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Review

Aging-suppressor Klotho: Prospects in diagnostics and therapeutics

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ABSTRACT

Introduction: The protein Klotho (KL) was first discovered in KL-deficient mice, which developed a syndrome similar to premature aging in humans. Since then, KL has been implicated in multiple molecular signaling pathways and diseases. KL has been shown to have anti-aging, healthspan and lifespan extending, cognitive enhancing, anti-oxidative, anti-inflammatory, and anti-tumor properties. KL levels decrease with age and in many diseases. Therefore, it has been of great interest to develop a KL-boosting or restoring drug, or to supplement endogenous Klotho with exogenous Klotho genetic material or recombinant Klotho protein, and to use KL levels in the body as a marker for the efficacy of such drugs and as a biomarker for the diagnosis and management of diseases.

Abbreviations: AAV, Adeno-associated virus; ASK1, Apoptosis signal-regulating kinase 1; ABI, Ankle-brachial index; AD, Alzheimer's disease; ADAM, A Desintegrin and Metalloproteinase; AF, Atrial fibrillation; Akt, Ak strain transforming; APOE4, Apolipoprotein e4; BACE1, Beta-Amyloid Precursor Protein Cleaving Enzyme 1; Bcl2, B-cell lymphoma 2; bFGF, basic fibroblast growth factor; BiP, Binding immunoglobin protein; BPD, Bronchopulmonary dysplasia; CAC, Carotid artery disease; CAD, Coronary heart disease; CAPD, Continuous ambulatory peritoneal dialysis; CAMKII, Calcium/calmodulin-dependent protein kinase II; ccRCC, Clear cell renal cell carcinoma; cDNA, Complementary DNA; CERA, Continuous erythropoietin receptor activator; CI, Cerebral infarction; CHO, Chinese hamster ovary; CHOP, CCAAT/enhancer-binding protein homologous protein; CI, Confidence interval; CIMT, Carotid intima-media thickness; CIS, Clinically isolated syndrome; CKD, Chronic kidney disease; CKD1, Chronic kidney disease stage 1; CKD2, Chronic kidney disease stage 2; CKD3, Chronic kidney disease stage 3; CKD4, Chronic kidney disease stage 4; CKD5, Chronic kidney disease stage 5; CNS, Central nervous system; CPB, Cardiopulmonary bypass; CPPs, Calciprotein particles; CR, Cardiorespiratory exercise; CREB, Cyclic adenosine monophosphate response element binding protein; CRF, Chronic renal failure; CSF, Cerebrospinal fluid; CVR, Cardiovascular remodeling; DCIS, Ductal carcinoma in situ; DDR, DNA damage response; DS, Depressive symptoms; EB, Epoetin-β; ECT, Electroconvulsive therapy; ER, Endoplasmic reticulum; eGFR, Estimated glomerular filtration rate; ERK, Extracellular signal-regulated kinase; ESRD, End-stage renal disease; ELISA, Enzyme-linked immunosorbent assay; FGF, Fibroblast growth factor; FGF19, Fibroblast growth factor 19; FGF21, Fibroblast growth factor 21; FGF23, Fibroblast growth factor 23; FGFR, Fibroblast growth factor receptor; FL-KL, Full-length Klotho; FOXO, FoxO forkhead transcription factor; FRS2, Fibroblast growth factor receptor substrate 2; FRS2α, Fibroblast growth factor receptor substrate-2α; G3PDH, Glyceraldehyde-3-phosphate dehydrogenase; H, Human; HCC, Hepatocellular carcinoma; HD, Hemodialysis; HDF, Hemodiafiltration; HEK-293, Human embryonic kidney 293; HF, Heart failure; IBL, Immuno-Biological Laboratories; IDC, Invasive ductal carcinoma; IGF-1, Insulin-like growth factor 1; IGF-2, Insulin-like growth factor 2; IGF-1R, Insulin-like growth factor 1 receptor; IL-12α, Interleukin-12 subunit alpha; IL-1β, IgG, Interleukin-1 betaImmunoglobin G; IP-IB, Immunoprecipitation-immunoblot; IPF, Idiopathic pulmonary fibrosis; IQR, Interquartile range; IRE1, Inositol requiring enzyme 1; IS, Ischemic stroke; JNK, c-Jun N-terminal kinase; kD, Kilodalton; KL, Alpha-Klotho protein; LOAD, Late-onset Alzheimer's disease; LPS, Lipopolysaccharide; M, Mouse; MAPK, Mitogen-activated protein kinase; MCI, Mild cognitive impairment; MDD, Major depressive disorder; MHD, Maintenance hemodialysis; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mRNA, Messenger RNA; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide; OPC, Oligodendrocyte progenitor cells; PAH, Polycyclic aromatic hydrocarbon; PBC, Peripheral blood cell; PD, Parkinson's disease; PH, Pulmonary hypertension; PI3K, Phosphatidylinositol-3-kinase; PARP, Poly (ADP-ribose) polymerase; PKA, Protein kinase A; PPMS, Primary progressive multiple sclerosis; PSA, Prostate-specific antigen; PTSD, Post-traumatic stress disorder; RA, Rheumatoid arthritis; RBA, Receptor-binding arm; ROS, Reactive oxygen species; RRMS, Relapsing-remitting multiple sclerosis; SCAD, Stable coronary artery disease; SD, Standard deviation; sgRNA, Single-guide RNA; shKL, Shed Klotho; siRNA, Small interfering RNA; SIRT1, Sirtuin 1; sKL, Secreted KL; SNP, Single-nucleotide polymorphisms; SOD, Superoxide dismutase; SOD1, Superoxide dismutase 1; SOD2, Superoxide dismutase 2; SPMS, Secondary progressive multiple sclerosis; SQI, Seizure quality index; SS, Signal sequence; SSRI, Selective serotonin reuptake inhibitor; ST, Strength exercise; T1D, Type 1 diabetes; T2D, Type 2 diabetes; TG, Thapsigargin; TGFβ, transforming growth factor beta; TM, Transmembrane; TNF-α, Tumor necrosis factor alpha; TRF, Time-resolved fluorescence immunoassay; Trx/Prx, Thioredoxin/peroxiredoxin; TU, Tunicamycin; VBR, Ventricle to brain volume ratio; VD, Vascular dementia; Wnt, Wingless/Integrated; XBP-1, X-box binding protein 1.

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Objective: The goal of this study was to provide a comprehensive review of KL levels across age groups in individuals who are healthy or have certain health conditions, using four sources: blood, cerebrospinal fluid, urine, and whole biopsy/necropsy tissue. By doing so, baseline KL levels can be identified across the lifespan, in the absence or presence of disease. In turn, these findings can be used to guide the development of future KL-based therapeutics and biomarkers, which will heavily rely on an individual's baseline KL range to be efficacious.
Methods: A total of 65 studies were collected primarily using the PubMed database. Research articles that were published up to April 2022 were included. Statistical analysis was conducted using RStudio.
Results: Mean and median blood KL levels in healthy individuals, mean blood KL levels in individuals with renal conditions, and mean blood KL levels in individuals with metabolic or endocrine conditions were shown to decrease with age. Similarly, CSF KL levels in patients with AD also declined compared with age-matched controls.
Conclusions: The present study confirms the trend that KL levels in blood decrease with age in humans, among those who are healthy, and even further among those with renal and endocrine/metabolic illnesses. Further, by drawing this trend from multiple published works, we were able to provide a general idea of baseline KL ranges, specifically in blood in these populations. These data add to the current knowledge on normal KL levels in the body and how they change with time and in disease, and can potentially support efforts to create KL-based treatments and screening tools to better manage aging, renal, and metabolic/endocrine diseases.

1. Introduction

1.1. Objectives

This review will first provide a brief background on the α-Klotho protein, which will now be referred to as KL, explaining its discovery, various isoforms and functions, and relevance. A discussion on its wide-ranging functions, methods of regulation, clinical implications will then be provided. More specifically, this work will highlight the ability to utilize KL as a biomarker or therapeutic tool for various diseases and health outcomes. To do this, we will provide a comprehensive review of previously reported KL levels in human blood, cerebrospinal fluid (CSF), urine, and whole biopsy or necropsy tissue, particularly in relation to different disease states and across age.

1.2. Discovery of Klotho

The gene encoding KL protein was discovered by Kuro-o et al. (1997) in a transgenic mouse, termed the kl/kl mouse. Mice homozygous for the insertional mutation of a transgene manifested phenotypes resembling premature aging in humans, such as "arteriosclerosis, osteoporosis, age-related skin changes and ectopic calcifications, together with short life span and infertility" (Kuro-o et al., 1997). Further changes included hypokinesis, slowed growth, abnormal gait, severe atrophy of the thymus, emphysema, atrophic growth hormone-, luteinizing-hormone, and follicle-stimulating-hormone-producing cells in the anterior pituitary, slightly increased serum calcium levels, hypoglycemia, decreased insulin in the pancreas, and decreased ratio of lymphocytes to leukocytes.

The mutant mouse was aptly named Klotho, after Clotho, the Greek goddess who spins the thread of life in humans. The structure of KL gene, i.e., the gene associated with the aging phenotype, was elucidated and it was discovered that the deletion in the mutant allele was in the "5' upstream region" (Kuro-o et al., 1997). KL gene was shown to be predominantly expressed in the kidney (distal convoluted tubules) and brain (choroid plexus). Further investigation predicted that the gene product is a type-I transmembrane protein. Kuro-o and his group also successfully isolated the human homologue of KL gene, isolating human complementary DNA (cDNA) encoding for a protein with 86% similarity to the mouse protein. As no premature-aging syndrome genes had been previously assigned to the chromosomal localization of KL gene, this represented a novel locus that could potentially influence aging and its manifestations, specifically in humans. Indeed, the KL mouse was the first laboratory animal model at the time to have various phenotypes mirroring premature aging in humans, caused by a single gene mutation.

Remarkably, two strains of transgenic mice with the homozygous KL gene deletion (i.e., two strains of kl/kl mice) significantly improved with exogenous expression of KL cDNA: "all disorders observed in kl/kl mice were improved by exogenous kl gene expression according to macroscopic, histological and blood analyses" (Kuro-o et al., 1997). This helped confirm that KL gene determined the KL phenotype in mice and certainly highlighted the potential of the KL mouse model to provide new insight into human aging and its underlying mechanisms.

The life-extending potential of KL was further demonstrated by studies conducted by Kurosu et al. (2005). This group found that over-expressing KL in mice led to longer lifespans, without changes in diet, oxygen consumption, or growth compared to wild-type mice. Notably, KL overexpression resulted in the production of less offspring, as well as resistance to insulin and insulin-like growth factor 1 (IGF-1). Inhibition of insulin and IGF-1 signaling was thought to be a mechanism by which KL decelerates aging.

1.3. Homologous Genes β-Klotho and γ-Klotho

There are three homologs of KL: α-KL (Kuro-o et al., 1997), β-KL (Ito et al., 2000), and γ-KL (Ito et al., 2002). α-KL is a co-receptor for fibroblast growth factor 23 (FGF23) and is therefore needed for the execution of FGF23's biological effects (Kurosu et al., 2006). α-KL forms complexes with fibroblast growth factor receptor (FGFR) 1c, 3c, and 4 in the kidneys and parathyroid glands, serving as high affinity receptors for FGF23. On the other hand, β-KL is needed for the activity of fibroblast growth factor 19 (FGF19) and fibroblast growth factor 21 (FGF21) (Kurosu et al., 2007; Ogawa et al., 2007). β-KL forms complexes with FGFR1c and 4 in the liver and adipose tissue, primarily binding FGF19 and FGF21. Lastly, γ-KL forms complexes with FGFR1b, 1c, 2c, and 4, in the eye, connective tissue, and kidney, and binds FGF19 (Tacer et al., 2010). β-KL's amino acid sequence is 41.2% identical to that of α-KL (Ito et al., 2000). γ-KL shares 37–38% identity with the first internal repeats of α-KL and β-KL (Ito et al., 2002).

1.4. α-Klotho's role in aging

KL, first and foremost, has been implicated in the process of aging. Since the publication of the seminal findings of Kuro-o et al. in 1997 and Kurosu et al. in 2005, much progress has been made in elucidating the mechanisms and roles of KL. Notably, Duce et al. (2008) linked gene expression changes associated with aging, with changes in brain white matter of the otherwise healthy rhesus monkey. Prior to then, genetic analysis of pure white matter had not been performed in the context of understanding aging. Duce et al. reported several genes of interest from microarray analysis of brain white matter (2008). In particular, there were age-related changes in expression of KL gene, as well as in telomeric repeat binding factor 1 and mitochondrial polymerase gamma, all of which are associated with metabolic conditions leading to

degenerative changes in the brain and reduced life span. Further, KL gene expression was found to be downregulated with age in brain white matter by approximately 1.5-fold. According to immunoblot analysis results, KL protein expression was also significantly reduced with age (by approximately 2-fold. Additionally, KL protein expression was only reduced in brain white matter, not gray matter, thereby lending support for KL's role in aging in white matter specifically. Even more, the white matter of aged rhesus monkey brains showed evidence of greater oxidative stress and neuroinflammation (Duce et al., 2008), pointing to the loss of the antioxidative, protective effects of KL.

When recombinant KL was added to primary oligodendrocyte progenitor cells (OPCs) in vitro, the cells underwent enhanced maturation into myelinating oligodendrocytes (Chen et al., 2013). Moreover, KL knockout mice exhibited grossly abnormal myelin by electron microscopy with only 10% of axons myelinated in the optic nerve compared to 90% in the wild type littermates (Chen et al., 2013). Experiments in a mouse cuprizone demyelinating model of multiple sclerosis (MS) showed that KL has a critical role in remyelination (Zeldich et al., 2015). Later, the protective effect of KL on neurons was evidenced as well, in vitro and in vivo, in mouse models of Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) (Dubal et al., 2015; Zeldich et al., 2014, 2019).

Chen et al. (2013) also found that KL knockout mice displayed abnormalities in both gray and white matter in the brain. There was evidence of hippocampal degeneration and loss of motor neurons.

