



Cognitive Vitality Reports® are reports written by neuroscientists at the Alzheimer's Drug Discovery Foundation (ADDF). These scientific reports include analysis of drugs, drugs-in-development, drug targets, supplements, nutraceuticals, food/drink, non-pharmacologic interventions, and risk factors. Neuroscientists evaluate the potential benefit (or harm) for brain health, as well as for age-related health concerns that can affect brain health (e.g., cardiovascular diseases, cancers, diabetes/metabolic syndrome). In addition, these reports include evaluation of safety data, from clinical trials if available, and from preclinical models.

Klotho

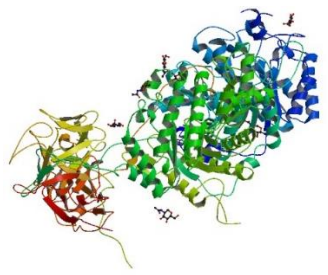
Evidence Summary

Preventing the age-related decline in Klotho may prevent age-related diseases by mitigating oxidative stress and inflammation, and regulating metabolism. Human-validated therapies are not available.

Neuroprotective Benefit: Higher Klotho is associated with cognitive reserve and a lower rate of dementia, and may mitigate the effects of ApoE4 on AD risk. Effects may be mediated by its antioxidant, anti-inflammatory, and lipid homeostatic activities.

Aging and related health concerns: Klotho levels decline with age, but Klotho maintenance may extend lifespan, preserve metabolic flexibility, and protect against age related diseases such as kidney disease, cardiovascular disease, inflammation, and cancer.

Safety: As an endogenous protein, Klotho itself is likely safe, but excessively high levels may increase FGF23 and alter phosphate and calcium mineral balance. The optimal form of Klotho, route of administration, and dosing schedule have not been established.

<p>Availability: Recombinant protein and viral expression vectors for research use.</p>	<p>Dose: Not yet determined</p>	<p>Chemical formula: Protein MW:135kDa (membrane)</p>
<p>Half-life: Varied 7.5 hours to 12 hours (estimated)</p>	<p>BBB: Not penetrant</p>	<p>65, 70, 130 kDa (cleaved/secreted)</p>
<p>Clinical trials: Serum Klotho levels are currently being used a biomarker for kidney function, and the ability of exercise to induce Klotho is being investigated. No trials testing Klotho therapies in progress.</p>	<p>Observational studies: Many studies show that Klotho levels decline with age and are associated with kidney function. Associations have also been found with lifespan, cognitive decline, hypertension, and cancer.</p>	

What is it?

Klotho (α -Klotho) is located on Chromosome 13, and has been described as an anti-aging gene. It has two major isoforms, a 135 kDa transmembrane protein that serves as an obligatory co-receptor for FGF23, and a 70 kDa secreted form. The transmembrane form is primarily expressed in the kidneys, and can be cleaved by the metalloproteinases ADAM10 and ADAM17 to produce a soluble 130 kDa form that is detectable in the serum that contains both the K1 and K2 domains, and can be further cleaved by beta and gamma secretases to produce a shorter form containing only the K1 domain [1]. The secreted form is most highly expressed in the brain, and is produced primarily by the choroid plexus, at least in rodents [2]. The soluble form of Klotho includes both the cleaved (majority) and secreted (minority) forms and acts as a hormone with pleiotropic functions. While several candidate interacting partners have been identified, the receptor(s) for the soluble form has not yet been definitively identified [3].

Klotho expression is induced by aerobic exercise and declines with age. Variants in the Klotho gene have been associated with lifespan extension and risk for cardiovascular disease. A measure of the circulating levels of Klotho has been proposed as a biomarker of kidney function. Supplementation of Klotho in the form of gene therapy or recombinant protein has shown promise in preclinical models and is currently being developed to target age-related diseases.



Neuroprotective Benefit: Higher Klotho is associated with cognitive reserve and a lower rate of dementia, and may mitigate the effects of ApoE4 on AD risk. Effects may be mediated by its antioxidant, anti-inflammatory, and lipid homeostatic activities.

Types of evidence:

- 20 observational studies
- Numerous laboratory studies

Human research to suggest prevention of dementia, prevention of decline, or improved cognitive function?

Prevention of cognitive decline: Potential benefit

While there have been no studies examining the effects of Klotho supplementation in humans, some observational studies indicate that **higher levels of Klotho circulating in the plasma or cerebrospinal fluid (CSF) are associated with higher cognitive function and a lower incidence of dementia** during the aging process [4; 5]. However, most of the studies regarding the relationship between Klotho and human cognition examine people with different genetic variants of the α -Klotho gene, and it is not clear how most of these polymorphisms alter Klotho expression or regulation in the brain.

Relationship between Klotho levels and cognitive decline

CSF: In an observational study (n=70) of older adults (average age 76) with and without Alzheimer's disease (AD) and younger adults (average age 30), CSF levels of Klotho were found to be affected by age, sex, and cognitive impairment, based on Mini mental status examination (MMSE) [4]. CSF Klotho was higher in men (899 pg/mL, 95% Confidence Interval (CI) 8144 to 983) compared to women (716 pg/mL, 95% CI 632 to 801) (P = 0.002). It was higher in young adults (992 pg/mL, 95% CI 884 to 1100) than older adults (766 pg/mL, 95% CI 658 to 874) (P=0.005), and higher in older adults without AD (776 pg/mL 95% CI 705 to 828) than those with AD (664 pg/mL, 95% CI 603 to 725) (P = 0.02). Overall, **CSF Klotho levels were found to decrease with age** (Spearman correlation of age and Klotho: -0.37, P = 0.003) **and during cognitive decline** (Spearman correlation of MMSE score and Klotho: 0.30, P = 0.02).

Plasma: In the InCHIANTI Italian cohort study of adults \geq 55-year-old (n=833), people with Klotho plasma concentrations greater than 669 pg/mL were, on average, younger, consumed fewer alcoholic drinks, had less depression, and were more likely to be female than participants with lower klotho concentrations (\leq 669 pg/mL), all P values $<$ 0.05 [5]. Individuals with higher Klotho levels had higher MMSE scores at the 3-year (P=0.002) and 6-year (P = 0.07) follow-up timepoints. Each additional natural



logarithm of klotho (pg/mL) was associated with 35% lower risk of meaningful decline in MMSE, defined as decline exceeding three points.

In a study of older adults with different types of cognitive impairment (n=320), having the lowest level of plasma Klotho (1st tertile) was associated with older age, higher prevalence of coronary heart disease and stroke, and higher levels of creatinine, homocysteine, and high-sensitivity C-reactive protein [6]. The **risk for vascular dementia was highest for those with the lowest Klotho levels** (≤ 514.8 pg/mL, Odds ratio OR: 3.54, 95% CI 1.05 to 11.93), and there was an increased risk for everyone with levels ≤ 680 pg/mL. Klotho levels were not found to be associated with the risk for AD (without vascular dementia) in this study.

Low plasma Klotho levels (≤ 400 pg/mL) have also been found to be associated with increased risk for Grade 2 (OR: 1.38) and Grade 3 (OR: 2.94) ($P < 0.05$) cerebral deep white matter lesions on MRI, and MMSE scores were correlated with α -Klotho levels ($P < 0.05$) in a study of community-dwelling people in Japan (n=280, average age 71)[7].

Schizophrenia: Plasma levels of Klotho are elevated in individuals with schizophrenia (1233.25 ± 382.25 pg/mL) relative to controls (844.49 ± 302.94 pg/mL) [8]. Cognitive dysfunction is a common feature of schizophrenia, and this study suggests that elevated Klotho may be a compensatory factor for preserving cognitive function. Klotho levels did not significantly correlate with psychological symptoms, but Klotho levels were positively correlated with measures of cognitive function. Klotho levels correlated with measures of attention, working memory, verbal memory, and executive function.

These studies suggest that systemic levels of circulating Klotho decrease with age, and that very low levels of Klotho correlated with cognitive decline and vascular dysfunction. Notably, the sex difference showing higher CSF Klotho in men and higher plasma Klotho in women suggests that the two populations of Klotho are independently regulated.

