Association Between a Functional Variant of the KLOTHO Gene and High-Density Lipoprotein Cholesterol, Blood Pressure, Stroke, and Longevity

Dan E. Arking, Gil Atzmon, Albert Arking, Nir Barzilai, Harry C. Dietz

Abstract—We previously identified a functional variant of KLOTHO, termed KL-VS, that is associated with human aging and early-onset occult coronary artery disease. Here, we determine whether the KL-VS allele influences cardiovascular disease risk factors, cardiovascular events, and ultimately, mortality. A total of 525 Ashkenazi Jews composed of 216 probands (age ≥95 years) and 309 unrelated individuals (ages 51 to 94) were genotyped for the KL-VS allele. In concordance with our previous data in Czech individuals (age ≥79; P<0.01), a heterozygous advantage for longevity was observed for individuals ≥79 years of age (P<0.004). Combined analysis indicates a 1.57-fold (95% CI, 1.23 to 1.98) increased odds ratio (OR) for 5-year survival in two independent populations (P<0.0002). Cardiovascular disease risk factors were assessed through multivariate regression analysis, demonstrating that high-density lipoprotein cholesterol (HDL-C; P<0.05) and systolic blood pressure (SBP; P<0.008) are associated with KL-VS genotype. History of vascular events was analyzed using logistic regression, indicating that after adjustment for traditional risk factors, heterozygous individuals were at significantly lower risk for stroke than wild-type individuals (OR, 5.88; 95% CI, 1.18 to 29.41), whereas homozygous KL-VS individuals had the highest risk (OR, 30.65; 95% CI, 2.55 to 368.00). Similarly, prospective analysis of mortality in probands using Cox regression indicates that wild-type individuals have a 2.15-fold (95% CI, 1.18 to 3.91) and homozygous KL-VS individuals a 4.49-fold (95% CI, 1.35 to 14.97) increase in relative risk for mortality after adjusting for potential confounders. Thus, cross-sectional and prospective studies confirm a genetic model in which the KL-VS allele confers a heterozygous advantage in conjunction with a marked homozygous disadvantage for HDL-C levels, SBP, stroke, and longevity. (Circ Res. 2005;96:412-418.)

Key Words: aging ■ cardiovascular genomics ■ genetics ■ lipids ■ hypertension

A role for KLOTHO (MIM 604824) in human aging and cardiovascular disease (CVD) was postulated initially on the basis of the observation that Klotho-deficient mice manifest a syndrome resembling accelerated human aging and display extensive and accelerated arteriosclerosis.1 Additionally, they exhibit impaired endothelium-dependent vasodilation2 and impaired angiogenesis,3 suggesting that klotho protein may protect the cardiovascular system through endothelium-derived NO production. Subsequent studies in humans have identified a functional variant of KLOTHO, termed KL-VS, that has been associated with longevity4 and early-onset occult coronary artery disease (CAD).5 This allele is common in the general population (frequency =0.16) and harbors three mutations in the coding region, of which one is silent, and two code for missense mutations F352V and C370S, which alter klotho metabolism.

Recent work has demonstrated that klotho is a novel β-glucuronidase capable of hydrolyzing steroid β-glucuronides.6 Klotho has been shown to be a circulating factor detectable in serum that declines with age,7 although recent evidence suggests that klotho may also act cell-autonomously.8 Although the specific biological targets of klotho have not been identified, klotho likely plays a role in calcium homeostasis,9 and klotho levels have been demonstrated to alter gene expression of angiotensin-converting enzyme (MIM 106180)10 and plasminogen activator inhibitor type 1 (PAI-1 [MIM 173360]),11 genes involved in CVD.12,13

In the present population-based study of 525 Ashkenazi Jews, we used cross-sectional methods to examine the effect of the KL-VS allele on longevity, cardiovascular risk factors, and cardiovascular events. In an elderly subset of this population (age ≥95 years; n=216), we were able to perform prospective studies for the effect of the KL-VS allele on mortality.
Materials and Methods

Study Populations

The Czech subjects represent an expanded cohort from a study described previously and consists of 435 elderly individuals (≥75 years) drawn at random from the Bohemian Czech population.14 Ashkenazi Jews were recruited as described previously.15,16 Briefly, 216 probands with exceptional longevity (95 to 109 years of age) were recruited to participate in the study. Probands were required to be living independently at 95 years of age as a reflection of good health, although at the time of recruitment, they could be at any institution or level of dependency. Additional samples consisted of spouses of the offspring and age-matched Ashkenazi Jewish individuals recruited from the Einstein Aging Study,17 for a total of 309 subjects (51 to 94 years of age). Informed written consent was obtained in accordance with the policy of the committee on clinical investigations of the Albert Einstein College of Medicine.

