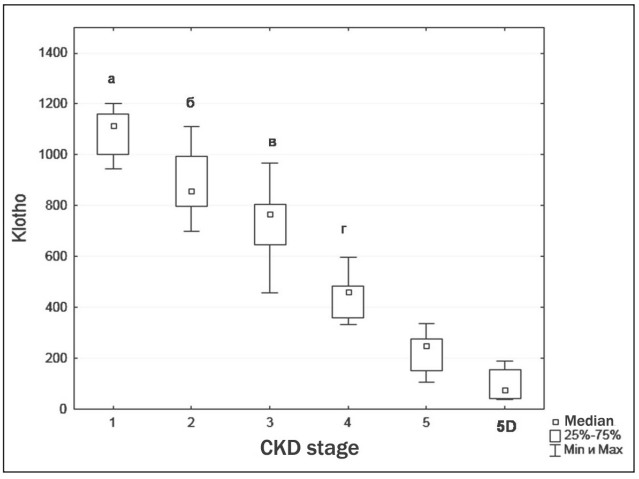
Statistical analysis was performed using SPSS 10 for Windows, STATISTICA 10.0, and MS Excel. We used nonparametric tests: Mann-Whitney test — to compare two groups, and Kruskal-Wallis test — for multiple comparisons. Correlations were assessed by Spearman rank correlation coefficient (r).

In case of normal distribution, the data are presented as M ± m (where M is an arithmetic mean, m — standard deviation). In case of non-normally distributed variables, results are presented as median and interquartile scope. The results were considered statistically significant at the level of $p < 0.05$.

## Results

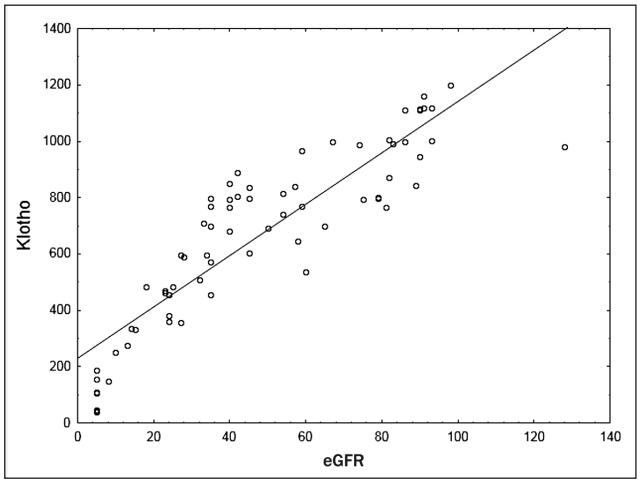
Serum Klotho levels differ between the groups of patients with different CKD stages. The high-

