

## Klotho in cardiovascular disease: Current and future perspectives

Javier Donate-Correa, Ernesto Martín-Núñez, Carmen Mora-Fernández, Mercedes Muros-de-Fuentes, Nayra Pérez-Delgado, Juan F Navarro-González

Javier Donate-Correa, Ernesto Martín-Núñez, Carmen Mora-Fernández, Juan F Navarro-González, Research Unit, University Hospital Nuestra Señora de Candelaria, 38010 Santa Cruz de Tenerife, Spain

Mercedes Muros-de-Fuentes, Nayra Pérez-Delgado, Clinical Biochemistry Service, University Hospital Nuestra Señora de Candelaria, 38010 Santa Cruz de Tenerife, Spain

Juan F Navarro-González, Nephrology Service, University Hospital Nuestra Señora de Candelaria, 38010 Santa Cruz de Tenerife, Spain

**Author contributions:** Donate-Correa J, Martín-Núñez E, Mora-Fernández C, Muros-de-Fuentes M and Pérez-Delgado N cooperated in the design and the writing; Navarro-González JF revised and polished the text.

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**Correspondence to:** Javier Donate-Correa, PhD, Research Unit, University Hospital Nuestra Señora de Candelaria, Ctra. Gral. del Rosario, 145, 38010 Santa Cruz de Tenerife, Spain. [jdonate@ull.es](mailto:jdonate@ull.es)  
Telephone: +34-922-602061  
Fax: +34-922-600562

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### Abstract

Protein Klotho, beyond its role as a regulator of the phosphatemia, is also involved in the maintaining of the cardiovascular health, being associated its alterations with the development of cardiovascular damage and increased morbi-mortality. For all this, nowadays Klotho is the subject of a thorough research which is focused on uncover its intimate mechanisms of action, and in analyzing the utility of its modulation as a potential strategy with clinical applicability. Molecular mechanisms of Klotho are not well understood but an emerging research area links Klotho deficiency with vascular pathology. Changes in this protein have been associated with cardiovascular-related complications like inflammation, vascular calcification, and endothelial dysfunction. All this is particularly relevant if considering the recent discovery of Klotho expression in vascular tissue.

**Key words:** Klotho; Cardiovascular disease; Chronic kidney disease; Mechanism of action; Therapeutics

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**Core tip:** Protein Klotho, beyond its role as a regulator of the phosphatemia, has also been involved in the maintaining of the cardiovascular health. For all this, is the subject of a thorough research which focused on uncover its intimate mechanisms of action, and in analyzing the utility of its modulation as a potential strategy for clinical applicability. Emerging research links Klotho deficiency with vascular pathology. Changes in this protein have been associated, among others cardiovascular-related

complications, with inflammation, vascular calcification, and endothelial dysfunction.

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## INTRODUCTION

The protein Klotho (formerly called  $\alpha$ -Klotho to distinguish it of a homologue subsequently discovered and called  $\beta$ -Klotho) has opened an extraordinarily wide research area because of its implication in diverse biological processes, many of them related to human longevity. Although the molecular mechanisms of action of Klotho are not well understood, but current knowledge points to the high pleiotropy of this protein with diverse biological functions and downstream targets. Functions of Klotho include regulation of energy metabolism, anti-inflammatory and anti-oxidative effects, modulation of ion transport, and regulation of mineral metabolism<sup>[1]</sup>. Many of these effects have been related to the maintaining of the vascular health and the mismatch of Klotho levels has been related with the onset of cardiovascular disease (CVD).

In the organism, protein Klotho is present as a transmembrane form, which participates in the signal transduction of the phosphatonin fibroblast growth factor (FGF) 23, and as an endocrine soluble factor, detectable in blood, urine, and cerebrospinal fluid<sup>[2]</sup>. Soluble form predominates in humans<sup>[2]</sup>, declines with age<sup>[3]</sup>, and it can be generated through different ways including an alternative RNA splicing<sup>[2,4]</sup>, which generates a short soluble form of 65 kDa, and a proteolytic cleavage by membrane-anchored A Disintegrin and Metalloproteinases (ADAM)-17 and ADAM-10 and by the beta-site amyloid precursor protein-cleaving enzyme 1<sup>[5,6]</sup> which generates a longer soluble form of 130 kDa. This longer form predominates in humans.

