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Genetic variants in *KLOTHO* associate with cognitive function among the oldest old

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Running title: *KLOTHO* and cognitive function

Abstract

Decline in cognitive abilities is a major concern in aging individuals. A potential important factor for functioning of the central nervous system in late life stages is the *KLOTHO* (*KL*) gene. *KL* is expressed in various organs including the brain, and is involved in multiple biological processes e.g. growth factor signaling. In the present study, nineteen tagging gene variants in *KL* were studied in relation to two measures of cognitive function, a 5-item cognitive composite score (CCS) and the Mini Mental State Examination (MMSE), in 1,480 Danes 92-100 years of age. We found that heterozygotes for the previously reported KL-VS had poorer cognitive function than non-carriers. Two other variants positioned in the 5'end of the gene, rs398655 and rs562020, were associated with better cognitive function independently of KL-VS, and the common haplotype AG was associated with poorer cognition, consistently across two cognitive measures in two cohort strata. The haplotype effect was stronger than that of KL-VS. Two variants, rs2283368 and rs9526984 were the only variants significantly associated with cognitive decline over seven years. We discuss an age dependent effect of *KL* and the possibility that multiple gene variants in *KL* are important for cognitive function among the oldest old.

Introduction

The goddess Clotho, who in the ancient Greek mythology was known to spin the thread of life, has lend her name to a gene, which, when mutated in mice, leads to multiple disorders resembling human aging and to a shortened life span (1). In addition, genetic *klotho* reduction in mice during embryogenesis has been shown to cause early postnatal death, hypomyelination (2), synaptic attrition (3), and cognitive impairment (4), suggesting that klotho is required for brain maturation. The mouse *Klotho* gene encodes a single-pass transmembrane protein that is predominantly expressed in the choroid plexus of the brain, distal tubule cells of the kidney, and parathyroid glands (5). The extracellular domain of the klotho protein is subject to ectodomain shedding, and as a result, the Klotho protein exists in 2 forms: membrane and secreted klotho (5). The secreted protein acts as a humoral factor with pleiotropic activities in the mouse, including maintenance of vascular integrity and inflammation (6), but also suppression of both growth factor signaling and oxidative stress, the latter with homology in human cells (7). Redundant mouse/human homology was found for the soluble form of klotho, which circulates in serum and cerebrospinal fluid throughout life and declines with aging (8-11). In parallel to the putative emergence of cognitive deficits, it is possible that klotho also fulfills important functions in the central nervous system in both early and later life stages.

The human homologue of the *KLOTHO* gene (*KL*), is composed of 5 exons and covers more than 50 kb on chromosome 13q12 (1). Variants in *KL* show a large degree of pleiotropic associations including associations to carotid atherosclerosis (12), cardiovascular risk factors such as fasting glucose, lipid levels and blood pressure (13-18), kidney stones (19), and bone related conditions (20-22). Additionally, age-related changes in the frequency of *KL* gene variants have been reported in several studies (15, 18, 23-26).

There are three variants in the human *KL* gene that have been analyzed in numerous association studies. A common haplotype, KL–VS, consists of six sequence variants, two of which are located in exon 2 and result in the amino acid substitutions, F352V and C370S. Due to the presence of perfect linkage disequilibrium (LD) across the six KL-VS SNPs, the variant F352V (rs9536314) has often been used to tag the KL–VS haplotype. KL-VS influence both the functional trafficking and the catalytic activity of KLOTHO (15) and it has been associated with intelligence in individuals assessed both as tweens and as elderly (27). Recently, consistency was found in three independent cohorts of primarily Caucasians without dementia or cognitive complaints, where the KL-VS heterozygotes were found to have significantly increased cognitive Z-scores (28). Also, heterozygous carriers of the KL-VS haplotype had higher serum KLOTHO levels than non-carriers, which correlated with better cognition (28), thus supporting the relevance of KLOTHO in cognitive functioning. Another well-studied variant, C1818T (rs564481), is a synonymous variant located in the fourth exon and it is therefore not likely to be functional by itself. However, the variant may, nevertheless, be a clinically relevant marker as it has been found to associate with cardiovascular risk factors such as fasting glucose, lipid levels and blood pressure, although so far mainly in populations of Asian descent (13, 16). The third variant, G395A (rs1207568) (19, 22), is located in the promotor region and may indeed be a functional variant, since it alters the DNA-protein affinity in cultures of human kidney cells (20). The interest in these two latter mentioned genetic variants has of course been driven by their association with biological conditions, but also for their abundancy in all three major population groups

i.e. Asians, Africans and Caucasians. In contrast, the KL-VS variant is very rare in the Asian populations (20, 29, 30). To our knowledge C1818T (rs564481) and G395A (rs1207568) have not previously been reported in relation to cognitive function.

In this study, we first investigated whether the minor allele of the KL-VS variant, rs9536314 (F352V), is associated with a higher cognitive level and a slower cognitive decline in a large population of oldest old Danes. We also tested nineteen tagging variants, of which no a priory hypothesis was applied for eighteen variants in relation to cognition, thereby covering the majority of the genetic variation in *KL*. The study population comprised three birth cohorts of oldest old Danes, who were assessed by two panels of cognitive tests. In one cohort, the 1905 birth cohort, cognitive tests were assessed repeatedly with up to seven years of follow-up, and in this cohort the effect of the *KL* gene variants were studied both at a cross-sectional cognitive level and by the longitudinal change in cognition.