Relationship between Klotho genetic variants and cognitive decline

The genetic and epigenetic regulation of Klotho is complex and varies with different ethnic groups. Overall, **the basic trend appears to involve alterations that decrease Klotho expression being associated with a greater risk of cognitive impairment**, although the particular mechanisms may vary. For example, in one ethnic Chinese population where the KL-VS variant is present in a similar frequency to Caucasians, the risk for mild cognitive impairment (MCI) was largely related to the presence or absence of the KL-VS associated single nucleotide polymorphisms (SNPs) [9]. Whereas in another ethnic Chinese population without this variant, MCI risk was better associated with the methylation status of



the Klotho promoter [10]. Consequently, a personalized approach may be needed in order to effectively boost Klotho expression levels in a given person using 'Klotho enhancing therapy'.

KL-VS variant: KL-VS is the most well-studied genetic variant of Klotho. It is a haplotype containing 6 SNPs and is present in approximately 15% of Caucasians [11]. Two of the SNPs located in exon 2 lead to amino acid substitutions (F352V and C370S). *In vitro* studies suggest that the F352V substitution, which takes place in a highly conserved residue decreases transmembrane shedding and half-life, but is compensated for by the C370S substitution [12]. Additionally, the KL-VS variant of Klotho has a lower propensity to homodimerize, and higher propensity to heterodimerize with FGF receptors, which may enhance signaling. There is a dosage effect to this genetic variant, in that homozygotes tend to have shorter lifespans, greater risk for cardiovascular disease, and greater rates of cognitive decline, whereas the opposite was found for heterozygote carriers [13]. However, there also appears to be an age-dependent effect, as the protective effects are seen during the middle age and early elderly period [14]. In contrast, the extreme elderly (≥ 90 years old) KL-VS carriers were found to have worse cognitive function (β : -0.59, $P = 0.046$) and a faster rate of decline than noncarriers [15].

The early cognitive protection afforded to KL-VS carriers may stem from these individuals having both higher initial cognitive reserve and higher circulating levels of Klotho. KL-VS heterozygosity was found to be associated with larger volumes in the right dorsolateral prefrontal cortex (rDLPFC), which is an area important for executive function, in two independent cohorts of adults aged 55-85 in the US (Cohort 1: $P = 4.07 \times 10^{-5}$, $n=222$ and Cohort 2: $P = 0.02$, $n=200$) [16]. Heterozygotes were also found to have greater intrinsic connectivity within the rDLPFC, and this was associated with higher serum Klotho levels ($P_{\text{uncorr}} < 0.001$) [17]. Meanwhile, KL-VS homozygotes tend to have smaller rDLPFC volumes and lower serum Klotho levels [14; 16]. In the Lothian Birth Cohort 1921 study ($n=464$), people with the V/V (homozygotes) genotype (F352V) were found to have lower verbal reasoning ability at both age 11 and age 79, which was due to lower IQ [18]. The KL-VS haplotype does not prevent cognitive aging, rather, the slower rate of cognitive decline in the KL-VS heterozygotes is thought to be related to their higher baseline cognition. These studies suggest that **the Klotho genotype may be more important for the development of cognitive reserve capacity than for cognitive aging per se.**

G-395A variant: This SNP is located in the promoter region of the Klotho gene. It is thought to alter DNA-protein binding affinity, and has been predicted to be a missense variant [11]. Two observational studies indicate that there may be a slight age-dependent benefit to the SNP in terms of cognitive decline. A study in China ($n=706$) found that having the G/A SNP was associated with a lower risk of cognitive impairment (OR: 0.66; 95 % CI 0.44 to 0.98) in those over age 90 [19]. In a Japanese study ($n=2234$), people aged 60 to 79 with the SNP had slightly higher IQ (102.6 ± 0.8 vs. 99.8 ± 0.5) and higher scores on



the JWAIS-R – Information, Similarities, and Picture Completion task. However, there were no SNP dependent differences in individuals younger than age 60 [20].

CSF Klotho inducing therapy

In a small study of 8 geriatric patients (age 70.3 ± 8.65), electroconvulsive therapy (ECT) was shown to increase levels of CSF Klotho from 792.5 pg/mL to 991.3 pg/mL, ($P=0.0020$) [21]. The increase was positively correlated with the number of ECT sessions ($F= 7.84$, $P=0.031$), however there was no correlation between CSF Klotho and depression-relief response to ECT. Notably, serum levels of Klotho were not affected by ECT, as they are derived from the kidney, whereas CSF levels are derived from the choroid plexus.

Human research to suggest benefits to patients with dementia: None

Mechanisms of action for neuroprotection identified from laboratory and clinical research:

Neurogenerative diseases (pre-clinical models): Potential benefit

The secreted form of Klotho, generated through alternative splicing, is expressed at the highest level in the brain, particularly in the choroid plexus. The level of the secreted form is 10X higher in the brain than the kidney [2]. This expression decreases with age [22; 23], and decays more rapidly in animals with neurodegenerative disease [2]. It remains to be established whether this alternatively spliced form is similarly elevated in the human brain, since another study has shown that this splice form undergoes nonsense mediated decay in the kidney [24]. Alternative forms of soluble Klotho can also be generated as a result of differential cleavage [25]; thus, the shorter (70 kDa) form of soluble Klotho could also be abundant in the human choroid, but produced via a different mechanism. The major cleavage sites in human Klotho have recently been identified [26]. The alpha1 site, LGSGLTGRF, cleaves the transmembrane form to produce the 130 kDa soluble form containing both the KL1 and KL2 domains. The alpha2 site is located within the linker region between KL1 and KL2, PPLPENQPL, and produces a form of soluble Klotho (70 kDa) containing only the KL1 site. Preclinical studies in cells and rodents have shown that the longer (130 kDa) and shorter (70 kDa) forms of soluble Klotho can have different biological effects, which likely stems from differential capacity to regulate FGF23 [27]. It will be necessary to determine the predominant form of active Klotho in the human brain in order to develop a therapeutic form of Klotho for neurodegenerative diseases. Since Klotho is not BBB penetrant, it is unclear whether peripherally administered or produced Klotho would impact brain levels or brain function. The majority of preclinical models use transgenic or viral mediated production administered directly to the CNS. In rodent models, Klotho supplementation or overexpression can prevent or reduce



cognitive decline. Klotho's ability to regulate glutamate signaling, antioxidant activity, metabolism, neurogenesis, and inflammation may underlie its neuroprotective effects.

Alzheimer's disease: Klotho is a physiological target of amyloid precursor protein (APP), and is regulated by soluble APPs β [28]. It has been speculated that Klotho may play a role in protecting against A β -mediated toxicity. In hAPP transgenic mice, Klotho expression in the dentate gyrus of the hippocampus was 62% lower than in wildtype mice [29]. Transgenic overexpression of Klotho enhanced spatial learning and memory increased the abundance of the GluN2B subunit of NMDA glutamate receptor at the synapse, and NMDA receptor-dependent synaptic plasticity [29]. A prior study found that transgenic Klotho overexpression was able to improve spatial memory task performance in 4-7 month-old and 10-12-month-old wildtype mice, and that this cognition enhancing effect was dependent on an increase in the expression of the GluN2B subunit of the NMDA receptor in the hippocampus [14]. Lentiviral expression of full-length mouse Klotho cDNA following intracerebroventricular administration at 7 months of age restored brain and serum Klotho levels in 13-month-old APP/PS1 AD mice [30]. The upregulation of Klotho was associated with reduced A β burden, synaptic loss, and cognitive deficits in the AD mice, whereas there were no significant differences in cognitive measures in wildtype mice overexpressing Klotho. In the AD mice, the restoration of Klotho levels also reduced NLRP3-mediated inflammation as well as the associated transformation of microglia into a pro-inflammatory state.

