Klotho: The Longevity Peptide

A naturally occurring peptide & critical regulator of aging

This groundbreaking molecule has been shown to influence a wide range of biological processes, from repair to cognitive function, offering promising therapeutic applications in anti-aging, chronic disease management, and regenerative medicine.

Klotho is a protein encoded by the KL gene and exists in two forms: a membrane-bound form primarily expressed in the kidneys and brain, and a circulating soluble form found in the bloodstream. Its functions include regulation of insulin signaling, calcium-phosphorus homeostasis, and oxidative stress reduction. **Soluble Klotho levels naturally decline with age, contributing to the development of various age-related conditions, including cognitive decline, chronic kidney disease, and cardiovascular dysfunction.**

Cognitive Enhancement: Studies suggest that increasing soluble Klotho levels improves cognitive function, particularly in aging populations and neurodegenerative diseases.

Renal Protection: Klotho's renal protective effects make it a potential therapy for chronic kidney disease and related complications.

Cardiovascular Health: By preventing vascular calcification and oxidative stress, Klotho reduces the risk of atherosclerosis and other cardiovascular diseases.

Longevity and Anti-Aging: Klotho supplementation has been shown to extend lifespan in animal models, with potential translation to human healthspan.

Klotho multifaceted role in health, neuroprotection, and systemic balance provides clinicians with a novel therapeutic target for mitigating the effects of aging and chronic disease.

- **Anti-Oxidative Properties:** Reduces oxidative stress and DNA damage
- **Neuroprotective Effects:** Enhances memory, learning, and neuron protection
- **Mineral Regulation:** Maintains mineral balance, prevents vascular calcification
- **Anti-Inflammatory Effects:** Lowers inflammation by reducing cytokine activity

As a pivotal regulator of longevity pathways,
Klotho is a promising tool in advancing both lifespan and healthspan.

Enhanced Cellular Transport with FG-Coupling

To improve Klotho's effectiveness, a phenylalanine-glycine (FG) sequence has been added to the peptide, enhancing its transport into cells and improving its interaction with nuclear structures. This modification allows Klotho to efficiently enter the nucleus, optimizing its biological activity.

Enhanced Delivery with Exosomes

Exosomes, nanosized extracellular vesicles (EVs), further enhance Klotho delivery. These vesicles can be loaded with medium sized molecules and play a critical role in regenerative medicine by facilitating the transport and cell delivery of their inside cargo.

The Klotho association with exosomes benefits its entry to cells, increasing its solubility in aqueous solutions, as well as its distribution within the body. Exosomes also enable more precise delivery by targeting specific cells that express ligands to proteins present in the exosomal surface, thus enhancing the potential therapeutic efficacy.

Benefits and Outcomes when combined together

- **Enhanced Energy and Neuroprotection:** Klotho boosts ATP production, reduces oxidative stress, and protects neurons by regulating antioxidant defenses. This dual action improves energy production and cognitive resilience.
- **Kidney Function Maintenance:** Klotho plays a critical role in maintaining phosphate balance, essential for proper nephron and kidney function.
- **Comprehensive Antioxidant Defense:** Klotho enhances the expression and activity of antioxidant enzymes, reducing oxidative damage, inflammation, and protecting mitochondria—the primary energy-producing organelles.
- **Improved Tissue Regeneration and Repair:** Exosomes enhance Klotho delivery while carrying additional regenerative factors, promoting targeted tissue repair and recovery.
- **Anti-Inflammatory Effects:** Klotho's anti-inflammatory properties, combined with exosome-mediated immune modulation, reduce chronic inflammation and support overall health.

Compound Overview

Klotho: An anti aging protein with important role on kidney function

Biological Roles and Mechanisms

The alpha-Klotho (or simply Klotho) protein is a transmembrane protein that regulates the metabolism of phosphate, calcium, and vitamin D [31, 32]. The membrane-anchored form of Klotho is an essential component of endocrine Fibroblast Growth Factor (FGF) receptor complexes, as they form required complexes for the high-affinity binding of FGF19, FGF21 and FGF23 to their cognate FGF receptors (FGFRs) [31, 31]. Moreover, the secreted form (s-Klotho) can exert hormonal activities with unknown cell receptor(s) [31, 32]. In preclinical models, treatment with Klotho extends the life span in animals [31, 31].

When the Klotho protein is inactivated by gene mutation, animals and humans suffer with hypertension, hyperphosphatemia, hypervitaminosis D and kidney disease, suggesting that Klotho may be essential to the maintenance of normal renal function.

Connection to Aging and Kidney Health

Klotho and FGF23 have phosphaturic activity, meaning that these both peptides increase phosphate excretion per nephron which is fundamental for keeping the kidney healthy [37, 38]. Nowadays, we know that Klotho increases the expression and activity of cell-protective antioxidant enzymes and inhibits important signaling pathways involved to aging, such as Transforming growth factor β (TGF-β), insulin-like growth factor 1 (IGF-1), Wnt and NF-κB. In the last few years, several preclinical trials started with this molecule and the overall results indicate an amelioration of renal, cardiovascular, diabetes-related and neurodegenerative diseases [31].

Understanding Klotho: From history to clinical trials

Discovery and Early Research

In Greek mythology, Klotho (Clotho), is the youngest goddess of the Three Fates or Moirai who spins the thread of life and controls the ultimate destiny of humans. This name with vital functions was given to a protein identified by Kuro-o and collaborators in 1997 after they observed that deletion of the *klotho* gene in mice led to a syndrome resembling aging [33].

Briefly, those alpha-Klotho-deficient animals presented short lifespan, infertility (gonadal dysplasia), arteriosclerosis, skin atrophy, osteoporosis (and calcification of soft tissues), severe hyperphosphatemia and emphysema, in a degree only found in very old persons [33].

Klotho-Deficient Animals: Lessons Learned

More interesting was the fact that insertion of a non-mutated gene (*wild-type gene*) for Klotho protein into the gene-depleted animals has rescued all of the macroscopic phenotypes to the levels of the wild-type/non-depleted mice, clearly showing that the *klotho* gene mutation was the responsible for all physiological changes [33]. In a brilliant study, these authors also reported that overexpression of Klotho (Kl) protein in animals slowed the aging process and the life span extended by 20–30% [33, 34]. These both discoveries were supposed to be enough for awakening general interest in this protein, but later results reporting that serum levels of human Klotho decrease with age (mainly after age 40 yrs-old) in healthy individuals, strengthened the scientific focus on these protein effects [35, 36].