Klotho protein deficiency was first related to CVD in the Klotho-deficient mice aging-model, which manifests many human progeroid symptoms including accelerated arteriosclerosis, associated with extensive medial calcification of the aorta, and both medial calcification and intimal thickening of medium-sized muscular arteries<sup>[7]</sup>. In addition, they exhibit impaired angiogenesis<sup>[8]</sup> and endothelial dysfunction<sup>[9]</sup> which can be ameliorated by *in vivo* gene delivery of the *Klotho* gene or by parabiosis with the Klotho wild-type specimen<sup>[10,11]</sup>.

Most of the research in Klotho has been focused in its role as renal cofactor for the binding of FGF23. The presence of Klotho in the kidneys elicits the phosphaturic effect of FGF23 and the inhibition of the synthesis of the active form of the vitamin D<sup>[12,13]</sup>. However, the

existence of a soluble form of Klotho<sup>[4,5]</sup> and, importantly, its recently discovered expression in vascular tissue<sup>[14,15]</sup> and in blood<sup>[16]</sup>, has allowed to consider this molecule as a novel factor able to exert important effects in multiple organs including the cardiovascular (CV) system.

## KLOTHO AND CVD

The kidneys are probably the main source of soluble Klotho and are also the target of multiple mineral metabolism-related effects of Klotho. Interestingly, both genetic and chronic kidney disease (CKD) Klotho deficiencies generate very similar systemic manifestations including CV lesions. This has prompted the recent appearance of numerous investigations focused on the association of Klotho deficiency with the extremely high CV morbidity and mortality in the renal patient<sup>[17,18]</sup>. This is a particularly relevant research area if we consider that mortality from CVD disease in a 20-year-old dialysis patient is the same as in a 80-year-old person not on dialysis<sup>[19]</sup>, and that the traditional risk factors, highly prevalent in CKD, are not enough to account for this incidence. In any case, studies focused on studying the relationship of Klotho with CVD should consider the presence of many confusing factors including age, kidney function, active vitamin D, FGF23, parathyroid hormone (PTH), medications, and Ca<sup>2+</sup> and Pi levels which affect blood soluble Klotho levels in these patients.

CVD is the leading cause of mortality in CKD patients which universally suffer from vascular calcification, inflammation, endothelial dysfunction and oxidative stress<sup>[20,21]</sup>. Reduced Klotho, in addition to traditional and CKD-related risk factors, has been proposed as a novel contributor to the appearing of these complications<sup>[22-24]</sup>.

The absence of Klotho in murine models causes accelerated aging syndrome and atherosclerosis, vascular calcifications<sup>[15]</sup>, and defects in angiogenesis<sup>[8]</sup>, and endothelial dysfunction<sup>[23]</sup>, which is reversed by administering the *Klotho* gene or by parabiosis with the wild-type. More recent studies have confirmed the protective effects of Klotho on the vascular system, including its participation in the maintenance of endothelial homeostasis and vascular functionality<sup>[9,23]</sup>, correlating their absence with the appearance of endothelial dysfunction and vascular calcifications<sup>[23,24]</sup>. Clinical studies have shown that low serum Klotho is associated with arterial stiffness in CKD patients<sup>[25]</sup> and independently associated with severity of coronary artery disease (CAD) in patients with normal kidney function<sup>[26]</sup>. In addition, genetic variation studies have demonstrated that Klotho gene polymorphisms might be also associated with longevity and CAD<sup>[27-29]</sup>. In particular, the KL-VS allele, characterized by six SNPs in a region of 800 bp in exon 2 and flanking sequence, is prevalent in the population and is associated with a reduced longevity<sup>[27]</sup>. In a study where two different groups of healthy siblings were tested, Arking *et al.*<sup>[28]</sup> found that this functional variant of *Klotho* gene is an independent risk factor for CAD. This risk is modulated by

modifiable risk factors, such as hypertension, increased high-density lipoprotein cholesterol levels or smoking<sup>[28]</sup>. Likewise, in an Ashkenazi Jew group it was found that homozygous KL-VS individuals were at higher risk of stroke than wild-type subjects<sup>[30]</sup>. In the case of G-395A polymorphism, the A allele has been found to be an independent predictor of atherosclerotic CAD but not of vasospastic angina in Japanese population<sup>[29]</sup>. This polymorphism affects the promoter of the *Klotho* gene, so that the G→A substitution impairs protein binding to the region and consequently affects gene expression<sup>[31]</sup> and soluble Klotho levels. Similarly, Jo *et al.*<sup>[32]</sup> observed an association of the G-395A allele with CAD but not with coronary artery calcification in Korean patients. Besides, subjects with the T allele for the C1818T polymorphism (located in exon 4) have lower prevalence of CAD than those with CC genotype<sup>[33]</sup>.

