Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
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Klotho and GPLD-1: Predictive Biomarkers for Development of Alzheimer's Disease in Older Adults

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Supervisor: Ulrik Wisløff
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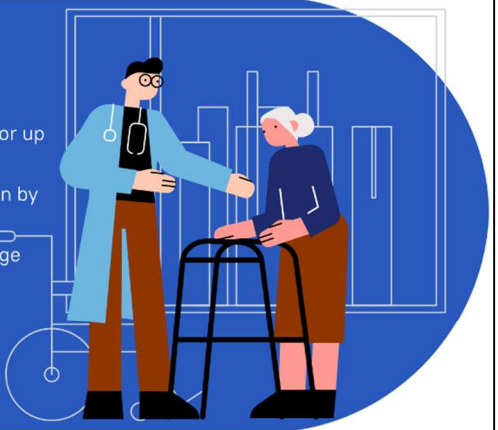
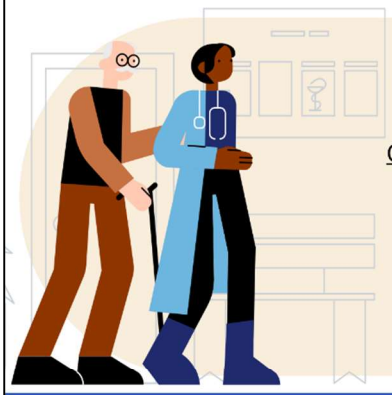
Background

- Alzheimer's Disease (AD) is the most common form of Dementia, accounting for up to 80% of cases
- The worldwide prevalence of dementia is predicted to increase up to 152 million by 2050, becoming one of the greatest health challenges of our generation.
- Physical inactivity, heredity and genes (especially APOE4 genotype) have a huge impact on the risk of developing AD.
- AD pathology has previously been linked to the proteins Klotho and Glycosylphosphatidylinositol-specific phospholipase D-1 (GPLD-1), which has associations with a range of physiological processes, encompassing neuroprotection, cognition, and aging.



Aim:

- Investigate Klotho and GPLD-1 's potential as predictive biomarkers for AD in serum
- Measure their possible correlations with cardiorespiratory fitness (measured as peak oxygen uptake, VO2-peak)



Methods:

Case-control study: (n = 81)

1. **AD Group**: (n = 27)
   Mean age: 72.4 ± 2.2
   Women 14 (45%)
   Mean VO2-peak: 29.9 ± 6.2
2. **Healthy Controls**: (n = 54)
   Mean age: 72.8 ± 2.2
   Women: 27 (50%)
   Mean VO2-peak: 35.1 ± 8.6

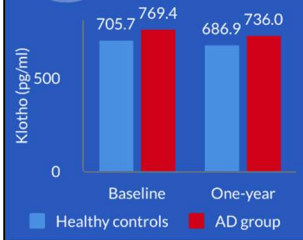
Participant inclusion

- All participants were healthy at baseline testing and the one-year-follow-up
- AD group developed AD in the follow-up period from 2013 to 2022.
- AD diagnosis was confimed by physician

Analysis:

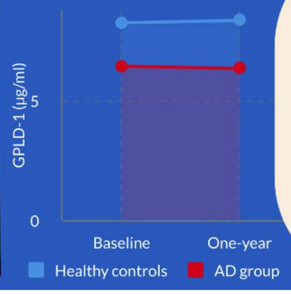
- Serum taken at baseline and one-year-follow-up was analyzed for Klotho and GPLD-1
- Protein levels were analyzed using the ELISA sandwich method

Results



Klotho

No significant difference between the AD group and healthy controls at baseline or after one year ($p > 0.05$).
Healthy controls had 2.7% decrease from baseline to one-year-follow-up, and an association with VO2-peak was found at both baseline (Sig. = 0.036) and after one year (Sig. = 0.042).
The AD group had neither the decrease from baseline to one-year, or the association with VO2-peak at either timepoint.



GPLD-1

The AD group had 22% lower, at BL ($p=0.009$), and 24% lower, at 1yr ($p=0.001$), levels of GPLD-1 when compared to that of the healthy controls.
Associations with GPLD-1 and AD at both BL (Sig.<0.001, R=-0.292) and 1yr (Sig.<0.001, R=-0.360) was found, even when adjusted for VO2-peak ($p=0.001$)
An inverse correlation between VO2-peak and GPLD-1 was also present (Sig.=0.035, R=-0.238)

Summary:

By observing and investigating the proteins' modified levels in pre-clinical AD patients, it's clear that Klotho and GPLD-1 has potential as biomarkers for pre-clinical AD. The correlation loop created between GPLD-1, AD diagnosis, and VO2-peak needs further elucidation. Further research into reference values for these proteins, and the exact biomolecular mechanisms of their effects on AD pathogenesis may clarify many unanswered questions about disease progression of AD, and may provide an avenue for early detection.

Abstract

Background: Alzheimer's disease (AD) is a neurodegenerative disease, and the most common form of dementia. In 2023, dementia affects more than 55 million people worldwide and is predicted to increase to 152 million by 2050. Yet, AD remains one of the most difficult disorders to diagnose. The disease progresses in the brain, prior to symptom debut, and the most used diagnostic tool is an invasive spinal tap to measure proteins in the cerebral spinal fluid. AD pathology has previously been linked to the proteins Klotho and Glycosylphosphatidylinositol-specific phospholipase D-1 (GPLD-1), which has associations with a range of physiological processes, encompassing neuroprotection, cognition, and aging. However, research regarding their potential as predictive biomarkers for AD has yet to happen. Thus, in this thesis, I intended to investigate the potential of the two proteins, Klotho and GPLD-1, as predictive biomarkers for AD in serum. I also viewed correlations with Cardiorespiratory fitness, measured as peak oxygen uptake (VO2-peak). I hypothesised that the AD group, when compared to the healthy controls, will have modified regulations for both Klotho and GPLD-1, in the form of lower mean values and disrupted difference from baseline (BL) to one year later (1yr).

Methods: Blood samples were collected from older adults that were healthy at baseline (BL) and after one year (1yr) of participating in the Generation 100 Study, and that in the follow-up period up to 2022, developed AD (n=27, age: 72.4±2.2 years, women/men: 14/13). These were matched with healthy controls in a case-control study (n=54, age: 72.8±2.2, women/men: 27/27). The Generation 100 study is a large-scale randomized controlled trial (n=1567), which investigated the effects of long-term exercise on overall health and longevity in a population of older adults aged 70 to 77. Using the ELISA sandwich method, the proteins were analysed in the serum of the participants.

Results: The results revealed a modified regulation of Klotho and GPLD-1 in those that developed AD during follow-up. The AD group had 22% lower, at BL ($p=0.009$), and 24% lower, at 1yr ($p=0.001$), levels of GPLD-1 when compared to that of the healthy controls. There was no significant difference between the BL values and the 1yr values for GPLD-1 for either group ($p>0.05$). For Klotho, no significant difference between the AD group and the healthy controls, at BL, was observed ($p>0.05$). However, the expected 2.7% decrease in Klotho levels from BL to 1yr shown in the healthy controls ($p=0.042$), was not present for the AD group. Correlation analysis revealed associations with GPLD-1 and AD at both BL (Sig.<0.001, R=-0.292) and 1yr (Sig.<0.001, R=-0.360), even when adjusted for VO2-peak ($p=0.001$); showing that a decrease in GPLD-1 values increase odds of developing AD later in life. Interestingly, an unexpected inverse correlation between VO2-peak and GPLD-1 was present (Sig.=0.035, R=-0.238). The healthy controls had an association with VO2-peak and Klotho at both BL (Sig.=0.036) and 1yr (Sig.=0.042). Klotho levels for the AD group, had no significant associations with VO2-peak at either BL or 1yr (Sig.>0.05).

Conclusions: This study demonstrated Klotho's and GPLD-1's potential as biomarkers for pre-clinical AD, by observing and investigating the proteins' modified levels in pre-clinical AD patients. Further research into reference values for these proteins, and the exact biomolecular mechanisms of their effects on AD pathogenesis may clarify many unanswered questions about disease progression of AD, and may provide an avenue for early detection.

Sammendrag

Bakgrunn: Alzheimers sykdom (AD) er en nevrodegenerativ sykdom og den vanligste formen for demens. I 2023 påvirker demens over 55 millioner mennesker globalt, og det er forventet å øke til 152 millioner innen 2050. Likevel forblir AD en av de vanskeligste lidelsene å diagnostisere. Sykdommen utvikler seg i hjernen før symptomene vises, og det mest brukte diagnostiske verktøyet er en invasiv ryggmargsprøve for å måle proteiner i cerebrospinalvæsken. Tidligere forskning har vist en sammenheng mellom AD-patologi og proteinene Klotho og Glycosylphosphatidylinositol-spesifikk fosfolipase D-1 (GPLD-1), som har assosiasjoner med en rekke fysiologiske prosesser, inkludert nevrobeskyttelse, kognisjon og aldring. Imidlertid har forskning på deres potensial som prediktive biomarkører for AD enda ikke blitt gjennomført. Hensikten til denne oppgaven er derfor å undersøke potensialet til de to proteinene, Klotho og GPLD-1, som prediktive biomarkører for AD i serum. Jeg så også på korrelasjoner med kardiorespiratorisk form, målt som topp oksygenopptak (VO2-peak). Min hypotese var at AD-gruppen, sammenlignet med de friske kontrollene, ville ha endrede reguleringer for både Klotho og GPLD-1, i form av lavere gjennomsnittsverdier og forstyrrede forskjeller fra utgangspunktet (BL) til ett år senere (1yr).

Metoder: Blodprøver ble samlet inn fra eldre voksne som var friske ved BL og 1yr med deltakelse i Generation 100-studien, og som i oppfølgingsperioden frem til 2022 utviklet AD (n=27, alder: 72,4±2,2 år, kvinner/menn: 14/13). Disse ble matchet med friske kontroller i en kasus-kontrollstudie (n=54, alder: 72,8±2,2, kvinner/menn: 27/27). Generation 100-studien er en stor randomisert kontrollert studie (n=1567), som undersøkte effekten av langvarig trening på generell helse og levetid hos en eldre voksenpopulasjon i alderen 70 til 77 år. Ved hjelp av ELISA-sandwichmetoden ble proteinene analysert i serumet til deltakerne.

Resultater: Resultatene viste en endret regulering av Klotho og GPLD-1 hos de som utviklet AD i oppfølgingsperioden. AD-gruppen hadde 22% lavere nivåer av GPLD-1 ved BL ($p=0,009$) og 24% lavere nivåer ved 1yr ($p=0,001$) sammenlignet med de friske kontrollene. Det var ingen signifikant forskjell mellom BL-verdiene og 1yr-verdiene for GPLD-1 for noen av gruppene ($p>0,05$). For Klotho ble det ikke observert noen signifikant forskjell mellom AD-gruppen og de friske kontrollene ved BL ($p>0,05$). Imidlertid var ikke den forventede 2,7% reduksjonen i Klotho-nivåer fra BL til 1yr ($p=0.042$) til stede i AD-gruppen ($p>0.05$). Korrelasjonsanalysen viste sammenhenger mellom GPLD-1 og AD både ved BL ($p<0,001$, $R=-0,292$) og 1yr ($p<0,001$, $R=-0,360$), selv når det ble justert for VO2-peak ($p=0,001$). Dette viser at en reduksjon i GPLD-1-verdier øker sjansene for å utvikle AD senere i livet. Interessant nok var det en uventet omvendt korrelasjon mellom VO2-peak og GPLD-1 ($p=0,035$, $R=-0,238$). De friske kontrollene hadde en sammenheng mellom VO2-peak og Klotho både ved BL ($p=0,036$) og 1yr ($p=0,042$). Klotho-nivåene hos AD-gruppen hadde ingen betydelige sammenhenger med VO2-peak verken ved BL eller 1yr ($p>0,05$).

Konklusjon: Denne studien viste potensialet til Klotho og GPLD-1 som biomarkører for preklinisk AD ved å observere og undersøke de endrede nivåene av proteinene hos pasienter med preklinisk AD. Videre forskning på referanseverdier for disse proteinene og de nøyaktige biomolekylære mekanismene for deres effekter på AD-patogenesen kan bidra til å klargjøre mange ubesvarte spørsmål om sykdomsforløpet til AD og kan gi en mulighet for tidlig påvisning.

Contents

Definitions

Amyloid-β is a peptide which is formed due to the cleavage of amyloid precursor protein, a transmembrane protein, into β- and y- secretase. The peptide can aggregate into amyloid-β plaques that are neurotoxic (2).

Amyloid Precursor Protein is a transmembrane protein implicated in regulatory functions like formation of synapses and neuronal plasticity. It is expressed in both neurons and other tissue (3).

Alzheimer's Disease is a progressive and debilitating neurological disorder accounting for up to 80% of cases for dementia. It greatly affects brain areas related to memory controls and other executive and cognitive functions. It's predominant pathology includes, death of neurons, inflammation, build-up and subsequent aggregation of amyloid-β into plaques and tau into neurofibrillary tangles (4).

Cardiorespiratory fitness is the ability of consumption, distribution, and utilization of oxygen during moderate- to high intensity training over a period of time, by the respiratory, circulatory, and muscular systems (5).

Dementia is a catch-all term for neurological diseases with symptoms of impaired memory, cognition, and executive functions that hinders daily life. It is better described as a syndrome, rather than one particular disease, as dementia can be causes by several diseases, Alzheimer's disease being the most predominant (4).

Maximal oxygen uptake is the highest possible rate at which, during dynamic work with large muscle mass, someone is able to transport and utilise oxygen. When measuring maximal oxygen uptake, it is commonly expressed with volume of oxygen, in millilitres, per kilogram of body mass per minute (ml/kg/min).

Peak oxygen uptake is a term often used interchangeably with maximal oxygen uptake. This is due to the fact that maximal physical exertion may not qualify as ones maximal oxygen uptake. This can be due to a number of factors, some of which may be lack of motivation, inability to maximally push oneself etc.

Synaptic plasticity is a term used for changes in signalling strength in synapses. It is usually as a response to altered synaptic activity, such as repeated activation or longer periods of inactivity (6).

Tau is a microtubule associated protein which is responsible for stable function of microtubule. In Alzheimer's disease, tau can become phosphorylated and cause microtubule instability and neuronal death. Phosphorylated tau proteins can aggregate into neurofibrillary tangles which furthers pathology of the disease (7).

List of abbreviations

AD	*Alzheimer's Disease*
KL	*Klotho*
GPLD-1	*Glycosylphosphatidylinositol-specific phospholipase D 1*
APP	*Amyloid Precursor proteins*
Aβ	*Amyloid beta*
MCI	*Mild Cognitive Impairment*
FAD	*Familial Alzheimer's Disease*
SAD	*Sporadic Alzheimer's Disease*
PSENs	*Presenilin 1 & 2*
NFT	*Neurofibrillary tangles*
CSF	*Cerebral Spinal Fluid*
MAP	*Microtubule Associated Protein*
MTBP	*Microtubule Binding Proteins*
ROS	*Reactive Oxygen Species*
PET	*Positron emission*
SPECT	*Single-Photon Emission Computed Tomography*
MRI	*Magnetic Resonant Imaging*
NMDA	*N-methyl-D-aspartate*
CKD	*Chronic kidney disease*
CVD	*Cardiovascular disease*
M	*Mean*
SD	*Standard Deviation*
CI	*Confidence interval*
BL	*Baseline samples*
1yr	*One year samples*
VO2-max	*Maximal oxygen uptake*
VO2-peak	*Peak oxygen uptake*

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1. Background

1.1 Alzheimer's Disease

AD is a progressive neurodegenerative disorder that primarily affects older adults. It is the most common cause of dementia, accounting for up to 80% of all dementia cases (4). As the world´s population is aging, prevalence of dementia is predicted to increase to an estimated 152 million people affected by the year 2050 (1)(figure 1). Currently, there is no cure for AD, and the available treatments only provide symptomatic relief. Diagnosing early AD development in patients is therefore paramount to shift the focus on preventative measures rather than symptomatic relief.