Clinical Assessment

Clinical evaluation was performed as described previously.15,16 Lipids analysis was performed by standard automated methods at the clinical laboratories of Montefiore Medical Center. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) subclass levels and mean particle sizes were determined for all participants by NMR spectroscopy at Liposcience, Inc.

Genotyping

Genotyping for the KL-VS allele was performed as described previously.5 Briefly, DNA was amplified using polymerase chain reaction and then digested with Mae III (Roche). The KL-VS allele was characterized by diagnostic Mae III restriction fragments of 265 and 185 bp. Selected genotypes were verified by Applied Biosystems TaqMan assays (primer sequences available on request).

Statistical Methods

Statistical analyses were performed using SPSS version 11.0. For association with age, KL-VS allele frequencies were plotted as 5-year averages in 1-year increments. Data points with <30 samples were not plotted. Significance for trend was determined using logistic regression with KL-VS allele status (heterozygous versus all others) as the dependent variable and age as the independent variable. For the Ashkenazi cohort, only individuals aged 63 to 104 years were included for this analysis, and for Czech, only individuals aged 75 to 94. These age cutoffs were chosen so that there was no missing data for a given age. The age cutoffs for the uprend and downtrend in each population were selected to maximize effect and statistical significance. Combined analysis included a term in the logistic regression model to account for allele frequency differences between the populations.

Univariate analyses were assessed by ANOVA for continuous variables and by the χ² test for dichotomous variables. Multivariate linear regression was performed for CVD risk factors with suggestive univariate associations (P<0.10). Because the data did not fit traditional genetic models, genotypes were treated as independent variables. In addition to traditional CVD risk factors, a term for recruitment status (proband versus control) was included to account for any differences attributable to sample collection. The overall significance of the KL-VS allele, Probust, was determined by comparing the explained variance, R², before and after inclusion of the KL-VS allele terms. Multivariate analysis of history of stroke was analyzed using multiple logistic regression with independent variables for each KL-VS genotype. Overall significance, Pprobust, was calculated by comparing log-likelihood ratios before and after inclusion of the KL-VS allele terms into the model.

Mortality was analyzed using Cox regression survival analysis, with survival measured in 1-year increments. Independent variables and calculations of overall significance were identical to those used for stroke.

Results

KL-VS Genotype Frequencies and Their Association With Age

The KL-VS allele was genotyped and found to be in Hardy–Weinberg equilibrium (P=0.38) in the entire Ashkenazi cohort, as well as when stratified by proband status (P>0.15). No genotype frequency differences were observed between males and females in the entire Ashkenazi cohort (P>0.59) nor in the probands alone (P>0.90). Figure 1 displays the 5-year average frequency of heterozygous individuals plotted in 1-year increments. For a comparison, we plotted 5-year moving averages for Czech elderly individuals reported previously to exhibit a heterozygous advantage for longevity.4 In Ashkenazi and Czech populations, a significant increase in the frequency of KL-VS heterozygous individuals is observed for ages ≥79 years (P<0.004 and P<0.01, respec-

![Figure 1. Frequency of KL-VS heterozygous individuals plotted as 5-year averages in 1-year increments. Blue line represents Ashkenazi samples. Red line represents Czech samples.](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.116.310524)
When the Ashkenazi cohort is restricted to the same age range as the Czech (79 to 94 years), the heterozygous advantage is still significant ($P < 0.007$), and the magnitude of the effect, as indicated by the estimates, is remarkably similar (0.092 versus 0.034).

Combining these two data sets, we find a 1.094-fold (95% CI, 1.043 to 1.146) increased odds ratio (OR) for survival per year for heterozygous individuals from age 79 to 94 years in two independent populations. This corresponds to a 1.57-fold (95% CI, 1.23 to 1.98) increased OR for 5-year survival ($P = 0.0002$).

In the Ashkenazi population, a decrease in the frequency of heterozygous individuals between the ages of 63 to 81 years is observed (OR, 0.908; 95% CI, 0.852 to 0.968; $P = 0.003$). Although not noted previously, the Czech population shows a similar decrease in frequency of heterozygous individuals 75 to 81 years of age (OR, 0.845; 95% CI, 0.695 to 1.028; $P = 0.092$). When the Ashkenazi cohort was restricted to the same age range (75 to 81 years), the negative trend for frequency of heterozygous individuals was not statistically significant ($P = 0.69$), nor was the trend significant in the combined data set ($P = 0.11$).