Overexpression and the Role in Longevity

Currently we know that the α-*Klotho* gene is highly conserved in humans, mice, rats and in other species, such as *Danio rerio* and *Caenorhabditis elegans* [32]. Taken different preclinical studies together, absence of alpha-Klotho resulted in phosphate imbalance and hyperphosphataemia (owing to impaired urinary phosphate excretion), poor growth, atrophy of multiple organs (for example, the gonads, thymus and skin), vascular calcification, sarcopenia, cardiac hypertrophy and fibrosis, osteopenia, emphysematous lung, hearing disturbances, cognitive impairment and shortened lifespan [31, 32, 37, 40]

Klotho Gene and Protein Variants

Three Variants: Alpha, Beta, and Gamma Klotho

Klotho gene encodes for 3 proteins: the α-Klotho protein, the β-Klotho protein and Klotho-related protein (Klrp or Lctl or γKlotho) [32, 37]. In this text, we will focus only in α-Klotho (alpha-Klotho) and its soluble form (s-Klotho or s-Kl), since the function of the others are poorly understood so far. Of note, we will omit the alpha letter (α) of the name throughout the text, thus Klotho means α-Klotho.

Focus on Alpha-Klotho and Its Soluble Form

In humans, Klotho protein has 2 isoforms: a full-length protein that is anchored and crosses the cell plasma membrane (a transmembrane protein), and a soluble form (called s-Klotho) which is result of the cleavage of the membrane-bound protein (shedding) [31].

Klotho Expression and Regulation

Distribution in the Body

The Klotho transmembrane protein is distributed in different body organs, such as arterial (blood vessels), epithelial (skin), endocrine (pancreatic β cells), reproductive and neuronal tissues [31, 89]. The most extensive Klotho expression seems to be the distal tubule epithelial cells of the kidney, parathyroid glands and in the brain choroid plexus [32, 37].

Factors That Modulate Klotho Levels

Its expression is modulated by internal and external factors such as acute inflammatory stress, long-term hypertension and vitamin D, but also by some pathological conditions such as diabetes mellitus, chronic renal failure and vascular problems [32].

The most known factors that increase Klotho expression are vitamin D, activation of Peroxisome proliferator–activated receptor γ (PPARγ), erythropoietin, ras homolog gene family A, rapamycin, statins, fosinopril, physical activity and losartan [32, 37]. Among the factors that decrease the Klotho expression are Angiotensin II, activation of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), metallopeptidase 9, tissue inhibitor of metallopeptidase 1 and plasminogen activator inhibitor-1 [32, 37].

Impact of Aging on Klotho Expression

Curiously, several of these down-modulator factors are elevated in aged persons [31, 32, 37].

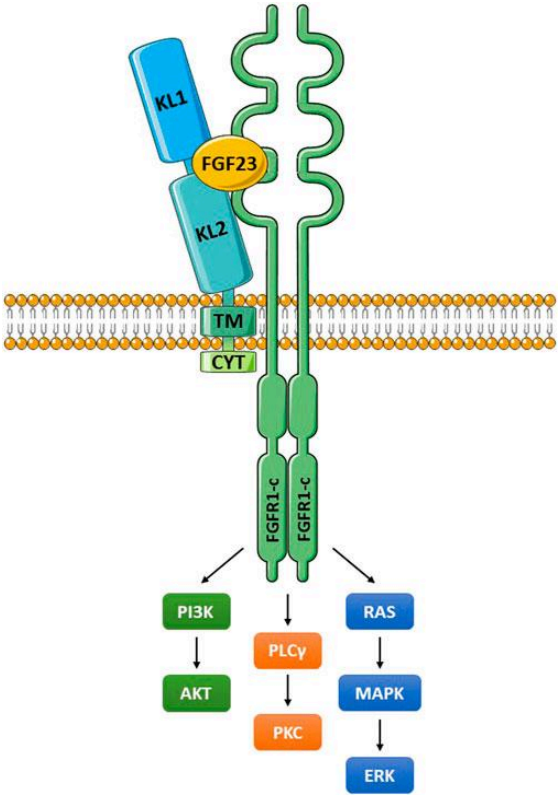
Klotho and Fibroblast Growth Factor (FGF) Signaling

Interaction with FGF Receptors

Inserted in the plasma membrane, Klotho has two extracellular domains (KL1 and KL2) that complexes with components of the fibroblast growth factor receptors, therefore being fundamental for different Fibroblast Growth Factors (FGF) signaling pathways. FGFs are fundamental in several physiological responses, such as activation of the sympathetic nervous system and the hypothalamus–pituitary–adrenal axis (eg. FGF 21), satiety after feeding (eg. FGF 19) and phosphate

regulation (eg. FGF 23), indicating that absence of Klotho can compromise these responses mediated by interaction of FGFs with their cognate receptors. [31, 31]. The importance of Klotho in kidney functions was already observed in preclinical and clinical models, and the deterioration of renal and other functions observed in elderly persons can be directly correlated with the insufficiency of Klotho [31, 37, 38]. For instance, Klotho deficiency increases osteoporosis, arterial calcification, hypertension, renal fibrosis, cardiac hypertrophy, and neurodegenerative diseases, as well as decreases insulin production [31, 37].

As FGF23, Klotho is crucial in the renal functions as stated above. FGF23 is a bone-derived phosphaturic hormone which acts on the kidney to suppress reabsorption of phosphate (Pi) and synthesis of active vitamin D (1,25-dihydroxy vitamin D3 or calcitriol). This is why deficiency of Klotho predisposes to Chronic Kidney Diseases, since this peptide permits the right accommodation of FGF23 in its cognate receptor, thus the disruption of receptor signaling predisposes to Pi retention [37] (see Figure [1] on the side). Curiously, transmembrane Klotho has different actions in two anatomical sites of the kidney: in the proximal tubule, the protein promotes a phosphaturic effect and inhibits vitamin D production, whereas in the distal tubule it enhances Ca^{2+} reabsorption (Figure [1]). Deterioration of Klotho expression by aging usually leads to renal dysfunction as well as lowers active vitamin D levels [37, 38]. Notably, the several conditions observed in *klotho* gene-deleted mice are the same related to mice deficient of the hormone FGF23, reinforcing that Klotho absence disrupts the renal FGF23 signaling [39]



The Role of Soluble Klotho (s-Klotho)

Release Mechanisms and Circulatory Role

The soluble Klotho (s-Klotho) occurs when its anchor to the membrane-inserted segment (transmembrane or TM, represented as green in the side figure) is cleaved by α- and β-secretases (usually ADAM-10 or ADAM-17 enzymes) and by insulin stimulation. Indeed, in experimental

[1] **Legend to figure:** This illustration is showing the FGF receptor (in green) inserted into the cell plasma membrane (small orange circles with tails). The Klotho protein is also shown with the two extracellular domains, KL1 and KL2 (in blue). The both domains permit a high affinity binding of FGF23 hormone to its FGF receptor (green). Without Klotho, FGF23 can not stay bound for a time enough to trigger the signaling pathway from FGFR1-c (represented as PI3K, AKT, PLCγ, PKC, etc). Extracted from Prud'homme GJ et al. (2022) *Front. Aging* **3** and reproduced without changes under the terms of Creative Commons Attribution License.

models, administration of specific inhibitors to these secretases mediating this proteolytic cleavage drastically reduced s-Klotho levels in the circulation [31]. Now this free form can work as an endocrine mediator with many functions, although its receptor in the plasma membrane is not fully identified [37].