In humans, Yamazaki et al. (2010) were the first to develop a sandwich enzyme-linked immunosorbent assay (ELISA) to measure circulating serum KL. They found serum KL to be inversely associated with age in a cohort of 181 healthy Asian volunteers. Specifically, healthy children had statistically significantly higher KL levels than adults ($p < 0.001$).

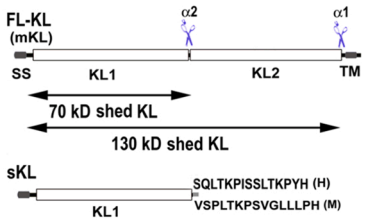
1.5. Isoforms of Klotho protein

There are several isoforms of KL, as initially studied by Shiraki-Iida et al. (1998). This group identified the structure of the mouse KL gene, its promoter, and two transcripts encoding either a membrane or secreted form of KL. Matsumura et al. (1998) then isolated the human homolog of the KL gene, identifying two transcripts that encode either the transmembrane form of KL or the secreted form of KL. Since then, it has been determined that KL exists in three forms, two produced by differential splicing – full-length transmembrane KL (FL-KL) and secreted KL (sKL) – and one circulating form produced by the cleavage of the FL-KL from the membrane, shed KL (shKL) (Chen et al., 2007, 2020; Bloch et al., 2009; see below). FL-KL has a signal sequence, followed by two homologous domains, KL1 and KL2, that share homology to glycosidases (Henrissat and Davies, 1997), a transmembrane domain, and short intracellular domain.

FL-KL and shKL act as a co-receptor for FGFR1 and FGF23 signaling in the kidney. shKL also functions as a hormone on remote tissues. Both the presence and shedding of KL are important for developmental, inflammatory, and pathologic processes in humans (Kuro-o, 2012; Wolf et al., 2008; Yamamoto et al., 2005). Please see Fig. 1 below for a summary of the structure of KL.

1.6. Function of Klotho at the cellular and molecular level at baseline and in aging

As described above, KL is involved in FGF signaling (Kurosu et al., 2006). Several years after its discovery by Kuro-o et al. (1997), Kurosu et al. (2006) showed that KL binds to various FGFRs and is a necessary cofactor for FGF23 activation of FGF signaling. In human embryonic kidney 293 (HEK-293) cells expressing FL-KL, termed 293KL cells, and transfected with expression vectors for various FGFR isoforms, KL bound to FGFR1, FGFR3, and FGFR4 with high efficiency. Additionally, FGF23 was pulled down with FGFR1c, − 3c, and − 4 only in the presence of KL.



- Transmembrane Klotho
  - Small intracellular domain of no known function
  - Large extracellular domain with two repeats (KL1 and KL2) that contain β-glycosidase homology (Kuro-o *et al.*, 1997, Nature)
- Shed Klotho and secreted Klotho
  - Klotho can be cleaved by α- and β-secretase to release extracellular domain (Chen *et al.*, 2007, Proceedings of the National Academy of Sciences, Bloch *et al.*, 2009, FEBS Letters)
  - Shed form found in blood, CSF and urine (Imura *et al.*, 2004, FEBS Letters)
  - Secreted Klotho found predominantly in brain (Massó *et al.*, 2015, PLoS ONE, 2018, Molecular Psychiatry)

Fig. 1. Structure of KL. The three forms of KL are summarized here. Findings from Kuro-o et al. (1997), Chen et al. (2007), Bloch et al. (2009), Imura et al. (2004), and Massó et al. (2015); (2018) are included. FL-KL is the transmembrane form, known also as mKL. SS, signal sequence; TM, transmembrane; H, human; M, mouse.

However, the extracellular domain of KL alone did not pull down FGF23, suggesting that FGF23 preferentially binds the KL-FGFR complex over KL or FGFR alone. Further, FGF23 did not induce phosphorylation of FGF receptor substrate-2a (FRS2a) or extracellular signal-regulated kinase 1/2 (ERK1/2) in HEK-293 cells, but phosphorylation was seen in 293KL cells, indicating that KL enhanced cellular sensitivity to FGF23. Both the extracellular domain of KL and FL-KL bound to exogenously expressed FGFRs in the same pattern, leading to the conclusion that both FL-KL and the extracellular domain of KL are needed cofactors for efficient FGF signaling via FGF23. Lastly, Chinese hamster ovary (CHO) cells that expressed FL-KL acquired the ability to respond to FGF23, and KL did not enhance HEK-293 or 293KL cells' ability to activate FGF signaling in response to FGFs other than FGF23. Taken together, these findings show that KL is necessary for the initiation of FGF signaling by FGF23, specifically through binding to FGFR1c, 3c, and 4. Because KL is expressed in the distal convoluted tubules of the kidney and the extracellular domain of KL is shed and soluble, KL was suggested to act on the adjacent proximal tubules and contribute to inhibition of phosphate reabsorption, a known function of FGF23 (Baum et al., 2005).

Please see Fig. 2 below for a structural schematic of how KL forms a complex with FGF23 and FGFR1c (Kuro-o, 2019).

1.6.1. Functions and mechanisms of shed Klotho

shKL has specifically been implicated in insulin and IGF-1 signaling (Dalton et al., 2017). Kuroso et al. (2005) found that shKL suppresses ligand-stimulated autophosphorylation of insulin and IGF-1 receptors in a dose-dependent fashion. shKL also suppresses activation of events downstream of receptor activation, such as the tyrosine phosphorylation of insulin receptor substrate 1 and 2, and association of the subunit of phosphoinositide 3-kinase p85 with insulin receptor substrate proteins. Notably, Kuroso et al. (2005) also found that inhibiting insulin and IGF-1 signaling improved survival and alleviated age-related pathologies in KL mice.

Furthermore, shKL may have an important role in cancer, a disease associated with aging. There are several papers that suggest that the insulin/IGF-1 signaling pathway can affect cell proliferation, apoptosis, and cancer (Pollak et al., 2004; Ray et al., 2014; Dalton et al., 2017). Pollak et al. (2004) has suggested that the growth of some cancers is stimulated by IGF-1 or IGF-2 in endocrine, autocrine, or paracrine manners. Ray et al. (2014) have highlighted that treatment of diabetes with insulin or insulin secretagogues increases the risk of developing solid cancers. On the molecular level, insulin and IGF-1 binding to their

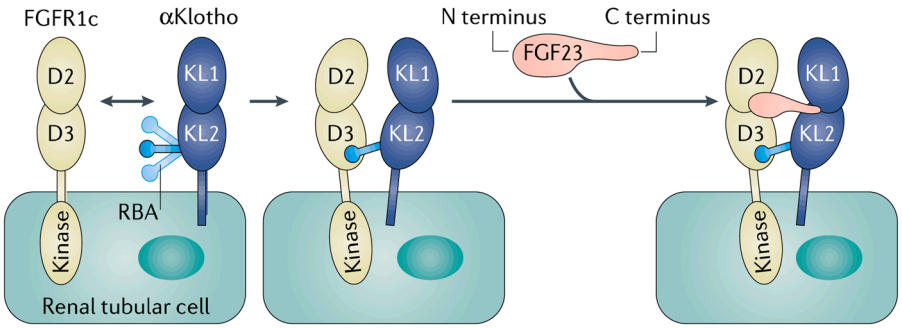


Fig. 2. KL-FGF23-FGFR1c complex. This schematic shows how KL complexes with FGFR1c for FGF23 signaling. KL's KL2 domain has a receptor-binding arm (RBA) that binds to the D3 domain of FGFR1c. FGF23 then fits into a groove generated subsequent to KL and FGFR1c binding. Figure taken from (Kuro-o, 2019).

respective receptors activates insulin receptor substrate proteins, which activate downstream signaling pathways that regulate normal cell development and maintenance, such as the phosphatidylinositol-3-kinase (PI3K)/Ak strain transforming (Akt) and MAPK/ ERK1/2 pathways (Dalton et al., 2017). It would thus follow those defects in these pathways would contribute to abnormal cell growth and consequently cancer.

Interestingly, shKL has also been tied to the physiologic reaction to oxidative stress in mammals, specifically in pathways involving superoxide dismutase (SOD) and catalase. Yamamoto et al. (2005) reported that in HeLa cells treated with paraquat, an agent that generates superoxide, administration of shKL inhibited paraquat-induced lipid oxidation. Further, in CHO cells treated with paraquat, shKL treatment inhibited apoptosis, with similar results seen in HeLa cells. It was thus postulated that shKL imparts a degree of resistance to oxidative stress to mammalian cells. Yamamoto et al. (2005) also studied the molecular basis of this resistance, showing that shKL treatment resulted in increased levels of superoxide dismutase 2 (SOD2) in vitro in HeLa cells and in vivo in mice. This was found to specifically result from suppression of insulin/IGF-1/PI3K/Akt signaling, which allowed for the activation of FoxO forkhead transcription factors (FOXOs) that upregulated expression of SOD2 (Kops et al., 2002). Indeed, shKL reduced Akt and FOXO phosphorylation in HeLa cells, indicating greater activation of FOXOs. As SOD2 is actively involved in the removal of reactive oxygen species (ROS), it was thought that KL conferred some level of resistance to oxidative stress by modulating SOD2 levels (Yamamoto et al., 2005). Another enzyme, catalase, also removes ROS and has been found to extend life span in mice when overexpressed (Schriner et al., 2005).

1.6.2. Klotho signaling via phosphorylation

Experimental studies have also shown KL signaling to be mediated via phosphorylation. Wolf et al. (2008) described the effect of KL on IGF-1 pathway activation in MCF-7 breast cancer cells, which express high IGF-1 receptor (IGF-1R) levels. MDA-MB-231 breast cancer cells, which express lower levels of IGF-1R, were also studied. Interestingly, KL overexpression in these cells was associated with reduced phosphorylation of IGF-1R and its downstream targets, including ERK1 and ERK2. In essence, KL overexpression inhibited IGF-1 signaling. Similar effects were noted on the insulin pathway in MCF-7 cells. Treatment with soluble KL also inhibited phosphorylation and activation of the IGF-1R in MCF-7 cells. Furthermore, KL knockdown in MCF-7 cells led to a statistically significant 2.2-fold increase in Akt phosphorylation following IGF-1 stimulation. KL overexpression also reduced phosphorylation of ERK1 and ERK2 in HEK-293 cells, indicating KL inhibited activation of the FGF pathway (Wolf et al., 2008). However, KL enhanced FGF pathway activation in breast cancer cells (Wolf et al., 2008).

Later in 2013, Chen et al. reported proteins in the ERK1/2 and Akt pathways to be phosphorylated in rat OPCs following KL treatment. FGF receptor substrate 2 (FRS2) was also phosphorylated following KL treatment, further implicating KL in FGF signaling. Notably, when OPCs were treated with KL in the presence of Akt and ERK inhibitors, ERK inhibition reduced, and Akt inhibition completely eliminated, the effects of KL on OPC maturation, indicating that KL enhanced OPC maturation via both Akt and ERK1/2 signaling. Chen et al. (2013) were also interested in identifying the potential transcription factors involved in the KL-induced maturation of OPCs. KL treatment was noted to lead to increased phosphorylation of transcription factor STAT3, thereby leading to greater STAT3 activity.

Finally, as further described below, KL-induced phosphorylation of the PI3K/Akt pathway has been linked to inhibitory phosphorylation of the transcription factor forkhead box O3a, FOXO3a, and induction of peroxiredoxin-2 (Prx-2) (Zeldich et al., 2014). Exogenous KL increased phosphorylation of both Akt/PI3K- and ERK-mediated pathways in rat primary hippocampal neurons, even as soon as 15 min following treatment with KL.

1.6.3. Klotho in endoplasmic reticulum stress and autophagy

A relatively newer area of study is the role that KL plays in endoplasmic reticulum (ER) stress responses and autophagy. Banerjee et al. (2013) studied whether KL levels were associated with ER stress in vitro. In HK-2 human kidney cortex proximal tubular control and KL overexpressing cells treated with potent ER stress-inducing agents thapsigargin (TG) and tunicamycin (TU), KL overexpression attenuated expression of ER stress markers phospho-inositol requiring enzyme 1 (IRE1), X-box binding protein 1 (XBP-1 s), binding immunoglobulin protein (BiP), CCAAT/enhancer-binding protein homologous protein (CHOP), phospho-c-Jun N-terminal kinase (JNK), and phospho-p38, which were all initially elevated in response to TG and TU. This was also observed in alveolar epithelial A549 cells in terms of the level of XBP-1 s, BiP, and CHOP in response to TG. On the other hand, silencing of KL in HEK-293 cells via small interfering RNA (siRNA) led to enhanced levels of phospho-IRE1, XBP-1 s, and BiP in response to TG. Lastly, KL overexpression in A549 cells inhibited TG-induced caspase and poly (ADP-ribose) polymerase (PAPR) cleavage, resulting in improved cell viability. This was confirmed with a cell survival essay where KL overexpressing cells had significantly greater cell survival compared to control cells across a wide range of TG doses. This all led the authors to conclude that KL does indeed have a role in the regulation of ER stress. While the loss of KL is causally associated with ER stress-induced apoptosis, overexpression lessens the ER stress response.

Mytych et al. (2019) were the first to study KL silencing via siRNA in normal human fibroblasts exposed to a non-toxic dose of lipopolysaccharide (LPS), which is known to impact the wound healing process. KL silencing in LPS-treated cells significantly slowed down the wound healing process and significantly increased cell cycle arrest in the G0/G1 phase, compared to treated, non-silenced cells. Further, KL silencing led to a significant decrease in 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide (MTT) activity, independent of LPS treatment,

compared to non-silenced cells, indicating a decrease in cellular metabolic activity. Of note, KL silencing did not lead to statistically significant differences in cyclic adenosine monophosphate response element-binding protein (CREB), considered the master regulator of mitochondrial biogenesis, so the decrease in MTT activity was attributed to decreased cell or mitochondrial number. In LPS-treated cells, KL silencing also led to significantly increased caspase 3 activity alongside a decrease in B-cell lymphoma 2 (Bcl2) protein, indicating activation of the apoptotic pathway, compared to treated, non-silenced cells. Calnexin is also known to be involved in apoptosis induced by ER stress, and there was a significant increase in its expression following KL silencing and LPS treatment, compared to non-treated, non-silenced cells. ER stress has been linked to genomic instability, and significantly increased micronuclei formation was observed in LPS-treated cells following KL silencing, compared to treated, non-silenced cells. There was a significant increase in DNA double strand breaks observed in these cells as well, compared to treated, non-silenced cells. Lastly, LPS-treated cells without KL silencing showed a significant increase in autophagy activation, compared to LPS-treated cells with KL silencing, suggesting that silencing led to autophagy repression. This was confirmed with a significant downregulation in synthesis of the ATG16L protein needed for autophagosome formation in treated, silenced cells, compared to LPS-treated cells without silencing. Overall, Mytych et al. (2019) provide key evidence that KL plays a role in both ER-mediated stress responses and autophagy in fibroblasts, as its silencing in LPS-treated cells led to poorer wound healing outcomes compared to non-silenced LPS-treated cells.