CAD is mainly caused by established coronary arteriosclerosis derived from endothelial dysfunction which could be developed by low Klotho levels. Stimulation of nitric oxide (NO) synthesis by Klotho, verified in experimental models<sup>[34]</sup>, could be essential for this endothelial protective effect. Consistent with this, the deficiency in Klotho expression in rats reduces the ability of arterial vasodilatation, with a decrease in the excretion of NO urinary metabolites<sup>[11]</sup>. Furthermore, this situation can be reversed by parabiosis with wild-type specimens, and also through administration of the *Klotho* gene<sup>[9,11]</sup>. Moreover, *Klotho*-deficient mice exhibit impaired angiogenesis which depends on endothelium-derived NO<sup>[35]</sup>. The mechanism by which Klotho regulates NO synthesis remains to be determined.

Klotho protein also appears to be associated with the inflammatory process. Thus, the proinflammatory cytokines tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and TNF- $\alpha$  cause the NF $\kappa$ B-dependent decrease in the expression of Klotho, both *in vitro* and *in vivo*<sup>[36]</sup>, while in human umbilical endothelial cells, the addition of Klotho is able to suppress the expression of adhesion molecules in the endothelium induced by TNF- $\alpha$ <sup>[37]</sup>. Moreover, the soluble form of Klotho is capable of inhibiting the Wnt signaling<sup>[38]</sup>. Although this signal is essential to ensure the proliferation and survival of stem cells, an excess exposure to it can contribute to depletion and accelerated senescence<sup>[39]</sup>. Therefore, the attenuation of the Wnt signal by Klotho contributes to the anti-aging properties of this protein.

Ectopic calcifications in soft tissues, including cardiac valves and aorta, have been also related to low Klotho soluble levels<sup>[7,24,40]</sup>. Vascular calcification is prominent in the Klotho mutant mice, similarly to CKD subjects, and can be reversed by Klotho overexpression through adenoviral delivery of *Klotho* gene<sup>[10]</sup>. Albeit in CKD the release of Ca<sup>2+</sup> and Pi, by disturbed PTH, low calcitriol and soluble Klotho and high FGF23, may trigger or accelerate vascular calcification<sup>[41]</sup>, the existence of direct effects of Klotho and also of FGF23 is the subject of extensive research at present.

## PLAUSIBLE MECHANISMS OF ACTION OF KLOTHO IN THE VASCULATURE

Many vascular molecular actions are proposed to explain Klotho vascular-protective effects. Klotho is expressed in the vasculature<sup>[14,15]</sup> and is probably that the formation of both, the membrane and the soluble form, coexists in the vascular beds. Since membrane Klotho protein has an extremely short intracellular domain, it is unlikely that displays a signal transduction by itself. More probably, membrane Klotho solely serves as a coreceptor for the binding to FGF receptors (FGFRs) and as a source of a soluble form<sup>[42]</sup>. However, it is still unknown what is the contribution of the vascular bed to the blood levels, and which are the regulatory mechanisms of Klotho shedding. Serum Klotho levels determined by ELISA varies between 200 and 740 pg/mL<sup>[43]</sup>.