Materials and Methods

Subjects

The participants included in this study were drawn from three population-based nationwide surveys conducted at the University of Southern Denmark: The Danish 1905 birth cohort Study (31), the Danish 1910 birth cohort Study (unpublished data) and the Danish 1915 birth cohort Study (32). The Danish 1905 birth cohort Study is a prospective investigation of an entire Danish birth cohort. The survey was initiated in 1998, when the participants were 92-93 years old and followed by three follow-up studies of the participating survivors in 2000, 2003 and 2005. Of the 3,600 individuals still alive at intake, 2,262 participated, and 1,651 provided either a blood spot sample or a cheek swap at their first assessment in 1998. The Danish 1910 and 1915 birth cohort studies include Danes born in 1910 and 1915, respectively, who were alive and living in Denmark on September 1st 2010. Among 400 invited participants from the 1910 birth cohort study, 273 participated and 176 provided blood samples. In the 1915 birth cohort study, 2,509 individuals were identified as eligible participants when they were 95 years old, 1,584 individuals participated and 1,165 individuals provided biological samples (32). Each of the surveys in the cohort studies comprises multidimensional face-to-face interviews and assessments of cognitive and physical functioning. Written informed consents were obtained from all participants and all three surveys were approved by the The Regional Scientific Ethical Committees for Southern Denmark.

Assessment of cognitive function

Cognitive functioning was assessed by using a 5-component cognitive composite score and the Mini Mental State Examination (MMSE) (33). The 5-component cognitive composite measures were originally selected to represent tasks that are sensitive to normative age changes, and which could be reliably and briefly assessed by lay interviewers. The cross sectional decline in cognitive function was estimated to approximately two and a half standard deviation from age 45 to 90 years (34). The specific tasks included a fluency task, which involved the number of animals an individual could name in a 1-minute interval, forward and backward digit span, and immediate and delayed recall of a 12-item list. The cognitive composite score (CCS) was computed separately for each cohort by taking the sum of the five standardized measures, separately from each cohort (33). The widely used MMSE ranges from 0 to 30 and can be graded as severely impaired for scores between 0 and 17, mildly impaired for scores between 18 and 23 and normal for scores between 24 and 30.

Genotyping and quality control

DNA from 1905 birth cohort participants was either extracted from blood spots using the QIAamp DNA Mini and Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, or from whole blood using a salting out method (35).

DNA from participants in the 1910 and 1915 cohorts was extracted from dried blood spots as previously described (36).

KL genotype data for the 1905 birth cohort were drawn from a previous study investigating the known genetic variation of a large number of candidate aging-related genes (24). Tagging SNPs with a minor allele frequency of at least 5%, and covering the majority of the common variation in the coding region plus 5,000 bp upstream and 1,000 bp downstream of the gene were selected and genotyped using the Illumina GoldenGate platform (Illumina Inc, San Diego, CA, USA). Genotype data for a total of nineteen KL tagging SNPs in 1,088 individuals was available for the present study.

For the 1910 and 1915 birth cohorts, *KL* genotype data was drawn from an existing genome wide data set previously acquired using the Illumina HumanOmniExpress BeadChips (Illumina, San Diego, CA, USA) (36). Eight of the nineteen tagging SNPs in the KL region were present in this data set and passed the quality control (QC) criteria; rs398655, rs562020, rs495392, rs2283368, rs9526984, rs2320762, rs657049, and rs648202. Two additional SNPs, rs9527024 and rs683907, were used as proxies, since they were in perfect LD with for the initially selected tagging SNPs, rs9536314 and rs564481, respectively. The pair-wise LD estimates were obtained using the SNAP database (Broad institute, MIT) based on the 1000 Genomes pilot 1 data set and the CEU population panel (37). Genotype data were retrieved for a total of 405 study participants, who were all older than 96 years of age.

Statistical analyses

Statistical analyses were performed using STATA 10.1 (StataCorp, Texas, USA). The influence of *KL* variants were assessed by linear regression analysis applying an additive genetic model and using intake measures adjusted for age, sex and time (birth year from 1905 coded as 0, 5 and 10). Data were analyzed in the combined 1905, 1910, and 1915 cohort group with 1 df on genotypes recoded 0, 1 and 2, where 0 are homozygotes for the major allele, 1 are heterozygotes, and 2 are homozygotes of the minor allele. Additionally, stratified analyses were performed by including the 1905 birth cohort and the 1910-1915 cohorts independently. Both the CCS and the MMSE were analysed as continuous variables. Subsequently, the KL-VS genotypes were analysed as categorical variables to test a non-additive model. Post-hoc linear regression analyses was performed on selected SNPs using the additive genetic model with and with-out the inclusion of an interaction term. Haplotypes of rs562020 and rs398655 were analysed on the combined cohort using linear regression analyses. Interactions were studied in categories of low vs. high cognition and presence/absence of the the rs562020 and rs398655 AG haplotype and the KL-VS genotypes. Odds ratios were calculated using logistic regression adjusted for age, age and birth year.

The 1905 cohort participants were assessed by cognitive measures in 1998, 2000, 2003 and 2005. A random effects model was applied to this cohort to perform analyses of the associations between *KL* variants and both the intercept and the slope of cognitive functioning separately within the 1905 birth cohort, since this cohort was assessed repeatedly with a follow-up time of up to seven years. By using a random effects model we considered the time from

intake and the fact that some participants were assessed 2-4 times. The annual linear slope of decline was estimated for both CCS and MMSE among the 1905 cohort participants. Analyses included additive genetic modes with genotypes coded as 0, 1 and 2 as written above for regression analysis. Sex and time from intake was included as covariates. The intercept was defined as the level of the cognitive functioning at the age of 93 as described in details elsewhere (38).