All in all, the mechanisms underlying KL's anti-aging, protective effects can potentially include autophagy regulation (Zhou et al., 2021). KL can influence Beclin 1/Bcl2 expression, as shown by Mytych et al. (2019), IGF-1/PI3K/Akt/mammalian target of rapamycin (mTOR) signaling, and aldosterone levels, which all have downstream effects on autophagy activity. See Fig. 3.

1. Regulation and Processing of Klotho

In order to yield shKL, KL must be processed via shedding, yielding shKL (Chen et al., 2007). Chen et al. (2007) specifically found that

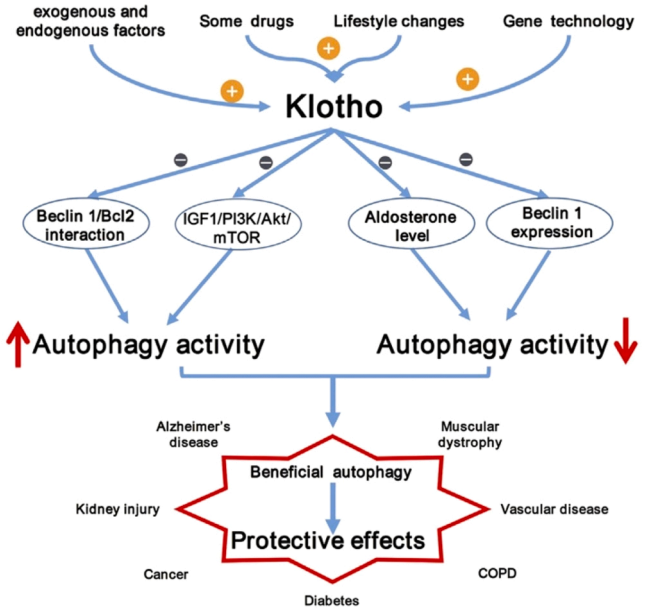


Fig. 3. KL's potential role in autophagy regulation. This schematic shows how upregulation of KL can affect multiple signaling pathways that influence autophagy activity. Beneficial autophagy may have protective effects against a variety of pathologies. Figure taken from (Zhou et al., 2021).

alpha-secretases of the ADAM (A Desintegrin and Metalloproteinase) family, ADAM10 and ADAM17, could cleave the extracellular domain of KL, producing 130-kilodalton (kD) and 68-kD fragments. Insulin was also found to promote KL shedding, likely through protein trafficking. Further, Bloch et al. (2009) found that shKL is generated from the cell surface not only by the alpha-secretases ADAM10 and ADAM17, but also by the beta-secretase BACE1 (Beta-Amyloid Precursor Protein Cleaving Enzyme 1) and gamma-secretase.

Moreover, there are two primary cleavage sites in the KL protein that are recognized by sheddases, known as alpha1 and alpha2 (Chen et al., 2014b). alpha1 is located near the juxtamembrane region, and alpha2 is located between the KL1 and KL2 domains. It was found that mutating the alpha1 site in mouse KL led to a decrease in both the 130 kilodaltons (kD) and 70 kD fragments of the protein, indicating that KL cleavages at the alpha1 and alpha2 sites are related to each other (Chen et al., 2020). It was further found that mutation in the alpha2 site affects alpha1 cleavage, in both human and mouse KL.

In order to understand the full therapeutic potential of KL, it is essential to study the different functions of its isoforms and how they are processed. It would be especially interesting to produce a transgenic mouse model with an uncleavable KL to fully characterize the functions of FL-KL, without the presence of shKL. It would be equally interesting to knock in or perform gene editing utilizing CRISPR techniques to only have shKL present, to study what happens in the absence of FL-KL, and whether shKL will fulfill the functions attributed to FL-KL.

1.6.4. Downregulation of Klotho expression in aging

The mechanisms by which KL gene expression is downregulated have also been studied in depth. King et al. (2012) reported that in aged rhesus monkeys, KL protein levels were 20% reduced in white but not grey matter of the dorsolateral prefrontal cortex, compared to young monkeys, consistent with the earlier findings of Duce et al. (2008) ($p < 0.04$). Additionally, King and colleagues (2012) showed that there was a statistically significant 0.4% increase in KL promoter methylation in the dorsolateral prefrontal cortex white matter of aged rhesus monkeys, compared to young monkeys ($p < 0.02$). This lends support that KL levels are epigenetically modified, at least in the white matter of older rhesus monkeys. Notably, Lee et al. (2010) found the complete loss of KL mRNA to occur in the late phase of tumorigenesis of cervical cancer in vitro, and this was associated with hypermethylation of the KL promoter. Similarly, Pan et al. (2011) found KL gene expression to be absent or downregulated in colon cancer in vitro, and this was associated with KL promoter hypermethylation as well.

Recently, Kuro-o (2021) described one possible mechanism through which KL deficiency can lead to aging. As is known, mice that are deficient in KL exhibit a syndrome that mirrors aging in humans (Kuro-o et al., 1997). It is also known that KL is necessary for FGF23 signaling (Kurosu et al., 2006). As FGF23 facilitates increased urinary phosphate excretion, mice with KL deficiency face both accelerating aging and phosphate retention. Since the extracellular fluid is supersaturated in phosphate and calcium ions, an increased phosphate concentration can result in the precipitation of calcium-phosphate. This precipitated calcium-phosphate is then adsorbed by serum protein fetuin-A to form calciprotein particles (CPPs), which can cause cell damage, calcification, and inflammation. Thus, a lack of Klotho can lead to increased levels of CPPs, which can ultimately contribute to aging.

1.7. KL-VS heterozygosity

The first description of KL-VS heterozygosity was provided by Arking et al. in 2002. They had identified an allele, KL-VS, that was defined by six single nucleotide polymorphisms that were present in an 800 base-pair region spanning exon 2 and flanking sequence. These six sequence variants were in complete linkage disequilibrium, and two of them led to amino acid substitutions F352V and C370S. Among a Bohemian Czech cohort, KL-VS heterozygosity was more prevalent in the

elderly ($p < 0.04$), a 1.43-fold (95% confidence interval [CI] [1.02, 2.01]) increased survival rate to age 75 was associated with heterozygosity, and a 2.70-fold (95% CI [0.84–8.69]) survival disadvantage was associated with KL-VS homozygosity.

2. Clinical implications

2.1. Aging

KL has been shown to play a crucial role in aging time and again, since its initial discovery by Kuro-o et al. (1997). Indeed, it has been consistently demonstrated that KL levels decline with age and that preservation of high KL levels may extend lifespan and provide protection against age-related diseases (Cognitive Vitality and Alzheimer's Drug Discovery Foundation, 2018). Serum KL levels have been found to decrease with age in humans (Shardell et al., 2019) and in bonobos and chimpanzees (Behringer et al., 2018). Researchers have also found that KL levels are lower in older AD patients compared to those without AD ($p = 0.02$), and lower in older adults compared to younger adults ($p = 0.005$) (Semba et al., 2014). In the subsections below, we expand on how Klotho levels change in a variety of diseases, all of which are associated with aging.

2.2. Renal conditions

As KL is produced in the kidneys, a main area of interest is the role of KL in the realm of renal diseases. The number and size of glomeruli, the filtration unit of the kidney, have been shown to significantly decrease with age (Nyengaard and Bendtsen, 1992). Additionally, hemodynamic and biochemical changes occur in the kidney with age that further predispose the aged kidney to disease (Weinstein and Anderson, 2010). Indeed, glomerular sclerosis incidence increases with age (Kaplan et al., 1975).

Thus, the implications of KL in the clinical management of renal conditions are manifold (Cognitive Vitality and Alzheimer's Drug Discovery Foundation, 2018). The greatest expression of FL-KL is seen in the kidneys, where the promoter component of the KL gene is largely unmethylated in renal tubular cells, compared to 30–40% methylation in non-KL expressing cells (Azuma et al., 2012). Of particular interest is the use of KL as a biomarker in kidney disease. Wang et al. (2018) found in their meta-analysis of nine studies of 1457 patients with chronic kidney disease (CKD) that soluble KL levels were statistically significantly associated with estimated glomerular filtration rate (eGFR), an indicator of kidney function ($r = 0.35$, $p < 0.05$), and inversely correlated with FGF23 ($r = -0.10$, $p < 0.05$). While high levels of FGF23 can have toxic effects, KL can suppress FGF23 levels, thereby promoting healthy kidney function, which is reflected in the eGFR (Lu and Hu, 2017). Known harmful effects of high FGF23 levels include impaired vasodilation and the induction of left ventricular hypertrophy (Faul et al., 2011). Taken together, these findings suggest that KL can potentially be used as a biomarker for kidney function.

Furthermore, KL has potential as a therapeutic in renal conditions. Indeed, Zhou et al. (2015) reported that in mice that underwent 5/6 nephrectomy, exogenous KL expressed in vivo via hydrodynamic-based gene delivery reduced levels of albuminuria and serum creatinine, morphological lesions, and levels of renin-angiotensin system components, reflecting protective effects on kidney function. Systolic blood and mean arterial blood pressure were also normalized in these mice following exogenous KL expression.

The use of KL as a biomarker for kidney function and treatment for kidney disease is of great interest. KL has the potential to alleviate the burden of renal conditions, especially for older individuals.

2.3. Cardiovascular disease

KL also has great potential to transform the clinical management of cardiovascular disease, which is also associated with aging. With increasing age, the heart's elasticity and ability to respond to changes in compliance decreases (Stern et al., 2003). The increase in vascular resistance amplifies the amount of work needed for the heart to pump blood. Angina pectoris, acute coronary syndromes, heart failure, and irregular heartbeats all increase in frequency among older populations.

There is evidence that KL can protect against harmful cardiovascular states by regulating ion transport, endothelial function, inflammation, lipid homeostasis, and oxidative stress (Cognitive Vitality and Alzheimer's Drug Discovery Foundation, 2018). In a cohort of hemodialysis (HD) patients who were at risk of developing cardiovascular events, serum KL levels were significantly reduced compared to non-dialysis CKD patients or healthy controls (Marçais et al., 2017). Patients with higher KL levels had less cardiovascular events and better survival rates compared to those with lower KL levels. Among patients with low KL levels, more cardiovascular events and worse survival were only observed when serum FGF23 was also high.

In mice, KL has been found to confer cardioprotection through the downregulation of TRPC6 calcium channels in the heart (Xie et al., 2012). More recently, Sun et al. (2022) conducted a Mendelian randomization study to assess the causal relationship between KL concentrations and cardiovascular disease, namely coronary artery disease (CAD), atrial fibrillation (AF), heart failure (HF), stroke, ischemic stroke (IS), and IS subtypes. Five single-nucleotide polymorphisms (SNPs) were associated with circulating KL levels and explained 7.68% of the variance in circulating KL concentrations. In the meta-analysis, for each unit increase in circulating KL, there was 3% lower odds of CAD with a 95% CI of (0.94, 1.00) and p-value of 0.044; for each unit increase in circulating KL, there was 4% lower odds of AF with a 95% CI of (0.93, 0.99) and p-value of 0.005. There was no causal relationship between KL levels and HF, stroke, and IS, and IS subtypes.

2.4. Pulmonary disease

KL research can also be extended to the realm of pulmonary disease. With age, the volume of the thoracic cavity decreases, lung volumes decrease, and the muscles that facilitate respiration are altered (Lowery et al., 2013). Of note, clearance of particles from the lung via the mucociliary elevator is reduced. As a result, the elderly population is vulnerable to developing pulmonary pathologies. The prevalence of chronic obstructive pulmonary disease is especially high among older adults (Buist et al., 2007).

Batlahally et al. (2020) studied preterm infants who were born at less than 29 weeks of gestation, and found that those with bronchopulmonary dysplasia (BPD) or both BPD and pulmonary hypertension (PH) had significantly lower plasma KL levels in cord blood compared to those without BPD or PH. Additionally, Q. Huang et al. (2020) identified KL to be a potential regulator of multiple pathological processes underpinning idiopathic pulmonary fibrosis (IPF). Isolated pulmonary fibroblasts from bleomycin-exposed mice, a model for IPF, showed reduced KL mRNA and protein levels. Promisingly, exogenous administration of recombinant KL was shown to alleviate pulmonary fibrosis *ex vivo* by affecting the activation and migration of pulmonary fibroblasts, production of extracellular matrix, and expression of known fibrotic genes. It will be interesting to evaluate, if this type of therapy, when it becomes available, has beneficial effects in older humans.

2.5. Dysfunctional Metabolic/Endocrine States

The endocrine system also undergoes changes in the face of aging. This includes alterations to the secretory patterns and sensitivity of the hypothalamic-pituitary axis (van den Beld et al., 2018). Further, glucose homeostasis is impaired, bone and muscle mass decreases, and fat mass increases. As the hypothalamic-pituitary axis has a diverse variety of target organs, multiple downstream effects can occur as one ages. Indeed, insulin resistance increases and pancreatic islet function

decreases with age, and diabetes is highly prevalent among older adults (Kirkman et al., 2012).

Subfamilies of Klotho have been implicated in conditions of the body involving the endocrine system and metabolism. Notably, Lan et al. (2017) further elucidated the role of FGF19, FGF21, and β-KL in regulating body weight and blood sugar levels. They had found β-KL, the co-receptor for FGF19 and FGF21, to be needed for both molecules to cause lower weight, glucose, and insulin levels, specifically in neurons, not adipocytes or hepatocytes. In addition, BonDurant et al. (2017) found that mice without β-KL in adipose tissue lacked FGF21's acute insulin-sensitizing effect, although this was not required for chronic metabolic effects. Overall, it is clear that β-KL plays a significant role in the maintenance of metabolic homeostasis, and that defects with the protein itself or pathways involving the protein may be linked to problematic metabolic/endocrine states, such as obesity and diabetes, especially in older populations.