Parkinson's disease: In the hSYN alpha-synuclein overexpression model of PD, peripheral administration (10 μ g/kg i.p.) of a recombinant version of the cleaved (shed) form of Klotho (amino acids 35-982) improved motor learning and cognitive deficits [31]. It was also able to boost spatial and working memory in aged (18-month-old) WT mice. The effect appears to be related to altered glutamate receptor signaling, and increases in the cleaved form of the glutamate GluN2B NMDA receptor subunit. However, the mechanism underlying its protective effect is unclear, since it is not BBB penetrant.

In the 6-OHD model of PD, pretreatment with recombinant Klotho (10 μ g, 30 min prior to 6-OHD) via intracerebroventricular injection, mitigated drug-induced rotational behavior, and improved performance on the narrow beam test [32]. It also reduced markers of oxidative stress and protected against neuronal degeneration in a PKA and CAMKII dependent manner.

Age-related cognitive decline: Exercise is a potent inducer of Klotho [33]. Continuous moderate exercise can slow the rate of cognitive decline in mice, but it cannot prevent or reverse cognitive decline [2]. In brain tissue (white matter) from rhesus monkeys, the level of Klotho promoter methylation increased with age [34]. These epigenetic modifications may decrease the ability to endogenously induce Klotho expression with age, and suggest that exogenous supplementation may be required to maintain high

levels throughout life. Overexpression of the secreted form of Klotho via adenovirus (AVVrh10) delivery into the cerebral ventricles of 6 and 12-month-old mice, improved age-related motor declines and cognitive performance (Morris water maze $P=0.0018$), when tested 6 months later [35].

Amyotrophic lateral sclerosis: Transgenic overexpression of Klotho modestly delayed the onset of progression by around 6 days and extended survival approximately 11 days in the SOD^{G93A} mouse model of ALS, though the protective effects were primarily seen in females [36]. The increased Klotho was accompanied by reduced inflammation and motor neuron loss, as well as increased expression of endogenous antioxidants. Considering the marginal nature of the benefits and that Klotho levels were systemically elevated throughout the lifespan of these animals, it appears unlikely that Klotho supplementation after the onset of disease would offer clinically meaningful benefit in this population.

Mechanisms of neuroprotection:

Antioxidant activity: Lentivirus mediated expression of full-length Klotho into the cerebral ventricles of 7-month-old senescence-accelerated prone (SAMP8) mice led to increases in levels of both transmembrane and soluble Klotho three months later, and reduced memory deficits, neuronal loss, and synaptic damage [37]. These effects may have been mediated by its **induction of an antioxidant response** by regulating the phosphorylation of Akt and FOXO1. The FOXO class of transcription factors are involved in the regulation of oxidative stress and are negatively regulated by the P13K/Akt pathway.

In cell culture with hippocampal neurons, pre-treatment with recombinant Klotho (0.4 $\mu\text{g}/\text{mL}$) reduced glutamate mediated cytotoxicity from 65% to 40%, and protected neurons against A β -mediated toxicity [38]. This neuroprotective effect was partially mediated by the induction of the thioredoxin/peroxiredoxin redox control system.

Neurogenesis: Local downregulation of Klotho in the hippocampus reduces adult hippocampal neurogenesis in mice, and leads to decreased performance on hippocampal-dependent memory tasks [39; 40]. Transgenic Klotho overexpression [39] or supplementation [40] with shed klotho protein can increase the proliferation rate of neural progenitors and rescue these effects. Recombinant Klotho (0.4 $\mu\text{g}/\text{mL}$) can also increase the maturation of rat oligodendrocyte precursor cells (OPCs) in cell culture, and involves the Akt and ERK1/2 signaling pathways [41].

Glutamate signaling: In addition to affecting levels of glutamate NMDA receptor subunits in the hippocampus, Klotho also affects the expression of glutamate reuptake transporters (EAAT transporters) [42]. Treatment with recombinant Klotho increases EAAT3 and EAAT4 expression (by modulating PI3K signaling), which could help prevent glutamate-mediated excitotoxicity.



Metabolism: Klotho expression in neurons is modulated by glutamatergic and insulin signaling, and may play a role in the metabolic coupling between neurons and astrocytes. Soluble Klotho was shown to enhance astrocytic aerobic glycolysis and the release of lactate to be taken up and used as fuel by nearby neurons [43].

Inflammation: The age-related reduction of Klotho expression and secretion from the choroid plexus may be a key driver of neuroinflammation. The expression of major histocompatibility complex (MHC) Class II, which is involved in antigen presentation, was found to be 5.6-fold higher in 22 to 23-month-old mice, compared to 2-3-month-old mice [23]. There was also an increase in adhesion molecules, such as ICAM-1, which could promote the infiltration of immune cells into the CNS through the blood-CSF barrier. Indeed, lower Klotho levels promote macrophage infiltration and microglia activation. The **anti-inflammatory** effects of Klotho appear to be partially mediated through the inhibition of the NLRP3 inflammasome in a Klotho-FGF23 signaling dependent manner.

APOE4 interactions:

Klotho affects the risk for cognitive impairment independently from ApoE4 status, but there is increasing evidence that **Klotho may act as a resilience factor in ApoE4 carriers**. In the Aberdeen birth cohort of 1936 longitudinal study, the KL-VS variant's associations with white matter volume, rDLPFC volume, and rate of cognitive decline were not affected by ApoE4 status [44]. In the Wisconsin Registry for AD prevention (n=325), late middle-age KL-VS heterozygotes had less ApoE4-associated cortical thinning [45].

In an analysis of 22 AD cohorts including 20,928 individuals, KL-VS heterozygosity was associated with reduced risk for AD conversion in ApoE4 carriers (Hazard ratio HR: 0.64, 95% CI 0.44 to 0.94, P = 0.02) [46]. This effect was primarily driven by those in the 60 to 80 years age range, which is the period when the effect of ApoE4 on AD risk is most prominent. The presence of ApoE4 is associated with increased A β burden, but the presence of KL-VS heterozygosity mitigated the effect of ApoE4 on A β load, without affecting A β load in non-ApoE4 carriers. The protective effect of KL-VS on A β load in ApoE4 carriers has also been demonstrated in other studies. In a study of 309 late middle-aged adults in Wisconsin, ApoE4 carriers without KL-VS had higher A β burden than ApoE4 noncarriers based on CSF (CSF: t = -5.09, P < 0.001) amyloid PET (t = 3.77, P < 0.001) measures, while KL-VS heterozygotes with ApoE4 did not have elevated A β relative to ApoE4 noncarriers (CSF t = -1.03, P = 0.308; PET t = 0.92, P = 0.363) [47]. In a study of 581 cognitively normal older adults in Australia, the trajectory toward cognitive decline was only mitigated in KL-VS carriers with reduced A β burden [48].



Although the mechanism by which KL-VS protects against ApoE4-mediated AD risk has not been established, it may stem from the ability of Klotho to influence lipid metabolism. ApoE4 promotes lipid storage, which can lead to the pathological buildup of lipids in cells and tissues, while Klotho has been shown to promote lipid oxidation/utilization. Therefore, in ApoE4 carriers, KL-VS may restore lipid homeostasis, reducing cellular/oxidative stress, and ultimately leading to reduced A β burden.

Aging and related health concerns: Klotho levels decline with age, but Klotho maintenance may extend lifespan, preserve metabolic flexibility and protect against age related diseases such as kidney disease, cardiovascular disease, inflammation, and cancer.

Types of evidence:

- 5 meta-analyses (longevity n=4; n=29 studies, Cardiovascular/kidney n=9; n=6 studies, Cancer n=18)
- 17 observational studies
- Numerous laboratory studies

Lifespan: Potential benefit

Circulating serum levels of Klotho decrease with age in humans and other long-lived primates [5; 49]. Animal studies suggest that the maintenance of high Klotho levels could slow the aging process and extend lifespan. Rodent models initially identified the relationship between Klotho levels and lifespan, and studies of people with different genetic variants of Klotho suggest that this association may extend to humans.