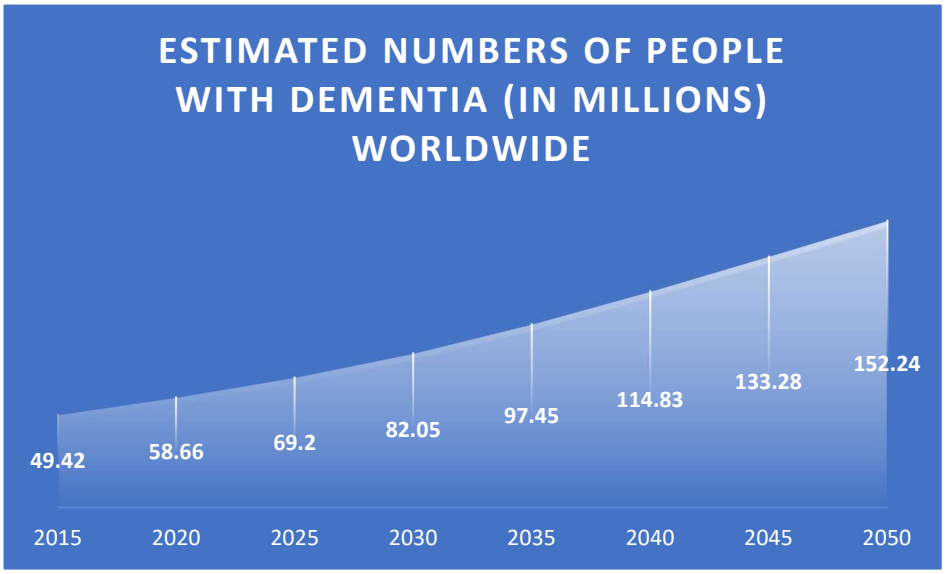

Figure 1: Estimated numbers of people with dementia (in millions) across the world. Created from data in Alzheimer's Disease Internationals (1).

Although the aetiology of AD is not fully understood, several risk factors have been identified, including age, genetics, and lifestyle factors (4). It's believed that about 35% of all cases of AD are preventable through lifestyle changes from early to late life, these are referred to as potentially modifiable risk factors for AD. Among these, physical inactivity is regarded as one of the most significant (8). Studies have shown that exercise can effectively mitigate the onset and progression of dementia by reducing brain damage and inflammation, and increasing the cognitive reserve (8). Cardiovascular disease (CVD) and low physical fitness have also been identified as modifiable risk factors for AD (9). Non-preventable risk factors for AD are subsequently referred to as potentially non-modifiable risk factors, the most significant among these being a genetic predisposition for AD, age, and sex (8, 10).

Most AD development is sporadic (SAD), however, some hereditary gene mutations increase the risk for AD development, referred to as familial AD (FAD). Gene mutations in the amyloid precursor protein (APP), Presenilin 1 and Presenilin 2 (PSENs), and APOE4 are among the most significant (2, 10). There can be numerous types of mutations of APP and PSENs genes, some of which seem to provide lessened amyloid beta (Aβ) burden (10).

1.1.1 AD progression and diagnosis

The high percentage of failure when it comes to AD drug trials are partly due to try-outs on later AD phases (11). Therefore, many researchers are focusing on preclinical and predementia phase of the disease (12). The preclinical stage of AD is recognized as the

period in which there are no apparent symptoms, yet it is possible to detect the disease (13). Mild cognitive impairment (MCI) is an early clinical phase characterized by cognitive impairment not affecting activities of daily living, which some clinicians and researchers see as a middle step between healthy aging and dementia, where it's possible to delay AD development. AD is considered as a clinical and biological continuum (Figure 2), from the preclinical phase to early predementia phase/MCI, to dementia (13). It is therefore imperative to clarify in AD research, what diagnostic criteria is used for separate groups in a study. There are seven stages in which AD progresses (Figure 2), for a more in depth description of these stages, see Sheppard & Coleman (14).

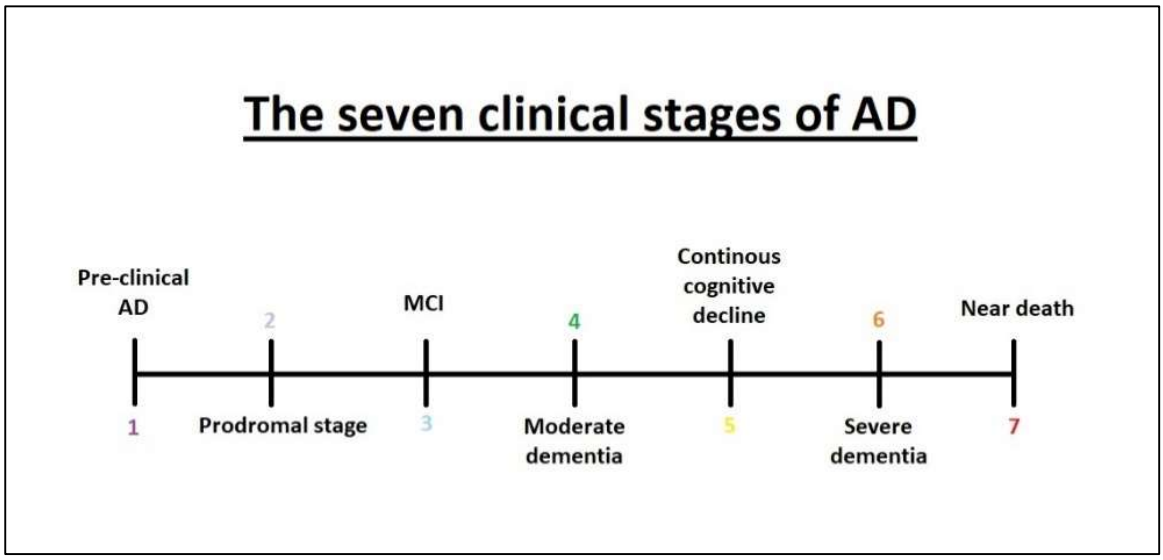

Figure 2: Simplified version of the seven clinical stages of AD. Adapted from Sheppard & Coleman, 2020 (14). Distance between points are not representative of time passed.

One of the most commonly used diagnostic tools for diagnosing AD is measuring biomarkers $A\beta_{40\text{-}42}$, T-tau, and P-tau_{181} in cerebral spinal fluid (CSF)(15) or by positron emission tomography (PET imaging)(16). Collection of CSF is done by lumbar puncture, an invasive test where a needle is inserted between the bones in the lower spine. PET is expensive and available in only high-income countries. Even though these tests can help diagnose AD, they do not provide an insight in disease progression or prognosis . In addition, there currently does not exist any blood-based diagnostic markers for AD, with any prognostic or diagnostic usefulness.

1.1.2 AD pathogenesis

AD is characterized by the accumulation of Aβ and tau in the brain, leading to the formation of Aβ-plaques and neurofibrillary tangles (NFT), with subsequent neuroinflammation and neurodegeneration (figure 3) (2, 3). The pathogenesis of AD is complex, and several hypotheses have been proposed to explain the disease mechanisms. One of the leading hypotheses is the amyloid cascade hypothesis, which suggests that the accumulation of Aβ protein in the brain is the primary event that leads to the neurodegeneration and cognitive decline in AD (2). Another hypothesis is the tau hypothesis, which suggests that the abnormal aggregation of tau protein in the brain is the primary event in the development of AD (17).

APP is present in the membrane, and subsequently cleaved into fragments, some of which contains Aβ (3). The Aβ peptides will aggregate into fibrils, which will in turn form

extracellular Aβ- plaques that cause oxidative stress (3), inflammation, disrupted mitochondria function (18, 19), and neuronal death (Figure 3).The aggregation cascade will partake in phosphorylating tau, and also impair APP metabolism, contributing to Aβ production, creating a positive feedback loop (20, 21, 22).

Tau is a microtubule binding protein (MTBP). Microtubules provide the path in which intracellular transport follows, create larger structures in the cell, and importantly provide an intracellular framework (7). In AD, phosphorylation of tau will aggregate into neurofibrillary tangles (NFT) (figure 3). This will cause microtubule instability, loss of intracellular transport, and eventual neuronal death (23, 24, 25).

1.2 Biomarkers

One promising approach to detect a disease at an early stage and to monitor its progression, is the use of biomarkers. Biomarkers are promising tools for the early detection and diagnosis of diseases like AD. Biomarkers are objective measures of disease-related processes or molecules that can be used to detect the disease at an early stage, monitor its progression, and evaluate the efficacy of interventions (26). In the context of AD, biomarkers can provide valuable information on the underlying pathophysiology of the disease and can help to identify individuals who are at high-risk of developing AD.

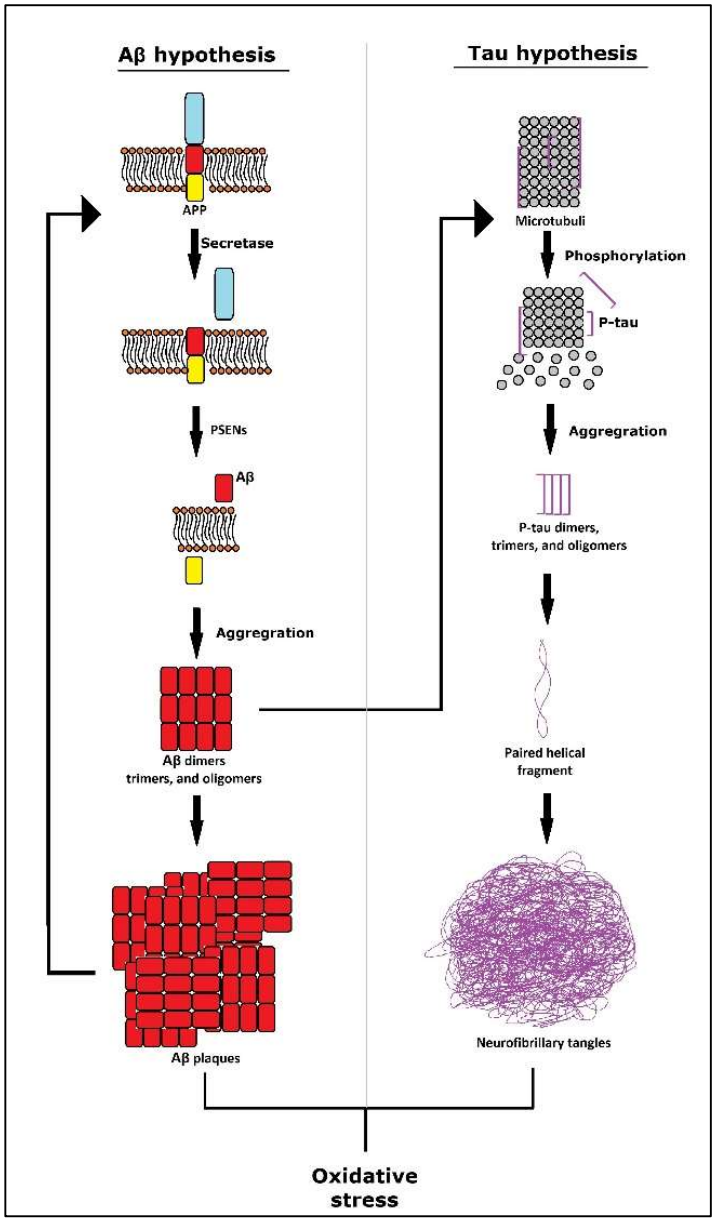

Figure 3: AD pathogenesis: A simplified Aβ and tau hypothesis

There exists two categories into which AD biomarkers can be classified at the present stage; those that directly reflect AD pathology, and those with an indirect or nonspecific measure of AD pathology (16). Direct biomarkers would be proteins such as Aβ and tau in CSF, as well as signs of neural injury (27).

Other direct measures of AD pathology are imaging techniques such as positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonant imaging (MRI) (16), as brain atrophy is a common symptom

seen in AD patients. AD related degeneration is also believed to be mediated by other factors such as cell death, synaptic damage, creation of reactive oxygen species (ROS), inflammation, and oxidative stress (16).

1.2.1 Klotho

Klotho is a protein that has been identified with potential as a biomarker for AD. Klotho was first discovered in 1997 as a putative anti-aging gene and protein (28). It is predominantly expressed in the brain, kidneys, and parathyroid glands (29), and its levels have been found to be reduced in the CSF and serum of AD patients (30). In the brain, Klotho is involved in several physiological processes, including synaptic plasticity, neurogenesis, and cognition (29). Klotho has been shown to have neuroprotective effects against Aβ-induced toxicity, oxidative stress, and inflammation (29). It has also been found to regulate the activity of the N-methyl-D-aspartate (NMDA) receptors, which is involved in learning and memory (29). It has also been found that an overexpression of Klotho in mouse AD-models improved clearing of ameliorated Aβ clearance indicating Klotho's potential for therapeutic treatment for AD (31).

There has been found a positive correlation between exercise and Klotho levels in serum, in men (32). No significant difference was found regarding sex; similar Klotho concentrations were observed in men and women (33). In healthy participants, an inverse relation was observed between Klotho levels and age; the older one is, the lower their Klotho levels (33). It is important to note however, an AD brain and an healthy aging brain are quite different, and it is important to not use them interchangeably. Several studies have reported a reduction in Klotho levels in CSF and serum of AD patients compared to controls (30, 34). Low Klotho levels in the CSF were associated with a higher risk of developing AD related dementia in older adults (34). A normal Klotho level for healthy adults has been described as 562 ± 146 pg/ml (mean ± SD) (33).

Dubal et al. (2014) found that an overexpression of Klotho in mice improved their results in tasks related to learning and memory (6). The increased levels of Klotho seemed to enhanced the NMDA receptor function by enhancing a NMDA receptor subunit, causing an elevated level of long term potentiation, a form of learning related synaptic plasticity (6). They also found that Klotho levels were related to cognitive ability, and not necessarily age (6), indicating that Klotho may be a measurement for cognitive capability rather than an age correspondence. Klotho manipulations in mice on an area of the brain related to cognitive ability, the hippocampus, showed various results, depending on age (35). Klotho restriction caused increase plasticity in mice younger than 5 weeks, but the plasticity evened out and matched wild-type mice by week 7 (35). An overexpression of Klotho in 4-6 month old mice showed increased memory and dentate gyrus plasticity (35). It would seem that Klotho has a multi-regulatory role in plasticity and isn't just related to potentiation.

Despite the promising results, there are several challenges that need to be addressed before Klotho can be used as a biomarker for AD. Studies are needed to validate the sensitivity and specificity of Klotho as a diagnostic and prognostic biomarker. Furthermore, the relationship between Klotho and other AD biomarkers, such as Aβ and tau, needs to be elucidated. Therefore the mechanisms underlying the reduction of Klotho levels in AD patients need to be further investigated.