Results

Study population and genotypes

The study population comprised three cohorts of oldest old born in 1905, 1910 or 1915. Descriptives of the cohorts; mean age, assessment date and mean cognitive performance CCS and MMSE are presented in Table 1. The estimated annual linear slope of decline for CCS and MMSE among the 1905 cohort is described in details elsewhere (38). Descriptives of the nineteen *KL* tagging SNPs in the 1905 birth cohort and the ten equivalent SNPs or proxy SNPs in the 1910 and 1915 birth cohorts are shown in Table 2. All gene variants, except rs397703 and rs1207362, were in Hardy-Weinberg equilibrium (HWE) ($p>0.05$).

Cross-sectional analysis of KL variants and cognitive function

Carriers of the functional KL-VS variant, as determined by either rs9536314 or the proxy rs9527024, had a poorer cognitive function than non-carriers when assessed using the MMSE (β: -0.59, p=0.046). This association was, however, only borderline significant and not reflected in the CCS (β: -0.06, p=0.74). Of note those homozygous for the KL-VS variant (N=38) had a better cognitive function than non-carriers, thus we subsequently applied a restricted model suggested by Dubal and co-workers that leaves out the homozygous KL-VS carriers from the analysis. This model was slightly better than the additive model, as heterozygous carriers of KL-VS had 1/10 of a SD poorer cognitive function in the CCS (β: -0.40, p=0.06) and 1/7 of a SD poorer cognitive function in the average MMSE (β: -0.81, p=0.02) than non-carriers.

In contrast, two variants, rs398655 and rs562020, that are located in the 5' end of the gene region were found to associate with both MMSE and CCS. The rs398655 variant is located upstream from the coding region, and carriers of the rs398655 C allele had better cognitive function than non-carriers both on the CCS (β: 0.35, p=0.008) and the MMSE (β: 0.64, p=0.003). Regarding the other variant, rs562020, which is located in the 5'end of the first intron, carriers of the A allele were found to have better cognitive function than non-carriers based on both the CCS (β: 0.30, p=0.03) and the MMSE (β: 0.54, p=0.02) (Table 3).

The effects of the rs398655 and rs562020 variants were investigated using post-hoc statistical regression models both including and leaving-out an interaction term. When repeating the analyses conditioning on the KL-VS variant, the associations between the rs398655 variant or the rs562020 variant and MMSE or CCS remained virtually unchanged, suggesting that these variants associate to cognition independently of KL-VS. Also, there was no interaction between the KL-VS variant and the variants rs398655 and rs562020. However, there was significant interaction between rs562020 and rs398655 ($p<0.004$), but the two variants are only in partial LD ($D'=0.62$, $R^2=0.27$). Thus, haplotype

analyses were carried out and as illustrated in the combined analysis (All) in Table 4 the analyses revealed that the haplotype, rs398655/rs562020 AG, i.e. the haplotype not carrying any of the minor alleles, was significantly associated with poorer CCS equivalent to 1/5 of a standard deviation (β: -0.76, p=0.001) and 1/5 of a standard deviation poorer MMSE (β: -1.10, p=0.003). Thus the haplotype effect was larger than that of KL-VS in the oldest old. Also the haplotype association was strikingly consistent as the associations remained statistical significant across two cognitive measures in the two cohort strata (Table 4). As displayed in Table 5 more individuals are cognitive impaired among carriers of the AG haplotype than non-carriers equivalent to an increased risk (Odds Ratio) of 45% for being severely impaired (MMSE <18) and an 37% higher risk of having a CCS lower then the mean (CCS <0.21) among non-carriers of the KL-VS variant (WT). The genotype restricted analyses not only illustrate the effect of the AG haplotype, but they also show that the AG haplotype is not an underlying effect of the KL-VS variant. In contrast the risk of the AG haplotype was much smaller and did not reach significance among heterozygotes KL-VS carriers.

Longitudinal analysis of KL variants and cognitive function

The effect of *KL* variants on change of cognitive functioning was studied among the 1905 birth cohort participants, who were 92-93 years of age when entering the study and were followed until their 100 year birthday. No significant associations with cognitive decline were observed for the KL-VS variant, the rs398655 variant or the rs562020 variant. However, there was a tendency for the rs398655C allele to be associated with a steeper rate of cognitive decline as compared to non-carriers (slope: 0.08, p-value: 0.08). Similarly, a more rapid cognitive decline was observed for carriers of the rs22833368 G allele compared with non-carriers with respect to the CCS (slope: -0.19, p-value: 0.008). In addition, carriers of the rs9526984 G allele declined more rapidly than non-carriers when assessed on the MMSE (slope: -0.38, p-value: 0.02) (Table 6).

Discussion

The *KL* gene has recently been proposed to be relevant for normal cognitive functioning. In the present work most of the common genetic variation in *KL* was tagged using nineteen polymorphic variants of which ten variants were studied across three cohorts of oldest old. Here we observed several associations between cognitive function and the minor alleles of gene variants in the coding region and upstream of *KL*, which indicates that *KL* may be relevant in cognitive function among the oldest old. Our most noteworthy results are the consistent association with cognition across two measures of cognitive performance, CCS and MMSE, of the upstream positioned rs398655 variant, and the rs562020 variant, situated in the far 5' end of the first intron of *KL*. Also it is noteworthy that the effect of the combined AG haplotype of these variants was larger than the effect of the most intensively studied variant in *KL*, the KL-VS variant, in the oldest old. In addition, the rs2283368 and rs9526984 variants were significantly associated with the slope of decline, but these were also restricted to only one of the two cognitive measures.