Regulation of Ion Channels and Transporters

Based on some studies, scientists believe that soluble Klotho (s-Klotho) regulates the activity of several ion channels and transporters, including the calcium-selective channel TRPV5 and the ATP-sensitive inward rectifier potassium channel 1 (ROMK1) [37, 38]. It is believed that s-Klotho promotes the interaction of these above mentioned transporters and channels with galectins, therefore enhancing their activity in calcium reabsorption and potassium secretion, respectively [37, 38].

Impact on Kidney Health

Once again, controlling these mineral levels is fundamental for filtration activities performed by the kidneys, as they participate in the formation of kidney stones.

Currently we know that ADAM-10 and ADAM-17, the secretases that release the Klotho from the membrane, are augmented by the action of insulin, growth factors and cytokines, suggesting that these conditions can increase the levels of circulating Klotho [31]. At least one drug, ligustilide, is able to induce cleavage and release of s-Klotho.

Once in the circulation, the cell receptor for s-Klotho is not fully elucidated. By studies with colocalization analysis of proteins and lipid rafts in the presence of Klotho, at least 2 different studies have speculated that s-Klotho could use surface monosialogangliosides GM1 and GM3 as receptors in the cell membrane [60, 61]. In the Klotho soluble form (s-Klotho), KL1 region can remain associated with KL2 or both domains can be separately presented, with KL1 domain likely involved in the binding of monosialogangliosides [31, 61]. In the soluble form, Klotho can be present in plasma and excreted in urine [31]. The principal source of circulating Klotho is the kidneys based on preclinical findings with animals that *klotho* gene mutation, specifically in the kidney, led to the reduction of 80% in the levels of the circulating form when compared with non-mutated controls [38]. Noteworthy is the fact that distal organs that do not express the transmembrane Klotho also suffer with the harmful effects of soluble Klotho absence. Of note, s-Klotho can bind to some plasma membrane-inserted proteins but this interaction usually inhibits the signaling, whereas a receptor is defined as a protein that transduces a signal [38].

Klotho as a Renoprotective Agent

Protection Against Kidney Injury

Among the best-known beneficial effects of s-Klotho are protection against vascular calcification due to the inhibition of phosphorus uptake and its influence on the enzymatic activity that controls phosphorus transporters [31, 32]. The protective effect of soluble Klotho in models of kidney injury is probably related to the regulation of the activity of several growth factors and ion channels and/or transporters in an FGF23-independent manner [37, 38]. Together, the results indicate that s-Klotho has important renoprotective effects.

Klotho's Broader Biological Functions

Antioxidant and Anti-Inflammatory Effects

The action of Klotho goes beyond these dependent FGF23 action: Klotho also has antioxidant and anti-inflammatory activities; anti-apoptotic and anti-senescence functions and stem cell preservation.

Anti-Senescence and Stem Cell Preservation

These Klotho-mediated actions led several researchers to investigate its function in diseases that are initiated or maintained by these aforementioned conditions. Two important Klotho-mediated activities are important for keeping cells healthy and alive: inhibition of insulin-like signaling and antioxidant properties, both initially observed when Klotho-deficient mice were produced by a japanese group.

Klotho and Cell Signaling Pathways

Insulin/IGF-1 Pathway Inhibition

Injecting s-Klotho in the animals lacking the protein resulted in inhibition of intracellular insulin/IGF-1 signaling pathways, with alleviation of aging-like phenotypes and extension of life span [34].

Klotho seems to be important in the vasculature system as well. Aging is accompanied by several changes in the vasculature, such as ectopic calcification. Using genetically engineered mice to express different levels of Klotho, a study provided evidence that Klotho acts on vascular smooth muscle cells, mainly in mice with chronic kidney disease [87] Later, the same researchers observed resistance against oxidative stress when Klotho was overexpressed in animals, followed by subsequent *in vitro* studies with different cells indicating that s-Klotho suppresses

autophosphorylation of insulin/IGF-1 receptors and downstream signaling events that include tyrosine phosphorylation of insulin receptor substrates (IRS) [34].

These reports stimulated researchers to wonder if these both properties are somehow connected to each other. Indeed, this insulin/IGF-1 pathway is also linked to the production of oxidative molecules: for example, inhibiting this signaling pathway induces activation of FoxO forkhead transcription factors (FOXOs) that upregulates genes that encode antioxidant enzymes, such as mitochondrial manganese superoxide dismutase (MnSOD), thus creating a resistance to oxidative stress promoters. In addition, s-Klotho activates another antioxidant pathway mediated by nuclear factor erythroid 2-related factor 2 (Nrf2) in renal, cardiovascular and neurological preclinical disease models [40]. Nrf2 is a transcription factor that protects against oxidative injury and, importantly, inhibits the inflammatory NF-κB pathway that will be discussed in the next paragraph.

For instance, Nrf2 induces expression of an antioxidant cellular defense, such as heme oxygenase-1 (HO-1), Superoxide Dismutase 2 (SOD2), catalase (CAT), and glutathione peroxidase (GPX) [40]. As we can see from these above explanations, the effect of Klotho on cell signaling encompasses several mechanisms acting together and that will culminate in the production of a natural and powerful antioxidant system which protects our cells from death (apoptosis). This has been clearly illustrated when Klotho was overproduced in a mouse cell line: high amounts of Klotho reduced the cell-death induced by oxidative stress injury (exposure to reactive oxygen compound hydrogen peroxide) [42]. Analyzing all together, it seems that aforementioned pathways stimulated by Klotho probably account for the many successful outcomes observed after s-Klotho was used as adjuvant in the treatment of diseases associated with kidney, cardiovascular and diabetic kidney disease, among others [40].

Curiously, some marketed antioxidant drugs, like statin, angiotensin II inhibitor and N-acetylcysteine augments the transmembrane Klotho expression, but currently is premature to affirm that these medications only act through increment of Klotho since they are also able to interfere in pathways that are not stimulated by Klotho [40, 41].