Though Klotho is known as the anti-aging hormone and is closely related to brain function, it also has other functions in the body. As stated earlier, Klotho is expressed in the kidney, and is thus related to chronic kidney disease (CKD) (36). Klotho levels decrease steadily the older someone gets, and is therefore related to many diseases stemming from seniority (36). A reduction of Klotho in humans is associated with, but not limited to calcification, endothelial disfunction, stiffening of the arteries, intime hyperplasia, hypertension, compromised angio- and vasculogenesis (37). s

1.2.2 GPLD-1

Another biomarker has also shown a potential association with AD pathology, Glycosylphosphatidylinositol-specific phospholipase D 1 (GPLD-1). Recent studies have suggested that GPLD-1 may play a role in the pathogenesis of AD. GPLD-1 overexpression in mice AD models supress production of Aβ (38). In addition to its role in inhibiting production of Aβ, GPLD-1 has also been shown to be involved in the regulation of neuronal survival and differentiation, a form of plasticity (39).

A study looking at exercise and its improvements of cognitive function found that exercise increases the amount of cytokines in the brain, cytokines like GPLD-1 (40). An increased level of GPLD-1 in plasma was correlated with improved cognitive function (40). Transfusion of blood factors from exercised rats to sedentary rats found that some benefits of exercise was seen in the sedentary rats (40). A significant factor that led to these benefits was found to be GPLD-1 (40). That same study increased the production of GPLD-1 in mice using the liver, by in-vivo transfection, lead to an increased level of GPLD-1 in serum, which in turn lead to better performance in the radial water maze and fear-conditioning (40), indicating that GPLD-1 plays an important role in the maintenance of neuronal function and learning. Another study, using long-lived mutant mice, found that GPLD-1 levels were elevated in plasma and liver, and that brain-derived neurotrophic factor (BDNF) and doublecortin (DCX) levels were elevated in hippocampus in these mice, suggesting that GPLD-1 probably increased these brain proteins (39). BDNF and DCX are proteins related to neuronal changes and neurogenesis, respectively. The study also seems to indicate that elevated GPLD-1 reflects selective mRNA translation in liver (39).

1.3 AD and cardiorespiratory fitness:

Cardiorespiratory fitness measured as maximal oxygen uptake, VO2-max, may be pertinent to predict AD development. A high VO2-max has exhibited associations with several physiological and cognitive benefits, including increased cardiovascular health, improved cognitive function, and decreased risk of age-related diseases such as AD (9). Higher levels of physical activity and VO2-max were associated with decreased risk of developing AD and other types of dementia (41). VO2-max was inversely associated with Aβ deposition in the brain, a hallmark feature of AD (42). Thus, maintaining elevated levels of physical fitness and VO2-max may be an important protective factor against AD.

The potential mechanisms underlying the association between VO2-max and AD risk are not well understood. Physical exercise and high VO2-max levels may promote the clearance of Aβ from the brain by increasing cerebral blood flow and the delivery of oxygen and nutrients to brain cells (9). Physical exercise may also stimulate the production of neurotrophic factors, such as BDNF, which promotes the survival and growth of neurons and may protect against AD pathology (43).

The VO2-max in AD patients was significantly lower when compared to controls, and was associated with decreased cognitive function and increased Aβ deposition in the brain (9). As well as lower levels of physical activity and VO2-max being associated with greater Aβ deposition and increased cognitive decline in AD patients (9), indicating that a low VO2-max could be a risk factor for AD.

The potential utility of VO2-max as a diagnostic biomarker for AD has also been investigated. There was found a significant correlation between the VO2-max and cognitive function and Aβ deposition in the brain in a group of AD patients (44). Patients with mild cognitive impairment (MCI) also had significantly a lower VO2-max when compared to healthy controls (45)

1.4 Generation 100 Study

The Generation100 Study (Gen100), a large-scale research initiative that aimed to investigate the effects of exercise on aging and longevity. The study enrolled more than 1,500 participants aged 70 to 77 years (46). It was the first randomized trial, at that scale, attempting to investigate the effect different levels of exercise has on morbidity in the elderly population (46).

The Generation 100 Study included participants born from 1936 to 1942, including only those who could participate in the planned exercise programs. Participants were excluded from the study if they had one or more of the following:

- *"Illness or disabilities that preclude exercise or hinder completion of the study.*
- *Uncontrolled hypertension*
- *Symptomatic valvular, hypertrophic cardiomyopathy, unstable angina, primary pulmonary hypertension, heart failure or severe arythmia.*
- *Diagnosed dementia*
- *Cencer that makes participation impossible or excersice contraindicated. Considered individually, in consultation with physician.*
- *Chronic communicalbe infectious diseases*
- *Test results indicating that study participation is unsafe*
- *Participation in other studies conflicting with participation in Generation 100"* (46)

In 2012/2013, Baseline (BL) assessments were made and variables were collected. This included blood samples, VO2-peak, cognitive screening, etc. To view full list of assessments and variables collected, refer to Stensvold et al. 2015 (46). A one-year-follow-up (1yr) was completed in 2013/2014 where the same information and variables were gathered. Similar follow-ups were completed after 3 years (2015/2016), 5 years (2017/2018), and 10 (2022/2023) years.

The participants were divided in three exercise groups, moderate intensity, high intensity, and a control group. The control group was instructed to follow the Norwegian recommendation for physical activity (46). Several studies have already reported on the findings of the Generation100 study, indicating the potential for the identification of biomarkers for Alzheimer's disease. For example, higher physical fitness levels were associated with improved cognitive function in older adults (47, 48). A different study revealed that elevated levels of cardiorespiratory fitness granted protective benefits

against age-related structural changes in vulnerable areas associated with aging and age-related diseases (49).

1.5 Objectives and Aim

Due to the difficulty in treatment of AD and the importance of early detection, research examining non-invasive techniques of disease identification is paramount. The objective of this master's thesis is to showcase the correlation between potential biomarkers, GPLD-1 and KL, with AD and Vo2-peak using serum collected from the Generation 100 Study, from participants who developed AD later in life. The aim is to clarify the following at two different timepoints, baseline and after one year:

- Compare GPLD-1/Klotho values from AD participants and healthy controls at baseline and after one year of the study protocol
- Identify potential correlations with GPLD-1/Klotho and VO2-peak or AD
- Investigate whether changes in GPLD-1/Klotho are correspondent with changes in VO2-peak

Finally, there is an intention to investigate whether GPLD-1, Klotho, both, or neither has the potential to be used as predictive biomarkers for development of AD later in life.

2. Methods:

2.1 Study design

This is a case-control study; a comparison of two similar groups. One group consists of people who developed AD later in life, and the other group consisting of people that did not develop AD. Participants, along with their data and serum, were included using their BL and 1yr results from the Generation 100 Study. Klotho and GPLD-1 were chosen as proteins of interest to further elucidate their potential associations with AD.

2.1.1 VO2-peak

Cardiopulmonary exercise testing was conducted at the Core Facility NeXt Move at NTNU (Norwegian University of Science and Technology) at both BL and 1yr. The test was performed to measure the maximal oxygen uptake, measured as VO2-max (ml/kg/min), by increasing either incline by 2% or speed by 1 km/h every minute, until either criteria for a VO2-max test was met or at voluntary exhaustion. The criteria, as defined by the Generation 100 Study, is when the VO2-value is no longer increasing by more than 2ml/kg/min even with a higher workload, and the respiratory exchange ratio is greater than or equal to 1,05. The VO2-max was determined by the mean value of the three highest measurements. Since not all the participants in Generation 100 Study reached the criteria needed for a VO2-max test, their tests are regarded as a VO2-peak, the highest value at voluntary exhaustion. For simplicity, the term VO2-peak includes both VO2-max tests and VO2-peak tests in this thesis.

The tests at both BL and 1yr were performed on either a stationary bicycle (Monark Ergomedic 839 E, Sweden) or a treadmill (Woodway USA Inc., Waukesha, WI, USA), using two different systems. One was the Oxycon Pro (Erick Jaeger, Hoechberg, Germany) and the other was the Cortex MetaMax II (Leipzig, Germany). Firstly both systems were calibrated against a motorized standardized mechanical lung (Motorized Syringe with Metabolic Calibration Kit; VacuMed, AkuMed AS, Oslo) for ambient air- and a reference gas mixture of known content (15.00% O_2, 5.00% CO_2). If ambient air measurements were rejected, the gas calibration was performed again. Repeated calibrations occurred prior to each test day and after every 5^{th} test. Preceding each test, a calibration of volume and the inspiratory flowmeter was completed with a 3L volume syringe (Hans Rudolph Inc., Kansas City, MO, USA). Prior to the VO2-max test itself, all participants had a 10-minute warm-up and familiarization of treadmill or bicycle.

2.2 Criteria for inclusion/exclusion

2.2.1 AD diagnosis

All participants were healthy with no AD presenting symptoms at baseline and 1-year-follow-up. Only participants with confirmed diagnoses of Alzheimer's disease were included in the AD group. To do this, the participant's medical records were reviewed. Only those that meet the criteria of Albert (16) or McKhann (50) from 2011 for AD were included. Only those who debuted cognitive impairment from 01.01.2014 and later were considered for the AD group.

Records, in the form of a contact list, discharge summary, and journal notes were reviewed for following diagnosis codes: F00-F03, G20, G30-G31, F10.7, R41.8, G12.2. Following this, these words were searched in free text in Norwegian:

Kognitiv, hukommelse, demens, dement, alzheimer, Alzheimers, normaltrykkshydrocephalus, «rote», «roter», glemsk, glemmer, desorientert, orientert, delir, forvirring, kolinesterase, cholinesterase, Donepezil, Aricept, Galantamin, Reminyl, Rivastigmin, Exelon, Memantin, Ebixa, Nemdatine

Approximate English translation:
Cognitive, memory, dementia, demented, Alzheimer's, normal pressure hydrocephalus, "confused", "confusing", forgetful, forgets, disoriented, oriented, delirium, confusion, cholinesterase, cholinesterase, Donepezil, Aricept, Galantamine, Reminyl, Rivastigmine, Exelon, Memantine, Ebixa, Nemdatine.

A neurologist reviewed the journals of participants that had one of the forementioned diagnosis codes or search terms in, or prior to, 2022. In participants that were suspected to have a neurodegenerative condition or dementia, relevant information from their clinical history was registered in an excel document. Diagnostic assessment was done by reviewing their clinical symptoms, results from medical imaging and spinal fluid analyses for AD biomarkers. An audit of whether the participants were taking any AD medication was additionally conducted. Following this, it was evaluated whether they fulfilled the Albert or McKhann criteria for AD, as mentioned above.

If participants had any other neurodegenerative conditions in their journals prior to 2012 and in the follow-up period 2012-2022 they were excluded. Participants with active cancer in the period of 2012-2013 were also excluded.

2.2.2 Healthy controls

The healthy controls (HC) were chosen from those participants that did not have the diagnosis codes or search terms, mentioned in 3.2.1, in their journals, and then matched by age and sex. Due to simplicity, there was chosen twice as many HC as there were in the AD group. A neurologist then examined their journals to exclude the existence of cognitive impairment, dementia, or other severe neurological afflictions prior to BL and in the follow-up period, up until 2022. Participants with active cancer in the period of 2012-2013 were also excluded.

2.3 ELISA

2.3.1 Blood samples

Blood samples were collected from the randomized controlled study, Generation100, in Trondheim, Norway, BL and after one year. The blood samples were collected in 2012/2013, centrifuged, frozen, and the serum was subsequently sent for storage at -80°C. In 2023, the serum was thawed and homogenized by vortex mixing the samples. They were once again frozen in -80°C after ELISA analysis (3.3.2). A select few of the samples were re-thawed to be analysed anew, and once again frozen.

2.3.2 Elisa analysis

Enzyme-linked immunosorbent assay (ELISA) was used for analysis of both GPLD-1 and Klotho in serum. There exists different ELISA methods; Direct, indirect, competitive and sandwich. For analysis of GPLD-1 and KL, the sandwich-ELISA was used. A plate consisting of 96 wells are coated with a capture antibody (51). Samples and standards are added to the wells and bind to the coating. A detection antibody is added and bind to the samples and standards in the wells, creating a "sandwich", hence the name (51). A substrate is added to the sandwich complex which causes a detectable reaction. After some time in incubation, a stop solution is added, and the fluorescence is measured

(51). Amount of incubation time, wash steps and detection wavelength depends on which substrate one is trying to detect, and should be adjusted as such. The standards with known concentration values are used to create a standard curve, from which you calculate the concentration of the samples. This was automatically done by the analysis machine: Dynex DS2, two-plate Automated ELISA Processing System (Dynex Technologies, Chantilly, USA).

2.3.3 Klotho analysis

In 3.3.5 the setup used for samples, blanks and standards is shown. The Human Soluble Alpha Klotho Assay Kit was purchased from IBL America (Catalog nr. 27998), lot 2H-110. Analysis followed the protocol that was provided with the test kits (Appendix). The analysis was completed in two parts. Part 1 was prior to sample insertion in the ELISA machine and Part 2 was after the samples had been inserted into the ELISA machine, for automated analysis by the machine. For more analysis details and specifications see (Appendix).

Part 1: *Preparation*
Prior to the beginning of analysis, the Wash buffer solution was made. The provided Wash Buffer concentrate (50 ml) was diluted 40-fold with deionized water. Secondly the standards were prepared. This was one by adding 0,5 ml of deionized water to the provided standard powder until it completely dissolved. The concentration of this solution was at 12000 pg/ml. 230 µl of provided EIA Buffer were added to 7 tubes marked 1-7 respectively. The previously made concentration was added to tube 7, this served as the highest standard. After thorough mixing, 230 µl of the mixture from tube 7 was added to tube 6. This was also thoroughly mixed and 230 µl of it was subsequently added to tube 5. This process of creating a series standard dilution was completed when each tube had reached their intended concentrations; Standard 7 (6000 pg/ml), Standard 6 (3000 pg/ml), Standard 5 (1500 pg/ml), Standard 4 (750 pg/ml), Standard 3 (375 pg/ml), Standard 2 (187.5 pg/ml), and Standard 1 (93.75 pg/ml). The labelled antibody was not prepared until a few minutes prior to its application in the machine.

Part 2: *Automated sample analysis*
100 µl of the standards were dispensed in their allotted positions. 50 µl of EIA buffer was subsequently added to all sample wells. Following this, 50 µl of serum from each participant was pipetted in their allotted space. This created a 1:2 dilution of the samples. 100 µl of EIA buffer was used as Reagent blanks. The plate was the incubated for 60 minutes at room temperature. A was step consisting of 500 µl of wash buffer added to each plate and promptly aspirated was completed 4 times. The newly made Labelled Antibody, 100 µl, was added to each well and left to incubate for 30 minutes at room temperature. The same wash step as described above, with the same volume of wash buffer, was then completed 5 times. 100 µl of provided Chromogen was then added to each well and the plate was left for incubation for 30 minutes at room temperature once again. After incubation, provided Stop Solution was added and the plate was immediately read at 450 using a microplate reader.

Prior to calculating the concentration of participant samples in serum, the highest standard (standard 7) was removed. This was attributed to the fact that the measured absorbance of standard 7 was higher than what could be measured, due to its high concentration. The measured absorbance of standard 7 was shown as 'OVER', and was therefore automatically placed in the value of 6000 pg/ml, as that is the highest

standards concentration. If we were to keep standard 7, the K concentration in the participant samples would be slightly inaccurate.

Using the modified standard curve, the concentration for the participant samples were calculated. Prior to plotting in SPSS, the values were multiplied by 2, the concentration factor. The Klotho values were measured in the unit of pg/ml. Three different variables were created for each participant, respectively. BL Klotho values, 1yr Klotho values, and Delta Klotho values (The BL Klotho value subtracted from the 1yr Klotho value)

2.3.4 GPLD-1 Analysis

The setup described in 3.3.5 was used as to determining which well in the plate was reserved for blanks, standards, and samples. Pre-coated 96 well strip plate was purchased from antibodies-online (Catalog nr: ABIN7062719), lot: L230208174. Followed the protocol that was provided with the test kits (Appendix). To see what reagents and materials were included in the purchased kits, see appendix. Each 96-well strip could analyse 80 samples when standards are taken into account. As each analysis used half a kit, only 32 samples could be analysed each time, as standards are needed for each analysis. For more analysis details and specifications see (Appendix).