In the present work the KL-VS variant was associated with poorer cognitive performance on the MMSE scale and this was further supported when applying the Dubal and co-workers model restricted to heterozygous KL-VS variant carriers and non-carriers, which led the results in the CCS to become borderline significant. Nonetheless, these results are contrary to those found recently by Dubal and co-workers (28). We can not rule out that the results in the present work may simply be chance findings, but we must emphasize that both CCS, MMSE and Dubal and co-workers cognitive tests are combined tests that share elements of working memory in addition to specific elements i.e. speeded tasks in CCS and basic orientation tasks in MMSE. Another explanation for the difference in the two studies is that the association is age-dependent; This is supported by the notion that our observations were made among 92+ year old individuals, whereas the initially report by Dubal and co-workers was observed among middle aged and elderly individuals with a maximum age of 85 years. Secondly, Dubal and co-workers reported a potential regression toward the mean with age, a tendency that could continue to the oldest old ages thereby supporting the discordance between the two studies (28). Thirdly, an age dependent effect of the KL-VS variant has analogously been observed in longevity studies (26). Thus the KL gene variant may exemplify that a genetic effect can be age-dependent not only for mortality, but also for aging phenotypes such as cognitive function.

The novel finding that the rs562020 variant is associated with cognitive performance could be of substantial interest, since the variant is situated in a potential regulatory element. Also, rs562020 is not in LD with other variants with obviously functional effects according to the HaploReg V2 database (Broad institute, MIT). Additionally, rs562020 uniquely modifies a binding site for the zinc-finger transcriptional repressor protein, RP58, which in mice is a key regulator of neuronal migration (39). The rs398655 variant may likewise be important in gene regulation, but it seems more obvious that the association is caused by one or several other variants in LD with rs398655. Many of these variants were not investigated in the present study and are both located in enhancer elements and/or modify one or

several transcriptional binding sites. An alternative hypothesis is that rs398655 causes a missense variation, H585Q, in a yet only hypothetically predicted transcript (XM_005266617) that expectedly encodes an uncharacterized protein LOC101927403 (NCBI database). Similarly, the variant G395A (rs1207568), which is important in other age-related phenotypes, is predicted to be a missense variant, P85S, in the same transcript, but in the present work it was not found to be associated with cognition (by proxy rs397703). The complexity of the *KL* gene variants, including rs398655 and rs562020, i.e. whether they themselves are functional or whether they are proxies for functional variants, is difficult to deduce. One way of understanding the complexity is by functional studies that characterize the regulatory elements of the KL gene as well as the possible role of the hypothetical protein LOC101927403. Another inform came from our haplotype analyses that suggest a combined role of rs398655 and rs562020, since the AG haplotype, which is composed of the major alleles of the two variants, was robustly associated with a poorer cognitive level across both cognitive measures and cohort strata and even independent of KL-VS. Since the effect was even stronger than that of KL-VS we suggest that this haplotype is a risk factor for cognitive deterioration and function in the oldest old. The notion that multiple genetic variations in KL are important is supported by other genetic variants, C1818T and G395A, which were previously associated with various phenotypes.

There are several advantages to this study. First of all, we used tagging SNPs to cover the major part of the genetic variation in *KL*, and thus we were able to detect novel associations with SNPs other than the most obvious candidate SNPs, C1818T, G395A and KL-VS. Secondly, the span of cognitive function varies more at extreme old ages compared with at younger ages, and thus there is likely a gain of power by studying oldest old participants. Thirdly, it is possible that some gene variants associate more strongly with cognition in selected populations, such as the oldest old, compared to less age-selected populations. If these scenarios indeed are the reasons why we observed associations between novel *KL* variants and cognition, then our study set-up might be ideal for identifying other novel gene variants in relation to cognitive functioning.

Our study also included a longitudinal analysis enabling us to examine the genetic effect of cognitive decline at the very old ages. We did not find a consistent impact of any of the *KL* variants on the slope of cognitive decline, but a tendency towards an association between a higher cognitive level and a more rapid decline was observed for the rs398655 C allele. This is similar to an observation found for variants in the *CLU* gene, and it probably indicates a regression towards the mean. We previously suggested that this trend could be explained by individuals with a high initial cognitive level having more room for declining compared to those with a lower initial cognitive level (38). In contrast, Deary and co-workers stressed that their longitudinal cohort study showed that the KL-VS gene variant associated with intelligence similarly in early and old age, thus suggesting that the KL-VS gene variant may impact on cognition in a large span of life (27). However, their study participants were younger than participants in the present work and their findings may therefore not necessarily apply to the oldest old.

There are also several drawbacks to our study that should be mentioned. First of all, the study only includes oldest old Danes, thus our novel findings may not necessarily be generalised to younger cohorts. Also our results may only apply to participants who are within the normal range cognitively and can not necessarily be extrapolated to clinically relevant functional decline. Secondly, there may be population differences that could influence the effects of the association between *KL* variants and cognition when generalised to other populations. One such explanation could be that the KL-VS variant is common in European populations and almost non-existing in East Asian populations.

In conclusion, we suggest that several *KL* variants are important for normal-range cognitive function in the oldest old. Also, we present evidence that suggests the genetic effect of KL-VS carriers on cognitive function is dependent on the ages of the elderly individuals. Thus our results suggest age is a crucial factor both when exploring novel associations between gene variants and age-related phenotypes and when exploring known gene candidates in associations with new phenotypes.

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Reference List

1. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T*, et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature. 1997;**390**:45-51.

2. Chen CD, Sloane JA, Li H, Aytan N, Giannaris EL, Zeldich E*, et al.* The antiaging protein Klotho enhances oligodendrocyte maturation and myelination of the CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2013;**33**:1927-1939.

3. Shiozaki M, Yoshimura K, Shibata M, Koike M, Matsuura N, Uchiyama Y*, et al.* Morphological and biochemical signs of age-related neurodegenerative changes in klotho mutant mice. Neuroscience. 2008;**152**:924-941.