Overall, it has compelling evidence that reducing oxidative stress and preserving mitochondrial health is one key factor to soften some chronic diseases and also the symptoms of aging. Notably, several of these Klotho actions are pivotal for the physiology of skeletal muscles (muscles involved in movement), such as muscle regeneration, mitochondrial biogenesis, endothelial function, oxidative stress, and inflammation.

For instance, clinical data from the Health, Aging, and Body Composition (Health ABC) study showed that older adults with high plasma Klotho (>747 pg/mL) exhibited higher knee extension strength compared with those with low doses, which is similar to recent studies showing a positive

correlation between sKlotho and handgrip strength, knee extension peak torque, and lean mass index in middle-aged adults [57]. Probably these results in skeletal muscle functions are a consequence of several Klotho actions cited here, since antioxidant, anti inflammation, muscle regeneration and cardiovascular capacity always favor muscle capacities [57].

TGF-β and NF-κB Pathway Modulation

Soluble Klotho also intervenes with TGF-β and NF-κB signaling pathways. In common, these both ways can promote sustained inflammation: TGF-β is involved in the promotion of cellular senescence, stem cell decline, immunologic impairment, fibrosis, while NF-κB can induce and sustain chronic, low-grade inflammation which may lead to permanent tissue damage [31, 40].

In both cases, presence of s-Klotho is able to inhibit these signaling pathways in several cells. To better illustrate, s-Klotho can interact with TGFβ-, IGF- and FGF-receptors to inhibit their signaling pathways [40, 52].

Klotho in Neuroprotection and Brain Health

Impact on Cognitive Decline and Aging Neurons

Markedly, an interesting study showed that Klotho inhibited senescence in human brain organoids, whereas established senescence in human neurons is associated with the reduced expression of Klotho [83]. In this case, the anti-senescence property of Klotho is linked to its ability to induce anti-senescence genes (eg. *sparcl1*, *col20a1*) and the inhibition of genes associated with TGF-β-induced pathway (eg. *serpind1*, *slc6a13*) [83]. With similar results but in an alternative approach, a different study investigated the deficiency of Klotho in cell senescence and reported that silencing Klotho expression upregulates p53/p21 proteins, which induces cell cycle arrest and consequently leads to premature senescence in human primary fibroblast cells [90] Also fascinating is the fact that a preclinical study showed that clearance of senescent cells by Senolysis results in the increment of Klotho in urine, brain, kidney [84]. Analyzing together this set of results, we conclude that keeping a good amount of Klotho in the cells could avoid premature senescence with cell division arrest.

Klotho and Cancer Research

Tumor Suppressor Pathways

Finally, some cell signaling triggered by s-Klotho are recognized as Tumor Suppressor pathways. Klotho expression is downregulated in some tumors, such as colorectal, pancreatic, gastric, oesophageal, breast, hepatocellular, ovarian, and renal [52]. Some of these Tumor Suppressor

pathways have been already discussed in this text (eg. insulin/IGF-1, wnt, TGF-β), and in general, inhibition of these pathways can protect against tumor initiation [52].

For example, aberrant activation of the wnt signaling pathway is associated with the development of numerous human cancers, and s-Klotho is able to inhibit this pathway in melanoma cells when it interacts with Dishevelled receptors [52]. However we must emphasize that results in cancer were done *in vitro* or in preclinical conditions, thus we do not suggest the use of Klotho or our product for cancer treatment.

Klotho's Role in Mitochondrial Function

Regulation of Mitochondrial Health and Bioenergetics

Klotho is involved in mitochondrial dysfunction commonly observed in aging and in several chronic diseases (some are aging-related diseases) [40]. Accumulated studies have shown that an increased number of proteins that regulate mitochondria functions can be directly or indirectly regulated by Klotho, such as mitochondrial uncoupling protein 1 (UCP1), B-cell lymphoma-2 (BCL-2), Wnt/β-catenin signaling pathway, and mitochondrial-related transcriptional regulators including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 alpha), transcription factor EB, (TFEB) and peroxisome proliferator-activated receptor gamma (PPAR-gamma) [40].

Prevention of Muscle Degeneration

Mitochondrial dysfunction occurs in aged muscles (cardiac and skeletal muscles) impacting the mitochondrial bioenergetics and production of ATP: in muscle progenitor cells, age-related decline of Klotho drove the cell mitochondrial dysfunction and impaired muscle regeneration, while *klotho* gene-silencing in young progenitor cells recapitulate what occurred in the old cells [43].

In general, s-Klotho administration in different models ameliorated mitochondrial dysfunctions by inhibiting oxidative damages or by modulation of factors directly associated with apoptosis (cell death), such as upregulation of anti-apoptotic Bcl-2 proteins [40]. Taken together, soluble Klotho can significantly reduce the harmful effects of aging and reactive oxygen species (ROS) on mitochondria.

Advanced Delivery Strategies for Klotho

FG-Coupled Klotho for Nuclear Entry

Finally, our offered Klotho is chemically linked to a sequence of Phenylalanine-glycine (FG) amino acids. This strategy significantly increases the chance of Klotho peptide to reach the interior of the cell nucleus, since FG sequences molecularly interacts with similar ones in the proteins (nucleoporins) of the nuclear pore complex, which are crucial mediating the entry of compounds into the cell nucleus [86]. Once inside the nucleus, Klotho can interact with our chromosomes (DNA) and other intranuclear molecules, improving its biological function. As mentioned above, experiments with human kidney cells exposed to Klotho indicated that peptide entry in the cell nucleus is important for exerting its biological functions [58].

Therapeutic Potential of Klotho

Addressing Age-Related Declines

Considering the circulating Klotho declines in human serum after 40 yrs-old, all these listed results together suggest that supplementation with **s-Klotho can be a novel strategy for mitigating several of the pathophysiological conditions involved in aging or age-related diseases.** Next we will cite some ongoing or concluded clinical trials with Klotho.

Clinical evidence

Overview of Klotho in Clinical Studies

Several clinical studies are being conducted with Klotho: the vast majority investigates the association of soluble Klotho levels in human blood with some symptoms of aging or diseases, and few studies evaluating Klotho levels in the outcomes of certain diseases. We will exemplify this topic with few clinical studies in men and women.

Cognitive and Muscle Function in Older Adults

An investigation with 103 older individuals living in 10 nursing homes detected that low s-Klotho levels were associated with a lower score in cognitive tests and in the low maintenance of body balance/muscle rigidity (assessed by body falls) [44].

Regarding frailty, clinical studies with middle-age and senior subjects have corroborated this positive correlation between s-Klotho levels and the improvement in muscle functions, such as handgrip strength, knee extension peak torque, lean mass index and [57].

Klotho Levels in Specific Populations

The concentration of s-Klotho in blood of children with type 1 diabetes (T1D) was lower than in the controls; and preterm infants with bronchopulmonary dysplasia (BPD) had also reduced levels of s-Klotho in their cord when also compared to controls [45, 46].