**Figure 1. Serum Klotho levels in patients with different stages of chronic kidney disease**



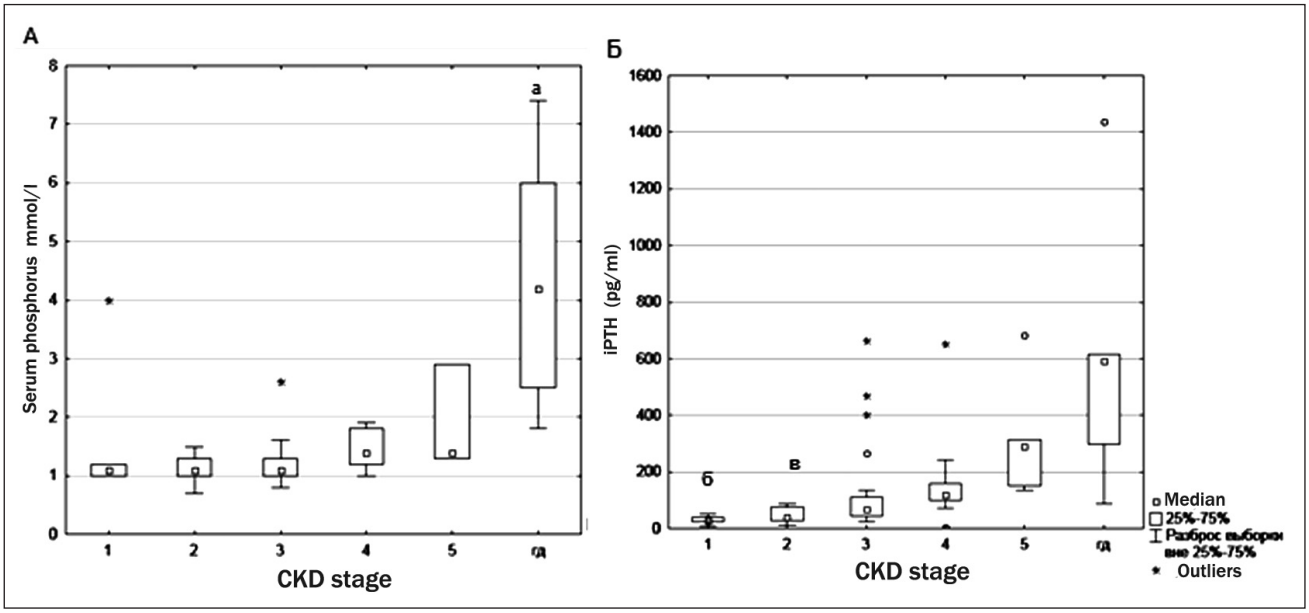

**Note:** CKD — chronic kidney disease. Letters show statistically significant differences between groups ($p < 0.05$) in the pair-wise comparison: a — CKD stages 3, 4, 5, 5D; b — with CKD stages 4, 5, 5D; c — CKD stages 5, 5D; d — CKD stage 5D.

**Figure 2. Relation between serum Klotho level and estimated glomerular filtration rate (ml/min/1.73 m$^2$)**




**Note:** eGFR — estimated glomerular filtration rate. In patients with chronic kidney disease stage 5D reduction in serum Klotho level inversely correlated with the duration of hemodialysis treatment ($r = –0.760$, $p < 0.05$).

**Figure 3. The change in the serum levels of inorganic phosphorous (A) and the intact parathyroid hormone (B) at different stages of chronic kidney disease**




**Note:** CKD — chronic kidney disease; iPTH — intact parathyroid hormone; A — significant differences ($p < 0.05$) in the pairwise comparison with chronic kidney disease stages 1, 2, 3; B — significant differences ($p < 0.05$) in the pairwise comparison with chronic kidney disease stages 3, 4, 5 and hemodialysis; in — significant differences ($p < 0.05$) compared to hemodialysis.

est concentrations were observed in patients with stage 1 CKD, and they decreased in individuals with more severe CKD with the lowest levels recorded in patients with stage 5 CKD (Fig. 1). There was a direct correlation between eGFR and Klotho concentration ($r = 0.92$, $p < 0.05$) (Fig. 2).

The levels of inorganic phosphorus significantly differed in patients with early stages of CKD (CKD 1, CKD 2) and in those with severe CKD (CKD 5–5D) (Fig. 3A). iPTH concentrations also differed significantly in patients with CKD 1 and those with CKD stages 3–5 ($p < 0.05$). There were no differences in iPTH levels in patients with more advanced (higher than stage 3) CKD stages (Fig. 3B).

Thus, we suggest that serum level of Klotho protein is an early marker of CKD progression, the changes in its serum levels are evident already at CKD stage 3 and further worsen with the CKD progression. The reduction in Klotho level precedes the increase in serum concentrations of phosphorus and iPTH and correlates with GFR decline in CKD patients.

Reduction in Klotho level was less profound in patients with CKD stages 3B-4, who were treated with paricalcitol, compared to patients treated with alfacalcidol, as adjusted for the degree of renal failure. It should be noted that the treatment groups did not differ by the degree of serum iPTH reduction, which suggests a protective, possibly stimulating effect of paricalcitol on Klotho production (Fig. 4).

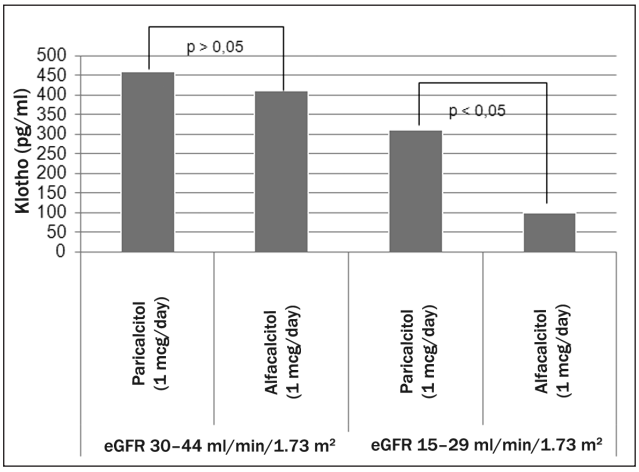
In normotensive patients with normal thickness of the posterior left ventricular wall (up to 0.9 cm, $n = 14$), Klotho level was greater than in patients with an initial left ventricular hypertrophy (thickness of the posterior left ventricular wall ≥ 1.0 cm, $n = 5$; $p < 0.05$) (Fig. 5).

In hypertensive patients, Klotho level negatively correlated with the thickness of the posterior left ventricular wall ($r = –0.594$, $p < 0.05$), with pulse wave velocity ($r = –0.537$, $p < 0.05$), and maximal systolic BP ($r = –0.603$, $p < 0.05$), and positively correlated with left ventricular ejection fraction ($r = 0.68$, $p < 0.05$).