4. Nagai T, Yamada K, Kim HC, Kim YS, Noda Y, Imura A*, et al.* Cognition impairment in the genetic model of aging klotho gene mutant mice: a role of oxidative stress. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003;**17**:50-52.

5. Kuro-o M. Klotho and aging. Biochimica et biophysica acta. 2009;**1790**:1049-1058.

6. Shimada T, Takeshita Y, Murohara T, Sasaki K, Egami K, Shintani S*, et al.* Angiogenesis and vasculogenesis are impaired in the precocious-aging klotho mouse. Circulation. 2004;**110**:1148-1155.

7. Yamamoto M, Clark JD, Pastor JV, Gurnani P, Nandi A, Kurosu H*, et al.* Regulation of oxidative stress by the anti-aging hormone klotho. The Journal of biological chemistry. 2005;**280**:38029-38034.

8. Imura A, Iwano A, Tohyama O, Tsuji Y, Nozaki K, Hashimoto N*, et al.* Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. FEBS letters. 2004;**565**:143-147.

9. Duce JA, Podvin S, Hollander W, Kipling D, Rosene DL, Abraham CR. Gene profile analysis implicates Klotho as an important contributor to aging changes in brain white matter of the rhesus monkey. Glia. 2008;**56**:106-117.

10. Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C*, et al.* Plasma klotho and mortality risk in older community-dwelling adults. The journals of gerontology Series A, Biological sciences and medical sciences. 2011;**66**:794-800.

11. Semba RD, Moghekar AR, Hu J, Sun K, Turner R, Ferrucci L*, et al.* Klotho in the cerebrospinal fluid of adults with and without Alzheimer's disease. Neuroscience letters. 2014;**558**:37-40.

12. Oguro R, Kamide K, Kokubo Y, Shimaoka I, Congrains A, Horio T*, et al.* Association of carotid atherosclerosis with genetic polymorphisms of the klotho gene in patients with hypertension. Geriatrics & gerontology international. 2010;**10**:311-318.

13. Rhee EJ, Oh KW, Yun EJ, Jung CH, Lee WY, Kim SW*, et al.* Relationship between polymorphisms G395A in promoter and C1818T in exon 4 of the KLOTHO gene with glucose metabolism and cardiovascular risk factors in Korean women. Journal of endocrinological investigation. 2006;**29**:613-618.

14. Yamada Y, Ando F, Niino N, Shimokata H. Association of polymorphisms of the androgen receptor and klotho genes with bone mineral density in Japanese women. Journal of molecular medicine. 2005;**83**:50-57.

15. Arking DE, Krebsova A, Macek M, Sr., Macek M, Jr., Arking A, Mian IS*, et al.* Association of human aging with a functional variant of klotho. Proceedings of the National Academy of Sciences of the United States of America. 2002;**99**:856-861.

16. Shimoyama Y, Nishio K, Hamajima N, Niwa T. KLOTHO gene polymorphisms G-395A and C1818T are associated with lipid and glucose metabolism, bone mineral density and systolic blood pressure in Japanese healthy subjects. Clinica chimica acta; international journal of clinical chemistry. 2009;**406**:134-138.

17. Jo SH, Kim SG, Choi YJ, Joo NR, Cho GY, Choi SR*, et al.* KLOTHO gene polymorphism is associated with coronary artery stenosis but not with coronary calcification in a Korean population. International heart journal. 2009;**50**:23-32.

18. Arking DE, Atzmon G, Arking A, Barzilai N, Dietz HC. Association between a functional variant of the KLOTHO gene and high-density lipoprotein cholesterol, blood pressure, stroke, and longevity. Circulation research. 2005;**96**:412-418.

19. Telci D, Dogan AU, Ozbek E, Polat EC, Simsek A, Cakir SS*, et al.* KLOTHO gene polymorphism of G395A is associated with kidney stones. American journal of nephrology. 2011;**33**:337-343.

20. Kawano K, Ogata N, Chiano M, Molloy H, Kleyn P, Spector TD*, et al.* Klotho gene polymorphisms associated with bone density of aged postmenopausal women. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2002;**17**:1744-1751.

21. Zarrabeitia MT, Hernandez JL, Valero C, Zarrabeitia AL, Ortiz F, Gonzalez-Macias J*, et al.* Klotho gene polymorphism and male bone mass. Calcified tissue international. 2007;**80**:10-14.

22. Tsezou A, Furuichi T, Satra M, Makrythanasis P, Ikegawa S, Malizos KN. Association of KLOTHO gene polymorphisms with knee osteoarthritis in Greek population. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2008;**26**:1466-1470.

23. Friedman DJ, Afkarian M, Tamez H, Bhan I, Isakova T, Wolf M*, et al.* Klotho variants and chronic hemodialysis mortality. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2009;**24**:1847-1855.

24. Soerensen M, Dato S, Tan Q, Thinggaard M, Kleindorp R, Beekman M*, et al.* Human longevity and variation in GH/IGF-1/insulin signaling, DNA damage signaling and repair and pro/antioxidant pathway genes: cross sectional and longitudinal studies. Experimental gerontology. 2012;**47**:379-387.

25. Ko GJ, Lee EA, Jeon US, Pyo HJ, Chin HJ, Chae DW*, et al.* The association of Klotho polymorphism with disease progression and mortality in IgA nephropathy. Kidney & blood pressure research. 2012;**36**:191-199.

26. Invidia L, Salvioli S, Altilia S, Pierini M, Panourgia MP, Monti D*, et al.* The frequency of Klotho KL-VS polymorphism in a large Italian population, from young subjects to centenarians, suggests the presence of specific time windows for its effect. Biogerontology. 2010;**11**:67-73.