Klotho and Chronic Kidney Disease (CKD)

Aging usually results in a deterioration of kidney/renal function, leading to chronic kidney disease (CKD). In humans, a graded reduction in urinary Klotho has been observed, starting at an early stage of CKD and progressing with loss of renal function [87]. It seems that Klotho administration could retard the appearance of CKD based on studies that mice with preserved Klotho expression have better renal functions when compared to the animals with Klotho peptide insufficiency [87]. In a clinical realized in Denmark, 24 CKD patients (in stages 1 to 5) were analyzed for circulating Klotho in blood, and the results indicated that patients with soluble Klotho (s-Klotho) below 204 pg/mL had higher age, lower phosphate clearance, and lower bone-specific alkaline phosphatase [88]. Even the treatment of CKD patients with Cholecalciferol was not able to change s-Klotho concentrations [88]. Combining the preclinical and clinical trials, we conclude that a decrease in Klotho negatively affects the renal functions.

Lifestyle Factors Impacting Klotho Levels

In a different study, alcohol consumption decreased the s-Klotho levels in a cross sectional study with middle-aged (40 to 60 years old) sedentary adults whereas exercises of moderate intensity training (MIT) induced Klotho secretion in middle-aged adult men and women [47,48, 49].

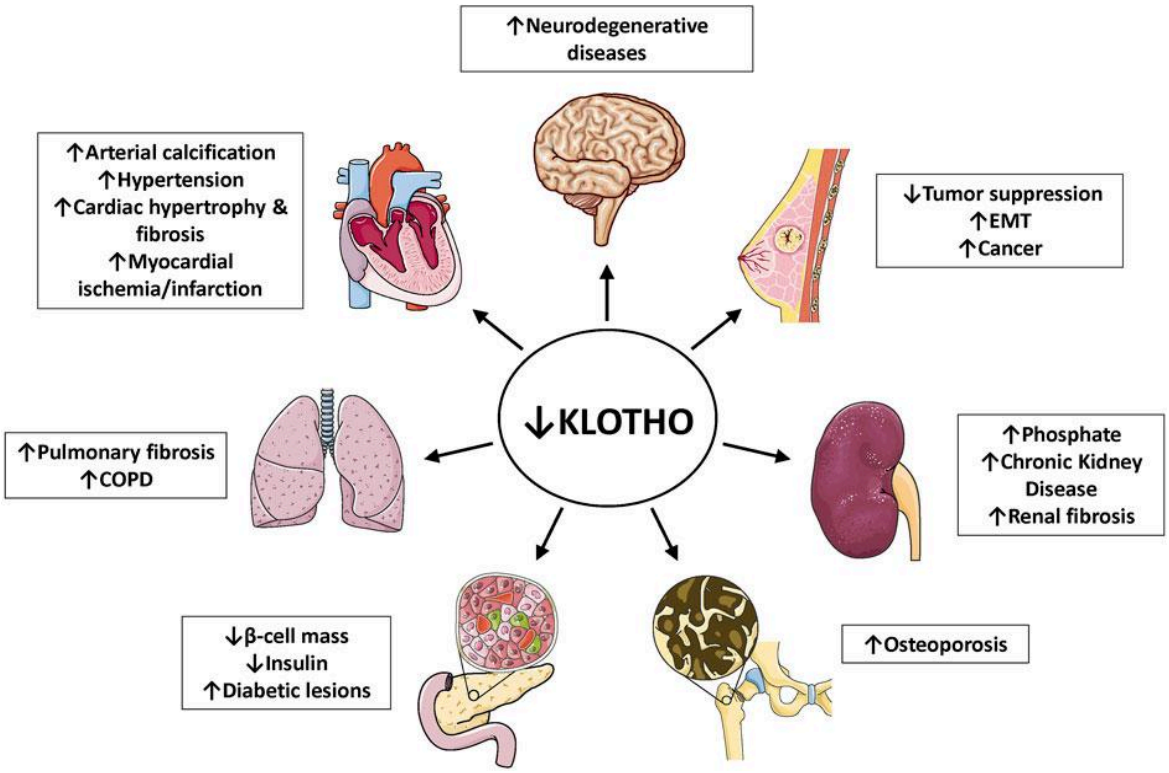
Genetic Mutations and Klotho Expression

A genetic polymorphism analysis of Japanese postmenopausal compared to premenopausal women, a mutation in the *klotho* gene, which impacts the protein expression, was significantly associated with bone loss of these volunteers [59].

Summary of Findings in Clinical Trials

In summary, human trials with Klotho (from a list of 80 total clinical published trials on pubmed.gov) suggest that the worst outcomes of several diseases are generally associated with reduced levels of circulating Klotho in blood or excreted in urine, such as acute kidney injury, ischemia, diabetic kidney disease, pituitary adenomas, atrial fibrillation, cigarette smoking and others. Finally, a clinical study with 3555 patients suffering from stable ischaemic heart disease has reported that low Klotho concentration is associated with an increased risk of CV death or heart failure hospitalization

[85]. From human trials or from animal models, the impact of Klotho deficiency in several organs



and tissues are summarized in the following illustration. (Figure [2])

[2] **Legend to figure:** This illustration depicts the consequence of low levels of circulating Klotho (arrow pointing down in central Klotho). Klotho deficiency impacts several organ and tissues, such as hyperphosphatemia, chronic kidney diseases, multiple cardiovascular conditions, neurodegenerative diseases and others. Adapted without changes from Prud'homme GJ et al. (2022) *Front. Aging* **3**. Reproduced without changes under the terms of Creative Commons Attribution License

Klotho-Rescuing Effects of Conventional Therapies

In some clinical trials, treatment with conventional therapies rescued the levels of soluble Klotho, such as in the treatment of artery diseases, heart failure, hemodialysis, type 2 diabetes, some human malignancies, growth hormone deficiency, sclerosis, chronic kidney disease, etc [50]. There are only preclinical studies with Klotho, and Klotho-based therapies in humans haven't reached clinical trials so far.