2.6. Cancer

The clinical applications of KL are far-ranging, from neurodegenerative diseases to renal disorders. Notably, KL is also known for its anti-tumor properties. This is important, as the incidence of most types of cancer increases with age, and KL levels decrease with age (White et al., 2014).

ONCOMINE is a tool that investigators can use to study differential gene expression across multiple types of cancer. It is essentially a cancer microarray database that collects results from gene expression analyses conducted by researchers across the world (Rhodes et al., 2004). ONCOMINE was the product of the efforts of Rhodes et al. (2004), who sought to facilitate easier access to all public cancer microarray data with an online platform. Such a tool was indeed needed at the time, as gene expression analyses of cancer samples were imperative to genetically profile a cancer and establish a trend in expression, but there did not exist an organized method to consolidate and publicize all of these results. As of September 2021, there have been 715 datasets from 86,733 samples uploaded to ONCOMINE. Notably, KL has been found to be downregulated in nearly all types of cancer on the ONCOMINE database (from www.oncomine.org).

Indeed, Chen et al. (2014a) performed a detailed search of the database, finding that KL gene expression is significantly decreased in many types of cancer, such as those afflicting the brain, bladder, lung, breast, prostate, skin, male germ cells, salivary gland, pancreas, T-cells, head and neck, ovary, and bone marrow. The only types of cancer in which KL levels were not significantly changed were thyroid and B-cell cancers. When studying brain cancers specifically, KL gene expression was found to be significantly reduced in glioblastoma ($p = 2.1e-9$), oligodendroglioma ($p = 2.8e-9$), and astrocytoma ($p = 2e-7$), compared to controls (Chen et al., 2014a). The results of Chen et al. (2014a) also highlight the prognostic potential of KL in the realm of breast cancer. A more severe downregulation of KL gene expression was associated with a worse prognosis, and vice versa.

The main objective of the study conducted by Chen et al. (2014a) was to investigate the effects of KL treatment on a hybrid line of human oligodendrocytic cells, known as MO3.13 cells. Interestingly, in a search

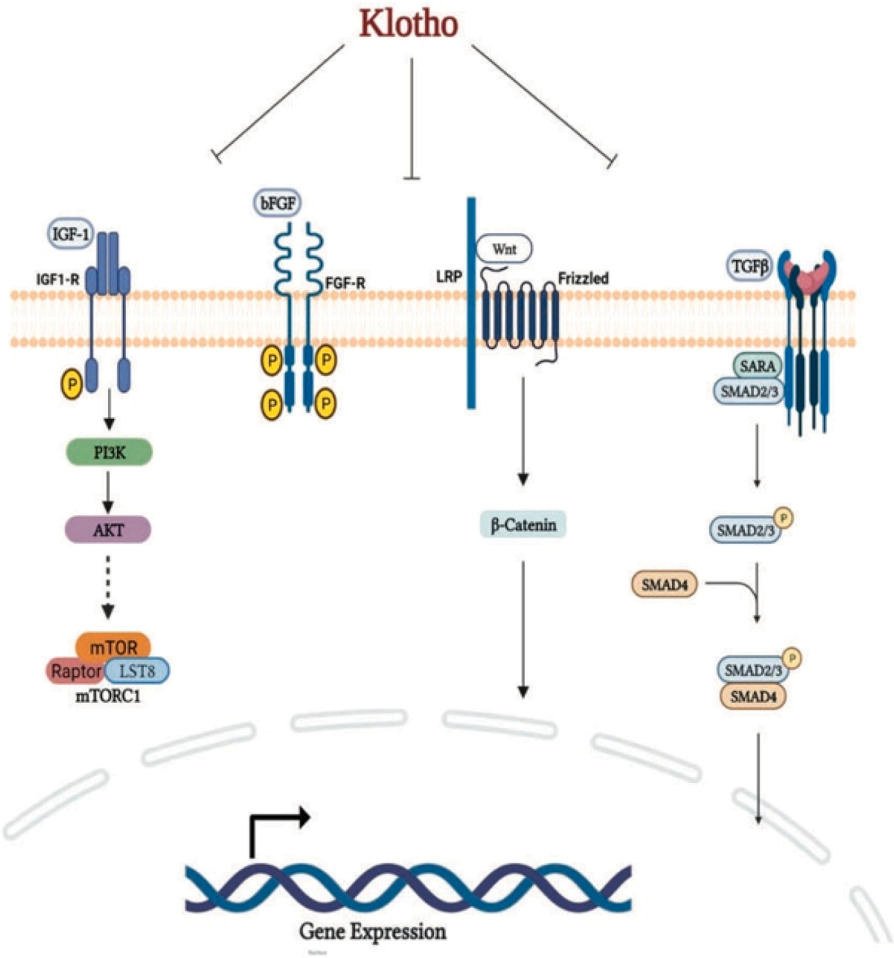


Fig. 4. KL's role in oncogenic pathways. An overview of KL's mechanisms of modulation of oncogenic pathways is provided here. Figure taken from (Ligumsky et al., 2022).

of 1186 chemical and genetic perturbation gene sets, it was found that differential signature genes from KL-treated cells were similar to those seen in "cancer, cardiovascular disease, stress, aging, and hormone-related chemical and genetic perturbations" (Chen et al., 2014a). Put together, these results suggest a role for KL in various physiological states, including cancer, stress, and aging.

Recently, Ligumsky et al. (2022) summarized the role of KL in cancer. In sum, KL has been implicated in the IGF-1, basic fibroblast growth factor (bFGF), Wingless/Integrated (Wnt), and transforming growth factor β (TGFβ) signaling pathways, which influence gene expression. Please see Fig. 4.

2.7. Neurodegenerative diseases

Not surprisingly, KL also has many implications in the realm of neurodegenerative diseases, for which aging is a major risk factor (Cognitive Vitality and Alzheimer's Drug Discovery Foundation, 2018; Wyss-Coray, 2016). Indeed, it has been observed that nearly all aged brains show changes that are linked to neurodegeneration.

Expression of sKL is ten times greater in the brain than in the kidney (Massó et al., 2015), although these results have not been replicated yet. The application of KL findings to therapeutics and diagnostics in AD and Parkinson's disease (PD) is especially exciting. Massó et al. (2015) found that sKL protein levels decrease with age in mice, and levels of mRNA transcripts for sKL and FL-KL declined even more rapidly in a mouse model of AD. In terms of human research, as mentioned previously, Semba et al. (2014) found that KL levels in CSF are lower in older AD patients compared to those without AD ($p = 0.02$). Promisingly, Dubal et al. (2015) found that increasing expression of KL in human amyloid precursor protein transgenic mice reduced premature mortality, decreased the occurrence of abnormal spike activity on intracranial electroencephalogram, improved cognitive and behavior performance in the Morris water maze, novel object recognition test, passive avoidance test, open field test, and elevated plus maze, and prevented degeneration of dendritic spines.

In vitro, the addition of shKL to primary mouse neurons was shown to protect the cells from glutamate-induced oxidative stress and from the toxic effects of oligomeric amyloid β peptide, known to be neurotoxic and synaptotoxic and to be one of the initiators of neurodegeneration in AD (Zeldich et al., 2014). The study provided evidence that the neuroprotective effects of KL were mediated via regulation of members of the redox system (thioredoxin/peroxiredoxin (Trx/Prx)). KL-induced phosphorylation of the PI3K/Akt pathway, a pathway important in apoptosis and longevity, was associated with sustained inhibitory phosphorylation of the transcription factor forkhead box O3a, FOXO3a, and was essential for the induction of Prx-2.

In the realm of PD, Sancesario et al. (2021) found serum KL levels to be significantly lower in PD patients compared to age-matched controls ($p < 0.05$), but CSF KL levels were significantly higher in the same patients compared to controls ($p < 0.05$). In contrast, in a study of PD patients with wild-type KL or the KL-VS haplotype, Zimmermann et al. (2021) found all PD patients to have significantly lower CSF KL levels compared to controls ($p \leq 0.001$). PD patients with the KL-VS haplotype had significantly higher KL CSF levels than those with wild-type KL ($p = 0.009$). These dissimilar results point to the need for more research that may further elucidate the role of KL in PD. Nonetheless, it is encouraging that Leon et al. (2017) found that peripheral administration of the shKL protein fragment improved motor learning and performance in rotarod testing, and improved spatial and working cognition in the two-trial Y-maze, in transgenic mice that overexpress wild-type human a-synuclein, a synuclein model of neurodegenerative disease. However, the KL protein fragment did not penetrate the blood-brain barrier, nor did it decrease steady-state levels of α-synuclein, tau, or their phosphorylated forms.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that is known to induce features of PD and target dopaminergic neurons (Sian et al., 1999). Brobey et al. (2015) found that transgenic mice overexpressing KL had less loss of striatal dopamine and nigral dopaminergic neurons in response to MPTP, compared to wild type mice. A strain of these mice, the *EFmKL48* mouse line, also did not show evidence of neurodegeneration on staining for tyrosine hydrolase activity, after a dose of MPTP, compared to mice administered saline. It was also revealed that neuroprotection conferred by KL is, in part, coordinated through the ROS-sensitive apoptosis signal-regulating kinase 1 (ASK1)/p38 mitogen-activated protein kinase (MAPK) pathway. The transgenic KL overexpressing mice had less p38 MAPK activation compared to KL knockout mice, showing that the dopaminergic neurons were less vulnerable to ROS-mediated oxidative stress in overexpressing mice. In turn, these mice had greater longevity and physical fitness.

The findings of Baluchnejadmojarad et al. (2017) further highlight KL's clinical potential in the treatment of PD. In their work, KL was found to improve movement behavior in the 6-hydroxydopamine rat model of PD, decrease striatal levels of deleterious compounds such as ROS and α-synuclein, and prevent neurodegeneration of tyrosine hydroxylase-positive neurons within the substantia nigra pars compacta. Furthermore, KL's mechanism of action was postulated to be dependent on protein kinase A (PKA) and calcium/calmodulin-dependent dependent protein kinase II (CAMKII)/CREB signaling, as inhibitors of PKA and CAMKII lessened the protective effects of KL.

Interestingly, CREB is a target of PKA and is activated via PKA phosphorylation at serine-133 (Yu et al., 2014; Zhu et al., 2019). Phosphorylation at this site is also modulated by CAMKII, calcium/calmodulin-dependent dependent protein kinase IV (CAMKIV), and p38 MAPK (Sheng et al., 1991; Xing et al., 1998).

Lastly, KL has been implicated in amyotrophic lateral sclerosis (ALS) in mice with a gain-of-function glycine 93 to alanine substitution in superoxide dismutase 1 (SOD1). Zeldich et al. (2019) reported that in this SOD1 mouse model of ALS, KL overexpression delayed onset and progression of ALS and extended survival. KL also decreased the expression of neuroinflammatory markers and proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-12 subunit alpha (IL-12a), and interleukin-1 beta (IL-1β), prevented neuronal loss, and increased the expression of antioxidative and promyelinating factors.

For a review on the role of KL in neurodegeneration, please see Abraham et al. (2016).

2.8. Psychiatric disorders

In addition to neurodegenerative diseases, KL may also have the potential to transform the treatment and diagnosis of psychiatric disorders. In 2020, the prevalence of any mental illness was highest among young adults aged 18–25 years (Mental Illness, 2022). However, a history of psychopathology has been associated with accelerated aging (Wertz et al., 2021). In a cohort of more than 1000 individuals, a history of mental disorder was associated with increased pace of biological aging at midlife, hearing impairment, cognitive difficulty, and an older-looking physical appearance. It will be interesting to observe if and how KL can be used in the treatment of psychiatric disorders, specifically to prevent the development of age-related pathologies later in life.

KL has been studied in the context of depression, stress, and schizophrenia thus far, as well as in the use of electroconvulsive therapy (ECT) in depression and schizophrenia. Notably, Gao et al. (2021) found patients with major depressive disorder (MDD) to have significantly lower plasma KL levels than age-matched healthy controls. Prather et al. (2015) found that women who were chronically high-stress maternal caregivers for a child with autism spectrum disorder had significantly lower serum KL levels compared to women who were low-stress caregivers of a typically developing child. Although Hoyer et al. (2018) found that CSF KL levels significantly differed in geriatric individuals with severe, therapy resistant depression before and after ECT, with KL

increasing with each ECT session, this did not translate into improved symptoms. Such an effect was not seen in serum. Further, Kranaster et al. did not find CSF KL to be a significant clinical predictor of therapeutic outcome in depressive patients undergoing ECT, both in 2019 and 2020. In the realm of schizophrenia, Turkmen et al. (2021) found that male schizophrenic individuals who self-discontinued medical treatment had significantly lower plasma KL compared to controls. After 20 days of standard medical therapy, plasma KL was not significantly different in these patients. Although the literature for KL in the realm of psychiatric disorders is less prolific than that for neurodegenerative and renal conditions, the potential for discovery is great. Still, it is important to note that research in KL in this context may be limited due to the lack of completely valid and reliable animal models for psychiatric conditions (Hitzemann, 2000).

2.9. Autoimmune diseases

KL has also been implicated in autoimmune diseases, such as MS and rheumatoid arthritis (RA). The incidence of various autoimmune diseases increases with age, including giant cell arteritis and RA (Goronzy and Weyand, 2012). Age is considered a risk factor for autoimmunity, as the immune system is less robust with age. Specifically, thymic T cell generation halts in the second half of adulthood.

The International Advisory Committee on Clinical Trials of MS have defined the four MS phenotypes (Lublin et al., 2014; *Types of MS*, www.nationalmssociety.org/What-is-MS/Types-of-MS): clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS). CIS is the first clinical presentation of disease that is characteristic of the inflammatory demyelination seen in MS, but that has not yet met the criteria of dissemination in time. RRMS is characterized by episodes of new or worsening neurologic symptoms, which are followed by incomplete or complete recovery. SPMS is characterized by a history of progressively worsening disease, which escalates from RRMS. PPMS is considered to be a less inflammatory form of MS and is characterized by the lack of exacerbations prior to disease progression.