### Analysis of CVD Risk Factors

Previous results have indicated that the KL-VS allele influences the risk for early-onset CAD, and therefore, we examined whether KL-VS allele genotype is associated with CVD risk factors. Table 2 displays CVD risk factors stratified by KL-VS genotype. Univariate analysis indicates that systolic blood pressure (SBP) levels were significantly associated with KL-VS genotype ($P < 0.001$), and suggestive results ($P < 0.10$) were seen for LDL-C, HDL-C, and DBP. Figure 2 indicates that for each of these measurements, the heterozygous individuals have the best CVD risk profile and homozygous KL-VS individuals the worst. These data do not fit traditional genetic models, and thus, linear regression was performed for each of these CVD risk factors, with each genotype entered as an independent variable (Table 3). HDL-C was associated significantly with KL-VS genotype after adjusting for potential confounders in a multivariate analysis ($P = 0.050$), with a decrease of 3.47 mg/dL (95% CI, 7.19 to 0.24) and 9.80 mg/dL (95% CI, 19.05 to 0.54) of HDL-C for homozygous wild-type and homozygous KL-VS individuals, respectively, relative to heterozygous individuals. In contrast, KL-VS allele status was not significantly associated with LDL-C levels after adjustment for potential confounders ($P = 0.28$).

SBP was significantly associated with KL-VS allele status after adjusting for potential confounders ($P < 0.008$), whereas suggestive data were seen for diastolic blood pressure (DBP; $P < 0.089$; Table 3). Individuals heterozygous for the KL-VS
allele had lower SBP (6.51 mm Hg; 95% CI, 0.97 to 12.05) and DBP (2.57 mm Hg; 95% CI, −0.05 to 5.19) than wild-type individuals. Remarkably, homozygosity for the KL-VS allele was associated with an increase of 18.97 mm Hg (95% CI, 5.18 to 32.77) in SBP relative to heterozygous individuals.

Medication data were available for 50% of our samples, with 33% taking cholesterol-lowering medication and 50% taking antihypertensives. To test for potential confounding attributable to medication, we substituted pulse pressure (defined as SBP-DBP) for SBP as the outcome in our regression analysis because this measure is robust to the effects of blood pressure medication18 and does not result in a loss of sample size. The overall effect of KL-VS genotype was significant (P = 0.039), again exhibiting a heterozygous advantage in conjunction with a homozygous disadvantage for the KL-VS allele. To examine the effects of cholesterol-lowering medication, we added a term into the regression model for medication status, and although the sample size was significantly reduced, the effect sizes for individuals homozygous for the wild-type or KL-VS alleles were increased, indicating that any potential confounding attributable to medication is more likely to lead to a false-negative result rather than a false-positive result.

Because recent work has demonstrated the importance of lipid particle size in CAD and longevity, we examined whether HDL-C and LDL-C particle size are associated with KL-VS allele status. Univariate regression analysis indicates that KL-VS allele status is a significant predictor of LDL-C particle size (P = 0.030) and HDL-C particle size (P = 0.023); however, in multivariate analyses, KL-VS allele status was not a significant predictor of LDL-C particle size (P = 0.17) or HDL-C particle size (P = 0.75; data not shown).

Table 3. Multivariate Linear Regression Analysis for Cardiovascular Risk Factors

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>P_value†</th>
<th>Genotype</th>
<th>β‡</th>
<th>SE</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C, mg/dL</td>
<td>0.28</td>
<td>FF</td>
<td>0.01</td>
<td>4.10</td>
<td>1.0</td>
<td>−8.06 to 8.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>14.82</td>
<td>10.18</td>
<td>0.15</td>
<td>−5.22 to 34.87</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>FF</td>
<td>−3.47</td>
<td>1.89</td>
<td>0.067</td>
<td>−7.19 to 0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>−9.80</td>
<td>4.70</td>
<td>0.038</td>
<td>−19.05 to −0.54</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>FF</td>
<td>6.51</td>
<td>2.82</td>
<td>0.022</td>
<td>0.97 to 12.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>18.97</td>
<td>7.01</td>
<td>0.007</td>
<td>5.19 to 32.77</td>
</tr>
<tr>
<td></td>
<td>0.089</td>
<td>FF</td>
<td>2.57</td>
<td>1.33</td>
<td>0.055</td>
<td>−0.05 to 5.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>5.15</td>
<td>3.32</td>
<td>0.12</td>
<td>−1.38 to 11.68</td>
</tr>
</tbody>
</table>

*Model is adjusted for age, sex, body mass index, LDL-C, HDL-C, triglycerides, SBP, DBP, and proband status, with the dependent variable excluded from each specific model. SBP and DBP are excluded from the analysis of blood pressure measurements.
†P_value refers to the overall significance of the KL-VS allele (see Material and Methods); ‡homozygous wild-type (FF) and KL-VS (VV) individuals are compared with heterozygous individuals (FV).