C. elegans: Two Klotho homologues have been identified in *C. elegans*. These are thought to represent an ancestral form of Klotho, which only encodes the K1 domain, and thus exerts its effects in an FGF23 independent manner. Knockdown of these Klotho genes reduces lifespan in worms, and the effect on lifespan appears to be mediated through modulation of insulin/IGF-1/PI3K/FOXO signaling [50]. The effect depends on FGF signaling, though the mechanism has not been elucidated. This form of Klotho has been hypothesized to act as an inhibitory decoy receptor, and/or it may modulate receptor activity via its sialidase activity. This study as well as a study in Klotho transgenic mice suggests that both FGF(23)-dependent and FGF23-independent effects are important for the effects of Klotho on lifespan.

Mice: Klotho knockout mice have a premature aging phenotype which includes atherosclerosis, decreased bone density, reduced skin thickness, abnormal lipid metabolism, and a shortened lifespan



averaging only 60.7 days [51]. In contrast, transgenic overexpression of **Klotho can extend life-span** in mice in a sex-dependent manner. In two different Klotho overexpression lines the lifespan of male mice was extended by 20% and 30.8%, while female lifespan was extended by 18.8% and 19%, respectively [52]. In these transgenic mice, the protein levels of Klotho are two to three-fold higher than in wildtype mice, with plasma levels ranging from approximately 200 to 250 pM in transgenic and 100 pM in wildtype 8-week-old mice of both sexes [52]. It is unclear whether this magnitude of lifespan extension is possible as a result of Klotho supplementation in humans, as well as the fold change in Klotho levels that would be needed to produce this outcome. The natural variation in plasma Klotho levels in adults has been shown to vary over 2-fold relative to the mean (i.e. range of 239 to 1266 pg/mL vs average 562 pg/mL) [53], suggesting that a 2-fold elevation may be insufficient for dependable lifespan extension in humans, perhaps due to heterogeneity in other longevity associated genes/factors in the human population. However, the data is not longitudinal, so the discrepancy may stem from the decline of Klotho levels with age in humans, and sustained elevated expression in the transgenic mice. This suggests that the maintenance of elevated Klotho levels throughout late adulthood may be essential for its effects on longevity, although studies from individuals with a tendency toward higher levels due to genetic variants suggests that high levels of Klotho early in life may also contribute to late-life effects on lifespan and healthspan.

KL-VS genetic variant: In a cohort of Italian adults aged 19 to 109 (n=1089), the proportion of individuals with the KL-VS heterozygous genotypes (who tend to have higher circulating Klotho levels) was significantly higher in the elderly (age 66 to 88) compared to younger adults (OR: 1.564, 95% CI 1.12 to 2.174, P=0.008), but there was no difference in genotype frequency between centenarians (age >88) and younger adults [54]. A meta-analysis hypothesis of 4 cohort studies (populations include: Bohemian Czech, US Caucasians, African Americans, Italians, and Indians) found that **KL-VS heterozygotes have slightly higher longevity** than non-carriers (OR=1.14, 95% CI 1.00 to 1.30, P=0.05) using a random-effects model [13]. KL-VS homozygotes show a non-statistically significant disadvantage during aging. Similar to what is seen with respect to cognitive function, KL-VS heterozygotes have a survival advantage that weakens over the course of the lifespan. The reason for this age-effect is unknown, but may be related to genetic-variant associated changes in Klotho expression and/or epigenetic regulation over time. A meta-analysis of Klotho polymorphisms for longevity found that, based on 2714 patients and 1831 controls, in the F352 V polymorphism the F allele was protective in determining human longevity (FF + FV vs. VV; OR: 1.51, 95% CI 1.01 to 2.26; P = 0.04) [55]. This is consistent with evidence that the F352V polymorphism may decrease Klotho stability, and it is only protective as part of the KL-VS haplotype.



Klotho SNPs and mortality: Two SNPs in Klotho were identified, rs9536314 and rs9527025, that are expected to reduce Klotho expression and protein stability. In those with the polymorphism, there were trends, but no significantly associations with elevated all-cause mortality or cardiovascular mortality (crude Hazard ratio HR: 1.72, 95% CI 0.96 to 3.07 in rs9536314; crude HR: 1.82, 95% CI 0.998 to 3.33 in rs9527025) or cardiovascular mortality (crude HR: 1.52, 95% CI 0.56 to 4.14 in rs9536314; crude HR: 1.54, 95% CI 0.55 to 4.33 in rs9527025) in a population of 2921 elderly men aged 69 to 81 years [56]. This suggests that **the effect of Klotho variants on lifespan may be modulated or compensated by the overall genetic background.**

Insulin/IGF-1 -mediated mechanism: The effect of Klotho on lifespan extension is mediated through its ability to inhibit insulin and IGF-1 signaling [57]. Klotho overexpression in rodents is associated with insulin resistance primarily in males and IGF-1 resistance in both sexes. It has been proposed that the ability of Klotho to extend lifespan may be related to its regulation of lipid homeostasis by preventing intracellular lipid overload and associated lipotoxicity [58].

Klotho-associated biomarkers:

Mineral homeostasis markers: Circulating Klotho levels are primarily derived from the kidney, thus the factors that are most associated with changes in Klotho levels are those that pertain to kidney function, such as eGFR. Since a major function of Klotho is the regulation of mineral balance in conjunction with FGF23, the levels of minerals, especially phosphate and calcium, are influenced by Klotho levels. Cross-sectional studies and preclinical animal studies suggest that these associations are most prominent in disease populations, particularly those with kidney disease (see Kidney disease biomarker section), cardiovascular disease, and metabolic dysfunction. In a healthy population, the associations between circulating Klotho levels and these biomarkers are weaker and less reliable. In a study of healthy Japanese adults (n=142) and children (n=39), the associations were age-dependent, and age was the most prominent characteristic that tracked with differences in serum Klotho levels [53]. In the adults (aged ≥ 20 years), Klotho levels were inversely correlated with creatine levels ($r=-0.183$, $P=0.030$), a measure of kidney function, but there were no significant associations of Klotho with gender, intact parathyroid hormone, 1,25(OH)2D- vitamin D, calcitonin, calcium, inorganic phosphate, blood urea nitrogen (BUN), or FGF23 based on simple regression analyses. When the children were included, Klotho levels correlated positively with inorganic phosphate, and inversely with age, creatine, BUN, and FGF23 using simple regression analysis. In a multiple regression analysis involving both adults and children, circulating Klotho levels were positively associated inorganic phosphate ($r=0.517$, $P<0.001$), and inversely associated with age ($r=-0.599$, $P<0.001$), FGF23 ($r=-0.350$, $P<0.001$), and calcium. Since these are cross-sectional studies, it is unclear how well movement on these measures tracks with Klotho levels



within an individual over time. It may be necessary to assess the entire panel of mineral homeostasis markers to determine which are most sensitive to changes in Klotho levels. The proper regulation of mineral balance via the Klotho-FGF23 axis can prevent the excessive mineralization of tissues, including vascular calcification, thus measures of vascular calcification could also potentially be influenced by Klotho levels.

Additionally, these studies assess endogenous Klotho levels and the markers may be differentially affected by exogenous Klotho treatment, and how the Klotho is administered.

Transgenic mice that overexpress Klotho have been shown to have alterations in mineral homeostasis markers such as FGF23, Vitamin D, parathyroid hormone, and aldosterone [59].

In disease models, exogenous Klotho can restore these markers toward normal levels, while in wildtype animals where the markers are already within healthy physiological range, exogenous Klotho treatment may produce little to no movement on these measures. For example, in mice with polycystic kidney disease, Klotho (10 ug/kg/day) reduced renal angiotensin II and systolic blood pressure, but in wildtype mice the same level of Klotho supplementation had no effects on blood pressure, renal function, or the renal angiotensin system [60]. Similarly, Klotho (0.01 mg/kg i.p. every 48 hours) blunted a decline in antioxidant enzymes in the streptozotocin-induced diabetes model in rats, but had no effect on antioxidant enzyme levels in healthy control rats [61].