KL has been tied to myelination in the central nervous system (CNS), which is a key concern in MS. Chen et al. (2013) found that KL treatment enhanced maturation of OPCs in vitro, and this was reflected in upregulation of myelin-associated gene and protein expression. Furthermore, in KL knock-out mice, there was evidence of significant impairment of myelination of the optic nerve and corpus callosum compared to control mice. Zeldich et al. (2015) have also studied KL in the context of CNS myelination. Specifically, they reported that in transgenic mice overexpressing KL who underwent cuprizone-induced demyelination, the number and density of remyelinated axons in the corpus callosum was significantly higher compared to wild-type mice. These findings suggest that KL has therapeutic potential in promoting remyelination in the CNS of those with MS.

RA is a debilitating chronic autoimmune and inflammatory disease that affects the synovial joints. As KL is known to have an anti-inflammatory role, studying the link between KL and RA may pave a new road for therapeutic development. Witkowski et al. (2007) found KL mRNA, protein, and enzymatic levels to be downregulated in $CD4^{+}$ lymphocytes from healthy elderly individuals, and even more so from RA patients. KL downregulation occurred with a concomitant downregulation of T cell costimulatory molecular CD28, which is known to be dependent on high levels of the inflammatory cytokine tumor necrosis factor alpha (TNF-α). Therefore, KL could serve as a point of modulation to attenuate the rampant inflammation seen in diseases such as RA.

2.10. Klotho as a therapeutic

Based on its broad clinical implications when downregulated during aging or disease, restoring or boosting endogenous KL or exogenous supplementation has tremendous therapeutic potential. Utilizing KL as a therapeutic agent in cancer is a promising prospect. Such a possibility has been explored by Wolf et al. (2008) for breast cancer and Abramovitz et al. (2011) in the context of pancreatic cancer. For the latter type of cancer, KL was found to be under-expressed in pancreatic adenocarcinoma compared to normal pancreas extracts, and treatment with overexpressed or soluble KL significantly reduced pancreatic cancer cell growth in vitro and in vivo. Treatment with KL also significantly augmented the activity of the common chemotherapeutic 5-FU in vitro; thus, less 5-FU was necessary when administered with KL, reducing undesired side effects of the drug. Of note, the KL1 domain had similar activity as FL-KL, but with a more desirable safety profile in vivo because it did not enhance FGF23 signaling. More recently, Rubinstein et al. (2021) explored the role of KL as a tumor suppressor in pancreatic ductal adenocarcinoma. Notably, pancreatic KL knockdown and Kras mutation in a novel genetic mouse model contributed to cancer progression and mortality, while administration of adeno-associated virus (AAV) particles with sKL in a xenograft model reduced pancreatic tumor load, and administration of soluble recombinant sKL in an established mouse model of pancreatic ductal adenocarcinoma improved survival. These findings certainly lend support for the inclusion of KL (and its domains) in the advancement of new diagnostic and therapeutic approaches for cancers.

Excitingly, KL and CRISPR technology have been studied jointly. Chen et al. (2018) studied the transcriptional activation of the KL gene via single-guide RNAs (sgRNAs) targeting the KL promoter region using a CRISPR-dCas9 complex. Two sgRNAs were determined to upregulate KL gene transcription. They were shown to increase both KL gene and protein levels in a Firefly Luciferase system, NanoLuc luciferase coincidence reporter system, a NanoLuc luciferase knock-in in the KL 3' untranslated region utilizing CRISPR genomic editing, and two human cell lines that express KL endogenously.

Overall, the potential uses of KL as a treatment for a myriad of health conditions is extremely promising. In the future, KL can be used in conjunction with other medical treatments and healthy lifestyle changes to substantially alleviate the burden of age-associated pathologies.

3. Use of Klotho as a biomarker

It is clear from Sections 7 and 8 that KL has transformative potential in the management of numerous diseases. KL may also have use as a biomarker for certain diseases. Abramovitz et al. (2011) found that KL expression is reduced in pancreatic adenocarcinoma, and Wolf et al. (2008) found reduced KL expression in breast cancer. Thus, the measurement and quantification of KL early on in a disease process may offer valuable insights to clinicians.

The use of KL as a biomarker is especially important in the diagnosis and management of age-related diseases, as these conditions may remain latent for long periods of time and may not have obvious signs and symptoms. For instance, AD is known to progress slowly from its onset to a full manifestation of symptoms. In a cohort of 200 patients, the incubation period for AD was estimated to last approximately 17 years (Villemagne et al., 2013). As such, the utilization of KL as a novel biomarker can prove extremely valuable, particularly in efforts to support preventive and screening health services.

4. Measurement of Klotho in various types of samples

The predominant method of measuring KL in blood samples is through performing immunoassays to measure soluble KL (Pedersen et al., 2013). Pedersen et al. (2013) compared two different immunoassays to detect differences in KL measurement in human serum: an ELISA kit from Immuno-Biological Laboratories (IBL) based in Japan, and a time-resolved fluorescence immunoassay (TRF) utilizing antibodies provided in an ELISA kit from Cusabio based in China and recombinant human KL as a standard. There was no correlation found between the two assays, which showed significant differences in

measured KL levels. The median and interquartile range (IQR) of the KL levels measured in the IBL assay was 471 (373−547) pg/mL; 80 (51−110) ng/mL for the TRF assay. As the levels of serum KL differed 1000-fold between the two assays, it was suggested that the two assays detect distinct forms of KL in serum. Furthermore, when the standard curves were switched from one assay to the other, no signals from the curves were obtained, suggesting that the two assays recognized different epitopes on soluble KL. As a significant portion of the literature's data is based on collected KL measurements utilizing an ELISA system, namely that provided by IBL, this thesis will focus on measurements collected using ELISA.

ELISA is heavily used to measure KL levels in human CSF and urine samples. However, whole tissue samples, immunoblot analysis, immunohistochemical staining, and mRNA quantification are also examples of methods that are utilized to determine the presence and amount of KL.

4.1. Klotho levels

Here, the KL measurements collected from the compiled studies are summarized. Please see Appendix for the KL measurements organized in a tabulated fashion, sorted by ascending age and stratified by sample and disease type. In these tables, KL concentrations are highlighted, alongside the studies' corresponding sample age group, sample type, and key findings. KL measured via ELISA was done so using the IBL kit, unless explicitly noted. In the tables where KL values are given as pg/mL, mathematical conversion was done for a handful of studies that gave measurements in other units to maintain consistency. There were also several studies for which KL levels are denoted "N/A," because values were not explicitly provided.

4.2. Blood samples

4.2.1. Healthy individuals

The measurements of KL in blood samples from healthy humans, or from individuals without any specified diseases, obtained from ELISA are summarized below in Appendix, Table 1. Overall, among healthy individuals, blood KL was inversely correlated with age, as expected. Indeed, the youngest individuals studied yielded the highest KL levels (Batlahally et al., 2020; Gkentzi et al., 2014; Ohata et al., 2011; Yamazaki et al., 2010), while the oldest individuals yielded the lowest KL levels (Iturriaga et al., 2021; Kresovich and Bulka, 2021; Ohata et al., 2011; Pedersen et al., 2013; Prather et al., 2015; Sartorius et al., 2019; Sugiura et al., 2011; Yamazaki et al., 2010; Yokoyama et al., 2017). Of note, KL levels have also been studied in the context of exercise in healthy individuals. Iturriaga et al. (2021) found that plasma KL significantly increased following acute cardiorespiratory (CR) exercise, and decreased immediately after the end of acute strength exercise but surpassed pre-exercise levels within 24 h. Morishima and Ochi (2021) also found that a single bout of resistance exercise increased serum KL 10 min following exercise, and that there was a positive correlation between KL and endothelin-1 in response to exercise. Huang and Wang (2021) conducted a large study of the general population of the United States by compiling data collected from the National Health and Nutrition Examination Survey (NHANES). Interestingly, there was a negative correlation found between sleeping duration and serum KL levels, and those in the highest tertile of sleep duration had slightly lower serum KL than those in the lowest tertile of sleep duration. More recently, Chen and Chen (2022) also reported on findings drawn from the NHANES, investigating the relationship between exposure to polycyclic aromatic hydrocarbons (PAHs) and serum KL levels. Among participants of the 2015–2016 NHANES, 2-napthol and 3-fluorene were significantly associated with decreased KL, adjusting for age, race, waist circumference, creatinine, albumin, medical history, and smoking history. Interestingly, when stratifying the sample by sex, there were no significant relationships between PAH exposure and serum KL found in women. Most recently, Espuch-Oliver et al. (2022) studied the serum KL levels of a cohort of healthy individuals in Spain. Of the three age groups between 18 and 85 years, KL levels were the lowest in the most senior group ($p < 0.001$). Of note, there were no significant differences in KL levels between sexes in each age category. There was also a statistically significant inverse correlation between age and KL levels ($p < 0.001$).

4.2.2. Renal conditions

The measurements of KL in blood samples from individuals with renal conditions, obtained from ELISA, are summarized below in Appendix, Table 2. Among these individuals, it was also observed that blood KL levels were generally inversely correlated with age. The typical pattern was that younger patients with kidney conditions had greater KL levels (Deng et al., 2018; Fischer et al., 2021; Gamrot et al., 2021; Guo et al., 2021). Another pattern observed was that blood KL levels were overall inversely correlated with severity of kidney disease (Otani-Takei et al., 2015; Donate-Correa et al., 2021; Gamrot et al., 2021; Guo et al., 2021; Milovanova et al., 2021). An exception to this trend was found in results from Sugiura et al. (2011), who reported that CKD patients had significantly greater serum KL levels compared to healthy controls. Yildirim et al. (2021) found KL levels to be higher in CKD patients who were on HD or peritoneal dialysis, compared to healthy controls, but the KL values found were extremely high compared to other studies, possibly due to the type of ELISA kit used. Interestingly, Jia et al. (2021) found that in CKD patients on maintenance hemodialysis (MHD) who also had severe hyperparathyroidism, KL levels increased following parathyroidectomy and auto-transplantation, although this increase was not significant. Liu et al. (2021) found KL levels to be weakly correlated with vascular calcification level among end-stage renal disease (ESRD) patients. Lastly, Uriol-Rivera et al. (2021) reported that among a cohort of patients on chronic HD, those who were on erythropoietin stimulation with continuous erythropoietin receptor activator (CERA) or conversion to weekly epoetin-b (EB) had a significant decrease in KL from 3 to 6 months of treatment, which tracked changes in transferrin, free serum iron, and red blood cell distribution width.

4.2.3. Cardiovascular Conditions

The measurements of KL in blood samples from individuals with cardiovascular conditions, obtained from ELISA, are summarized below in Appendix, Table 3. The general pattern observed was that blood KL was inversely correlated with cardiovascular disease risk and severity (Navarro-González et al., 2014; Xu et al., 2017; Shakked et al., 2021; Yeganeh-Hajahmadi et al., 2021). Younger persons also had higher blood KL compared to older individuals with cardiovascular disease (Shakked et al., 2021). Of note, Drew et al. (2021) found that in a cohort of well-functioning elderly adults, higher serum KL was associated with higher baseline diastolic blood pressure, lower rate of incident hypertension, and lower subsequent systolic and diastolic blood pressure in follow-up. Marçais et al. (2017) reported that among a group of chronic HD patients, those who had serum KL levels greater than the first quartile (280 pg/mL) had 61% lower odds of combined cardiovascular events and death, and this was significant at a 95% CI of (0.19, 0.78) and p-value of 0.08, in the unadjusted analysis. In the adjusted analysis, those with serum KL levels greater than the first quartile had 14% lower odds of cardiovascular combined cardiovascular events and death, and this was still significant at a 95% CI of (0.76–0.99) and p-value of 0.03.

4.2.4. Pulmonary conditions

The measurements of KL in blood samples from individuals with pulmonary conditions, obtained from ELISA, are summarized below in Appendix, Table 4. As expected, blood KL measured in infants were high (Batlahally et al., 2020), although these were infants who were born preterm with a gestation period of less than 29 weeks. It was also found among these infants that those with bronchopulmonary dysplasia (BPD) or both BPD and pulmonary hypertension (PH) had lower KL levels compared to preterm infants without these conditions.

4.2.5. Abnormal metabolic/endocrine states

The measurements of KL in blood samples from individuals with metabolic or endocrine dysfunction obtained from ELISA, are summarized below in Appendix, Table 5. Again, it was observed that infants had the highest KL levels (Wojcicki et al., 2018), compared to older children (Socha-Banasiak et al., 2020; Zubkiewicz-Kucharska et al., 2021). It was also found that KL levels were higher in obese children compared to overweight and normal children (Socha-Banasiak et al., 2020), and that KL levels were higher in insulin-resistant children compared to insulin-sensitive children (Socha-Banasiak et al., 2020). Additionally, Zubkiewicz-Kucharska et al. (2021) found that KL levels were lower in children with Type 1 diabetes (T1D) compared to healthy controls. More recently, Guarnotta et al. (2022) compared serum KL levels in children with growth hormone deficiency (GHD) treated with recombinant human growth hormone (rhGH) therapy for 12 months to sex-, age-, and pubertal status-matched control short children. They found that at baseline, children with GHD had significantly lower KL levels than control children ($p = 0.001$). Over 12 months of follow-up both controls ($p = 0.030$) and GHD children ($p < 0.001$) had a significant increase in their KL levels. Interestingly, at 12 months, GHD children compared to controls had significantly higher KL levels ($p < 0.001$). Among a cohort with type 2 diabetes (T2D), Ciardullo and Perseghin (2022) found patients with reduced serum KL levels to be significantly older ($p < 0.001$), more frequently male ($p < 0.001$), and more likely to have lower eGFR ($p < 0.001$) and lower hemoglobin A1c (HbA1c; $p < 0.001$). Finally, H.-J. Lee et al. (2022) studied the association between serum uric acid levels and serum KL levels among participants of the NHANES. KL was negatively associated with uric acid ($p < 0.0001$) and positively associated with eGFR ($p < 0.0001$). KL levels were also lower in individuals with hyperuricemia versus those without ($p < 0.0001$), individuals with a history of gout versus those without ($p < 0.0001$), and individuals with both hyperuricemia and a history of gout versus those with only a history of gout ($p = 0.0169$). Overall, there is a general trend of decreasing blood KL levels with age across the studies summarized in Table 5, excluding the substantially lower KL measurements provided by Socha-Banasiak et al. (2020), who used an ELISA kit for Klotho SEH757Hu from Cloud-Clone Corp.