27. Deary IJ, Harris SE, Fox HC, Hayward C, Wright AF, Starr JM*, et al.* KLOTHO genotype and cognitive ability in childhood and old age in the same individuals. Neuroscience letters. 2005;**378**:22-27.

28. Dubal DB, Yokoyama JS, Zhu L, Broestl L, Worden K, Wang D*, et al.* Life extension factor klotho enhances cognition. Cell reports. 2014;**7**:1065-1076.

29. Nolan VG, Baldwin C, Ma Q, Wyszynski DF, Amirault Y, Farrell JJ*, et al.* Association of single nucleotide polymorphisms in klotho with priapism in sickle cell anaemia. British journal of haematology. 2005;**128**:266-272.

30. Zhang F, Zhai G, Kato BS, Hart DJ, Hunter D, Spector TD*, et al.* Association between KLOTHO gene and hand osteoarthritis in a female Caucasian population. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2007;**15**:624-629.

31. Nybo H, Gaist D, Jeune B, Bathum L, McGue M, Vaupel JW*, et al.* The Danish 1905 cohort: a genetic-epidemiological nationwide survey. Journal of aging and health. 2001;**13**:32-46.

32. Christensen K, Thinggaard M, Oksuzyan A, Steenstrup T, Andersen-Ranberg K, Jeune B*, et al.* Physical and cognitive functioning of people older than 90 years: a comparison of two Danish cohorts born 10 years apart. Lancet. 2013;**382**:1507-1513.

33. McGue M, Christensen K. The heritability of cognitive functioning in very old adults: evidence from Danish twins aged 75 years and older. Psychology and aging. 2001;**16**:272-280.

34. Vestergaard S, Thinggaard M, Jeune B, Vaupel JW, McGue M, Christensen K. Physical and mental decline and yet rather happy? A study of Danes aged 45 and older. Aging & mental health. 2015;**19**:400-408.

35. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic acids research. 1988;**16**:1215.

36. Deelen J, Beekman M, Uh HW, Broer L, Ayers KL, Tan Q*, et al.* Genome-wide association meta-analysis of human longevity identifies a novel locus conferring survival beyond 90 years of age. Human molecular genetics. 2014;**23**:4420-4432.

37. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI. SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics. 2008;**24**:2938-2939.

38. Mengel-From J, Thinggaard M, Lindahl-Jacobsen R, McGue M, Christensen K, Christiansen L. CLU genetic variants and cognitive decline among elderly and oldest old. PloS one. 2013;**8**:e79105.

39. Ohtaka-Maruyama C, Hirai S, Miwa A, Heng JI, Shitara H, Ishii R*, et al.* RP58 regulates the multipolar-bipolar transition of newborn neurons in the developing cerebral cortex. Cell reports. 2013;**3**:458-471.

	1905 birth cohort				1910 birth cohort	1915 birth cohort
Assessment year	1998	2000	2003	2005	2010	2010
Age, years (SD)	93.14 (0.31)	95.26 (0.31)	97.63 (0.28)	99.64 (0.29)	100.25 (0.32)	95.26 (0.29)
Number of individuals	1651	901	383	182	162	158
Completed CCS	1578	786	274	109	162	158
Mean CCS (SD)	0.23 (3.47)	0.19 (4.15)	0.21 (3.51)	0.30 (3.72)	-0.55 (3.65)	0.84 (3.48)
Completed MMSE	1583	774	292	138	159	158
Mean MMSE (SD)	21.77 (5.78)	21.18 (6.27)	20.27 (6.60)	19.86 (6.36)	21.32 (6.57)	23.70 (4.98)

Table 1: Descriptives in the 1905 birth cohort at baseline in 1998 and follow up in 2000, 2003, and 2005. The 1910 birth cohort and the 1915 birth cohort at baseline in 2010.

SNPs	Position[1]	Variant location	1905 birth cohort		1910 and 1905 birth cohorts combined	
			Major/Minor allele	MAF	Major/Minor allele	MAF
rs397703[¤]	33,587,329	Upstream gene variant	A/G	0.21	-	-
rs398655	33,587,652	Upstream gene variant XM_005266617: missense variant H585Q	A/C	0.43	A/C	0.43
rs562020	33,592,070	Intron 1 variant	G/A	0.34	G/A	0.32
rs495392	33,592,193	Intron 1 variant	C/A	0.29	C/A	0.28
rs385564	33,592,409	Intron 1 variant	C/G	0.28	-	-
rs575536	33,592,777	Intron 1 variant	G/A	0.27	-	-
rs576404	33,593,100	Intron 1 variant	C/A	0.44	-	-
rs2283368	33,593,270	Intron 1 variant	A/G	0.11	A/G	0.13
rs9526984	33,609,937	Intron 1 variant	A/G	0.09	A/G	0.08
rs1207362[¤]	33,612,839	Intron 1 variant	C/A	0.25	-	-
rs2320762	33,617,174	Intron 1 variant	A/C	0.36	A/C	0.37
rs1888057	33,622,695	Intron 1 variant	G/A	0.20	-	-
rs657049	33,622,817	Intron 1 variant	A/G	0.28	A/G	0.31
rs683907*	33,624,175	Intron 1 variant	-	-	A/G	0.41
rs687045	33,624,889	Intron 1 variant	A/G	0.44	-	-
rs9536314[#]	33,627,138	Missence F352V exon 2	A/C	0.15	-	-
rs9527024[#]	33,627,693	Intron 1 variant	-	-	G/A	0.16
rs9527026	33,628,239	Synonymous variant K385K	G/A	0.16	-	-
rs522796	33,630,055	Intron 3 variant	A/G	0.41	-	-
rs564481	33,634,983	Synonymous variant H589H exon 4	G/A	0.44	-	-
rs648202*	33,635,463	Synonymous variant A749A exon 4	G/A	0.15	G/A	0.15

Table 2: Genotype distribution and minor allele frequency (MAF) estimations for the 1905 birth cohort and combined for the 1910 and 1905 birth cohorts.