Similarly, the mechanisms of action of soluble Klotho remain unknown and the existence of a receptor for this protein is a matter of debate. Because FGFRs are quite ubiquitous, the binding of soluble Klotho to these receptors has been proposed<sup>[44]</sup> as an explanation to the pleiotropic effects of Klotho. Theoretically, soluble Klotho can bind to this FGFRs but there is no evidence that this activates signalling events<sup>[44]</sup>. Another possibility is that soluble Klotho does not act as an hormone *sensu stricto*, since it lacks of a specific receptor. In this scenario, the hypothetical functions of soluble Klotho would be based on its putative enzymatic abilities. Different works point to the glycan-modifying activity of circulating Klotho. Thus, a putative activity as sialidase which removes terminal sialic acids from N-linked glycans of glycoproteins in the cell surface has been proposed to explain the effect of circulating Klotho in preventing the endocytosis of the transient receptor potential cation channel, subfamily V, member 5 (TRPV5) and the renal outer medullary potassium channel 1 in the kidneys<sup>[45,46]</sup>, thereby increasing renal reabsorption of Ca<sup>2+</sup> and excretion of K<sup>+</sup>, respectively. This enzymatic activity is also responsible of promoting the endocytosis of the NaPi-2a cotransporter in the renal proximal tubule<sup>[47]</sup>. But circulating Klotho acting as a glycan-modifying enzyme could also exert a broad range of effects beyond the kidneys; the addition of recombinant soluble Klotho protein to rat vascular smooth muscle cells (VSMCs) cultures is able to decrease high Pi-induced calcification by diminishing expression and activity of Na/Pi cotransporters type 3 (Na/Pi-3, also known as Pit 1 and Pit 2)<sup>[24,47]</sup>. Na/Pi-3 cotransporters are widely expressed in tissues such as intestinal epithelium, liver, lung, heart and VSMCs<sup>[48,49]</sup>, but to date there is no evidence for an effect of Klotho on Pit-1 and 2 in the vascular beds. Finally, the aminoacidic sequence of Klotho shares homology with a  $\beta$ -glucosidase. However, this glucosidase activity has not been confirmed<sup>[7,50]</sup>.

Another possible explanation for some of the CV disorders observed in situations of low levels of Klotho comes from FGF23. In this sense, vascular calcification might be an off-target effect of FGF23 levels. Lim *et*

*al*<sup>[15]</sup> proposed that vascular Klotho acts not only as an endogenous inhibitor of vascular calcification in VSMCs but also, as a cofactor required for vascular FGF23 signaling. In this study, restoration of Klotho expression in knock-down cells unmasked FGF23 anticalcific effects<sup>[15]</sup>. Finally, a second effect of FGF23 on CVD derives from the extremely high levels of this hormone observed in primary (mainly genetic) and secondary (commonly in CKD) Klotho deficiency states<sup>[7,24,51-55]</sup>. In CKD, uremic cardiomyopathy, characterized by cardiac hypertrophy and fibrosis, is a principal cardiovascular pathological feature and includes left ventricular hypertrophy (LVH). Novel risk factors for LVH in uremic cardiomyopathy are low levels of soluble Klotho and high levels of FGF23<sup>[55,56]</sup>. In fact, FGF23 causes LVH after intramyocardial and intravenous administration in wild-type mice<sup>[55]</sup>. Since Klotho is not normally expressed in the ventricles, the effects caused by Klotho deficiency might be due to other parameters. One of the parameters considered is FGF23. Some *in vivo* experiments show that Klotho influences FGF23 production<sup>[57]</sup>, however, there are no *in vitro* data supporting this effect.

Klotho is also involved in the modulation of inflammation. The mechanism of regulation of inflammation by Klotho may be based in the inhibition of NF- $\kappa$ B activity in the endothelium<sup>[37]</sup> by unknown mechanisms. Similarly, secreted Klotho protein is also able to suppress the Wnt biological activity<sup>[38,58]</sup>. In this case the binding of Klotho to various Wnt family members results in the signal inhibition.

Finally, Klotho has been related to the regulation of the intrinsic generation of reactive oxygen species (ROS) which play a role in aging and longevity determination. Yamamoto *et al*<sup>[59]</sup> determined that cell surface-bound Klotho inhibited FOXO3a phosphorylation and promoted its nuclear translocation. FOXO3a is a member of the O subclass of the forkhead family of transcription factors which are characterized by a fork head DNA binding domain. Nuclear translocated FOXO3a then became bound to the MnSOD promoter and upregulating its expression which results in suppressing ROS formation. Moreover, Ohta *et al*<sup>[60]</sup> investigated the effect of Klotho gene delivery on blood pressure and oxidative stress *in vivo* finding an upregulation of MnSOD expression and total SOD activity in the aorta of mice, enhanced NO production, and downregulated lipid peroxide concentration in serum of mice. It was concluded that Klotho gene infusion into the tail vein of mice and rats suppressed ROS formation in animals.