4.2.6. Elderly population

The measurements of KL in blood samples from elderly populations specifically, obtained from ELISA, are summarized below in Appendix, Table 6. As expected, this population has a relatively lower overall KL level compared to the findings above (Shardell et al., 2019). It was found that among the elderly, higher plasma KL is associated with lower odds of developing frailty (Shardell et al., 2019), and that those in lowest tertile of plasma KL were at the greatest risk of death while those in the highest tertile had the lowest risk of death (Semba et al., 2011). Semba et al. (2011) also discovered a negative correlation between plasma KL and age among the elderly. Recently, King et al. (2022) compared serum KL in younger versus older men during exercise in temperate and hot environmental conditions. As expected, absolute KL levels were higher in young versus older men in temperature conditions ($p = 0.032$). However, in hot conditions, the difference was not significant ($p = 0.064$). Moreover, prolonged exercise in temperate conditions did not lead to a significant KL response in either young or older men, but prolonged exercise in hot conditions did.

4.2.7. Cancer

The measurements of KL in blood samples from individuals with cancer, obtained from ELISA, are summarized below in Appendix, Table 7. So far, the literature has been scarcer in this specific area of KL research. Pako et al. (2020) found no significant difference in KL levels between lung cancer patients and healthy controls. There were also no significant differences in KL levels between different stages and types of lung cancer. Interestingly, in the realm of clear cell renal cell carcinoma (ccRCC), Gigante et al. (2015) did find serum KL to be significantly lower in non-metastatic and metastatic ccRCC patients compared to healthy controls. KL levels were also negatively correlated with tumor size, tumor grade, and tumor stage, suggesting a prognostic role for KL in this specific type of cancer. This group also performed real-time quantitative reverse transcription PCR and immunohistochemistry analysis to assess KL gene and protein expression, respectively, but did not provide an age range for the patients for whom this was performed. There was significantly decreased KL gene and protein expression found in ccRCC tissue compared to adjacent normal renal parenchyma.

4.2.8. Neurodegenerative diseases

The measurements of KL in blood samples from individuals with neurodegenerative diseases, obtained from ELISA, are summarized below in Appendix, Table 8. The general trend seen is that blood KL levels are usually lower in patients compared to healthy controls, as seen in Parkinson's disease (PD) (Sancesario et al., 2021), and multiple system atrophy (MSA) (Y. Guo et al., 2017). Brombo et al. (2018) also found patients with vascular dementia (VD) to have lower plasma KL levels compared to late onset Alzheimer's disease (LOAD) and mild cognitive impairment (MCI) patients. Gaitán et al. (2021) found no significant differences in serum KL levels across KL-VS non-carriers, heterozygotes, and homozygotes, in a cohort of individuals at risk for developing AD. This in contrast to Dubal et al.'s findings in 2014 that KL-VZ heterozygous, overall healthy individuals had higher levels of serum KL compared to non-carriers (data not shown). Lastly, in a cohort of older individuals at risk for AD, Czaplicki et al. (2021) found significant interactions between serum KL and ventricle to brain volume ratio (VBR) in terms for global cognition and executive function, where higher serum KL attenuated the adverse effects of VBR.

4.2.9. Psychiatric conditions

The measurements of KL in blood samples from individuals with psychiatric conditions, obtained from ELISA, are summarized below in Appendix, Table 9. Generally, individuals with these conditions had lower blood KL compared to healthy individuals, seen in depression (Gao et al., 2021), chronic psychological stress (Prather et al., 2015), and schizophrenia (Turkmen at al, 2021). Brunoni et al. (2020) specifically studied KL level differences in unipolar depression and bipolar depression (BD), and found that KL levels were higher in BD patients compared to those with unipolar MDD. In contrast, Sartorious et al. (2019) found no significant differences in serum KL between patients with depression and heathy controls at baseline, nor did they find KL levels to significantly differ before and after therapy among patients. Hoyer et al. (2018) also found no significant difference in serum KL levels in geriatric patients with severe, therapy-resistant depression, before and after ECT.

4.2.10. Autoimmune diseases

The measurements of KL in blood samples from individuals with MS, obtained from ELISA, are summarized below in Appendix, Table 10. Of note, Ellidag et al. (2016) found serum KL to be higher in patients with relapsing-remitting multiple sclerosis (RRMS) compared to healthy controls. However, Abraham et al. (unpublished data) found no significant differences in KL levels between all types of multiple sclerosis (MS) studied, and between patients with MS and healthy controls. Recently, Ercan et al. (2022) found baseline serum KL levels in RA patients to be higher than in controls. KL levels significantly increased in both groups following exercise.

4.2.11. Immunoprecipitation-immunoblot assay

The measurements of KL in human blood samples obtained from IP-IB assay are summarized here. Notably, Dhayat et al. (2019) found serum KL to be higher in those aged 30 years and younger, compared to those aged 60 years and older, which is consistent with previous findings. Barker et al. (2015) also found KL to decrease as the stages of CKD progressed, which is, again, consistent with prior findings obtained with

ELISA.

4.2.12. Time-resolved fluorescence assay

The measurements of KL in human blood samples obtained from TRF are summarized here. This assay was developed specifically by Pedersen et al. (2013). Consistent with prior findings, they found a negative correlation between serum KL and age.

4.3. Cerebrospinal fluid

4.3.1. Abnormal metabolic states

The measurements of KL in CSF samples from individuals with metabolic dysfunction, obtained from ELISA, are summarized below in Appendix, Table 11. Landry et al. (2020) found KL levels to be greater in lean subjects compared to overweight and obese individuals.

4.3.2. Neurodegenerative diseases

The measurements of KL in CSF samples from individuals with neurodegenerative disease, obtained from ELISA, are summarized below in Appendix, Table 12. As observed before, KL levels decreased with age (Semba et al., 2014). KL levels were lower in patients compared to controls in AD (Semba et al., 2014). Gaitán et al. (2021) found KL levels to be significantly elevated in KL-VS heterozygotes and homozygotes compared to non-carriers. The results were more conflicting in the context of PD. While Sancesario et al. (2021) found KL levels to be higher in patients compared to controls, Zimmermann et al. (2021) found PD patients to have lower KL than controls.

4.3.3. Psychiatric conditions

The measurements of KL in CSF samples from individuals with psychiatric conditions, obtained from ELISA, are summarized below in Appendix, Table 13. Patients with severe depression undergoing electroconvulsive therapy (ECT) have been of particular interest. As can be seen, CSF KL levels were not significantly correlated with antidepressant therapy outcomes of ECT in a 12-person study (Kranaster et al., 2019, 2020). However, in a previous 8-person study that enrolled geriatric patients, there were significant changes in CSF KL levels before and after ECT (Hoyer et al., 2018). In particular, CSF levels rose following ECT ($p = 0.0020$). However, this rise in KL was not associated with an improvement of clinical symptoms.

4.3.4. Multiple sclerosis

The measurements of KL in CSF samples from individuals with MS, obtained from ELISA, are summarized below in Appendix, Table 14. Aleagha et al. (2015) found that KL was decreased in RRMS patients compared to controls, and that there was a negative correlation between severity score and KL level, a trend that was not seen in blood measurements.

4.4. Urine

4.4.1. Renal conditions

The measurements of KL in urine samples from individuals with renal conditions, obtained from ELISA, are summarized below in Appendix, Table 15. Gamrot et al. (2021) found a significant positive correlation between serum KL and urine KL, although KL measurements were not provided.

4.5. Immunoblot

The measurements of KL in urine samples from individuals with renal conditions, obtained from immunoblot, have been reported on. Hu et al. (2011) found that KL levels decreased with worsening severity of CKD, which is consistent with prior results.

4.6. Whole tissue samples used in mRNA expression analysis

4.6.1. Renal conditions

The relative concentrations of KL in tissue samples from individuals with renal conditions, obtained via mRNA expression analysis, have been reported by multiple research groups. Donate-Correa et al. (2021) found KL mRNA levels in peripheral blood cells (PBCs) to be correlated with estimated glomerular filtration rate (eGFR), which is a measure of kidney function. Deng et al. (2018) also found that KL levels were lower in kidney donors who were aged 50 years and greater, and that their KL levels were negatively correlated with the recipients' eGFR 1 month following transplantation. Koh et al. (2001) found that KL was reduced in the kidneys of individuals with chronic renal failure (CRF) compared to control kidneys. Lastly, Gigante et al. (2015) performed real-time quantitative reverse transcription PCR on ccRCC tissue and matched adjacent non-tumor tissue, finding that KL mRNA was significantly under-expressed in tumor tissue compared to non-tumor tissue.

4.6.2. Cardiovascular conditions

The relative concentrations of KL in tissue samples from individuals with cardiovascular conditions, obtained via mRNA expression analysis, have been studied as well. Navarro-González et al. (2014) obtained thoracic aorta specimens from patients undergoing elective coronary artery bypass graft surgery or valvular replacement surgery. They found patients with significant coronary artery disease (CAD) to have reduced KL mRNA expression compared to those with non-significant CAD, which is consistent with ELISA findings.

2. Whole Tissue Samples Used in Immunohistochemistry Analysis

The relative expression of KL in tissue samples from individuals with cancer, obtained via immunohistochemistry analysis, are summarized here. Notably, KL expression has been found to be reduced in ovarian cancer (Yan et al., 2017), breast cancer (Wolf et al., 2008), liver cancer (Huang et al., 2021), and ccRCC (Gigante et al., 2015). KL expression was also correlated with survival in ovarian cancer (Yan et al., 2017) and liver cancer (Huang et al., 2021), and decreased with increasing grade of RCC (Gigante et al., 2015).

4.6.3. Study methodologies

Table 16 in the Appendix organizes the studies included in this meta-analysis by KL measurement methodology. Note that some studies utilized multiple measurement protocols and are thus shown in more than one column.

5. Trends in Klotho

Among healthy subjects whose KL levels were measured via ELISA, there was a clear trend in mean KL by age group. Infants (0–1 years) had the highest mean KL levels (Ohata et al., 2011), children (2–16 years) had intermediate mean KL levels (Gamrot et al., 2021; Guarnotta et al., 2022; Yamazaki et al., 2010;), and adults (18 + years) had the lowest mean KL levels (Abraham et al., unpublished data; Espuch-Oliver et al., 2022; Gao et al., 2021; Iturriaga et al., 2021; Ohata et al., 2011; Prather et al., 2015; Sancesario et al., 2021; Sartorius et al., 2019; Sugiura et al., 2011; Yamazaki et al., 2010; Yokoyama et al., 2017). There were significant differences in mean KL levels between infants and children ($t = 4.82$, difference of means=1914.04, standard error=397.08, $p = 7.92e\text{-}5$), between children and adults ($t = 18.00$, difference of means=554.19, standard error=30.79, $p = 1.77e\text{-}37$), and between infants and adults ($t = 6.23$, difference of means=2468.23, standard error=396.05, $p = 2.83e\text{-}6$). The mean serum values reported by D. Huang and Wang (2021) and Y.-Y. Chen and Chen (2022) were not included in the analysis, as both studies provided a mean value for a large, aggregated sample that was not stratified by healthy versus non-healthy. The mean serum value reported by Semba et al. (2011) was

not included for similar reasons.

Among healthy subjects whose KL levels were measured via ELISA, there was also a clear pattern in median KL by age group. Infants (0–1 years) had the greatest median KL levels (Batlahally et al., 2020), children (2–18 years) had intermediate median KL levels (Gkentzi et al., 2014), and adults (19 + years) had the lowest median KL levels (Brombo et al., 2018; Kresovich and Bulka, 2021; Pako et al., 2020; Pedersen et al., 2013; Shardell et al., 2019). It is important to note that Batlahally et al. (2020) studied preterm infants specifically; thus, their control group consisted of preterm infants without two conditions of interest, BPD and PH. This study assumes that preterm infants in the control group were otherwise healthy. Furthermore, the control median KL value reported by Brombo et al. (2018) was obtained from individuals who were cognitively intact, but complained of memory loss. This study assumes that these individuals were otherwise healthy. The median KL value reported by Drew et al. (2021) for a cohort of well-functioning older adults was not included in the analysis, as we were not able to obtain the primary data to ensure the separation of healthy well-functioning older adults from well-functioning older adults with significant pre-existing health conditions. Similarly, the median KL value reported by Navarro-González et al. (2014) for their control group was not included in this analysis, as these controls had non-significant CAD but still may have had other comorbidities, such as diabetes mellitus, hypertension, and dyslipidemia. Based on Mood's median test, there was a significant difference in median KL concentrations between infants and children (χ^2=161.54, df=1, p < 2.2e-16), children and adults (χ^2= 11,774, df=1, p < 2.2e-16), and infants and adults (χ^2= 10937, df=1, p < 2.2e-16).

Even among individuals with renal conditions, there is a clear decrease in median KL levels in older groups compared to younger groups. Among individuals with kidney conditions, those who are the youngest (child group, 5–20 years) tend to have highest median KL (Fischer et al., 2021). Those of intermediate age (adult group, 21–49 years) had intermediate median KL levels (Deng et al., 2018). Those who are the oldest (elderly group, 50 + years) tend to have the lowest median KL (Deng et al., 2018; Donate-Correa et al., 2021; Otani-Takei et al., 2015). Upon conducting Mood's median test, there is indeed a statistically significant difference in median KL levels between children and adults (χ^2=16.92, df=1, p = 3.89e-05), children and elderly (χ^2=281.01, df=1, p < 2.2e-16), and adults and elderly (χ^2=107.76, df=1, p < 2.2e-16). The median KL values reported by Uriol-Rivera et al. (2021) were not included in this analysis, as baseline KL levels were not reported in their study of chronic HD patients on erythropoietin stimulation.