[1]Genomic position according to genome build GRCh37/hg19.on chromosome 13.

[#] Proxy for KL-VS variant

* Proxy for rs564481

¤ Not in Hardy-Weinberg equilibrium

	1905 birth cohort				1910 and 1915 birth cohorts				All			
SNP	CCS		MMSE		CCS		MMSE		CCS		MMSE	
	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.
rs397703[¤]	0.13 (0.19)	0.51	0.48 (0.31)	0.13	-	-	-	-	-	-	-	-
rs398655[¤]	*0.41* (0.15)	*0.006*	*0.77* (0.25)	*0.002*	0.13 (0.26)	0.61	0.30 (0.44)	0.50	*0.35* (0.13)	*0.008*	*0.64* (0.22)	*0.003*
rs562020	0.21 (0.16)	0.19	0.44 (0.26)	0.09	0.54 (0.28)	0.05	0.82 (0.47)	0.08	*0.30* (0.14)	*0.03*	*0.54* (0.23)	*0.02*
rs495392	0.20 (0.17)	0.24	0.50 (0.28)	0.07	0.20 (0.29)	0.49	-0.02 (0.50)	0.97	0.21 (0.15)	0.16	0.35 (0.24)	0.15
rs385564	-0.02 (0.16)	0.92	-0.67 (0.27)	0.01	-	-	-	-	-	-	-	-
rs575536	-0.07 (0.17)	0.70	0.41 (0.28)	0.14	-	-	-	-	-	-	-	-
rs576404	0.02 (0.15)	0.89	0.24 (0.24)	0.32	-	-	-	-	-	-	-	-
rs2283368	0.44 (0.23)	0.06	0.04 (0.38)	0.91	0.14 (0.37)	0.71	0.68 (0.63)	0.29	0.36 (0.20)	0.07	0.24 (0.32)	0.46
rs9526984	0.31 (0.26)	0.24	-0.14 (0.43)	0.74	-1.08 (0.46)	*0.02*	-1.52 (0.79)	0.06	-0.04 (0.23)	0.85	-0.51 (0.38)	0.18
rs1207362	-0.04 (0.17)	0.80	-0.06 (0.27)	0.84	-	-	-	-	-	-	-	-
rs2320762	0.09 (0.15)	0.56	-0.05 (0.25)	0.83	-0.62 (0.27)	*0.02*	-1.06 (0.45)	*0.02*	-0.10 (0.13)	0.46	-0.32 (0.22)	0.14
rs1888057	0.23 (0.19)	0.23	0.28 (0.32)	0.38	-	-	-	-	-	-	-	-
rs657049	0.23 (0.17)	0.18	0.11 (0.28)	0.70	-0.26 (0.28)	0.35	-0.43 (0.47)	0.36	0.10 (0.15)	0.50	-0.05 (0.24)	0.84
rs687045	-0.15 (0.15)	0.32	-0.08 (0.25)	0.75	-	-	-	-	-	-	-	-
rs9536314[#] /rs9527024	0.07 (0.21)	0.75	-0.64 (0.34)	0.06	-0.40 (0.34)	0.24	-0.47 (0.59)	0.42	-0.06 (0.18)	0.74	*-0.59* (0.30)	*0.046*
rs9527026	0.07 (0.21)	0.75	-0.48 (0.34)	0.16	-	-	-	-	-	-	-	-
rs522796	0.07 (0.15)	0.65	0.31 (0.25)	0.22	-	-	-	-	-	-	-	-
rs564481/rs683907	-0.13 (0.15)	0.36	-0.01 (0.25)	0.97	0.49 (0.26)	0.06	1.05 (0.46)	*0.02*	0.02 (0.13)	0.86	0.28 (0.22)	0.21
rs648202	0.29 (0.22)	0.17	0.79 (0.35)	0.03	-0.17 (0.39)	0.66	-0.48 (0.66)	0.47	0.19 (0.19)	0.33	0.46 (0.31)	0.14

Table 3: Cross-sectional association study of *KL* gene variants and CCS and MMSE at intake of the participants in the 1905, 1910 and 1915 birth cohorts.

Analyses were conducted using a linear regression model and a genetic additive model adjusted for age, sex and time (year of birth). Significant results are in italic ($p<0.05$). se = Standard error

¤ Upstream of the KL gene

Tagging the KL-VS variant

		1905 birth cohort				1910 and 1915 birth cohorts				All			
Haplotypes	Estimated frequency	CCS		MMSE		CCS		MMSE		CCS		MMSE	
		Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.*	Coef. (se)	p-val.*
AG	0.50	*-0.63* (0.26)	*0.01*	*-0.95* (0.42)	*0.03*	*-1.08* (0.44)	*0.01*	*-1.48* (0.75)	*0.049*	*-0.76* (0.22)	*0.001*	*-1.10* (0.37)	*0.003*
AA	0.08	-0.19 (0.22)	0.38	-0.10 (0.36)	0.78	-0.55 (0.39)	0.15	-0.41 (0.65)	0.53	-0.28 (0.19)	0.13	-0.19 (0.31)	0.54
CG	0.16	0.22 (0.22)	0.32	0.59 (0.35)	0.10	*-1.08* (0.37)	*0.003*	*-1.28* (0.63)	*0.04*	-0.12 (0.19)	0.51	0.07 (0.31)	0.83
CA	0.27	0.14 (0.21)	0.51	0.42 (0.35)	0.23	0.08 (0.37)	0.82	0.25 (0.63)	0.69	0.13 (0.19)	0.48	0.36 (0.31)	0.24

Table 4: Cross-sectional haplotype study with CCS and MMSE at intake of the participants in the 1905, 1910 and 1915 birth cohort. Haplotypes were estimated from rs398655 and rs562020.