Metabolic markers: Klotho's effects on metabolic function may contribute to its effects on longevity, thus metabolic measures may provide an informative readout of whether exogenous Klotho treatment is producing a biologically meaningful impact. In the FIT-AGEING study (n=74, mean age (53.7 ± 5.1 years old), soluble plasma Klotho levels were associated with lean mass index ($\beta = 74.794$, $R^2 = 0.346$, $p < 0.001$) which may stem from the increased ability to utilize lipids as an energy source [62]. A similar effect was seen in mice fed a high fat diet, with those receiving recombinant mouse Klotho (0.2 mg/kg i.p. every 48 hours) showing decreased fat accumulation and a higher lean mass relative to their untreated counterparts [63]. The effect appears to be driven by the ability of Klotho to enhance metabolic flexibility, particularly the ability to increase fat oxidation in response to increase fatty acid availability, and to appropriately shift between fat and carbohydrate oxidation. In the FIT-AGEING study, the capacity to oxidize fat under basal and exercise conditions was positively associated with plasma Klotho levels [64]. There was an inverse association between Klotho levels and cardiometabolic risk in middle-aged sedentary adults (men $\beta = -0.658$, $R^2 = 0.433$, $P < 0.001$ and women $\beta = -0.442$, $R^2 = 0.195$, $P = 0.007$), which was also related to lean mass [65]. Aging, which involves a decline in Klotho levels, typically involves declines in lean mass and mitochondrial efficiency, along with a shift

toward increased carbohydrate oxidation [64]. Similarly, soluble Klotho levels are lower in individuals with type 2 diabetes, further supporting the relationship between declining Klotho levels and metabolic dysfunction [66]. The mechanisms by which Klotho influences metabolism have not been fully elucidated. Insulin can induce the shedding of Klotho from the transmembrane to the soluble form, where it can inhibit prolonged insulin signaling, inhibit Wnt signaling, and promote resistance to oxidative stress through the induction of endogenous antioxidant pathways. These studies suggest that declining levels of Klotho may lead to a shift in fat utilization resulting in increased lipid accumulation in tissues, resulting in elevated levels of oxidative stress, which promotes various aging-related diseases.

Frailty: Potential benefit

In the Italian InCHIANTI longitudinal cohort study of adults ≥ 65 years old, frailty status (n=744) and handgrip strength (n=804) were associated with plasma Klotho levels. Handgrip strength is an indicator of total body muscle strength and is a predictor of poor outcomes in older adults. Each increase in the natural logarithm of Klotho (pg/mL) was associated with lower odds of frailty versus robustness after adjustment for covariates (OR: 0.46, 95% CI 0.21 to 0.98, $P = 0.045$) [67]. **Grip strength was positively correlated with plasma Klotho** at threshold <681 pg/mL. In adults with plasma Klotho <681 pg/mL, each standard deviation increase in plasma Klotho was associated with increased grip strength ($\beta=1.20$, standard error = 0.35, $P = 0.0009$), after adjustment [68].

Kidney Disease: Potential benefit

The kidney has the highest Klotho expression. While the promoter is 30 to 40% methylated (silenced) in low or non-Klotho expressing cells, it is largely unmethylated in renal cells, which drives high expression in the kidney [69]. **The majority of the soluble fraction of Klotho circulating in the peripheral blood originates from the cleavage (shedding) of the transmembrane form in the kidney.** Unlike the CNS, the secreted form of Klotho does not appear to significantly contribute to this pool. In the kidney, the transcript for the alternatively spliced (secreted) form is a target for nonsense mediated mRNA decay [24]. Dysregulation of Klotho transcript splicing may contribute to the loss of Klotho protein that occurs in the context of kidney injury.

Kidney disease biomarker: A meta-analysis of 9 studies including 1457 patients with chronic kidney disease found that soluble α -Klotho levels were associated with the estimated glomerular filtration rate (eGFR), ($r=0.35$, 95% CI 0.23 to 0.46, $P<0.05$), and inversely correlated with FGF23 ($r=-0.10$, 95% CI -0.19 to -0.01, $P<0.05$) [70]. The eGFR is considered one of the more reliable measures of kidney function. Transmembrane Klotho is an obligate co-receptor for FGF23, and the level of circulating FGF23 itself is



associated with chronic kidney disease progression [71]. FGF23 is important for the regulation of phosphate and Vitamin D homeostasis by promoting the degradation of Vitamin D and inhibiting phosphate absorption [72]. High levels of FGF23 can lead to toxicity, which can be prevented by the presence of the soluble (cleaved) form or Klotho [73]. A meta-analysis of 6 cohort studies (n=655) found that a low Klotho level was associated with increased risk for all-cause mortality in patients with chronic kidney disease (Risk ratio RR 1.88, 95% CI 1.29 to 2.74), although the cutoff values for 'low' Klotho varied across the studies [74]. This suggests that serum Klotho levels could serve as a biomarker for kidney function. Some clinical trials are already incorporating serum Klotho levels into their outcome measures, however, its use in this capacity may be premature.

Assay development: A standardized assay that can reliably measure soluble Klotho has not yet been validated. This lack of standardization inhibits progress in this field by hindering reproducibility. One group analyzed the EC₅₀ of specific lots of recombinant Klotho from multiple vendors using an FGF23-dependent proliferation assay and found that recombinant Klotho is unstable and greatly affected by even minimal freeze-thaw activity, and thus each batch requires in-house validation before use [75]. Klotho activity was significantly reduced within one day of thawing and completely inactive within three days of thawing. Klotho antibodies have also been problematic, as they are primarily polyclonal, and thus promiscuous. Without selective antibodies for the different forms of Klotho, it is impossible to get a clear picture of the diversity of Klotho forms *in vivo*, and how this diversity influences their biological activity in different tissues. The ELISA assay has been the predominant immunoassay for detecting soluble Klotho in bodily fluids, but a lack of standardization has led to large variances in values across studies. A recent study suggests that the immunoprecipitation–immunoblot (IP–IB) assay is more sensitive and reproducible in detecting Klotho in human serum [76]. However, similar to recombinant Klotho, freeze-thaw cycles significantly impact the ability to reliably measure Klotho levels. Additionally, the lack of standardization has precluded the establishment of a threshold for Klotho levels indicative of disease/pathologically low, with different studies using different cutoff thresholds.

Kidney disease treatment: In addition to regulating phosphate homeostasis through FGF23, Klotho also helps regulate calcium homeostasis in the kidney through regulation of the calcium channel TRPV5. Klotho increases cell surface expression of TRPV5 in the renal epithelium by modifying sugar residues (removal of sialic acids) [77]. It is anticipated that the maintenance or restoration of Klotho levels could help restore mineral homeostasis and improve kidney function in the context of kidney disease. Animal studies provide proof-of-principle evidence to support this hypothesis.

Klotho gene therapy: Treatment of nephrotomized mice (starting 1 week after injury) with vector encoding the secreted form of Klotho via hydrodynamic based gene therapy injury, prevented induction



of the blood pressure regulating renin-angiotensin system (RAS), normalized blood pressure, and ameliorated renal fibrotic lesions at 6 weeks post-injury [78]. In the Streptozotocin-induced diabetes rat model, an intravenous injection of adenovirus containing full-length Klotho was found to prevent progression of renal hypertrophy and fibrosis in for at least 12 weeks [79]. In a mouse model of chronic kidney disease, retro-orbital injection of adenovirus containing the soluble cleaved form of Klotho reduced hyperphosphatemia and prevented vascular calcification [80]. These gene therapy approaches allowed for the maintenance of Klotho levels under conditions where they would normally decrease.