Initially the samples were mixed, to ensure homogeneity, and diluted 1:10; 12 µl serum and 108 µl of Phosphate buffer saline (PBS) solution. The was solution was created by adding 20 ml of Wash Solution concentrate with 580 ml of deionized water, and mixed thoroughly. Detection reagent A Working Solution was created by adding 50 µl Detection Reagent A with 4950 µl of Assay diluent A. Detection reagent B Working Solution was created by mixing with the same volumes, but with Detection reagent B and Assay diluent B respectively. The Standards 1-7 were created by first adding 1000 µl of Standards diluent to Reference Standard, which came in a stock, and had the concentration 100 ng/ml. 100 µl of the mixture was added to a tube marked '7', this served as the highest standard at 50 ng/ml. 50 µl of Standard diluent was added to each tube marked 1-6. 50 µl of the solution from Standard tube 7 was added to Standard tube 6. 50 µl from Standard tube 6 to 5, and so on until the last standard. Standards reached the concentrations of: 50 ng/ml (Standard 7), 25 ng/ml (Standard 6), 12.5 ng/ml (Standard 5), 6.25 ng/ml (Standard 4), 3.12 ng/ml (Standard 3), 1.56 ng/ml (Standard 2), and 0.78 ng/ml (Standard 1). Creation of standards were the last step prior to beginning the analysis.

The diluted samples were placed in the analysis machine where they were further diluted by 1:100. 10 µl of 1:10 diluted sample to 990 µl PBS, this created a 1:1000 dilution of the serum. 100 µl of the standards were then placed in their respective positions on the pre-coated plate. The positions for blank samples were manually placed with 100 µl of PBS. The following was completed autonomously by the analysis machine: 100 µl of the 1:1000 diluted sample were placed in their corresponding wells on the analysis plate. Excess liquid was aspirated from the wells. 100 µl of Detection Reagent A Working Solution was added to each well and incubated for 1 hour at 37°C. The solution was aspirated after incubation. 350 µl of Wash solution was added and left to soak for 1.5 minutes, the liquid was then aspirated, this washing procedure was completed a total of 3 times. 100 µl of Detection Reagent B Working Solution was then added and left to incubate for 30 minutes at 37°C. The same wash washing procedure as earlier was completed 5 times. After the wash, 90 µl of Substrate Solution was added to each well and incubated for 15 minutes at 37°C. After incubation, 50 µl of the Stop Solution was

added to each well. Measurements were immediately taken at 450 nm with a microplate reader.

Using the standard curves created by the measured absorbance of the standards, concentration in serum was calculated. Results were multiplied by 1000, the dilution factor. The results were then converted from ng/ml to µg/ml. The converted results were then manually plotted in the SPSS files with their respective Gen100 ID numbers. Three variables were created; BL values for GPLD-1; 1yr values for GPLD-1 and DELTA values for GPLD-1 (BL GPLD-1 values subtracted from 1yr GPLD-1 values).

2.3.5 Analysis setup

For setup analysis, which samples were re-run, and their respective Generation 100 numbers, see appendix (Figure C).

Basis for choosing which samples were re-run:

- Outliers or errors
- Samples with the highest and lowest values
- Large variance between BL and 1yr value on a single sample
- Randomly chosen samples

After the select samples were analysed for the second time, the average value for both analysis on the same sample was used for calculations. Interassay calculations were completed on the re-run samples to ensure replicable methodology.

2.4 Pilot analysis

To ensure quality of kits and the Elisa machine, several preliminary analysis were run on samples unrelated to Gen100 serum. Each preliminary analysis were referred to as a 'Pilot'.

2.4.1 Klotho pilot

There were carried out three pilots on the Klotho kits. Each pilot provided some insight into what aspects of the setup for the analysis should be improved (Table 1,2).

Table 1: Notable findings, identified errors, and modification made from Klotho pilots 1-3.

KL	Notable findings/deficiencies	Errors identified	Modifications made
Pilot 1	- Inconsistent results on the same sample	- Insufficient mixing/homogenizing of samples after thawing, prior to analysis	- Mix/vortex the samples thoroughly prior to analysis
Pilot 2	- Systematic reduction of measured concentration for each strip on a plate for identical samples	- Possible sedimentation of samples when dilution is done manually - Possible inconsistent incubation period	- Wash steps were changed from 'plate-wise' to 'strip-wise' - Incubation period started at first sample extraction, rather than last - Automatic dilution of samples
Pilot 3	- Consistent results on identical sample throughout the plate	- No errors identified	- No modifications made

2.4.2 GPLD-1 pilot

A single pilot was completed for the GPLD-1 analysis.

Table 2: Notable findings, identified errors, and modification made from GPLD-1 pilot.

GPLD-1	Notable findings/deficiencies	Errors identified	Modifications made
Pilot 1	- First half of the plate had inconsistent measured concentrations when compared to second half of the plate	- Possible unstable kit - Possible sedimentation of samples due to manual dilution - Possible inconsistent incubation period	- Only run half a plate at a time. Analyse 32 samples (half-plate) each run rather than 80 (full plate)

2.5 Mathematical equations and statistical analysis

After Klotho and GPLD-1 analysis in serum was completed, the results were plotted in an SPSS file containing all other variables as well. Version 28 of SPSS Statistics (IBM SPSS, New York, USA) was used to complete all statistical computations. MATLAB (ver. R2018b) was used for creating graphs. Prior to analysis, the data was tested for normality by making a bar graph and QQ-plots showing the distribution of each group for each timepoint separately (appendix, figure A).

Non-parametric correlation estimate was computed between Klotho and GPLD-1. Following this, independent sample t-tests using AD-diagnosis as the dependent variable with the proteins at each timepoint as test variables was conducted. A comparison of means at different timepoints was done with a paired sample t-test. Binary logistical regression was used to find the association between risk of developing AD with proteins, VO2-peak, and sex, respectively. It was also employed to investigate a potential effect of AD diagnosis with change in VO2-peak and protein levels. Linear regression was utilized for look at the potential influence of VO2-peak on the proteins and if the change in VO2-peak affects the change in proteins between BL and 1yr. A significance value of 0.05 or less (95% confidence interval) was chosen as statistically significant results.

2.6 Ethical considerations

This master's thesis is part of a larger study; "Project proposal Fitness and blood factors in Generation 100 and risk of dementia" (REK Midt, 183708). The main Generation 100 study already has approval from the Regional Ethical Committee (REK 2012/381). All participants in the study have given their written consent and are involved voluntarily.

To ensure the protection of sensitive information about participants, no identifiable information about them was sent via e-mail, messages or used outside of encrypted computers at St. Olav's Hospital. Each participant got assigned a number, referred to their 'Generation 100 number', to ensure they remain anonymous. A confidentiality agreement was signed prior to working with Gen100 participants or being granted access to their information.

3. Results

3.1 Descriptive Statistics

Table 3: Descriptive statistics for participants included in the study. All: All 81 participants included in the study.

	All (n = 81)	AD (n = 27)	HC (n = 54)
Age (years)	72.7 ± 2.2	72.4 ± 2.2	72.8 ± 2.2
Sex, women (%)	41 (51%)	14 (48%)	27 (50%)
Education level, college/university n(%)	53 (65%)	15 (56%)	38 (70%)
VO2-peak (ml/kg/min)	33.3 ± 8.2	29.9 ± 6.2	35.1 ± 8.6
Blood pressure (mmHg)			
Systolic	135 ± 16	136 ± 16	134 ± 16
Diastolic	75 ± 9	75.9 ± 9	75 ± 8
BMI (kg/m^2)	24.8 ± 3.3	25.5 ± 3.4	24.5 ± 3.2

Age, VO2-peak, blood pressure, and BMI presented as: Mean ± standard deviation. AD: The participants that developed AD later in life. HC: Healthy controls, the participants that did not develop AD later in life and did not have any other CVD or cancer.

Descriptive information about participants and information about risk factors for AD are calculated from BL data (table 3).

Klotho and GPLD-1 were treated as independent variables resulting from a correlation analysis (Spearman's rho), calculated with BL values, which showed the following:

ALL samples: Correlation coefficient = 0.111, sig. (2-tailed) = 0.111
AD group: Correlation coefficient = 0.178, sig. (2-tailed) = 0.384
HC group: Correlation coefficient = 0.072, sig. (2-tailed) = 0.603

One participant was excluded from all Klotho and GPLD-1 analyses due to inconsistent and abnormal results from analyses on their serum, they were part of the AD group, reducing their n from 27 to 26. One participant form the HC group did not have BL VO2-peak values and is therefore not included in the calculations requiring VO2-peak data.

3.2 Klotho

Table 4: Mean values and SD of Klotho and GPLD-1 at BL and 1yr for HC and AD group.

	Timepoint	HC	AD
Klotho (pg/ml)	BL	705.7 ± 175.1	769.4 ± 362.9
	1yr	686.9 ± 170.9	736.0 ± 342.6
GPLD-1 (µg/ml)	BL	8.2 ± 2.6	6.4 ± 2.9
	1yr	8.3 ± 2.4	6.3 ± 2.6

Data presented as: Mean ± standard deviation.

3.2.1 BL to 1yr

Klotho levels in healthy controls decreased by 2.7% (p=0.042) from BL to 1yr (table 4, Figure 4). In contrast, the AD group had no significant change in Klotho levels from BL to 1yr (Table 4)(Figure 4).

Comparing the Klotho levels between HC and the AD group, produced non-significant findings at both BL and 1yr. Comparing the groups at both timepoints gave the following results:

BL Klotho (pg/ml): Mean diff.= -63.6, 95% CI: [-183.0, 55.7]
1yr Klotho (pg/ml): Mean diff. = -49.1, 95 % CI: [-163.1, 64.8]

Showing that, at both timepoints, there is an insignificant difference between the HC and the AD group regarding their Klotho values.

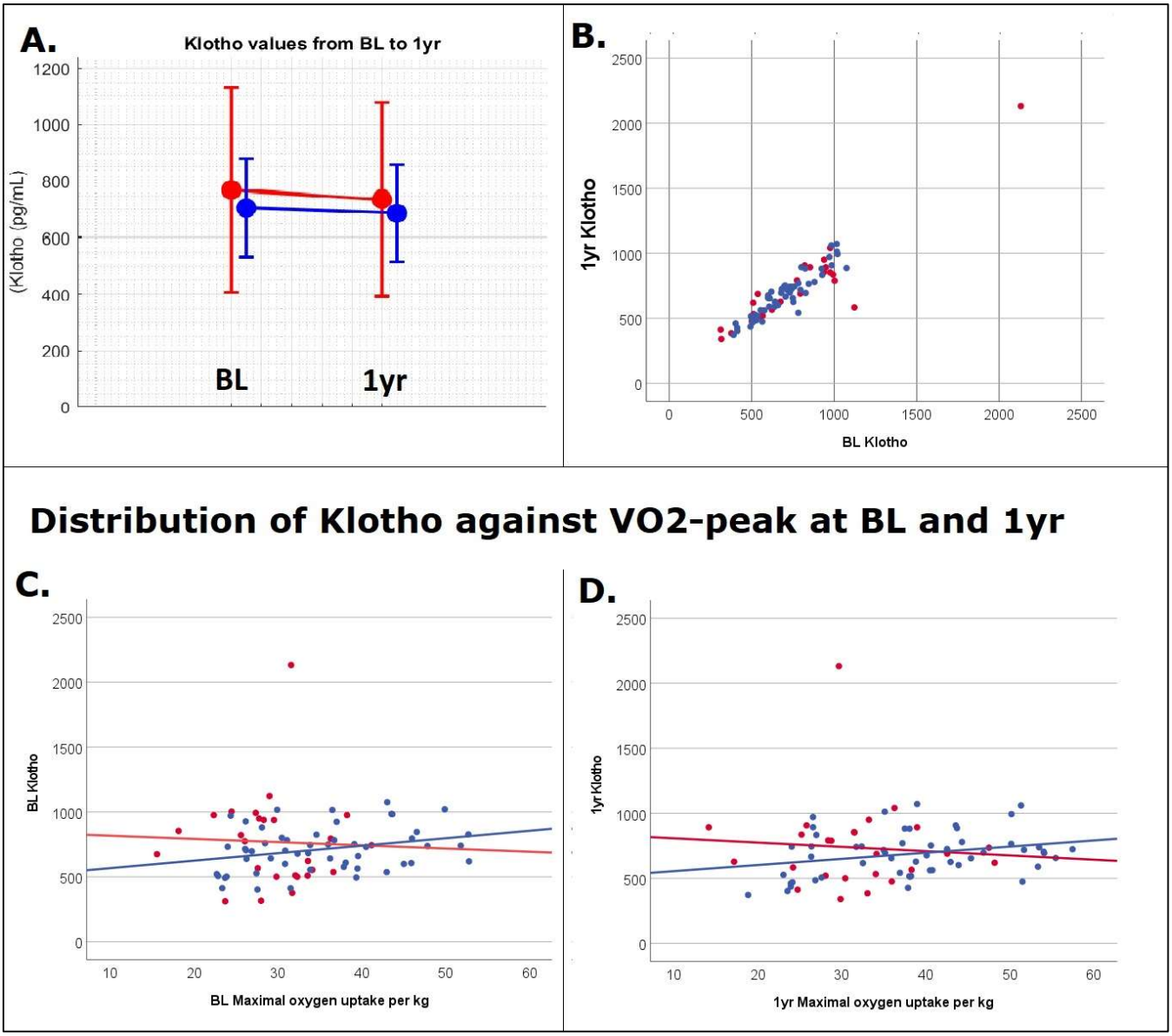

Figure 4: Distribution of Klotho values for HC and AD group at different timepoints. Red = AD group. Blue = HC group. A: Development of Klotho values for HC and AD group from BL to 1yr. B: BL Klotho against 1yr KL, in pg/ml C: VO2-peak against Klotho (pg/ml) at BL. D: VO2-peak against Klotho (pg/ml) at 1yr.

3.2.2 AD associations

Table 5: Associations between proteins; VO2-peak; sex, and odds of not developing AD at different timepoints.

Timepoint	Variable	1	2	3
BL	Klotho	0.999 (0.997 to 1.001)	0.999 (0.997 to 1.001)	-
	VO2-peak	1.120 (1.037 to 1.210)	-	1.106 (1.028 to 1.190)
1yr	Klotho	0.999 (0.997 to 1.001)	0.999 (0.997 to 1.001)	-
	VO2-peak	1.089 (1.025 to 1.158)	-	1.082 (1.021 to 1.148)
BL	GPLD-1	1.443 (1.151 to 1.809)	1.317 (1.073 to 1.617)	-
	VO2-peak	1.154 (1.058 to 1.258)	-	1.106 (1.028 to 1.190)
1yr	GPLD-1	1.546 (1.202 to 1.988)	1.438 (1.149 to 1.798)	-
	VO2-peak	1.115 (1.040 to 1.195)	-	1.082 (1.021 to 1.148)

Data presented as: *Odds ratio (95% confidence interval for odds ratio).* ***1****: AD diagnosis as dependent with proteins, VO2-peak, and sex as variables.* ***2****: AD diagnosis as dependent with proteins and sex as variables.* ***3****: AD diagnosis as dependent with VO2-peak and sex as variables.*

Klotho values at both BL and 1yr are not correlated with odds of developing AD later in life, shown by their CI (table 5). Independent correlation between odds of AD development and Klotho show an insignificant result, with a similar result observed even when controlled for VO2-peak (table 5).