Among individuals with metabolic or endocrine conditions, there is also an inverse relationship seen between KL levels and age. Among these individuals, infants (Wojcicki et al., 2018) had higher KL levels than children (Guarnotta et al., 2022), and children had higher KL levels than adults and elderly individuals (H.-J. Lee et al., 2022). This difference was statistically significant in an independent samples t-test between infants and children (t = 9.25, difference of means=1728.40, standard error=186.77, p = 5.03–15). Children versus adults/elderly individuals, and infants versus adults/elderly individuals, could not be compared in the statistical analysis because the 95% CI was provided for the adult/elderly sample's KL measurements instead of the standard deviation (SD).

Please see Fig. 5 for an overall summary of KL trends in health and disease.

6. Discussion

6.1. Klotho levels collected

6.1.1. Healthy individuals

As expected, KL levels in blood declined with age in overall healthy subjects, as illustrated by Figs. 2 and 3. These figures show an age-dependent decrease in KL, which starts with the highest level in infancy and reaches lowest levels in adulthood/elderhood. It is important to note that the age categories defined in this study are quite broad. Because of the diverse populations studied and categorizations utilized by the wide range of studies included in this analysis, we determined the most accurate ordering of age to be infant, child, and adult. As such, KL levels in younger adults and older adults cannot be separated. This meta-analysis still offers valuable insight into differences in KL levels at

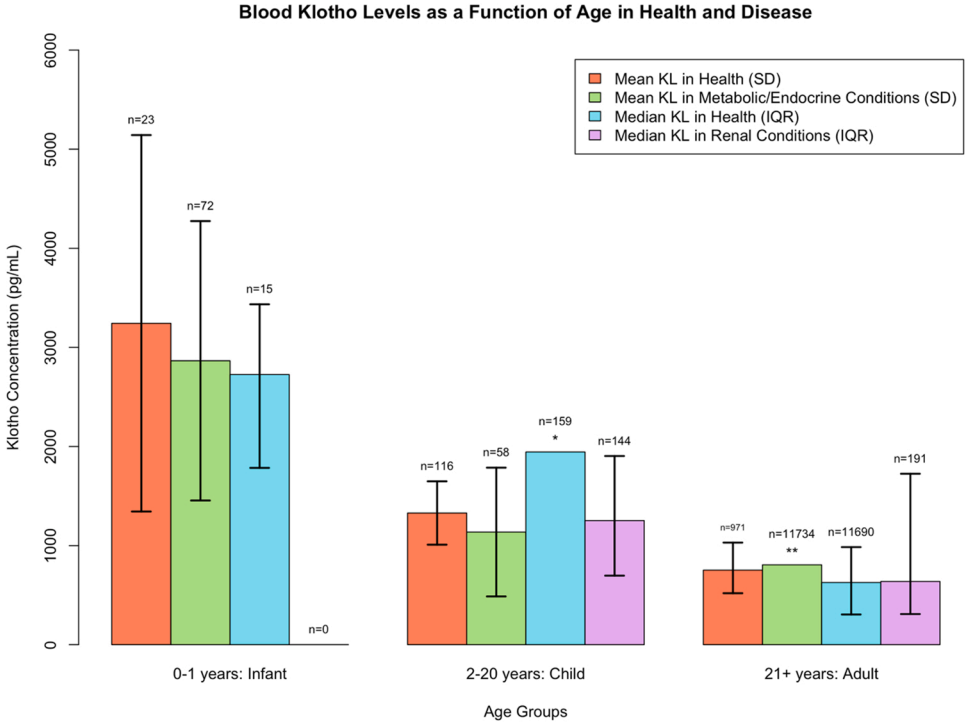


Fig. 5. KL measurements in blood samples from healthy individuals, individuals with metabolic/endocrine conditions, and renal conditions, ranging from infancy to adulthood, obtained from ELISA. Orange, mean KL obtained from healthy subjects with SD; green, mean KL obtained from subjects with metabolic/endocrine conditions with SD; blue, median KL obtained from healthy subjects with IQR; purple, median KL obtained from subjects with renal conditions with IQR. Age ranges are approximate. There is a clear decrease in mean KL expression with age demonstrated in health and disease. * denotes a study for which range was provided instead of IQR; * * denotes a study for which 95% CI was provided instead of IQR.

distinct age stages. The decline in KL with age matches the expected trend in KL as one grows older, as serum KL has been shown to decrease with age after 40 years (Xu and Sun, 2015).

6.2. Renal conditions

KL research in the realm of kidney disease has been extremely robust. As the kidneys are one of the main sources of KL in the body, it follows that kidney disease would impede KL production. This is seen in multiple studies which show that blood KL levels are inversely correlated with severity of kidney disease (Otani-Takei et al., 2015; Donate-Correa et al., 2021; Gamrot et al., 2021; Guo et al., 2021; Milovanova et al., 2021).

Among individuals with health conditions involving their kidneys, there is a clear trend in KL with age, as shown in Fig. 4. KL reaches its highest levels in childhood (defined as 5–20 years) and decreases further into adulthood (defined as 21–49 years) and even more in elderhood (defined as 50 + years). The range of median KL levels among individuals with renal conditions is also smaller than that among healthy individuals. Because KL found in blood is primarily produced in the kidneys, it is reasonable that those with kidney conditions would have a smaller range of KL levels compared to those who are healthy.

6.3. Metabolic/endocrine conditions

As KL clearly plays a role in the metabolic state through its various signaling pathways, research in this area has been of interest. Wojcicki et al. (2018) found cord blood serum KL levels to be inversely correlated with leptin ($p < 0.01$) and insulin ($p < 0.03$) among a group of Latino infants at risk for obesity. Socha-Banasiak et al. (2020) found serum KL levels to be significantly higher in obese children compared to normal-weight and overweight children, and to be higher in insulin-resistant children compared to insulin sensitive children. However, Landry et al. (2020) found CSF KL levels to decrease with increased body mass index and body weight.

Across the lifespan, mean KL levels in blood decrease with age, as can be seen in Fig. 5. KL levels were higher among infants at risk for obesity (Wojcicki et al., 2018) than children who had GHD (Guarnotta et al., 2022). Both these groups of infants and children had higher KL levels than adult participants of the NHANES survey from 2007 to 2016 (Lee et al., 2022).

In the context of T1D, Zubkiewicz-Kucharska et al. (2021) found serum KL to be significantly lower in children with T1D than in controls ($p = 0.0113$), and to be negatively associated with HbA1c ($p = 0.0066$). Interestingly, among T2D patients, Ciardullo and Perseghin (2022) found patients with reduced serum KL to be significantly older ($p < 0.001$), more likely to be male ($p = 0.046$), and more likely to have lower HbA1c ($p < 0.001$) and lower eGFR ($p < 0.001$). As serum KL has been correlated with HbA1c in opposite directions in T1D and T2D, this raises the question of potential differences in KL amount and activity between T1D and T2D patients. We speculate that these differences arise, at least in part, from medications taken by T2D patients, which have KL-raising effects. These include metformin, shown to raise serum KL levels among women with polycystic ovary syndrome (Saifi Novashnag et al., 2016); pioglitazone, shown to increase KL gene expression in unstimulated control Madin-Darby canine kidney cells, and reverse KL gene suppression caused by angiotensin II (Maquigussa et al., 2018); troglitazone, shown to enhance renal KL mRNA expression in Otsuka-Long-Evans Tokushima Fatty rats, a model of atherogenic disease (Yamagishi et al., 2001).

In the realm of hyperuricemia and gout, Lee et al. (2022) recently studied the link between serum KL levels and serum uric acid levels. Among a large cohort of adults in the United States, KL and uric acid were negatively correlated ($p < 0.0001$). KL levels were also lower in individuals who had hyperuricemia compared to those without ($p < 0.0001$), and in individuals who had a history of gout compared to those without ($p < 0.0001$). The results suggest that reduction of KL could be potentially involved in the progression of hyperuricemia and/or gout, although causality cannot be directly established.

Very recently, Guarnotta et al. (2022) found serum KL to be significantly lower in GHD children compared to control children at baseline ($p = 0.001$). Over 12 months of follow up of control children and treatment of GHD children with rhGH, both controls ($p = 0.030$) and GHD children ($p < 0.001$) had significant increases in KL. After 12 months, GHD children who underwent rhGH therapy had significantly higher KL levels than controls ($p < 0.001$). Of note, IGF-1 was found to be positively associated with KL levels in multivariable analysis, which was expected given IGF-1 has been shown to stimulate KL secretion (Caicedo et al., 2018; Rubinek et al., 2016). Interestingly, insulin has also been shown to increase KL release from the membrane (Chen et al., 2007).

6.4. Aging population

Among the ever-growing aging population, higher plasma KL levels have been associated with positive outcomes, such as lower odds of frailty (Shardell et al., 2019). On the other hand, lower plasma KL levels have been linked to more negative outcomes, such as greater mortality rate (Semba et al., 2011). The negative correlation between plasma KL and age has also been confirmed (Semba et al., 2011). Thus, it would be reasonable to infer that with age, KL decreases, and this consequently contributes to the various pathologies seen with aging. Interestingly, the relationship between age and serum KL has been explored in the context of exercise. King et al. (2022) found absolute serum KL levels in young men to be higher than in older men during exercise in room temperature conditions, but not in hot conditions.

6.5. Cardiovascular and pulmonary conditions

Generally, blood KL levels have been inversely correlated with cardiovascular disease risk and severity (Navarro-González et al., 2014; L. Xu et al., 2017; Shakked et al., 2021; Yeganeh-Hajahmadi et al., 2021). This makes sense in the context of renal disease as well, because kidney disease is usually associated with cardiovascular effects. Batlahally et al. (2020) also found blood KL levels to be decreased in preterm infants with BPD and BPD or PH, compared to preterm infants without these diseases.

6.6. Cancer

There have been less KL measurements made with ELISA in the realm of cancer, and more research done with immunohistochemical staining. Pako et al. (2020) found no significant differences in serum KL levels between lung cancer patients and healthy controls. However, in the context of kidney cancer, Gigante et al. (2015) did find serum KL levels to be statistically significantly lower in ccRCC patients compared to healthy controls. Gene and protein expression of KL were also significantly lower in tumor tissue compared to adjacent normal renal parenchyma, based on real-time quantitative reverse transcription PCR and immunohistochemistry analysis, respectively. Yan et al. (2017), Wolf et al. (2008), and Huang et al. (2021) found staining for KL to be reduced in ovarian cancer, breast cancer, and liver cancer, respectively. Therefore, although KL may not be a reliable biomarker or diagnostic tool for all types of cancer utilizing ELISA, the utilization of immunohistochemistry analysis may prove very useful in the clinical management of cancer.

Of note, KL measurements in pediatric cancer patients could not be found in the literature search. This could be due to how cancer is largely a disease that presents later in adulthood, while childhood cancers are relatively rare.

6.7. Neurodegenerative diseases

6.7.1. Alzheimer's disease

Interestingly, KL levels have also been measured to be statistically significantly lower in the CSF of older AD patients compared to age-matched controls ($p = 0.02$; Semba et al., 2014). On the other hand, blood plasma KL measurements in AD patients have been more elusive. Although Brombo et al. (2018) found a statistically significant and strong relationship between lower KL levels and development of vascular dementia (VD), there was no significant association identified with late-onset AD (LOAD).

6.7.2. Parkinson's disease

In PD, there have been some conflicting results in KL measurements in blood versus CSF. Sancesario et al. (2021) found serum KL levels to be lower in PD patients compared to controls, but found CSF KL levels to be higher in PD patients compared to controls. Even more, Zimmermann et al. (2021) found PD patients to have lower CSF levels compared to controls. As KL is produced both by the kidneys and in the brain by the choroid plexus and neurons, its levels may differ depending on the region of the body affected by the disease. It will be interesting to see more research on how KL levels differ between blood and CSF specifically in neurological diseases, as the compromised blood-brain-barrier may affect the correlation of blood and CSF KL levels.

6.7.3. Amyotrophic lateral sclerosis

Although it would be extremely interesting to see KL measurements in the context of ALS, a neurodegenerative disease, there have been no human studies done in this area to the author's knowledge.

6.8. Autoimmune diseases

As KL measurements employing the ELISA assay are generally more reliable than those based on other assays, the findings of Abraham et al. (unpublished data) suggest that KL would not be an ideal biomarker for MS, at least in the blood, as they do not differ significantly across controls and several types of MS studied (PPMS, RRMS, and SPMS). However, Aleagha et al. (2015) measured KL in CSF using the IBL assay, and found that KL levels were significantly reduced in RRMS patients compared to controls ($p < 0.0001$). Higher severity scores for RRMS patients were also associated with lower CSF KL ($p = 0.0058$). These results indicate that KL in CSF can potentially be used as a biomarker for MS and MS severity, which makes sense given KL's role in myelination (Chen et al., 2013) and remyelination (Zeldich et al., 2015) and the impairment of remyelination in MS.

Very recently, Ercan et al. (2022) reported on the effect of exercise on serum KL levels in RA patients. Serum KL was increased in RA patients compared to controls at baseline, and an acute bout of aerobic exercise induced an increase in KL levels in both groups. The levels of other anti-inflammatory cytokines were also increased in both groups, suggesting a protective role of exercise in not only individuals with disease, but those who are healthy as well. Additionally, one can speculate that KL levels are higher in RA patients compared to controls at baseline, possibly because as a compensation effect in these patients who are in a constant, chronic state of inflammation.

7. Effects of KL-VS and other heterozygosities

Of note, Dubal et al. (2014) linked a life-extending variant of the human KL gene, designated KL-VS, to improvements in cognition in heterozygous carriers. In this variant, two amino acids are substituted in exon 2: F352V (rs9536314) and C370S (rs9527025) (Arking et al., 2002). Dubal et al. (2014) showed that the KL-VS variant is tied to higher KL levels in serum and improved cognition among aging individuals who are heterozygotes for the variant: non-carriers ($n = 118$) had a mean serum KL of approximately 800 pg/mL and heterozygotes ($n = 38$) had a mean serum KL of approximately 900 pg/mL. Heterozygous KL overexpressing transgenic mice that had greater KL expression also exhibited better cognitive performance than control mice at all ages studied. It remains unknown whether the beneficial effects of KL-VS heterozygosity are due to the slightly elevated levels of the KL protein or due to the two amino acid substitutions which could affect its conformation and function.