Recombinant Klotho: In a sepsis-induced acute kidney injury model, i.p. injection of recombinant Klotho (0.01 mg/kg, 1-hour post-injury) attenuated renal dysfunction ($P < 0.05$) and partially restored endogenous renal Klotho expression ($P < 0.05$), but did not impact apoptosis or autophagy [81]. In an ischemia-induced acute kidney injury model, i.p. injection of recombinant Klotho (0.01 mg/kg/day for 4 days starting 24 hours post-injury), prevented the progression to chronic kidney disease, prevented cardiac remodeling, and restored Klotho levels long after the cessation of the therapy [82]. In a chronic kidney disease model, 0.3 mg/kg/day was delivered to mice via an osmotic pump starting 4-12 weeks after injury. When used as a treatment, rather than a preventative measure, Klotho was only partially effective in restoring renal function and reducing cardiac remodeling [82]. In db/db diabetic mice, daily treatment with 10 ug/kg recombinant soluble klotho (R&D SYSTEMS, 1819-KL-050) i.p. for 8 weeks altered metabolism and protected against mitochondrial damage [83]. The protection was mediated by downregulation of mTOR/TGF β and an upregulation of AMPK-PGC1 α signaling. In a mouse model of polycystic kidney disease (DBA/2-*pcy*), recombinant human klotho protein (PeproTech) at 10 μ g/kg/day subcutaneous from 8 to 20 weeks of age, reduced kidney fibrosis, systolic blood pressure, renal angiotensin II levels, while enhancing eGFR and renal Klotho expression [60]. The effects were associated with the inhibition of TGF β , collagen type 1, Akt, and mTOR.

These studies suggest that in order to be most beneficial, Klotho therapy would need to be used either prophylactically, or very early in the course of disease/injury. The early benefits likely stem from Klotho's antioxidant activity, which could help to mitigate the induction of inflammatory and oxidative stress damage.

Cardiovascular disease: Potential benefit

Klotho can help protect against cardiovascular disease by regulating endothelial function, lipid homeostasis, ion transport, oxidative stress, inflammation, and protecting against kidney damage. Klotho can be induced as a protective mechanism in the context of inflammation, thus individuals with



lower Klotho induction capacity are more likely to experience inflammation-associated adverse events. The risk for cardiovascular problems may be related to a decline in vascular expression of Klotho.

Serum Klotho and Cardiovascular disease risk: Several observational studies have found an association between **low Klotho levels and higher risk for cardiovascular disease**. Because serum Klotho levels are most closely associated with kidney function, the associations between Klotho levels and cardiovascular disease are strongest in people with impaired kidney function, such as those with diabetes or kidney disease. In the ARNOGENE cohort study of dialysis patients with kidney disease (n=238), individuals with levels ≥ 280 ng/L had a significantly reduced occurrence of cardiovascular events and cardiovascular death (OR: 0.39, 95% CI 0.19 to 0.78, $P = 0.008$) compared to patients with Klotho < 280 ng/L [84]. In type 2 diabetic patients in China (n=168), a high Klotho level was associated with a reduced risk of developing coronary artery disease and cerebrovascular accidents (adjusted OR: 0.397, 95% CI 0.227 to 0.696, $P = 0.001$) [85]. Notably, diabetic patients taking statins were more likely to have higher Klotho levels, and those taking fibrins were more likely to have lower Klotho. Meanwhile, in the LURIC Cardiovascular Healthy Study (n=2948), serum Klotho levels did not add predictive power to cardiovascular and mortality risk assessment in patients with normal renal function [86]. After adjustment for cardiovascular risk factors the hazard ratios in the fourth quartile compared to the first quartile of s-klotho were HR: 1.14 (95%CI, 0.94 to 1.38, $P = 0.187$) for all-cause mortality and HR: 1.03 (95%CI 0.80 to 1.31, $P = 0.845$) for cardiovascular mortality.

Mechanism: In mice, Klotho-associated cardioprotection is mediated by the down regulation of TRPC6 cation channels in the heart, via inhibition of IGF-1 and PI3K [87].

Cardiac Klotho and Cardiovascular disease: In a study of patients with chronic heart failure (n=287), plasma levels of FGF23, but not soluble Klotho, was associated with adverse cardiovascular outcomes, including death, heart transplantation or assist device implantation [88]. Those with the lowest levels of plasma FGF23 had reduced risk of adverse outcomes (HR per unit SD 1.44, 95%CI 1.19 to 1.74, $P < 0.001$), and high FGF23 was accompanied by significantly increased NT-proBNP, serum phosphate, parathormone, central venous pressure, mean pulmonary artery pressure, and pulmonary capillary wedge pressure levels, along with decreased 25(OH) vitamin D. While soluble plasma Klotho levels tended to be lower with elevated FGF23 and worse outcome, the association was not significant (HR per unit SD 0.39, 95%CI 0.09 to 1.79, $P = 0.227$). **The protective effect of Klotho in the heart appears to be related to the local increase of Klotho expression within cardiac tissue**, rather than from circulating kidney-derived Klotho. Klotho is expressed in cardiomyocytes and cardiac fibroblasts in the human heart. mRNA toward full-length Klotho and the proteases involved in Klotho cleavage were found

to be upregulated in the hearts with cardiomyopathy, and ultimately led to an increase in the doubly cleaved short form of Klotho (65 kDa).

Local cardiac Klotho appears to protect the heart from fibrotic damage, and may be a compensatory response in those with decreased systemic Klotho levels due to impaired kidney function, as the degree of cardiac fibrosis is inversely correlated with cardiac Klotho expression in patients with chronic kidney disease ($r=-0.456$ $p=0.025$) [89]. The protective effect is mediated by the ability of Klotho to inhibit TGF β 1 and TGF β 1-induced pro-fibrotic Wnt signaling.

It is unclear whether supplemental Klotho treatment would be similarly protective, though preclinical studies suggest it may have therapeutic potential, but that the outcome depends on the form of Klotho. In a mouse model of kidney disease-associated cardiomyopathy (male nephrectomized mice), treatment with murine recombinant Klotho (0.01 mg/kg/day i.p. 6 weeks) protected against altered Ca²⁺ cycling and related contractile dysfunction in the heart [90]. In cell culture, the effect of recombinant Klotho treatment on cardiac fibroblasts differed depending on whether the full-length form (130kDa) or the short, cleaved form (65 kDa) was used [27]. The 130 kDa form of soluble Klotho increased collagen synthesis, stimulated myofibroblast differentiation, and promoted proliferation. These effects were driven by ERK, TGF β and FGF mediated signaling. In contrast, the short 65 kDa form of soluble Klotho had an anti-fibrotic effect by inhibiting myoblast proliferation and collagen synthesis. It cannot be conclusively established that these differences are driven by the distinction between the 130 and 65 kDa forms per se, since the recombinant proteins were generated in different cell types (CHO vs HEK293), and a separate study has found that the effects of soluble recombinant Klotho differ depending on the cell type of origin, due to differential post-translational modifications [91].

These studies suggest **that local organ specific effects of Klotho may be driven by particular forms of Klotho**, and that different physiological and pathophysiological states may differentially utilize the various forms of Klotho. *In vivo*, it is unclear whether Klotho supplementation can influence endogenous Klotho production, and whether exogenous Klotho is differentially processed and/or utilized by different tissues.

Atherosclerosis: Klotho levels may impact risk for atherosclerosis through the modulation of lipid metabolism and inflammation. KL-VS variant SNPs were found to be associated with serum levels of hemoglobin, albumin, and high-density lipoprotein cholesterol (HDL-C), fasting insulin, and fasting glucose [92]. In the InCHIANTI Italian cohort study ($n=1023$), plasma klotho was correlated with HDL cholesterol ($r = 0.11$, $P = 0.0004$), and inversely correlated with C-reactive protein ($r = -0.10$, $P = 0.0008$) [93]. In a small study, patients with atherosclerosis ($n=27$) had lower Klotho serum concentrations than



healthy controls (n=11) (413 pg/mL, 95% CI (317 to 479) vs. 1481 pg/mL (1227 to 1889); $P < 0.0001$) [94]. Vascular (arterial) Klotho expression was also 1.725 (0.537 to 3.104) vs. 4.647 (2.255 to 7.002); $P < 0.0001$. **Higher vascular Klotho was associated with an anti-inflammatory profile** (high IL-10, low TNF α , and low LDL). Klotho expression was also found to be reduced in the cardiomyocytes of patients with atherosclerotic cardiovascular disease [95]. Therefore, vascular Klotho expression is likely to be a more reliable marker of cardiovascular disease risk than serum Klotho, and maintenance of high Klotho levels may be protective against the development of atherosclerosis.