Compound overview

Exosomes: Versatile Nanocarriers for Cellular Communication, signaling and Regeneration

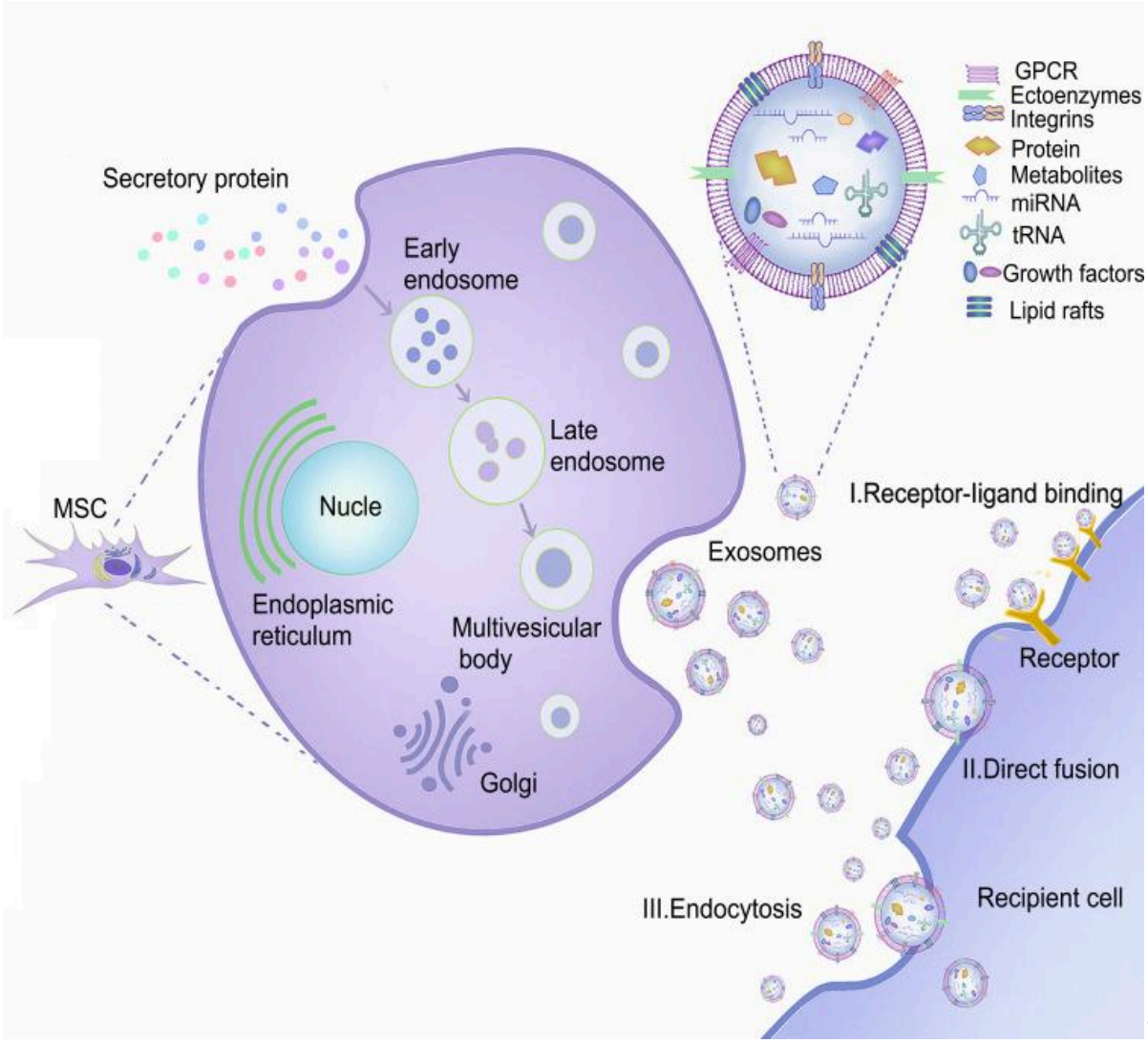
Structure and Composition

Exosomes (or Exos) are extracellular vesicles (EVs) with a nanoscale size (from 40 to 160 nanometers in diameter) that are produced and secreted by cells [63, 64, 66]. Potentially all cells of different species can produce and release exosomes in their living milieu.

Exosomes are single-membrane nanovesicles, with their external part composed of a lipid bilayer with proteins inserted in. The internal part of exosomes contains cell constituents and biomolecules, such as nucleic acids, amino acids, proteins and intracellular cell metabolites.

All exosomes constituents are acquired from their producer cells. Each exosome size, shape, and density are primarily determined by its specific content of protein, lipid, enzymatic, and mineral, consequently we find a heterogeneous population of these nanovesicles in every extracellular environment [63, 65].

Exosomes are found in almost all bodily fluids, such as urine, saliva, breast milk, blood and interstitial fluids [63, 64, 66]. When traveling in blood, they can reach target cells located far away from the region where they were originally secreted by their producers. Because of this ability to travel long distances, exosomes are very important mediators in cell-to-cell communication (also referred as intercellular communication), working like nano-messengers, since they are uptaken by specific target cells (also known as recipient cells) at their final destination [63, 64, 66] (see figure [3] below).



[3]

Exosomes from Mesenchymal Stem Cells (MSCs)

Therapeutic Potential of MSC Secretome

Recently too much focus is given on particular exosomes: the exosomes produced by Stem Cells, in particular those produced by Mesenchymal Stem Cells (MSCs). Firstly, it has

[3] Schematic representation of exosome-producer cells, exosome nanospheres and recipient cells. The producer cell (left) synthesizes and releases exosomes (in the middle). Exosomes are composed of lipid bilayer with several molecules encapsulated inside (eg. Protein, miRNA, Metabolites, tRNA, Growth Factors) and some inserted into the membrane (eg. GPCR, Ectoenzymes, Integrins). Once exosomes find their target cells (recipient cells), they are uptaken by endocytosis (III in the illustration), direct fusion (II in the illustration) or triggering a signaling pathway through receptor binding (I in the illustration). This illustration was adapted from Wang S et al (2022) *Int J Nanomedicine* **17**:1757-1781. Reproduced under terms of creative commons

been established that therapeutic effects mediated by Stem cells occur mainly by secreted factors (referred as secretome), with exosomes included as one of these [67].

Differentiation Capacity and Unique Properties

Secondly, MSCs have the ability of self renewal and also retain the capacity of differentiating into multiple cell lineages, such as cartilage (chondrocytes), bone (osteocytes), cardiac muscle (myocytes) and fat tissue (adipocytes), suggesting that their secretome are enriched of vital components for cells [66, 67].

Cultivation and Monitoring of MSCs

In general, MSCs can be isolated from bone marrow, fat, dental pulp, amniotic fluid, placenta, or umbilical cord [63, 64, 67] and they can also be expanded in lab flasks (*in vitro*). Differentially from the other differentiated cells, MSCs-derived exos usually carry several biomolecules that are only produced by them. Grow MSCs *in vitro* requires attention to some important details: cell purity; special medium formulation; retention of their differentiation capacity and telomere size. Of note, telomeres are the end region of each chromosome, composed by a repetitive DNA sequence. Each time a cell divides, the telomeres become slightly shorter due to the cell inability of copying DNA in these regions. In normal cells when telomere size is critical, cells stop dividing (called senescence) as a way to avoid genomic instability and possibly oncogenesis. Therefore telomere attrition should be accompanied during proliferation of MSCs in culture. Our MSCs are always followed up for these details.