7.1. Neurodegenerative diseases

Interestingly, the KL-VS haplotype has been shown to have a variable protective effect, seemingly mitigating disease symptoms in certain scenarios but not others. In a study of PD patients, Zimmermann et al. (2021) found that compared to individuals with the wild-type haplotype, KL-VS haplotype heterozygotes developed cognitive impairment earlier ($p = 0.019$ for females, $p = 0.046$ in males), and had higher severity scores at their last visit ($p = 0.006$). However, KL levels in CSF were significantly higher in individuals heterozygous for the KL-VS haplotype, compared to individuals with the wild-type haplotype ($p = 0.009$). On the other hand, there has been support for the protective effect of KL-VS heterozygosity in AD, specifically in AD patients who carry the apolipoprotein e4 (APOE4) allele, considered the strongest genetic risk factor for AD (Fleisher et al., 2013; Morris et al., 2010). Belloy et al. (2020) found that being heterozygous for the KL-VS haplotype was associated with a 25% decreased risk of AD in all APOE4 carriers aged 60 years or older ($p = 7.4e10–7$), and a 31% decreased risk of AD in APOE4 carriers specifically aged between 60 and 80 years ($p = 3.6e10–8$). One can speculate that, based on the findings of Zimmermann et al. (2021) and Belloy et al. (2020), dopaminergic neurons in PD may not be so dependent on KL, even at high concentrations in CSF. On the other hand, cholinergic neurons in AD may be more dependent on KL levels in order to function properly. This point is well-illustrated in recent findings reported by Gaitán et al. (2021), who found that CSF KL levels were elevated in KL-VS heterozygous and homozygous carriers compared to non-carriers.

7.2. Psychiatric diseases and conditions

KL polymorphisms have also been studied in the realm of psychiatry. Gao et al. (2021) primarily studied the rs9315202 KL genetic polymorphism in a cohort of individuals with MDD. Of note, plasma KL levels were higher in carriers of rs9315202 T alleles regardless of age and sex, and the rs9315202 T allele was negatively correlated with disease severity in elderly patients.

E. J. Wolf et al. (2019) examined multiple KL variants in association with post-traumatic stress disorder (PTSD) severity among military veterans. The rs9315202 and rs9563121 variants were found to interact with PTSD severity and sleep disturbance to predict advanced epigenetic age, as measured by DNA methylation. Another variant, rs398655, interacted with self-reported pain in relation to slowed epigenetic age. The rs9527025 (C370S) variant was also associated with slowed epigenetic age. The rs9315202 variant also interacted with PTSD to predict fractional anisotropy values in the right cingulum bundle/cingulate gyrus and in the right fornix/stria terminalis. These effects were more pronounced in older subjects in the study sample.

E. J. Wolf et al. (2020) on KL polymorphisms and their association with PTSD and KL DNA methylation in the same sample of military veterans used in their 2019 study. The rs9527025 variant interacted with PTSD severity such that the minor allele was associated with reduced methylation of a Cytosine-phosphate-Guanine site, cg00129557, among individuals with less PTSD symptoms. In contrast, there was greater methylation of cg00129557 among those with greater PTSD symptoms, and an association with higher levels of inflammation as indicated by C-reactive protein levels. These results support an interaction between the genetic KL variant rs9527025 variant and PTSD severity, via KL DNA methylation and mediation of inflammatory

pathways.

More recently, Wolf et al. (2021) studied the rs9315202 polymorphism in the context of PTSD, depression, and alcohol use disorders. Remarkably, PTSD was found to be associated with advanced epigenetic age in the motor cortex, as a function of the number of rs9315202 minor frequency allele A. This was found only in older individuals studied in the sample. Specifically, the rs9315202 polymorphism interacted with PTSD to predict decreased KL expression via DNA methylation: PTSD was associated with advanced DNA methylation age in those with the minor allele A.

Lastly, Paroni et al. (2017) have studied several KL variants in relation to the treatment of late-life MDD with selective serotonin reuptake inhibitors (SSRIs). Interestingly, the distribution of the rs9536314 (F352V) variant was significantly different across all three therapeutic response groups: responders, poor responders, and non-responders. A significantly higher frequency of the minor genotype of rs9536314 was found in the non-responding group. In addition, rs1207568 was shown to follow a dominant and additive model of inheritance, while rs9536314 followed a recessive model of inheritance. It was reported that those who carried at least one minor allele at rs1207568 had a significantly improved response to SSRI treatment compared to those who carried both major alleles. Further, those with rs9536314 homozygosity with two minor alleles had significantly worse responses to SSRI treatment, compared to those with at least one major allele.

Taken together, these results show the value of studying not only KL levels, but also KL polymorphisms and their downstream effects. Performing genetic testing to determine the presence of these polymorphisms may add to KL's diagnostic and therapeutic potential, especially in the treatment of psychiatric disorders within older populations.

7.3. Cerebrovascular disease

Very recently, Li et al. (2022) reported on the association between three nucleotide KL polymorphisms with cerebral infarction (CI). KL levels in peripheral blood were statistically significantly lower among CI patients than in controls ($p < 0.05$), and the rs192031 polymorphism was significantly correlated with the occurrence of CI ($p < 0.05$). There were also clinical differences between CI patients and controls: CI patients had significantly lower high density lipoprotein cholesterol ($p = 0.003$), higher blood glucose ($p = 0.001$), higher systolic blood pressure ($p < 0.0001$), and higher diastolic blood pressure ($p = 0.018$) than controls. More specifically, on allele analysis, CI patients with the TT genotype of the rs192031 polymorphism had significantly higher high density lipoprotein cholesterol ($p = 0.019$) and systolic blood pressure ($p = 0.001$) than controls.

7.4. Cancer

Intriguingly, KL-VS heterozygosity has also been tied to various types of cancer. Wolf et al. (2010) found that in a cohort of Ashkenazi Jewish women, carriers of the BRCA1 (185delAG, 5282insC) mutation who also had heterozygosity for the KL-VS haplotype had a statistically significantly increased risk of developing both breast and ovarian cancer, as well as risk of being diagnosed with breast cancer at a younger age. Therefore, one can speculate that the combination of KL-VS heterozygosity with some mutations can lead to certain vulnerabilities in health, at least in the context of breast cancer. It may also be possible that the BRCA1 mutation has such a potent effect that the protective effects of KL-VS heterozygosity are diminished or even reversed. Furthermore, it had been previously found that KL expression in vitro decreases the number and size of surviving pancreatic adenocarcinoma (Abramovitz et al., 2011). One can also speculate that in breast cancer, KL-VS heterozygosity may not be ideal for augmentation of apoptotic pathways.

In addition, KL polymorphisms have been studied in prostate cancer. Kim et al. (2014) found that the rs3752472 polymorphism was significantly associated with prostate cancer risk in a cohort of Korean men. rs211247 was also significantly associated with high levels of prostate-specific antigen (PSA), a well-used biomarker for prostate cancer, in the co-dominant and dominant models; rs1207362 was associated with high PSA levels in the recessive model. Conversely, rs385564 was significantly associated with low PSA levels in the co-dominant and recessive models; rs563925 was associated with low PSA levels in the recessive model.

Just as the effects of KL genetic polymorphisms differ among neurodegenerative diseases, such as PD and AD as noted above, they may vary among different types of cancer. More studies will need to be performed to validate any speculations.

7.5. Effects of sex on Klotho levels

In addition to age-associated changes in KL levels, differences in KL concentration and activity by sex, another non-modifiable risk factor, have also been of interest. Interestingly, Öz et al. (2007) found KL protein levels to be elevated in mice deficient in aromatase, the enzyme that converts androgens into biologically active estrogens. Increased KL protein was seen in the distal convoluted tubule in immunohistochemistry analysis, real-time PCR, and Western blot analysis. Following estradiol treatment of these mice, KL expression decreased in the kidney at the mRNA and protein levels. These findings suggest that KL differences by sex could be due, at least in part, to differences in steroidal hormonal levels.

Additionally, Zeldich et al. (2019) found that in the SOD1 mouse model of ALS, overexpression of KL led to effects that were differential by sex. Delayed onset and progression of disease, as well as prolonged survival, was more significant in female mice than male mice. There was also more of a delay in initial development of symptoms in females than males, but this difference was not statistically significant.

7.6. Implications of β-Klotho levels

This study reports primarily on levels of α-KL measured in humans, as they have been more commonly encountered in the literature, likely in part because α-KL was discovered before β-KL. Nonetheless, β-KL levels have also been studied (Wu et al., 2021). In a single-center study of individuals with stable coronary artery disease (SCAD), serum levels of β-KL were determined from SCAD patients with depressive symptoms (DS) and those without. Notably, those with DS had a significantly greater degree of coronary arteriostenosis ($p < 0.05$), and serum β-KL was significantly lower in SCAD patients with DS compared to those without ($p = 0.041$). These results yield insight into the potential for β-KL to have diagnostic and therapeutic value in the realm of cardiovascular disease and psychiatric symptoms. It would be interesting to see measurements of α-KL in this context as well.

7.7. Therapeutic potential of Klotho

It should now be abundantly clear that KL has the potential to transform the therapeutic management of a diverse variety of diseases and conditions. At the present time, research in KL is a robust field and fertile area of study for the development of KL-based pharmaceuticals. KLOTHO Therapeutics, Inc., had plans for developing recombinant KL, primarily for the treatment of CKD (KLOTHO Therapeutics, www.klotho.com) but it is unclear if it is still pursuing that strategy. Klogenix, LLC (Klogenix, www.klogenix.com) is pursuing two strategies: (i) upregulation of endogenous KL, and (ii) delivery of exogenous KL genetic material. Small KL boosting molecules, as well as advanced gene therapy modalities are also being developed by Klogenix. Unity Biotechnology (unitybiotechnology.com) is pursuing systemic delivery of recombinant KL for cognition enhancement.

7.8. Study strengths

One of the strengths of this study was the compilation of results reported globally. As there were no criteria to exclude studies done in certain geographical regions, the results are more likely to be generalizable to the broader population. This is important to consider, as there may be cultural and regional factors that contribute to KL differences across populations, such as through diet, physical activity, lifestyle choices, and exposure to environmental pollution.

Another strength of this study was the assessment of the relationship between KL levels and a variety of diseases, using multiple sample sources: blood, CSF, and urine. As KL can have diverse effects on the body through various signaling pathways, it is important to investigate these effects comprehensively.

7.9. Study limitations

There were aspects of this study that were limited by the nature of the study design. Because this study comprises a meta-analysis of published work, we rely heavily on work and data that are provided by other researchers. Not all data that were provided were comprehensive in all cases, and it was occasionally necessary to contact corresponding authors for further clarification or for more data. Because our study did not impose a lower time limit on published works to be included in this secondary data analysis, some data could not be obtained by virtue of the duration of time that has passed since study inception and publication. Additionally, there were also several instances in which studies could not be included because they were published internationally, thereby imposing accessibility and language barriers.

In addition, the lack of uniformity in KL measurement across studies was also a limitation for our review. As mentioned above, a standardized method to measure KL is needed so that consistent, readily comparable KL measurements can be made. Although the IBL assay has generally been used more frequently, at least in our search of the literature, there remains a great deal of studies that use disparate assays.

Furthermore, a disproportionate amount of data on KL levels was collected from blood samples from older populations, likely because blood samples are convenient to obtain, older individuals may be more amenable to giving blood samples, and more research has been done in the adult and elderly populations than in the infant and child populations. As KL is predominantly produced in the distal convoluted tubules of the kidney and choroid plexus of the brain (Kuro-o et al., 1997), it would also make sense that there has been greater scientific focus on KL in the context of renal and neurodegenerative conditions, compared to other types of diseases. These limitations and the ones listed above restricted the statistical analysis of this study to mean and median KL blood levels in healthy individuals, and mean KL blood levels in individuals with renal conditions.

8. Conclusions

It is important to note that KL present in blood is mostly produced by the kidney, while KL in CSF is largely synthesized by the choroid plexus (Kuro-o et al., 1997). Whether there is a correlation between KL levels in blood and in CSF in a single individual still remains to be discovered. A future database that collects KL measurements may be able to help identify such a relationship. However, the current data on KL levels may be disproportionately determined from blood measurements because a blood draw is considerably more convenient and feasible to do as opposed to a lumbar puncture to collect CSF. It will be interesting to see the relationship between KL in the CSF and KL in blood as more research is done.

Only recently, Kundu et al. (2022) reported CSF and serum KL levels to be highly correlated in a cohort of 94 individuals with AD ($r(92) = 0.572$, $p < 0.001$). This relationship remained statistically significant upon stratification by sex. Interestingly, both serum and CSF KL were strongly positively correlated with Mini-Mental State Examination scores ($r(91) = 0.348$, $p = 0.001$, and $r(91) = 0.457$, $p < 0.001$, respectively), indicating an association between serum and CSF KL and cognitive status. Conversely, both serum and CSF KL were strongly negatively correlated with clinical dementia ratings ($r(91) = -0.441$, $p < 0.001$, and $r(91) = -0.600$, $p < 0.001$, respectively). However, there was not a statistically significant relationship between KL and age, in either serum or CSF, as would be expected from the trends elucidated in the present meta-analysis. The authors did suggest that this could have arisen from the distribution of ages in their sample. As the mean age of the sample was 66 years, an age-related reduction in KL levels may have been seen if the sample had a wider age distribution, as the authors explain. Further, the sample only included individuals with AD, in contrast to data from Yamasaki et al. (2010), who reported from a healthy sample. It will certainly also be interesting to evaluate for the presence of serum and CSF KL level correlation in healthy individuals.

As we know more about KL, the possibility for a KL-boosting therapeutic becomes more real. Having baseline values of KL in healthy and diseased individuals across the lifespan will be essential for developing such treatments. This review adds to the current body of knowledge on KL by providing a comprehensive summary of typical KL values found in multiple populations organized by age and disease type, using a variety of specimen types: blood, urine, CSF, and whole tissue.

Declaration of interest

Dr. Carmela Abraham is the co-founder of Klogenix, a company that is seeking to develop Klotho-boosting therapeutics.

Data Availability

This is a meta analysis of 65 published articles. No new data added.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.arr.2022.101766.

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