Analyses were conducted using a linear regression model and a model adjusted for age, sex and time (year of birth). Significant results are in italic ($p<0.05$). Standard errors are abbreviated se

	Haplotype AG absent % severely impaired (N)	Haplotype AG present % severely impaired (N)	OR (95% CI)
WT	15 % (275)	20 % (772)	1.45 (1.00-2.13)
Heterozygous KL-VS	22 % (25)	22 % (314)	1.05 (0.49-2.25)
Homozygous KL-VS	0 % (3)	18 % (34)	
OR (95% CI)	1.72 (0.78-3.80)	1.21 (0.88-1.67)	

	Haplotype AG absent % with low cognition on the CCS (N)	Haplotype AG present % with low cognition on the CCS (N)	OR (95% CI)
WT	42 % (275)	49 % (772)	1.37 (1.03-1.80)
Heterozygous KL-VS	53 % (25)	50 % (314)	0.88 (0.46-1.66)
Homozygous KL-VS	0 % (3)	32 % (34)	
OR (95% CI)	1.61 (0.85-3.03)	1.05 (0.80-1.36)	

Table 5: Summery statistics of the proportion of cognitive impaired participants in the combined cohort. The upper table displays the percentage of severely impaired individuals (MMSE <18), and the lower table display the percentage of individuals with a cognition (CCS) lower than the mean (CCS less than 0.21). Total numbers are in brackets. The groups are stratified by the presence/absence of the AG, rs398655 and rs562020 haplotype and by the KL-VS genotype. Homozygous KL-VS are not included in Odds Ratio (OR) calculations. OR are adjusted for sex, age and birth year and presented in columns or rows with reference to the KL Wild Type (WT) or absence of the AG haplotype.

SNP	CCS Baseline		CCS Slope		MMSE Baseline		MMSE Slope	
	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.	Coef. (se)	p-val.
rs397703¤	0.10 (0.19)	0.60	-0.11 (0.06)	0.07	0.47 (0.31)	0.14	-0.17 (0.12)	0.14
rs398655¤	*0.41* (0.15)	*0.007*	-0.08 (0.05)	0.08	*0.80* (0.25)	*0.001*	-0.08 (0.09)	0.40
rs562020	0.17 (0.16)	0.29	-0.04 (0.05)	0.46	0.42 (0.26)	0.11	0.10 (0.10)	0.32
rs495392	0.14 (0.17)	0.41	-0.08 (0.06)	0.13	0.54 (0.28)	0.05	-0.07 (0.10)	0.48
rs385564	-0.04 (0.17)	0.80	-0.05 (0.05)	0.41	*-0.68* (0.27)	*0.01*	0.03 (0.10)	0.82
rs575536	0.03 (0.17)	0.84	0.08 (0.05)	0.16	0.44 (0.28)	0.12	-0.04 (0.10)	0.67
rs576404	-0.06 (0.15)	0.70	0.01 (0.05)	0.98	0.22 (0.24)	0.37	0.01 (0.09)	0.91
rs2283368	0.35 (0.24)	0.14	*-0.19* (0.07)	*0.008*	0.03 (0.38)	0.94	0.03 (0.14)	0.81
rs9526984	0.24 (0.27)	0.38	-0.14 (0.09)	0.10	-0.01 (0.43)	0.99	*-0.38* (0.16)	*0.02*
rs1207362	-0.02 (0.17)	0.91	0.08 (0.05)	0.13	-0.08 (0.27)	0.78	0.10 (0.10)	0.30
rs2320762	0.08 (0.15)	0.61	0.03 (0.05)	0.57	-0.02 (0.25)	0.94	-0.09 (0.09)	0.31
rs1888057	0.30 (0.20)	0.13	-0.09 (0.07)	0.18	0.24 (0.32)	0.46	0.08 (0.12)	0.53
rs657049	0.28 (0.17)	0.11	-0.10 (0.06)	0.09	0.13 (0.28)	0.65	-0.05 (0.10)	0.61
rs687045	-0.22 (0.15)	0.15	0.03 (0.05)	0.52	-0.11 (0.25)	0.65	0.08 (0.10)	0.39
rs9536314#	-0.01 (0.21)	0.99	-0.11 (0.07)	0.10	-0.62 (0.34)	0.07	-0.12 (0.13)	0.33
rs9527026	-0.03 (0.21)	0.90	-0.10 (0.07)	0.13	-0.51 (0.34)	0.13	-0.15 (0.13)	0.23
rs522796	0.16 (0.15)	0.29	0.04 (0.05)	0.39	0.35 (0.25)	0.16	-0.02 (0.09)	0.87
rs564481	-0.19 (0.15)	0.22	0.04 (0.05)	0.45	-0.03 (0.25)	0.90	0.09 (0.09)	0.34
rs648202	0.41 (0.22)	0.06	-0.05 (0.08)	0.48	*0.73* (0.35)	*0.04*	0.04 (0.13)	0.79

Table 6: The longitudinal association study of *KL* gene variants associated with CCS and MMSE by random effect models at intercept 93 years of age and yearly decline (slope) for participants in the 1905 birth cohort. Analyses were adjusted for sex and time from intake. Standard errors are abbreviated se. Significant results are in italic ($p<0.05$).

¤ Upstream of the KL gene

KL-VS variant