The reduced serum Klotho level was associated with greater frequency of cardiac and vascular calcification ($r = –0.584$, $p < 0.01$), increased arterial stiffness ($r = –0.454$, $p < 0.05$), and reduced blood flow in the arteries of the lower limbs (ankle-brachial index) ($r = –0.380$, $p < 0.05$).

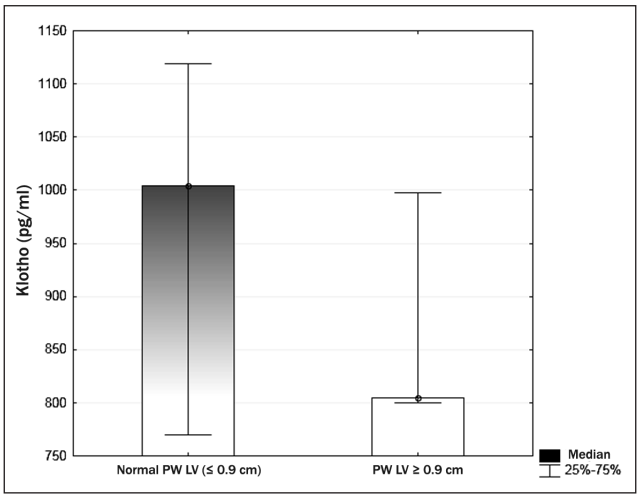
Moreover, patients with CKD stage 3B-4 who had a target BP level and were treated by ACE inhibitors showed higher Klotho levels

**Figure 4. Serum Klotho levels in patients with chronic kidney disease with hyperproduction of intact parathyroid hormone and treated with paricalcitol (n = 10) and alfacalcidol (n = 12)**




**Note:** eGFR — estimated glomerular filtration rate.

**Figure 5. Median serum levels of Klotho protein in patients with normal thickness of the posterior left ventricular wall and in patients with early left ventricular hypertrophy**




**Note:** PW LV — the thickness of the posterior wall of left ventricle.

($r = 0.509$, $p < 0.01$) and less pronounced myocardial remodeling.

## Discussion

Our results confirm the role of Klotho as an early marker of CKD progression. They also prove that the change in Klotho levels occur at the third stage of CKD and worsen with progression of renal failure [15, 16]. The reduction in Klotho level precedes an increase in serum concentrations of phosphorus and parathyroid hormone as the decline in eGFR progresses in patients with CKD. Reduced expression of the transmembrane form of Klotho in the kidneys in CKD patients correlates with the drop in eGFR, and reaches 5 % of normal levels in patients receiving dialysis treatment. We found a strong inverse relationship between the decrease in serum Klotho level and high cardiovascular risk in patients with CKD.

In our patients with CKD and hypertension Klotho deficiency correlated with higher BP levels, cardiac and vascular calcification, and an increase in arterial stiffness. On the other hand, experimental studies showed a significant reduction in angiotensin II level and proteinuria in hypertensive mice with CGN when expression of Klotho is increased [2–4]. In transgenic mice with CKD increased expression of Klotho was associated with normal phosphaturia, better kidney function and significantly lower calcification compared to wild-type mice with CKD and reduced production of Klotho [5]. Thus a beneficial effect of Klotho on vascular calcification was greater than its effect on renal function and phosphaturia that could be related to its direct influence on the vessels. There data showing the complex role of ADAM17/TGF-α/EGFR in the restructuring of the parathyroid glands and reducing Klotho expression in the kidneys, induced by activation of the renin-angiotensin system (RAS) and calcitriol deficiency [6, 7]. These data suggest the role of the effective RAS blockade and correction of D-hormone deficiency for the prevention and treatment of CVD in patients with CKD. In our study, higher rates of Klotho and less pronounced myocardial remodeling were observed in patients who achieved target BP and who were treated by ACE inhibitors.

Transgenic mice with CKD and high Klotho production demonstrated significantly lower degree of vascular calcification as compared to the wild-type mice with CKD and reduced Klotho production [15]. Favorable effect of Klotho on vascular calcification was greater than its effect on renal function and phosphaturia that is associated with the direct influence of Klotho on the vessels.

Klotho was shown to bind the receptor of transforming growth factor beta type 2 (Engl. TGF-β — transforming growth factor beta) and inhibit its downstream signals to slow interstitial fibrosis [2].

## Conclusions

Thus, in addition to role of circulating Klotho in mineral metabolism in CKD, its pleiotropic effects are now recognized and its role in the development of CVD (through its impact on cardiac and vascular calcification). We can hypothesize that serum Klotho level can be used as an early diagnostic marker for increased cardiac and renal risk. Further research is required for better understanding of the impact of ACE inhibitors, paricalcitol and other nephroprotective drugs on Klotho metabolism in patients with CKD and the possible medication-stimulated Klotho production. We assume that the elimination of Klotho deficiency is a promising cardio- and nephroprotective strategy in patients with CKD.

**Conflict of interest**

The authors declare no conflicts of interest.

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