Table 6: Comparison of difference in Klotho and GPLD-1 between groups when adjusted for, and not adjusted for VO2-peak

	Adjusted for VO2-peak	Not adjusted for VO2-peak
Klotho (pg/ml)	-7.7 (-51.4 to 35.9)	-8.1 (-50.8 to 34.7)
GPLD-1 (µg/ml)	0.59 (-0.52 to 1.23)	0.67 (0.28 to 1.32)

Data presented as: Mean difference (95% confidence interval)

A measure of the difference in values between BL and 1yr, Delta, was computed for Klotho, GPLD-1, and VO2-peak. Analysing correlations between Delta Klotho and AD diagnosis, while adjusting for-, and not adjusting for VO2-peak, insignificant results were found (table 6)

3.2.3 VO2-peak associations

Table 7: Associations between VO2-peak and proteins at different timepoints

	Timepoint	HC	AD
Klotho (pg/ml)	BL	705.7 (0.5 to 11.1)	769.4 (-28.5 to 23.6)
	1yr	686.9 (0.2 to 9.2)	736.0 (-21.4 to 14.9)
GPLD-1 (µg/ml)	BL	8.2 (-0.2 to -0.02)	6.4 (-0.44 to -0.09)
	1yr	8.3 (-0.12 to 0.01)	6.3 (-0.27 to -0.02)

Data presented as: Mean (95% confidence interval)

For the healthy controls, there was found a positive correlation between Klotho and VO2-peak, shown by their CI; corresponding results were seen for both BL and 1yr samples (table 7, figure 4C:D). For the AD group however, there were, at neither timepoint, any significant association with VO2-peak (table 7C:D).

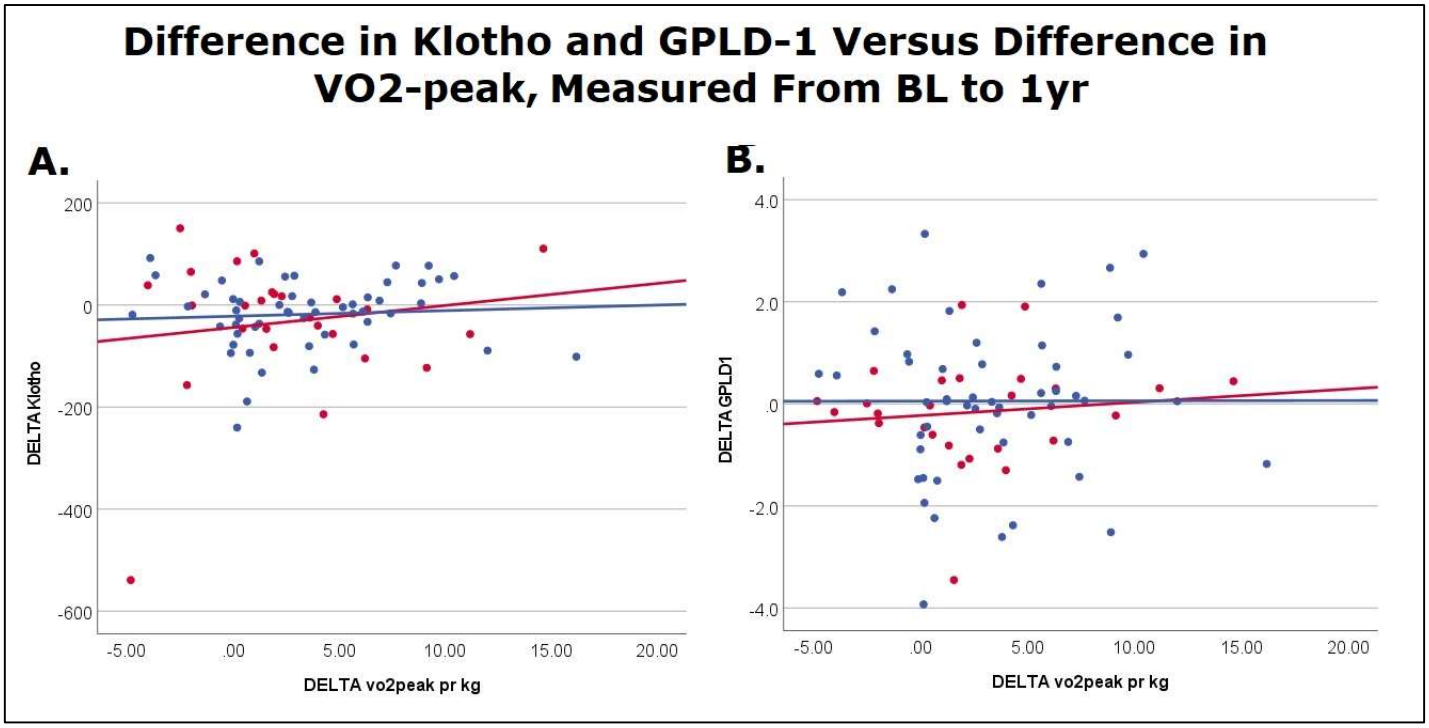

Figure 5: The difference in Klotho and GPLD-1 versus difference in VO2-peak, measured from BL to 1yr. Red: AD group. Blue: HC group. A: Delta (BL value subtracted from 1yr value) Klotho versus Delta VO2-peak. B: Delta GPLD-1 versus Delta VO2-peak.

Analysing the association between Delta Klotho and Delta VO2-peak gave a significance value of > 0.05 for both groups (figure 5), no association was found.

3.3 GPLD-1

3.3.1 BL to 1yr

GPLD-1 values showed no significant difference between their BL and 1yr results ($p>0.05$) for both the AD group and HC (table 4).

In contrast to the Klotho findings, a comparison of GPLD-1 values between the HC and AD groups, yielded significant findings at both BL and 1yr. Comparing the groups at BL and 1yr gave the following results:

BL GPLD-1 (µg/ml): Mean diff. = 1.8, 95% CI: [0.5, 3.0]
1yr GPLD-1 (µg/ml): Mean diff. = 2.0, 95% CI: [0.8, 3.2]

This demonstrates a significant difference in GPLD-1 values between the HC and the AD group, at both BL and 1yr, with the AD group having lower values than the HC (figure 6A:B).

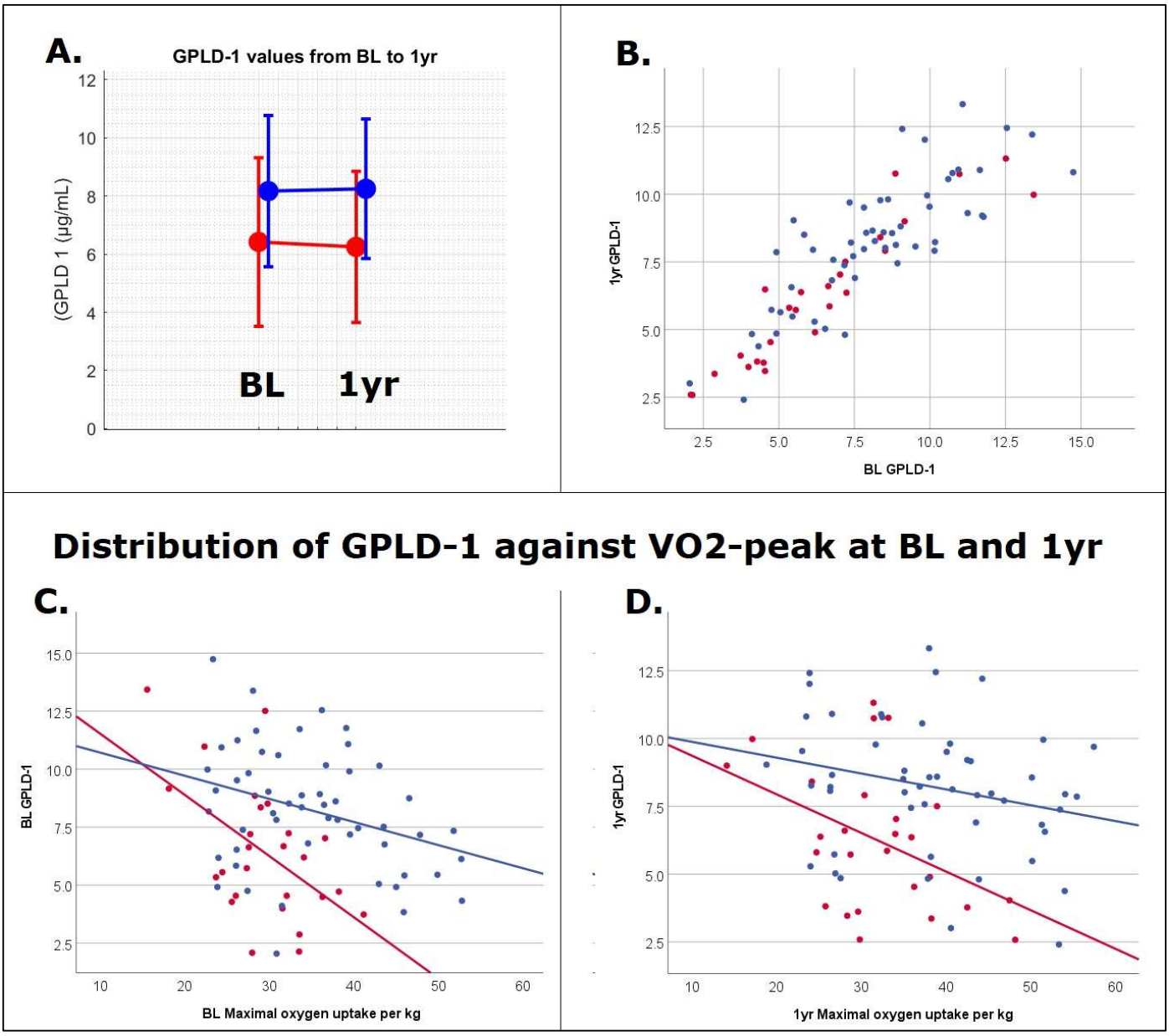

Figure 6: Distribution of GPLD-1 values for HC and AD group at different timepoints. Red = AD group. Blue = HC group. A: Development of GPLD-1 values for HC and AD group from BL to 1yr. B: BL GPLD-1 against 1yr GPLD-1, in µg/ml C: VO2-peak (ml/kg/min) against GPLD-1 (µg/ml) at BL. D: VO2-peak (ml/kg/min) against GPLD-1 (µg/ml) at 1yr.

3.3.2 AD associations

GPLD-1 values at BL and 1yr both showed a significant correlation with odds of developing AD at a later age (table 5). Analysing GPLD-1's correlation with AD, controlling only for sex, showed that an increased GPLD-1 value lowers odds of developing AD. When controlled for both sex and VO2-peak, a similar significant result was seen (table 5).

When investigating the difference in Delta GPLD-1 between the HC and the AD group, a significant result was seen when not adjusting for VO2-peak (table 6). When I adjusted for VO2-peak, the association was no longer present (table 6).

3.3.3 VO2-peak associations

GPLD-1 values showed an association with VO2-peak at both timepoints for the AD group, with Sig. < 0.05, but only at BL for the healthy controls. The 1yr GPLD-1 value for HC did not have a significant association with VO2-peak, as it surpassed the cut-off value with a Sig. = 0.072 (table 7).

An inverse relationship between GPLD-1 and VO2-peak was identified; as your VO2-peak increases, your GPLD-1 levels decreases (figure 6C:D).

Mirroring the same results as KL, analysis of the association between Delta GPLD-1 and Delta VO2-peak also gave a significance value of > 0.05 for both HC and the AD group (figure 6), no association was found.

3.4 Other findings

VO2-peak was shown to have an inverse correlation with risk of AD; the higher ones VO2-peak, the lower their risk for developing AD (table 5). The AD group had a significantly lower VO2-peak than the HC group (sig. < 0.05).

4. Discussion:

The aim of this thesis was to investigate the potential use of Klotho and GPLD-1 as predictive biomarkers for AD, by using serum from Generation 100 participants in the years preceding their AD diagnosis. As the molecular mechanisms of physical activity and the relation to AD risk may be a target point for future AD diagnosis and treatment, this study also investigated the association between the change in fitness levels with the change in protein levels. The results revealed that AD participants showed a modified regulation of Klotho and GPLD-1.

Specifically, GPLD-1 levels were significantly lower in AD participants at both BL and 1-year follow-up compared to healthy controls. However, there were no correlations between the two GPLD-1 values at BL and 1yr. At both timepoints in both groups, an inverse correlation was found with GPLD-1 values and VO2-peak. Except for the forementioned AD and VO2-peak correlations, no other GPLD-1 correlations of note were found.

In contrast, no significant differences were found in Klotho levels between AD participants and healthy controls at either BL or 1yr. Nevertheless, the change in Klotho levels between BL and 1-year follow-up was significantly altered for AD participants. In healthy controls, Klotho levels were decreased by 2.7% at 1-year follow-up compared to BL, but no such changes were observed in AD participants. Furthermore, a direct relationship between Klotho levels and VO2-peak was observed in healthy controls, but not in the AD group. Overall, these findings suggest that AD patients have altered regulation of Klotho and GPLD-1, which may contribute to the pathogenesis of AD.

The AD group had lower VO2-peaks than the healthy controls. VO2-peak was found to be an inverse correlation with AD-diagnosis, for both groups, at BL and 1yr, even when adjusted for either Klotho or GPLD-1, respectively (table 5); The higher ones VO2-peak, the lower their odds of developing AD.

4.1 Klotho

For healthy controls, a statistically significant decrease was observed from BL to 1yr (figure 2). These values were also correlated with the participant's respective VO2-peaks. Klotho levels for AD participants however, seem to be regulated differently. There was no significant decrease from BL to 1yr, as well as a lack of correlation with VO2-peak. This is consistent with findings from previous studies, which indicate an altered Klotho regulation for AD patients (30, 52). All the measured Klotho values, for both groups, were within the expected range for Klotho in humans (33). It is not possible to infer the odds of developing AD later in life with a single Klotho measurement. An irregular change in Klotho levels over long periods of time however, may be a better indication. This is attributed to the fact that the AD group had such a high standard deviation in their measurements, but also that their Klotho values did not significantly decrease from BL to 1yr, as stated earlier.

Investigating how Klotho effects the odds of developing AD yielded insignificant findings, even when controlled for VO2-peak (table 6). Previous studies found that Klotho values are significantly lower in AD patients compared to healthy controls (29, 34), my results do not indicate this. The mean Klotho level was higher in AD participants, but their standard deviation was also greater. This proved a higher variance of Klotho, but is likely not an indication of a trend, but rather irregular biological variation. This may be attributed to several factors. Klotho levels in serum are increased after cardiovascular

exercise, but decreased after strength exercise (53). Another study found that more than 7.5 hours of sleep each night decreased Klotho levels, but this may be related to time spent inactive rather than amount of hours slept (54). How far someone has come in the course of illness may also differ Klotho levels. There is a clear difference on how Klotho changes over time in AD patients compared to healthy controls, but the exact mechanisms on how AD pathogenesis effects Klotho remains to be elucidated. It is unknown whether the change in Klotho regulation for AD patients is due to a specific biochemical change, for instance an increased levels of Aβ or p-tau in the brain, or as a response to AD-caused neural degeneration.