## ABOUT THE THERAPEUTIC UTILITY OF KLOTHO

Injection of soluble Klotho produces biological effects in experimental animal models with kidney injury<sup>[24,61,62]</sup>, which is a state of Klotho deficiency, and in intact animals<sup>[44,47]</sup>. This supports the idea of a therapeutic approach based on the administration of soluble Klotho. In CKD, one of the

earliest events is Klotho deficiency, and establishing whether Klotho has therapeutic potential treating this disease and its complications is a critical step. However, no study has documented the therapeutic effect of Klotho in humans with renal disease.

Another approximation is based on the stimulation of Klotho expression. Experimental studies show that calcitriol administration promotes expression of Klotho *via* the activation of vitamin D receptor<sup>[63,64]</sup>. A recent work has demonstrated that administration of alfacalcidol, a vitamin D receptor activator, promoted an up regulation of Klotho gene expression in the kidney of nephrectomized spontaneously hypertensive rats<sup>[65]</sup>. According this, Klotho variants associated with lower Klotho gene expression have been associated with a decrease in survival of dialysis patients, more pronounced among patients not treated with active forms of vitamin D<sup>[66]</sup>.

Strategies aimed to increase extrarenal Klotho production might be of particular importance in end-stage renal disease patients with a significant loss of functional renal tissue. In these patients, administration or stimulation of synthesis of Klotho may potentially reverse or retard the disease progression. Therapeutic effects of Klotho may result from the modulation of circulating hormones, regulation of mineral parameters or from direct effects on target organs, leading to induction of phosphaturia, anticalcifying, antifibrotic, and antioxidative effects, and antagonism of angiotensin II effects.

Another therapeutic approach to increase Klotho levels could be based on the recently examined relationship between inflammation and Klotho expression, showing that the inflammatory cytokines TWEAK and TNF $\alpha$  promote the NF $\kappa$ B dependent downregulation of Klotho expression both *in vitro* and *in vivo*<sup>[36]</sup>. Activation of these inflammatory cytokines and the subsequent reduction of Klotho expression could contribute to organ damage. In this sense, regulation of Klotho expression by anti-inflammatory therapeutic treatments, including statins, could suppose a new approach to delay the progression of renal damage.

More specifically, Klotho expression in the vasculature may also become an important option in therapeutics. If resident vascular Klotho protects the vasculature against vascular disorders, either as a transmembrane or as a soluble form in paracrine and/or autocrine manner, upregulation of vascular synthesis could be reflected in the prevention of many vascular disorders such as endothelial dysfunction, arterial stiffness and vascular calcification.

## CONCLUSION

Nowadays, the major interest to the clinical application of Klotho research is related to CKD, where FGF23 and Klotho have been linked with CV morbidity and mortality independently of phosphatemia<sup>[67]</sup>. Importantly, these associations remain in population without impaired kidney function. Thus, Klotho has been proposed as a



potential therapeutic option. Another open question is the elucidation of the contribution of soluble vascular Klotho in advanced stages of CKD, where low serum Klotho levels are frequent. Thus far, measurements of vascular Klotho mRNA and protein in humans have been limited. It is unknown if vascular expression is also lowered in advanced CKD stages or remains at physiologic levels. Anyway, it is probably that vascular derived Klotho plays a more important role in these patients since renal contribution is dramatically diminished. What we know is that vascular expression of Klotho, along with blood soluble levels, are decreased in CAD patients without renal disease<sup>[26]</sup>.

Although depicted results clearly point to Klotho as a potential therapeutic agent in mineral-cardiovascular disorders, further studies are needed to evaluate the reliability and practical utility of this protein. Despite the controversies regarding Klotho expression and its effects, current data point to a pathogenic role of Klotho deficiency the CVD. Overall, the recent discovery of FGF23 and Klotho, and the elucidation of their regulatory effects on the phosphate homeostasis, has been accompanied by significant contributions to the CVD researching field. These discoveries will revert in the development of new targets and therapies with potential clinical applications.

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