Therapeutic Roles of MSC-Derived Exosomes

Roles in Tissue Regeneration and Disease Management

It has been well documented by preclinical studies that MSCs-derived exosomes can exert immunomodulatory effects (particularly in controlling chronic inflammations), can favor tissue regeneration, can work as carrier for molecules delivery (including drugs), can help in some cells differentiation, can participate in the restoration of mitochondrial function (mainly in kidney injury like in renal ischemia-reperfusion), can help in the activation of T cells, can help in the recovery of lung and liver injury, can exert beneficial role in neurodegenerative diseases (eg. Alzheimer's disease, multiple system atrophy, Parkinson's disease, narcolepsy, and autism), among others [66, 67].

Anti-Aging Capabilities

Markedly, exosomes have some anti-aging capacities, such as reverse of mitochondrial dysfunction, antioxidant enzymes, and cellular senescence [67]. Interestingly, antioxidant enzymes, such as peroxiredoxins (PRDXs), have been already reported inside exosomes released by Stem cells, and they are transferred to recipient cells, alleviating cellular aging phenotypes [68, 69]. Moreover, a growing number of studies suggests that cellular senescence can be attenuated or even partially reversed by EVs derived from stem cells through mechanisms that encompass regulation of specific genes and decrease of senescence-associated secretory phenotype [67, 68, 69].

Exosomes vs. Klotho: Overlapping and Distinct Effects

Comparison of Functions

Some effects of MSCs-derived exosomes (EVs) overlay those also generated by Klotho, such as control of inflammation, restoration of mitochondrial functions and anti-aging properties. As far as we know, there is no clinical data suggesting that Klotho and exosomes will synergistically act to improve the individual effect triggered by each one separately.

Potential Synergy

However, an analysis of molecule-target of each isolated compound revealed that they do not overlay to each other. For example, Klotho modulates cell signaling pathways and exosomes deliver biological molecules which control gene and protein expressions. Thus it is possible to envisage that all together could promote better results than each one individually. In addition, exosomes help in the peptide delivery to cells, as will be discussed in the next topic.

JuveXO®: MSC-Derived Secretome Product

GMP-Compliant Manufacturing and Quality Control

Finally, the MSCs-derived secretome of our product (JuveXO®) is produced in good manufacturing practice (GMP)-compliant process and quality control (QC). It is extensively tested for induction of specific proteins (Collagen I and Elastin) by human fibroblasts (see the science behind JuveXO), indicating that our EVs are fully functional.

Each lot is regularly tested for these above mentioned effects, and the EVs-producer cells (Mesenchymal Stem Cells) were isolated from Umbilical Lining Stem Cells of human cord blood and microbiologically tested for several pathogens, microbes (eg. bacteria, fungi and protozoa) and possible microbial molecules (eg. bacterial endotoxin) as contaminants.

Safety and Applications of MSC-Derived Exosomes

Applications in Skin Regeneration

MSCs-derived exosomes, including ours, have been used in skin regeneration for the past few years [67]. Cutaneous wound healing is a market that moves more than US$15 to $22 billion by 2024 and MSCs-derived exosomes are already in use for this purpose. As discussed, exos have ability to orchestrate all phases of skin wound healing (eg. modulate inflammation, activate migration and proliferation of various cells including immune cells, fibroblasts, and keratinocytes, and even ameliorate scarring) [67].

Safety Profile in Clinical Studies

Because of its singular nature as cell-secreted product found in blood and all body secretions (eg. tear, saliva, blood, sperm, sweat, etc), all the preclinical studies and ongoing human trials have already indicated that exosome administration is safe without significant collateral effects [67].

For example, in two clinical phase I studies of patients with Inflammatory Bowel Disease (IBD) followed by 6 months demonstrated that injection of exosomes isolated from MSCs is safe and a satisfactory therapeutic effect in the treatment of complex perianal fistulae [70, 71]. Indeed, exosome technology has raised more than U$ 386.2 million in investor funding by 2020 with several of these funds coming from big pharma companies [67].

Peptides and Exosomes: A Perfect Combination for a Better Response

Introduction to the Combination of Klotho and Exosomes

As aforementioned, cell-derived exosomes and the peptide Klotho have been preclinically used for the past 10 to 15 years to mitigate inflammation, for improving skin and to decrease the harmful effects of oxidation (mainly in the cell mitochondria). The constituents of our product provide a broad spectrum of actions since each one targets specific component(s) or/and signaling

pathway(s) that usually possess complementary actions on the cell metabolism. In addition, our chemical modification (FG coupling) is specially designed for a better entry of the peptide into the specific cell compartment, such as the nucleus.

Strategies for Improved Peptide Delivery

Role of Exosomes and Liposomes

In the past few years, different strategies have been tested to improve the efficacy and delivery of peptides to cells [53, 54]. Between these strategies, exosomes (EVs) and liposomes should be the most studied so far.

To contextualize, exosomes can be internalized by recipient cells, their surface molecules can signal through target-cell receptors or it can be used as a carrier for internal molecules that are protected against degradation by extracellular enzymes. Because they are natural products of cells, exosomes have elevated biocompatibility, stability in the circulation, biological barrier permeability, low immunogenicity, and low toxicity [53,54]. Certain exosomes types are potentially therapeutic for some diseases and for delivering drugs to specific target cells [53, 54]. For example, MSCs-derived EVs contain cytoprotective molecules captured from their producer/parent cells, as reported in preclinical studies of myocardial ischemia/reperfusion injury, hypoxia-induced pulmonary hypertension, brain injury, acute renal injury and liver fibrosis [54].