4.2 GPLD-1

There was found a statistically significant difference between the measured GPLD-1 values of healthy controls and those that developed AD later in life; The AD group had lower values than the HC. I found the same results at both BL and 1yr. This shows the potential of using GPLD-1 as a predictive biomarker for development of AD. Due to the novelty of GPLD-1 research, there currently exists no reference values, or expected values for normal GPLD-1 levels in humans. It also seems that GPLD-1 values remains fairly stable over a longer period of time. Because of this, it would be impetuous to claim that GPLD-1 can be used as a definitive biomarker, however, it displays great potential.

In light of the fact that there is a strong correlation between GPLD-1, VO2-peak and AD diagnosis, it is hard to say whether GPLD-1 correlates more with AD development than is does with VO2-peak. The change in VO2-peak not being correlated with the change in GPLD-1; though one changes, the other may not, indicates that GPLD-1 may tend towards a stronger correlation with AD rather than VO2-peak. Previous studies have found that exercise increases the levels of GPLD-1 in serum as well as lowering the risk of developing AD (55). An overexpression of GPLD-1 also promotes cognitive ability (40). A common finding in AD patients is a high Aβ-burden. Since GPLD-1 has been found to suppress Aβ-production (38), there seems to be an inverse relationship between GPLD-1 and Aβ. This could be inferred from my results on account of the AD group having lower GPLD-1 values, and therefore may have a higher Aβ-burden, thus progressing AD pathogenesis. An overexpression of GPLD-1 has, in mice, found to increase cognitive ability (40). A lower GPLD-1 level could then be related to reduced cognitive ability, a common finding in AD. Further research investigating the relationship between GPLD-1 levels, Aβ, and cognitive ability could be crucial in understanding the preliminary stages of AD pathogenesis.

In stark contrast to previous findings, I found evidence of an inverse relationship between GPLD-1 and VO2-peak, rather than a positive correlation. Past research shows that exercise leads to a higher GPLD-1 value (39, 40), my results show that the higher your VO2-peak, the lower your GPLD-1 values (Figure 6C:D). The relationship between GPLD-1, VO2-peak and development of AD must be investigated, considering that a sequence of self-contradictory correlations that cannot coexist, exists (Figure 7). Since this paper uses a case-control study, and isn't a randomized controlled trial, some results may be misrepresentative of the entire population.

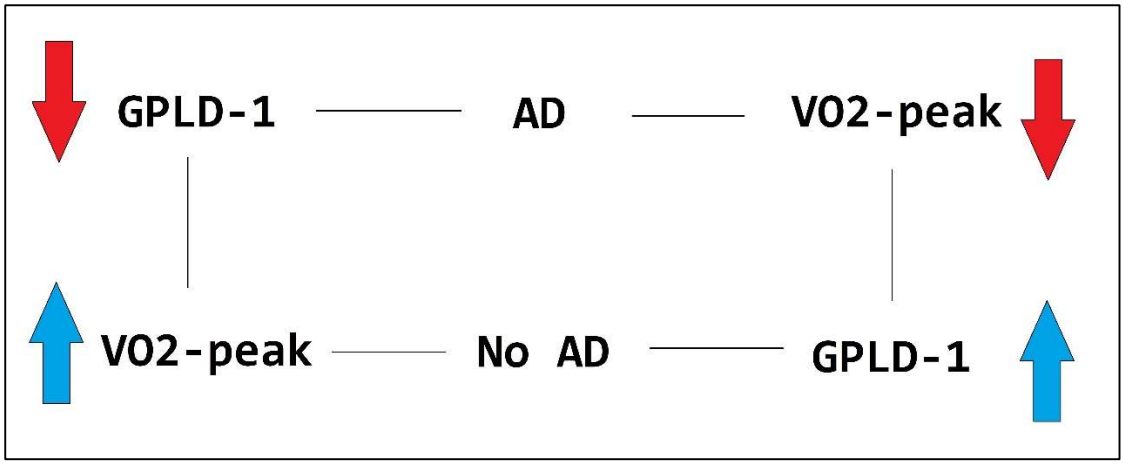

Figure 7: Correlational loop between GPLD-1, VO2-peak and AD diagnosis. Straight lines = correlations. Blue arrow pointing up: a high value. Red arrow pointing down = a low value.

Due to using such strict criteria for inclusion in the study there are few cases compared to how many participants were in the Generation 100 Study. However, this is also a positive feature of this study, since there are clear set criteria regarding who is and is not included. The AD cases present in this study has had their diagnosis validated by a physician. Their diagnosis has not solely been confirmed by using diagnosis codes as are seen in some other studies. The blood samples used were also taken years prior to symptom debut, the pre-clinical phase, allowing for an insight in the protein's predictive potential as compared to their reactive properties in a disease.

4.3 Limitations

It is difficult to define what a healthy control is, as they may suffer from other currently unknown ailments (56).

Using novel proteins without clear reference ranges makes it difficult comparing measured values with expected ones. In regards to GPLD-1, as mentioned earlier, there is no consensus on what is an expected range for healthy adults. This is crucial to identify, since it can mean the difference in being able to use GPLD-1 as a predictive biomarker for AD, or not. Another slight of using novel proteins is the fact that we don't fully understand their underlying regulatory mechanisms yet. There has been observed correlations with the proteins and other processes, i.e. Aβ or p-tau regulation, but how they modulate this is not yet understood. It is believed that there is no association between the APOE4 gene mutation and Klotho levels, however there remains a lack of research in regards to see if the gene mutations that increase the risk of AD changes the expression of GPLD-1, and how that may potentially affect the course of illness.

For the best results you'd want fresh samples. In this study I used sampled taken in 2012 and 2013. They were frozen for the good part of ten years and underwent 2-3 freeze/thaw cycles. What effects the long term freezer-storage and repeated freezing and thawing may have had on the results is unknown. As mentioned earlier, this thesis is a case control study. The results found here may therefore not necessarily be reflective for people that develop AD.

4.4 Future prospects

Assessing a reference range for GPLD-1 for healthy adults in serum is a critical first step to see whether we can use it as a biomarker. Further elucidation of the relationship between GPLD-1 and Klotho, and the Aβ and tau pathogenesis is necessary. Understanding this relationship may create possibilities or earlier detection and preventative medicines. Further investigating the relationship of exercise and training with the change in Klotho and GPLD-1, may grant an insight into the mechanisms of how exercise contributes to AD prevention. Looking at whether the irregular Klotho regulation is a response to AD pathogenesis or if the inverse is true. It could provide insights into what may be the first point of attack for the disease. Potential Klotho or GPLD-1 supplements in animal models, and further down the line for humans, may provide us with an advantage for potentially halting disease progression. Since the Generation100 project also has blood samples collected at later timepoints, after 3 years, 5 years, and 10 years, analysing these samples could provide further insights in Klotho and GPLD-1 regulation.

5. Conclusion

In this case control study investigating the potential of proteins, Klotho and GPLD-1, as predictive biomarkers for AD. They both showed great promise in distinct ways. The AD group had a modified regulation of Klotho over a period of 1 year when compared to healthy controls. For healthy controls one would also see a correlation with VO2-peak, but for the AD group this was not true, neither at baseline nor after one year. GPLD-1 values for the AD group were significantly lower than the healthy controls. There were also found correlations between GPLD-1 and both VO2-peak and AD diagnosis. This applied to both groups. The groundwork has been laid, and further investigation of these proteins and elucidation of their relationship with AD is imperative to early AD detection and potential treatment.

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Appendix

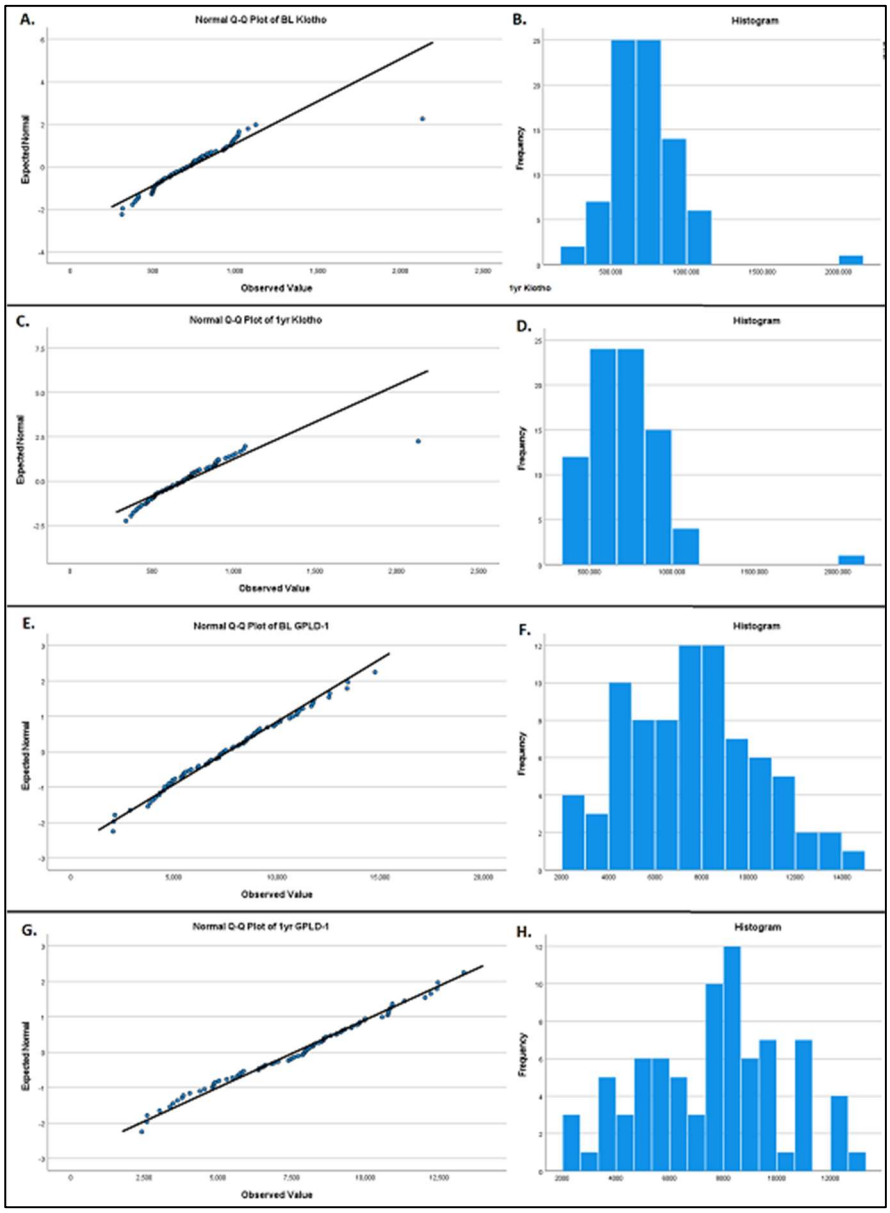


Figure A: QQ-plots and bar graphs showing that the GPLD-1 and Klotho data are approximately normally distributed. **A:** QQ-plot of BL Klotho values **B:** Bar graph showing distribution of BL KL. **C:** QQ-plot of 1yr Klotho values **D:** Bar graph showing distribution of 1yr KL. **E:** QQ-plot of BL GPLD-1 values. **F:** Bar graph showing distribution of BL GPLD-1. **G:** QQ-plot of 1yr GPLD-1 values. **H:** Bar graph showing distribution of 1yr GPLD-1.

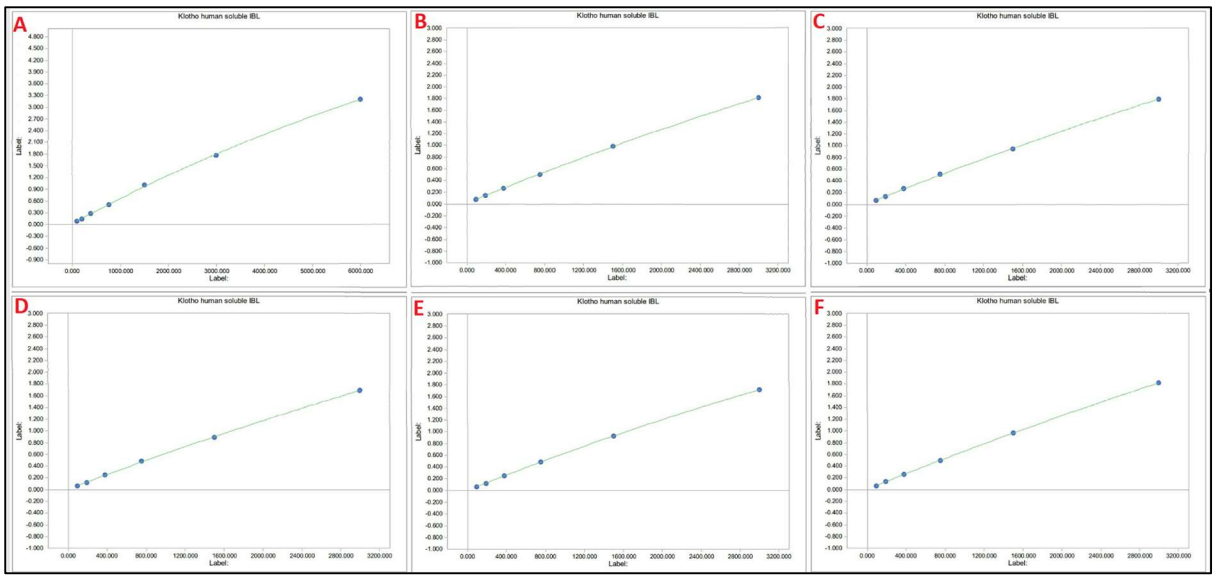


Figure B: Klotho standard curves. **A:** Standard curve for setup nr 1, sample 83-1559. **B:** Standard curve for setup nr 2, sample 1638-2969. **C:** Standard curve for setup nr 3, sample 2989-3983. **D:** Standard curve for setup nr 4, sample 4181-5469. **E:** Standard curve for setup nr 5, sample 5631-6747. **F:** Standard curve for setup nr 6, sample 6814 and all rerun samples

Setup 1		1	2	3	4	5	6
BL	A	-	STD 4	83	603	970	1351
1yr	B	-	STD 4	83	603	970	1351
BL	C	STD 1	STD 5	314	751	1072	1371
1yr	D	STD 1	STD 5	314	751	1072	1371
BL	E	STD 2	STD 6	363	781	1217	1482
1yr	F	STD 2	STD 6	363	781	1217	1482
BL	G	STD 3	STD 7	460	965	1244	1559
1yr	H	STD 3	STD 7	460	965	1244	1559

Setup 2		1	2	3	4	5	6
BL	A	-	STD 4	1638	1991	2431	2717
1yr	B	-	STD 4	1638	1991	2431	2717
BL	C	STD 1	STD 5	1673	1998	2470	2843
1yr	D	STD 1	STD 5	1673	1998	2470	2843
BL	E	STD 2	STD 6	1717	2096	2644	2931
1yr	F	STD 2	STD 6	1717	2096	2644	2931
BL	G	STD 3	STD 7	1936	2395	2689	2969
1yr	H	STD 3	STD 7	1936	2395	2689	2969

Setup 3		1	2	3	4	5	6
BL	A	-	STD 4	2989	3186	3338	3615
1yr	B	-	STD 4	2989	3186	3338	3615
BL	C	STD 1	STD 5	3078	3197	3350	3720
1yr	D	STD 1	STD 5	3078	3197	3350	3720
BL	E	STD 2	STD 6	3082	3243	3454	3966
1yr	F	STD 2	STD 6	3082	3243	3454	3966
BL	G	STD 3	STD 7	3160	3269	3587	3983
1yr	H	STD 3	STD 7	3160	3269	3587	3983

Setup 4		1	2	3	4	5	6
BL	A	-	STD 4	4181	4367	4614	5175
1yr	B	-	STD 4	4181	4367	4614	5175
BL	C	STD 1	STD 5	4184	4390	4740	5270
1yr	D	STD 1	STD 5	4184	4390	4740	5270
BL	E	STD 2	STD 6	4273	4459	5158	5317
1yr	F	STD 2	STD 6	4273	4459	5158	5317
BL	G	STD 3	STD 7	4355	4606	5160	5469
1yr	H	STD 3	STD 7	4355	4606	5160	5469

Setup 5		1	2	3	4	5	6
BL	A	-	STD 4	5631	5900	6233	6425
1yr	B	-	STD 4	5631	5900	6233	6425
BL	C	STD 1	STD 5	5666	6019	6244	6475
1yr	D	STD 1	STD 5	5666	6019	6244	6475
BL	E	STD 2	STD 6	5747	6103	6384	6724
1yr	F	STD 2	STD 6	5747	6103	6384	6724
BL	G	STD 3	STD 7	5814	6125	6386	6747
1yr	H	STD 3	STD 7	5814	6125	6386	6747

Setup 6		1	2	3	4	5	6
BL	A	-	STD 4	6814	1673	2989	4606
1yr	B	-	STD 4	6814	1673	2989	4606
BL	C	STD 1	STD 5	83	2470	3243	5175
1yr	D	STD 1	STD 5	83	2470	3243	5175
BL	E	STD 2	STD 6	314	2644	3615	6125
1yr	F	STD 2	STD 6	314	2644	3615	6125
BL	G	STD 3	STD 7	603	2931	4181	6425
1yr	H	STD 3	STD 7	603	2931	4181	6425

Re-run	AD	Standards

Figure C: Analysis setup for analysis of Klotho and GPLD-1. Columns: Numbers 1-6 reflect which strip on a half-plate. Rows: Grey rows, A, C, E, and G are the BL Gen100 samples. White rows: B, D, F, and H, are the 1yr Gen100 samples. The numbers 83-6814 are the Gen100 ID numbers for the participants to make sure they remain anonymous. Red outline: AD group. Orange background: Samples were re-run on Setup 6.