Hypertension: Klotho appears to be beneficial for **preventing and reducing high blood pressure** through regulation of endothelial function and inflammation. Klotho may be important for the vascular production of nitric oxide (NO), and the prevention of vascular calcification. An observational study (n=109) looking at the relationship between serum Klotho and hypertension found that older patients (average age 64) in China with hypertension had lower levels of Klotho protein (0.303 ± 0.096 vs. 0.489 ± 0.216 , $P < 0.01$) and NO (43.95 ± 21.85 $\mu\text{mol/L}$ vs. 62.63 ± 21.26 $\mu\text{mol/L}$, $P < 0.01$) than age-matched people without hypertension [96]. Another observational study of hypertensive patients in Egypt (n=80) found that patients treated with anti-hypertensives (angiotensin converting enzyme inhibitors) had higher levels of Klotho and NO and lower levels of carotid thickening and the oxidative stress marker malondialdehyde (MDA) [97]. The study found a positive correlation between Klotho and NO levels.

Mechanism: Mice with reduced levels of Klotho (Klotho heterozygotes) show evidence of endothelial dysfunction with an attenuated dilation response and reduced production of NO [98]. These mice develop spontaneous hypertension and are highly sensitive to salt-induced hypertension and an associated (CCR2-dependent) infiltration of macrophages and T-cells in the kidney [99]. Klotho can also prevent angiotensin-II mediated proliferation, migration and inflammatory signaling responses in vascular smooth muscle cells [100].

Klotho gene therapy: In a strain of rats that develop spontaneous hypertension, a single dose of adenoviral vector mediated full length (mouse) Klotho (2×10^8 particles per rat via tail vein i.v.) prevented the further increase in blood pressure past the baseline level for at least 12 weeks [101]. The Klotho treatment also induced an anti-inflammatory (IL-10) and antioxidant response (decreased Nox2, NADPH oxidase, and superoxide production) in the aorta and kidney. In senescence accelerated (SAMP1) mice, treatment with adenovirus mediated secreted-form of **Klotho inhibited inflammation** (macrophage infiltration) and attenuated fibrosis in the aorta [102]. In a rat model of atherosclerosis, there is a reduction in endogenous levels of Klotho. Adenovirus mediated Klotho expression improved the NO mediated endothelial relaxation response to acetylcholine (from $62 \pm 3\%$ to $82 \pm 5\%$), reduced blood



pressure to control levels, and reduced the perivascular fibrosis area (from 10995 ± 1303 mm to 6448 ± 986 mm, $P < 0.05$) [103].

Klotho-inducing small molecule therapy: Treatment of aged mice (24 to 27 months old) with a small molecule called Compound H (15 mg/kg, i.p. daily for 2 weeks) that induces Klotho expression by promoting its demethylation [104], attenuated age-related increases in arterial stiffness (pulse wave velocity) and blood pressure [100]. It reduced levels of fibrosis-associated molecules (MMP2, MMP9, TGF- β) and rescued the age-related downregulation of Sirt1 deacetylase.

Cancer: Potential benefit

In a meta-analysis of 18 studies (17 in Asians, 1 in Caucasians) regarding the relationship between cancer and Klotho expression, tissue klotho protein expression was found to be significantly lower in overall malignancies [105]. Furthermore, **higher expression of tissue klotho was associated with a good prognosis** (Overall OR: 0.482, 95% CI 0.379 to 0.613, $P < 10^{-4}$). The loss of Klotho expression in various cancers has been shown to be associated with extensive promoter hypermethylation (gene silencing) [106; 107; 108; 109]. These studies suggest that Klotho may act as a tumor suppressor by inhibiting multiple cancer-associated signaling pathways, such as the insulin/IGF-1 pathway, FGF pathway, Wnt signaling pathway, and transforming growth factor- β 1 pathway [3].

Safety: As an endogenous protein, Klotho itself is likely safe, but excessively high levels may increase FGF23 and alter phosphate and calcium mineral balance. The optimal form of Klotho, route of administration, and dosing schedule have not been established.

Types of evidence:

- Several laboratory studies
- 2 case studies

Thus far, methods to enhance or induce Klotho expression via small molecules, gene therapy, or recombinant proteins have only been tested in cell and animal models. At this point, the safety data is extremely limited, since most of the studies perform a one-time intervention and do not provide any information about potential toxicity. In the context of the Streptozotocin-induced diabetes model, adenovirus mediated expression of Klotho did not affect the blood glucose or body weight of the rats [79], and recombinant Klotho did not either exacerbate or ameliorate hyperglycemia in the diabetic



mice [110]. Mice with kidney disease that were chronically administered recombinant Klotho for 20 weeks did not show any evidence of overt toxicity [82]. Long-term effects are unknown.

Since Klotho is an endogenous protein, and high levels are positively associated with health in a variety of organ systems, the maintenance of chronically elevated Klotho is expected to be safe. However, there are some case reports to suggest that excessively high levels of Klotho could have detrimental effects if they produce or are accompanied by a global dysregulation of the Klotho-FGF23 mineral homeostasis system. An individual with a de novo translocation break adjacent to the Klotho gene had Klotho levels approximately 9 standard deviations above normal based on Western blot analysis and 5-fold higher based on β -glucuronidase activity [111]. This person had hyperparathyroidism, hypercalcemia, and hypophosphatemic rickets, which are thought to stem from the elevated Klotho. FGF23 levels were also markedly elevated 12-fold above normal. Although it may seem counterintuitive that elevated Klotho could lead to an elevation of FGF23, since they are typically inversely associated, there is evidence from rodent studies that overexpression of Klotho could have a stimulating effect on FGF23 [59]. The overexpression of soluble Klotho using AAV mediated gene therapy in mice can increase FGF23 levels and induce hypophosphatemia [112].

A separate case report found that a woman undergoing in vitro fertilization with an abnormally high level of Klotho of $1,971.12 \pm 201.11$ pg/mL during the follicular phase of preimplantation had a poor clinical outcome [113]. The resulting fetus was diagnosed with Klinefelter syndrome (XXY), and it is unclear whether the high Klotho levels contributed to this outcome.

Safety still needs to be demonstrated for the methods used to deliver exogenous Klotho, such as the vector and method of gene delivery in any type of gene therapy. The optimal form of the Klotho protein (full length, soluble cleaved, secreted), method of delivery, and dosing still need to be established for recombinant protein therapy.

Sources and dosing:

Dosing: A therapeutic level for Klotho treatment has not been established. The majority of rodent (both mouse and rat) studies use a dose of recombinant Klotho at 0.01 mg/kg, which translates to approximately 0.2 to 0.3 μ g for an average sized mouse, administered daily or every other day. One study found that this dosing increased the concentration of serum Klotho by 30 to 50% [114]. A separate study found that recombinant mouse Klotho (0.25 μ g i.p.) increased plasma Klotho levels from 0.4 ng/mL to 1 ng/mL in NOD diabetic mice when assessed 3 to 4 hours after administration [115]. This is the dose that is typically used to restore Klotho levels to 'normal' in animal disease models, but there



isn't clear evidence that this level of supplementation can further increase healthspan or lifespan in aging, but otherwise healthy, rodents. A study in healthy adults found that serum Klotho levels ranged from 239 to 1266 pg/mL, with a mean of 562 ± 146 pg/mL [53]. Levels below 400 pg/mL are often associated with disease states, and there are some case reports that levels 4 to 5 fold above average levels could be associated with adverse clinical outcomes [111].

The transgenic Klotho mouse which has an increased lifespan has Klotho levels 2 to 3 times higher than wildtype mice [52], but due to the wide range of Klotho levels in healthy humans, and variation over the course of the lifespan, it is not clear whether there is a level of Klotho supplementation that could impact human lifespan to a similar degree. Additionally, dosing with Klotho gene therapy will be challenging, as the levels cannot easily be titrated in a personalized manner.