Loading and Fusion Capabilities of Exosomes

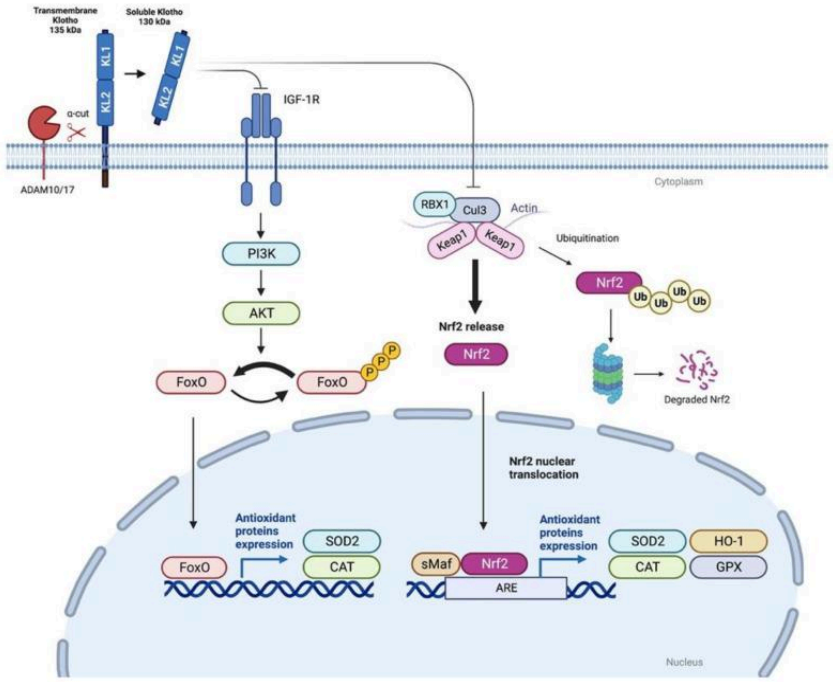
Exosomes can be loaded with exogenous molecules: due to their nature similar to liposomes, EVs are naturally permeable to small molecules, thus allowing some specific molecules to enter and be stored inside the exosomes. In this scenario, liposomes are being studied for several years because of their physico-chemical properties that allow complexation with proteins and nucleic acids [54, 55].

Lipid-Based Fusion with Cell Membranes

Exosomes, as liposomes, are composed of phospholipids commonly found in plasma membranes, thus molecules able to anchor and insert into the membrane phospholipids should do the same in isolated liposomes or exosomes. Finally, the lipophilic nature of liposomes and exosomes allows them to directly fuse with cell membranes, thus delivering their bound or inside components directly to the interior of recipient cells.

Evidence from Preclinical Studies Exosomes as Carriers

Even though Klotho's enter into the cell doesn't happen passively, Klotho can be delivered to cells by exosomes. This is illustrated in a study conducted by Grange and collaborators: the authors observed that exosomes in urine carries Klotho and that their intravenous injection accelerated renal recovery in a preclinical assay, with reduction in the expression of inflammatory and injury markers, and also restored endogenous Klotho loss in their used model of acute kidney injury [56]. Interestingly, exosomes absent from Klotho did not exert the same effect, indicating that the beneficial effects were mediated by Klotho intracellularly delivered to cells by exosomes or exosomes-carrying Klotho were able to trigger signals in renal cells [56]. In this scenario, it is important to mention that Klotho can exert its antioxidant activity through 2 distinct pathways: inhibiting the insulin/IGF-1/PI3K/Akt/FoxO signal, or inside the cells Klotho can induce expression of antioxidant molecules, such as SOD2, CAT, GPX and HO-1 (see Figure [4]) [40].



Therapeutic Potential of MSC-Derived Exosomes

Preclinical and Clinical Applications

Besides all these raised points, MSCs-derived exosomes confer regenerative effects in preclinical models of disease and tissue injury, and are in the phase I and II trials for limb ischemia, congestive heart failure and acute myocardial infarction [55]. This is based on the fact that exosomes can exert critical immunosuppressive and anti-inflammatory effects when tested *in vivo*, with positive results observed in extensive clinical trials for Crohn's and graft-versus-host diseases [55].

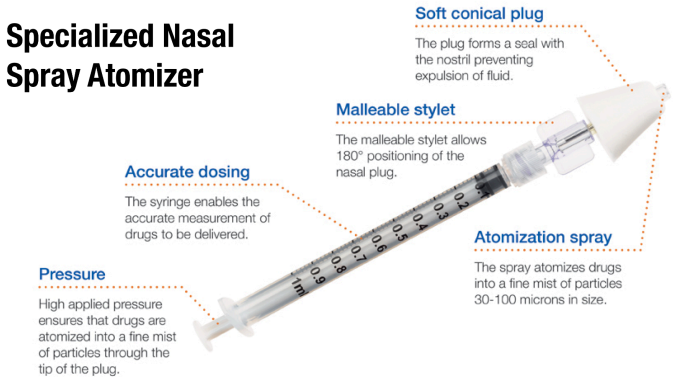
[4] Legend to figure: Membrane Klotho can be cleaved and released to extracellular milieu. Soluble Klotho can interact with cell membrane molecules and inhibits the insulin/IGF-1/PI3K/Akt/FoxO pathway, which permits expression of antioxidant proteins SDO2 and CAT. If Klotho enters the cells, it can induce the activation of the transcriptional factor Nrf2, which mediates the expression of antioxidant proteins. Reproduced from Donate-Correa et al. (2023) *Antioxidants* 12:239 [40], under the terms and conditions of Creative Commons Attribution license.

Role in Inflammaging and Skin Regeneration

MSCs-derived EVs, like those we have in our product, can be administered easily and safely for treatment of some inflammatory conditions, such as those observed in aged persons (inflammaging). As already discussed, our EVs have the capacity to induce secretion of collagen and elastin from human fibroblasts, which are important for skin regeneration and wound recovery. Due to all these explanations, combining all these components together (Klotho and exosomes) in a single product may promote a more powerful effect when compared to the activity exerted by each one separately. Importantly, our consumers must **not** replace any conventional therapy with our product and should follow doctor orientation for safety and better results.

The MAD Nasal™ Atomizer: Advanced Intranasal Delivery

The intranasal route enables compounds to **bypass the blood-brain barrier and first-pass metabolism, delivering significant amounts to both the systemic circulation and the brain.**



This method offers higher bioavailability compared to other routes like topical, intradermal, or intramuscular administration. Bioavailability refers to the fraction of an active substance that reaches systemic circulation in its unaltered form. For example, up to 80% of some medications can enter the bloodstream following intranasal delivery [ref]. This leads to faster and more efficient therapeutic effects while reducing side effects.

The MAD Nasal™ Device ensures **precise, controlled, and needle-free dosing, making intranasal administration painless, non-invasive, and free from needle stick injury risks.** This innovative system delivers compounds with accuracy and efficiency.

When paired with exosomes, intranasal-administered peptides via the MAD Nasal™ Device provide a comprehensive approach to enhancing vitality. This advanced delivery system maximizes absorption and effectiveness, offering patients cutting-edge care.

The combination of Klotho and exosomes, delivered via the MAD Nasal™ Device, introduces a novel method for improving energy, reducing oxidative stress, and supporting neuroprotection. Our specialized formulation targets multiple metabolic pathways, offering benefits such as:

- Enhanced mitochondrial function.
- Anti-inflammatory effects.
- Improved tissue regeneration.

Together, this innovative approach supports heart, kidney, and brain health, offering a promising and multifaceted solution for vitality and wellness.

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