Human soluble α-Klotho Assay Kit - IBL

96 Well

Please read carefully this instruction prior you use this assay kit.

INSTRUCTIONS FOR USE

This product is for research use only and is not intended for diagnostic use.

KIT COMPONENT

1	**Precoated plate:** (Anti- Human Klotho (67G3) Mouse IgG MoAb)	96Well x 1
2	**Labeled antibody conc.:** (30X) HRP conjugated Human Klotho (91F1) Mouse IgG)	0.4mL x 1
3	**Standard:** (Recombinant human soluble α-Klotho)	0.5mL x 2
4	**EIA buffer**	30mL x 1
5	**Solution for labeled antibody**	12mL x 1
6	**Chromogen:** TMB solution	15mL x 1
7	**Stop solution**	12mL x 1
8	**Wash buffer conc.**	50mL x 1

MEASURING SAMPLES

Human serum, EDTA plasma, Heparin plasma, citrated plasma, urine and cell culture supernatant.

PRINCIPLE

This kit is a solid phase sandwich ELISA (Enzyme-linked Immunosorbent Assay). As a primary antibody is coated on a plate, samples and standard are added into the wells for 1st reaction. After the reaction, HRP-conjugated secondary antibody is added into the wells for 2nd reaction. After washing away unbound the secondary antibody, Tetra Methyl Benzidine (TMB) is added to the wells and color develops.

OPERATING PRECATION

1 Test samples should be measured soon after collection. For storage of samples, store them frozen and do not repeat freeze/thaw cycles. Thaw the test samples at a low temperature and mix them completely before measurement.
2 Test samples should be diluted with “4, EIA buffer” contained in this kit.
3 Duplicate measurement of test samples and standards is recommended.
4 Standard curve should run for each assay.
5 Use test samples in neutral pH range. The contaminations of organic solvent may affect the measurement.
6 All reagents should be brought to room temperature (R.T.) and mixed completely and gently before use. After mixing them, make sure of no change in quality of the reagents.
7 Use only “8, Wash buffer conc.” contained in this kit for washing the precoated plate. Insufficient washing may lead to the failure in measurement.
8 Using a plate washer is recommended (wait time zero second). It should be washed by a plate washer immediately after each reaction. If you use a washing bottle instead of a plate washer, after filling wash buffer in each well, immediately turn the plate upside down and shake it off to completely remove the wash buffer. Repeat the number of times of wash defined in a table for measurement procedure described in section 3. It should be properly washed off as instructed in order to avoid any insufficient wash.
9 Carefully tap the plate against a clean paper towel without contacting with inside of each well to completely remove the washing buffer after repeated the determined number of wash.
10 “6, Chromogen - TMB solution” should be stored in the dark due to its sensitivity against light. It should be also avoided contact with metals. Required quantity should be prepared into a collecting container for each use.
11 After adding TMB solution into the wells, the liquid in the wells gradually changes the color in blue. In this process the plate should be in dark. Remained TMB solution in the collecting container should not be returned into the original bottle of TMB solution to avoid contamination.
12 Measurement of O.D. should be done within 30 minutes after addition of “7, Stop solution”.

OPERATION MANUAL AND DOSAGES

1. Materials needed but not supplied.

Plate reader	Micropipette and tip
Test tubes for dilution	Measuring cylinder and beaker
Deionized water	Plate washer or washing bottle
Paper towel	Collecting container (i.e. clean disposable test tube)

2. Preparation

(1) Preparation of wash buffer
Dilute “8, Wash buffer conc.” 40 fold with deionized water. The diluted one is used for the assay as a wash buffer. Adjust the required quantities if needed.
(2) Preparation of labeled antibody
Dilute “2, Labeled antibody conc.” 30 fold with “5, Solution for labeled antibody” using a prepared collecting container.

Example)
In case you use one strip (8 well), the required quantity of Labeled antibody is 800 µL. (Dilute 30 µL of “2, Labeled antibody Conc.” with 870 µL of “5, Solution for labeled antibody” and mix it. And use 100µL the mixed solution in each well.)
This operation should be done just before applying labeled antibody.
The remaining “2, Labeled antibody Conc.” should be stored at 4°C in a firmly sealed vial.

(3) Preparation of standard
Add 0.5 mL of deionized water into the vial of “3, Standard” and completely dissolve it. Concentration of the standard is 12,000 pg/mL. The standards enclosed in this kit can be frozen and stored after reconstitution. However the freeze-thaw shall not be repeated.
Prepare 7 test tubes for dilution of the standard and adding 230 µL of the EIA buffer into each tube.

Put 230 µL of 12,000 pg/mL standard into the tube 6,000 pg/mL (Tube-1) and gently mix it. Afterword, put 230 µL of the mixed liquid of tube-1 into the tube 3,000 pg/mL (Tube-2) and gently mix it. Dilute two fold standard solution in series to set up 7 points of diluted standard between 6,000 pg/mL and 93.75 pg/mL.

Tube-1	6,000	pg/mL
Tube-2	3,000	pg/mL
Tube-3	1,500	pg/mL
Tube-4	750	pg/mL
Tube-5	375	pg/mL
Tube-6	187.5	pg/mL
Tube-7	93.75	pg/mL

(4) Preparation of test samples
Dilute test samples with “4, EIA buffer” contained in this kit as follows.
Human serum, EDTA plasma, heparin plasma, citrated plasma, urine 2~4 fold.
Cell culture supernatant: more than 2 fold.

3. Measurement Procedure

(1) Add test sample blank
Determine wells for test sample blank. Put 100µL each of “4, EIA buffer” into the wells.
(2) Add prepared test samples and standard
Put 100 µL prepared test samples and 100 µL prepared standard into appropriate wells.
(3) Incubation with plate lid (1st reaction).
(4) Washing (Refer to No. 8 and 9 described in OPERATING PRECATION.)
Wash the plate with the prepared wash buffer and remove all liquid.
(5) Add prepared labeled antibody
Put 100 µL prepared labeled antibody into the wells.
(6) Incubation with plate lid (2nd reaction).
(7) Washing (Refer to No. 8 and 9 described in OPERATING PRECATION.)
Wash the plate with the prepared wash buffer and remove all liquid completely.
(8) Add “6, Chromogen - TMB solution”
Put 100 µL the TMB solution into the wells.
(9) Incubation in dark
(10) Add “7, Stop solution”
Put 100 µL the Stop solution into the wells.
(11) Determination of optical density (O.D.)
Remove any dirt or drop of water on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, measure the both O.D. of standard and the test samples against a test sample blank.
Measurement wavelength: 450 nm. In case of 2 wavelengths:
Main wavelength is 450nm. Sub-wavelength is between 600 and 650 nm.

Table for measurement procedure

Reagents	Test Sample	Standard	Test Sample Blank	Reagent Blank
	Test sample 100 µL	Diluted standard (Tube 1-7) 100 µL	EIA buffer (Tube-8) 100 µL	EIA buffer 100 µL
Incubation for 60 minutes at room temperature with plate lid				
4 times (wash buffer more than 350 µL) (Refer to No. 8 and 9 described in OPERATING PRECATION.)				
Labeled Antibody	100 µL	100 µL	100 µL	-
Incubation for 30 minutes at room temperature with plate lid				
5 times (wash buffer more than 350 µL) (Refer to No. 8 and 9 described in OPERATING PRECATION.)				
Chromogen	100 µL	100 µL	100 µL	100 µL
Incubation for 30 minutes at room temperature (shielded)				
Stop solution	100 µL	100 µL	100 µL	100 µL
Read the plate at 450nm against a Reagent Blank within 30 minutes after addition of Stop solution.				

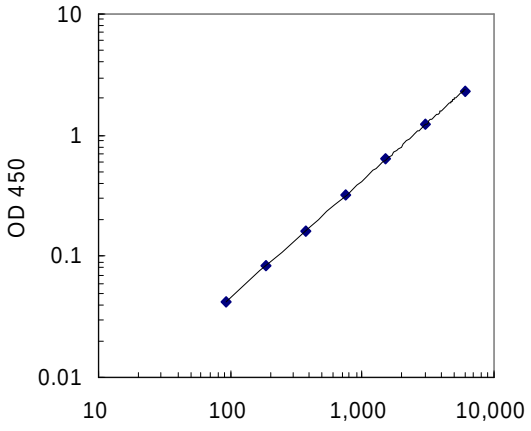
Manufacturer: Immuno-Biological Laboratories Co., Ltd.

CALCULATION OF TEST RESULT

1 Plot the concentration of the standard on the x-axis and its O.D. on the y-axis. Draw a standard curve by applying appropriate regression curve on each plot (i.e. quadratic regression of double logarithm conversion).
2 Read the concentration by applying the absorbance of the test samples on a standard curve.
3 Calculate the concentration of the test samples by multiplying dilution ratio of test samples on the value.

Example of standard curve and measured value

Conc. (pg/mL)	Absorbance (450nm)
6,000	2.307
3,000	1.245
1,500	0.635
750	0.324
375	0.166
187.5	0.087
93.75	0.046
0 (Test Sample Blank)	0.004

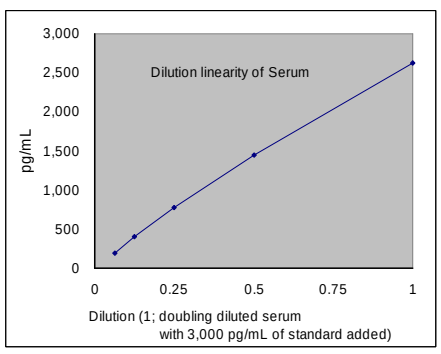


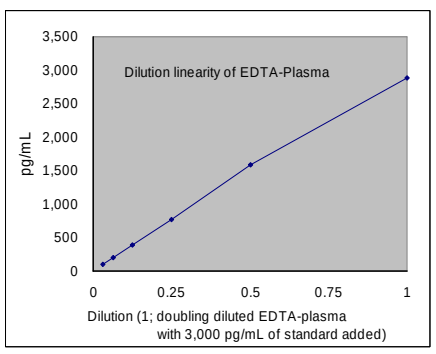
PERFORMANCE AND CHARACTERISTICS

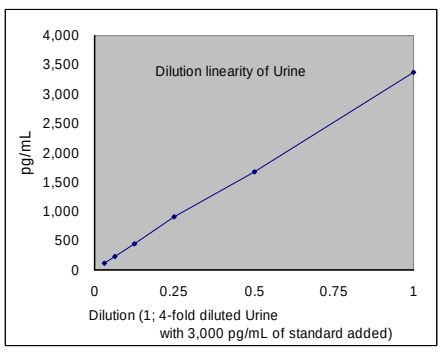
1 Sensitivity
6.15 pg/mL (Calculated by NCCLS method using the standard.)

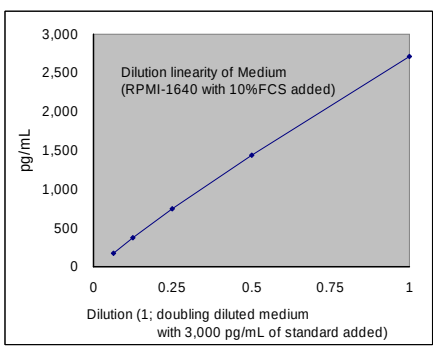
2 Measurement range
93.75 ～ 6,000 pg/mL

3 Dilution linearity









4 Added recovery assay

Specimen	Theoretical Value (pg/mL)	Measurement Value (pg/mL)	%
Human Serum (x4)	1764.53	1494.92	84.7
	1014.53	924.85	91.2
	639.53	602.29	94.2
Human Plasma (EDTA) (x4)	1770.47	1677.46	94.7
	1020.47	984.78	96.5
	645.47	629.21	97.5
Human Urine(x2)	1513.86	1490.76	98.5
	1138.86	1109.98	97.5
	951.36	929.40	97.7
10%FCS added RPMI-1640 (x2)	750.00	713.85	95.2
	375.00	355.67	94.8
	187.50	181.27	96.7

5 Intra-assay

Measurement Value (pg/mL)	SD value	CV value (%)	n
2968.78	92.26	3.1	24
757.34	20.65	2.7	24
186.64	6.62	3.5	24

6 Inter-assay

Measurement Value (pg/mL)	SD value	CV value (%)	n
2903.01	85.44	2.9	5
706.32	45.72	6.5	5
165.47	18.82	11.4	5

7 Specificity*

Compound	Cross Reactivity
Human α-Klotho	100 %
Human secreted α-Klotho	100 %
Human β-Klotho	＜0.1 %
Human osteopontin	＜0.1 %
Human VEGF (165)	＜0.1 %
Human PDGF	＜0.1 %

PRECAUTION FOR INTENDED USE AND/OR HANDLING

1 Precaution for handling (Hazard prevention)

(1) Treat the components carefully and wash hands after handling it.
(2) “7, Stop solution” is a strong acid substance (1N Sulfuric acid). Therefore, it should be careful for the treatment and do not contact your skin and clothes with it. It also needs to pay attention to the disposal of it.

2 Precaution for intended use

(1) “3, Standard” is lyophilized products. It should be careful to open this vial.
(2) All reagents should be stored at 2 - 8°C.
(3) Precipitation can be seen in “4, EIA buffer”, “5, Solution for labeled antibody” and “8, Wash buffer conc.”, however, it does not affect its performance.
(4) Do not mix or replace the reagents with the reagents from a different lot or kit.
(5) Do not use expired reagents.

3 Precaution for disposal

(1) Dispose used materials after rinsing them with large quantity of water.

STORAGE AND THE TERM OF VALIDITY

Storage Condition: 2 - 8°C
The expiry date is specified on the outer box.