One study found that the bioavailability of recombinant Klotho in rodents was approximately 47.5%, but this could be influenced by the degree to which the Klotho was modified, and may be significantly lower [91]. It is unclear the degree to which recombinant Klotho is taken up by tissues or the degree to which it may undergo processing in a manner that either increases or decreases its bioavailability and therapeutic utility.

Exercise: Aerobic exercise is the most potent natural inducer of Klotho, and may contribute to the life expectancy benefit of prolonged aerobic exercise [33]. Induction of soluble Klotho is related to fitness level, as those with higher aerobic capacity have greater induction. The induction of Klotho may occur as a mechanism to repair exercise associated skeletal muscle tissue damage, and related to level of exercise intensity [116]. In mice, Klotho is induced in muscle progenitor cells in response to muscle injury, but the induction is attenuated in older animals [117]. This regenerative effect on muscle involves the promotion of mitochondrial bioenergetics. In a study, younger women (age 36.0 ± 7.0) were able to boost their Klotho levels ($30.08 \pm 11.94\%$ vs $15.25 \pm 6.56\%$) in response to exercise to a higher degree than older women (age 68.3 ± 3.0) [116]. This may be due to a combination of both lower Klotho induction capacity and lower exercise intensity capacity for the older women.

Klotho inducing compounds: Many different compounds, particularly those with antioxidant activity, have been shown to induce Klotho expression. This list includes (but is not limited to): Vitamin D, curcumin, ginsenoside Rg1, statins, resveratrol, testosterone, EGCG, glitazars, ligustilide, rhein, HDAC inhibitors, and molecular hydrogen. However, most of the evidence comes from animal studies, and it is not clear whether they can appreciably boost Klotho levels in humans over a prolonged period of time.



The method of induction is also likely to impact long-term efficacy. The age-related decline of Klotho appears to be related, at least in part, to epigenetic silencing in the form of promoter methylation and histone deacetylation, and is likely to vary slightly from person to person. Therefore, agents capable of altering these epigenetic marks would need to be used in combination with agents that drive Klotho expression at the mRNA and protein levels. However, currently available broad-spectrum demethylating agents and HDAC inhibitors have pleiotropic effects.

Klotho replacement therapy: There are a couple companies working on developing Klotho replacement therapy.

[Klotho Therapeutics](#) is a virtual biotechnology company that is working on developing recombinant Klotho for the treatment of kidney disease. They have provided proof-of-concept in preclinical models of acute kidney injury and chronic kidney disease [82] (see Kidney disease treatment section). Notably, this study showed that Klotho treatment was most effective at early stages of the disease, and may be better suited for the prevention of kidney disease than for treatment of late-stage disease. In 2017, the company announced that it had obtained Series A financing to develop its recombinant Klotho for use in clinical trials for acute kidney disease, but no further information is available.

[Klogene Therapeutics](#) is a Boston based biotechnology company that is working on developing small molecules to upregulate Klotho, but little progress has been made to date. In late 2017, Klogene announced that it was merging with [Kogenix Therapeutics](#) (Barcelona), which specializes in the development of gene therapy-based approaches to Klotho enhancement. They are developing adenovirus (AAV) based gene therapy using CRISPR/dCas9 to activate the secreted form of β -Klotho. In early 2018, they published a study in which they identified two guide RNAs (sgRNAs) that bind in to the Klotho promoter (-1 to -300 bp region) and enhance expression of the Klotho gene [118]. In April 2020 they published a paper describing the design of zinc finger proteins targeting within -300 bp of the human Klotho promoter based on sequences from the Egr1 transcription factor, which is an endogenous activator of Klotho [119].

[Unity Biotech](#) (NASDAQ:UBX) entered into a worldwide license agreement with the University of California, San Francisco in 2019 for its IP related to alpha Klotho ([Press release](#)). No further progress has been reported as of late 2020.

Considerations for Klotho recombinant protein therapy:

Endogenous Klotho is produced as a transmembrane form that can undergo single cleavage to generate a long 130 kDa soluble form or double cleavage to form a shorter (65 to 70 kDa) soluble form, and they



appear to have overlapping as well as distinct functions. Since recombinant supplementation only supplies the soluble form, it needs to be determined whether the transmembrane form is essential for the prolongation of healthspan and lifespan. The ratios of the different forms in circulation and in various tissue types have also not been well characterized. It also needs to be clarified how the different types of recombinant Klotho are processed in the body, and whether they are cleaved, modified, etc., in a manner that promotes their utilization by various cell types in a similar manner as endogenous Klotho.

Source: A recent study by Pfizer has found that the modifications on recombinant Klotho are dependent on the cell type of production, and that the different modifications influence the biological activities and pharmacokinetic properties of the recombinant Klotho *in vitro* and *in vivo* [91]. The production of recombinant Klotho involves the transfection of DNA encoding the Klotho ectodomain into an expression cell line resulting in the production of a secreted form that can be purified. The use of Chinese hamster ovary (CHO) cells or human embryonic kidney (HEK293F) cells produces differently modified forms of Klotho. The Klotho from CHO cells is heavily modified through the sialylation of N-glycans, and has good *in vivo* PK properties in rats, including a terminal half-life of 12.2 hours and bioavailability of 47.5%. The Klotho from the HEK293F cells contains a rare LacdiNAc glycan modification at multiple N-linked sites, and was only detectable following the first 5 minutes after administration into rats, indicative of high systemic clearance. In cell culture, the sialylated Klotho from CHO cells was less potent at acting as a coreceptor for FGF23 and activating ERK signaling compared to the LacdiNAc modified Klotho from HEK293F cells. Klotho has also been reported to have glucosidase activity, and the sialylated Klotho was more potent in the activation of β -glucuronidase activity, whereas this activity was negligible with the Klotho from HEK293F cells.

This suggests that the expression system can be optimized to produce versions of Klotho biased toward particular biological activities and PK properties. At this point, however, it is not clear exactly what activities of Klotho are most critical for its effects on healthspan and lifespan. It also hints that the process of producing a secreted form of Klotho may induce modifications that alters its activity, since evidence suggests that the vast majority of soluble endogenous Klotho in humans is produced by shedding of the transmembrane form rather than expression of a secreted form.

Timing of administration: The response to Klotho may vary in an age-specific manner. Indeed, the effects of Klotho polymorphisms vary across the lifespan. It has not been established whether the dose or method of Klotho therapy would need to be adjusted in accordance with age. The extent of muscle regeneration in aged (22-24 months old) mice following recombinant Klotho treatment varied based on the timing and route of administration. Mice receiving osmotic pumps (324 pg/mL in saline vehicle) with Klotho starting 3 days prior to muscle injury and continuing for 14 days after, showed a 3.5-fold increase



in MyoD+ muscle progenitor cells and an increase in the number of regenerating fibers [117]. However, the timing of administration appears to be critical for functional recovery. When administered daily (10 ug/kg i.p.) from days 3 to 5 after injury, which mimics the rise in endogenous Klotho in young animals, Klotho treatment promoted regeneration and functional recovery. In contrast, when administered from days 1 to 6 after injury, functional recovery was impaired [75]. This suggests that there may be an optimal therapeutic window for injury or disease conditions.

Research underway: According to Clinicaltrials.gov, there are several trials using serum Klotho levels as a biomarker. There are currently no registered trials testing Klotho replacement therapy.

Search terms:

Pubmed, Google: Klotho + dementia, Alzheimer's, neurodegeneration, cognitive, aging, lifespan, genetics, cardiovascular, hypertension, atherosclerosis, kidney, cancer, inflammation, meta-analysis, safety, gene therapy, recombinant, exercise, enhancers, insulin, ApoE4, biomarker, clinical trials, companies

Websites visited for Klotho:

- [Clinicaltrials.gov](https://www.clinicaltrials.gov)
- [PubChem](https://pubchem.ncbi.nlm.nih.gov)

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