PACKAGE UNIT AND PRODUCT NUMBER

Package unit: 96 Well
Product number: 27998

REFERENCE

1.Yamazaki Y, Imura A, Urakawa I, Shimada T, Murakami J, Aono Y, Hasegawa H, Yamashita T, Nakatani K, Saito Y, Okamoto N, Kurumatani N, Namba N, Kitaoka T, Ozono K, Sakai T, Hataya H, Ichikawa S, Imel EA, Econs MJ, Nabeshima Y. Establishment of sandwich ELISA for soluble alpha-Klotho measurement: Age-dependent change of soluble alpha-Klotho levels in healthy subjects. Biochem Biophys Res Commun. 2010 Jul 30;398(3):513-8.

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antibodies-online™

ABIN7062719

Sandwich ELISA Kit for Glycosylphosphatidylinositol Specific phospholipase D1

Human

5 x 96 tests

For research use only
Not for use in clinical diagnostic procedures
Version Mar 2023

Intended use

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of GPLD1 in human serum, plasma.

Reagents and materials provided

- Pre-coated, ready to use 96-well strip plate, flat buttom
- Plate sealer for 96 wells
- Reference Standard
- Standard Diluent
- Detection Reagent A
- Detection Reagent B
- Assay Diluent A
- Assay Diluent B
- Reagent Diluent (if Detection Reagent is lyophilized)
- TMB Substrate
- Stop Solution
- Wash Buffer (30 x concentrate)
- Instruction manual

Materials required but not supplied

1. Microplate reader with 450 ± 10nm filter.
2. Precision single or multi-channel pipettes and disposable tips.
3. Microcentrifuge tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution
7. Incubator capable of maintaining 37 °C.
8. 0.01mol/L (or 1×) Phosphate Buffered Saline (PBS), pH7.0-7.2.

Storage of the kit

1. For unopened kit: All reagents should be stored according to the labels on the vials. The Standard, Detection Reagent A, Detection Reagent B, and 96-well Strip Plate should be stored at -20 °C upon receipt, while the other reagents should be stored at 4 °C.
2. For opened kits: the remaining reagents must be stored according to the above storage conditions. In addition, please return the unused wells to the foil pouch containing the desiccant and seal the foil pouch with the zipper.

.

Note:

It is strongly recommended to use the remaining reagents within 1 month, if this is done before the expiry date of the kit. Please refer to the label on the kit packaging for the expiration date of the kit. All components are stable until the expiration date.

Sample collection and storage

Sample type	Collection procedure
Plasma	Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2-8°C within 30 min of collection. Collect the supernatant and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles. Hemolysed samples are not suitable for ELISA assay!
Serum	Use a serum separator tube and allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 min at 1000×g. Collect the supernatant and assay freshly prepared serum immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4 °C, otherwise samples must be stored at -20 °C (≤ 1 month) or -80 °C (≤ 2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen should not be used.
3. When performing the assay, bring samples to room temperature.

Reagent preparation

1. Bring all kit components and samples to room temperature (18-25 °C) before use. If the kit will not be used up in one time, please only take out strips and reagents for present experiment, and leave the remaining strips and reagents in required condition.
2. Standard - Reconstitute the Standard with 1.0 mL of Standard Diluent, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution is 100ng/mL. First dilute the stock solution to 50ng/mL and the diluted standard serves as the highest standard (50ng/mL). Then prepare 7 tubes containing 0.5mL Standard Diluent and use the diluted standard to produce a double dilution series. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 50ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.12ng/mL, 1.56ng/mL, 0.78ng/mL, and the last tube with Standard Diluent is the blank as 0ng/mL.
3. Detection Reagent A and Detection Reagent B - If lyophilized reconstitute the Detection Reagent A with 150μL of Reagent Diluent, keep for 10 minutes at room temperature, shake gently (not to foam). Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute them to the working concentration 100-fold with Assay Diluent A and B, respectively.
4. Wash Solution - Dilute 20 mL of Wash Solution concentrate (30x) with 580 mL of deionized or distilled water to prepare 600 mL of Wash Solution (1x).
5. TMB substrate - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standards within 15 minutes before assay. Please do not dissolve the reagents at 37 °C directly.
3. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μL for one pipetting.
4. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be used only once.
5. If crystals have formed in the Wash Solution concentrate (30x), warm to room temperature and mix gently until the crystals are completely dissolved.
6. Contaminated water or container for reagent preparation will influence the detection result.

Sample preparation

- It is recommended to use fresh samples without long storage, otherwise protein degradation and denaturationmay occur in these samples, leading to false results. Samples should therefore be stored for a short periodat 2 - 8 °C or aliquoted at -20 °C (≤1 month) or -80 °C (≤ 3 months). Repeated freeze-thawcycles should be avoided. Prior to assay, the frozen samples should be slowly thawed and centrifuged toremove precipitates.
- If the sample type is not specified in the instructions, a preliminary test is necessary to determinecompatibility with the kit.
- If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibilityof causing a deviation due to the introduced chemical substance.The recommended dilution factor is for reference only.
- Please estimate the concentration of the samples before performing the test. If the values are not in therange of the standard curve, the optimal sample dilution for the particular experiment has to be determined.Samples should then be diluted with PBS (pH =7.0-7.2).

Assay procedure

1. Determine wells for the diluted standard, blank and sample.
   Add 100µL each of the dilutions of standard (see Reagent Preparation), blank and samples to the appropriate wells. Cover with a plate sealer. Incubate for 1 hour at 37°C.
2. Remove the liquid from each well, do not wash.
3. Add 100µL of Detection Reagent A Working Solution to each well, cover the wells with a plate sealer, and incubate at 37 °C for 1 hour.
4. Aspirate the solution and add 350µL of 1x Wash Solution into each well using a squirt bottle, multichannel pipette, manifold dispenser, or automated washer and let it soak for 1-2 minutes. Remove the remaining liquid from all wells completely by tapping the plate on absorbent paper. Wash a total of 3 times. After the last wash, remove all remaining Wash Buffer by aspirating or decanting. Turn the plate over and blot it against absorbent paper.
5. Add 100µL of the Detection Reagent B Working Solution to each well, cover the wells with a Plate Sealer and incubate for 30 minutes at 37°C.
6. Repeat the aspiration/washing procedure a total of 5 times as performed in step 4.
7. Add 90µL of Substrate Solution to each well. Cover with a new Plate Sealer. Incubate for 10-20 minutes at 37°C (do not exceed 30 minutes). Protect from light. The liquid will

turn blue with the addition of Substrate Solution.

8. Add 50µL of Stop Solution to each well. The liquid turns yellow due to the addition. Mix the liquid by tapping the side of the plate. If the color change does not appear even, gently tap the plate to mix thoroughly.
9. Remove all water droplets and fingerprints from the bottom of the plate and make sure there are no bubbles on the surface of the liquid. Then run the microplate reader and immediately take a measurement at 450 nm.

Test principle

The test principle applied in this kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to target. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to target. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain target, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of target in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Calculation of results

Average the duplicate readings for each standard, control, and samples and subtract the average of the measured optical density of the zero standard. Create a standard curve by plotting the mean OD value and concentration for each standard and draw a best-fit curve through the points on the graph or create a standard curve on log-log graph paper with concentration on the y-axis and absorbance on the x-axis. The use of plotting software is also recommended. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical data

In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the log of concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. The O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects).

Detection range

The detection range of the kit is 0.78 ng/mL - 50 ng/mL
The standard curve concentrations used for the ELISA's were 50ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.12ng/mL, 1.56ng/mL, 0.78ng/mL, 0.00ng/mL

Sensitivity

The minimum detectable dose of GPLD1 is typically less than 0.35 ng/mL.
The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.
It was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

Note:
Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between GPLD1 and all the analogues, therefore, cross reaction may still exist.

Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level of target were tested 20 times on one plate, respectively.
Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level of target were tested on 3 different plates, 8 replicates in each plate.
CV(%) = SD/meanX100
Intra-Assay: CV < 10%
Inter-Assay: CV < 12%

Stability

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5 % prior to the expiration date under appropriate storage condition. To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly monitored. It is also strongly suggested that the assay is performed by the same operator from the beginning to the end.

Assay procedure summary

1. Prepare all reagents, samples and standards,

2. Add 100μL standard or sample to each well. Incubate 1 hours at 37 °C,
3. Aspirate and add 100μL prepared Detection Reagent A. Incubate 1 hour at 37 °C,
4. Aspirate and wash 3 times,
5. Add 100μL prepared Detection Reagent B. Incubate 30 minutes at 37 °C,
6. Aspirate and wash 5 times,
7. Add 90μL Substrate Solution. Incubate 10-20 minutes at 37 °C,
8. Add 50μL Stop Solution. Read at 450nm immediately.

Important note

1. The kit is designed for research use only, we will not be responsible for any issue if the kit was used in clinical diagnostic or any other procedures.
2. Limited by the current conditions and scientific technology, we cannot perform a complete identification and analysis of the raw material used. Therefore, the use of the kit may be associated with some qualitative and technical risks.
3. We are only responsible for the kit itself, not for the samples used in the test. The possible amount of sample used in the whole test should be calculated in advance and sufficient sample material should be provided.
4. Each kit undergoes a very strict QC testing. Nevertheless, end-user results may differ from our internal results due to unexpected transport conditions or different laboratory equipment. Intra-assay deviations between kits from different lots can also be related to this.
5. The test results depend on the validity of the products, so the kit should be used before the expiration date and stored according to the instructions.
6. Even the same user may obtain different results in two separate experiments. To obtain reproducible results, each step of the assay should be controlled.
7. The standard of the kit and immunogen used for antibody preparation are commonly recombinant proteins, as different fragments, expression systems, purification methods might be used in recombinant protein preparation, we can not guarantee the kit could detect recombinant protein from other companies. So, it is not recommended to use the kit for the detection of recombinant protein.
8. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by the manufacturer.
9. Protect all reagents from strong light during storage and incubation. All reagent bottle caps should be tightly closed to prevent evaporation and contamination with

microorganisms. The TMB substrate should remain colourless until it reacts with the enzyme.

10. A freshly opened ELISA plate may show a water-like substance, which is normal and does not affect the test results. Return unused wells to the foil pouch and store as before.
11. Mistakes in reagent preparation and application, as well as incorrect parameter setting for the plate reader, can lead to incorrect results. A microplate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. at a wavelength of 450 ± 10 nm is suitable for absorbance measurement. Please read the instructions carefully and set up the instrument before the experiment.
12. Do not reuse the reconstituted standard and the prepared working solutions. The unused stock solutions should be stored according to the storage conditions.

Precaution

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Troubleshooting

Problem	Possible Source	Corrective Action
Poor Standard Curve	Improper standard curve preparation	Ensure accurate operation of the dilution
	Incomplete washing and aspiration	Adequate washing and adequate aspiration
	Inaccurate Pipetting	Check and Calibrate pipettes
Poor Precision	Incomplete washing of wells	Ensure sufficient washing
	Inadequate mixing and aspiration reagents	Adequate aspiration and mixing reagents
	Reused pipette tips, containers and sealers	Change and use new pipette tips, containers and sealers
	Inaccurate Pipetting	Check and Calibrate pipettes
Low O.D Values	Inadequate reagent volumes added to wells	Calibrate pipettes and add adequate reagents

Problem	Possible Source	Corrective Action
	Incorrect incubation times	Ensure sufficient incubation times
	Incorrect incubation temperature	Reagents balanced to room temperature
	Conjugate or substrate reagent failure	Mix conjugate and substrate, color should develop immediately
	No stop solution added	Follow the assay protocol in the kit manual
	Read beyond suggested reading time	Read within the time recommended in the manual
Sample Values	Improper Sample Storage	Store the sample properly and use the fresh sample
	Improper sample collection and preparation	Take proper sample collection and preparation method
	Low quantity of analyte in samples	Resample and repeat assay

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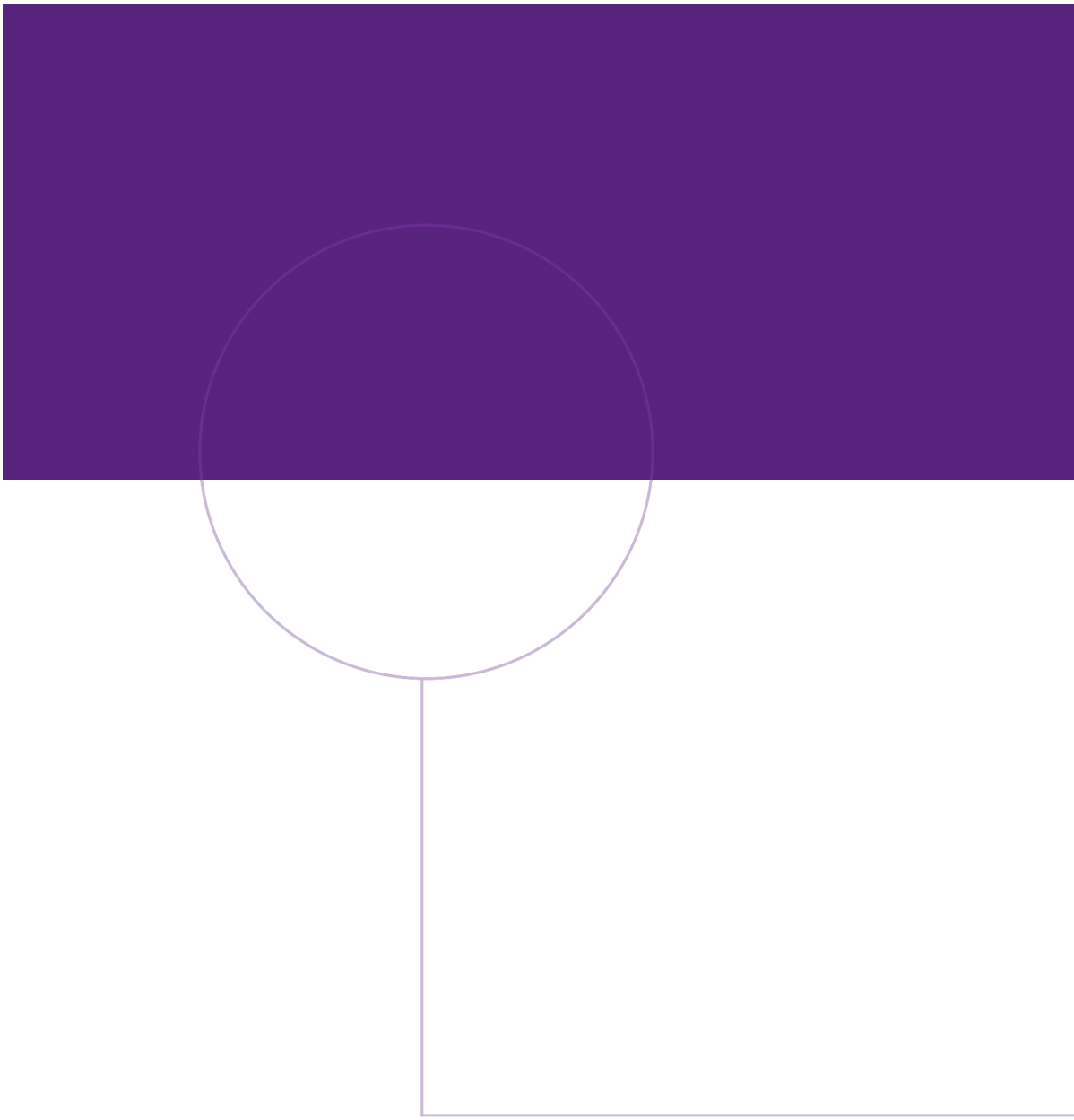

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