Analysis of CVD Events

A subset of samples had information on history of CVD events. A total of 11.1% reported having had a stroke (30 of 271) and 13.4% a myocardial infarct (MI) (39 of 292). Figure 3 depicts event data stratified by KL-VS genotype. No effect of KL-VS allele status on MI was observed (P = 0.79); however, KL-VS allele status was associated significantly with incidence of stroke (P = 0.026). Similar to the CVD risk factor data, heterozygous individuals have the fewest stroke events and homozygous KL-VS the most. Multiple logistic regression was performed incorporating traditional CVD risk factors, demonstrating that KL-VS allele status is an independent predictor of stroke (P = 0.009; Table 4). Wild-type individuals had a 5.88-fold (95% CI, 1.18 to 29.41) increased OR for stroke and homozygous KL-VS individuals a 30.65-fold (95% CI, 2.55 to 368.00) increased OR for stroke, relative to heterozygous individuals.

Prospective Analysis of Survival in Probands

Probands were recruited from 1998 to 2002 and were followed prospectively for mortality status; as of January 2004, 96 of 216 (44.4%) had died. Univariate logistic regression indicates that KL-VS allele status is a significant predictor of mortality (P = 0.016), and similar to the other phenotypes...
examined, heterozygous individuals have the lowest mortality and homozygous KL-VS individuals the highest (Figure 4). We performed Cox regression survival analysis, adjusting for age, sex, body mass index, SBP, DBP, HDL-C, LDL-C, and triglycerides, indicating that KL-VS allele status is associated significantly with mortality after adjusting for potential confounders (P < 0.01). Wild-type individuals had a 2.15-fold (95% CI, 1.18 to 3.91) increase in relative risk (RR) for mortality relative to heterozygous individuals. Homozygous KL-VS individuals displayed a 4.49-fold (95% CI, 1.35 to 14.97) increased RR compared with heterozygous individuals. These data strongly support the cross-sectional analyses indicating a heterozygous advantage for survival late in life.

**Discussion**

Survival is a product of the interaction between environmental and genetic effects, and mortality is determined through numerous mechanisms such as infectious disease, cancer, and CVD. In previous studies of the impact of *KLOTHO* on human longevity, we had identified a functional variant, KL-VS, for which homozygosity was markedly reduced in the elderly relative to infants, and in the Czech population, showed a heterozygous advantage late in life. Analysis of the frequency of KL-VS heterozygous individuals as a function of age in the current cohort of 525 Ashkenazi Jews demonstrated remarkable concordance with the initial study in Czechs, exhibiting a heterozygous advantage for ages ≥79 years. A combined analysis was performed for overlapping age ranges, demonstrating a 1.57-fold (95% CI, 1.23 to 1.98) increased OR for survival in two independent populations. No significant trend was observed for homozygous KL-VS individuals (data not shown), largely attributable to small sample size. However, the prospective analysis of mortality (discussed below) confirms the homozygous KL-VS disadvantage for survival.

The age range of the Czech data set did not allow for analysis of ages <75 years, and although a trend for a heterozygous disadvantage for survival was observed for ages 75 to 81 years, it was not statistically significant. The Ashkenazi cohort spanned a greater age range and exhibited a marked decrease in the frequency of heterozygous individuals 63 to 81 years of age. This apparent change in the effect of the KL-VS allele on mortality is consistent in both populations and raises questions as to the mechanism through which klotho exerts its effects on survival. It is possible that the KL-VS allele may increase the risk for causes of mortality earlier in life, such as cancer, but in later life, reduce the risk for CVD. Interestingly, the transition age at which the leading cause of mortality in Caucasian Americans switches between cancer and heart disease is ≈80 years. An alternate hypothesis is that those who have survived to age 80 years with the detrimental KL-VS allele have faced a stronger survival selection and are thus representative of a more robust cohort. However, if this were the case, one would expect that those who survive to age 80 years with two copies of the KL-VS allele would also exhibit this robust phenotype. Instead, these individuals have lower HDL-C, higher SBP, and the highest RR for mortality, as measured prospectively in the proband cohort.

The association of the KL-VS allele with the CVD risk factors SBP and HDL-C is particularly intriguing in light of previous work demonstrating that the effect of the KL-VS allele on early-onset occult CAD is modified by these two factors. A recent genome scan for quantitative trait loci influencing HDL-C concentration identified suggestive linkage (LOD, 2.36), with a marker 360 kb 3′ of *KLOTHO*, providing further evidence for a role for klotho in influencing HDL-C levels. The mechanism through which klotho affects these phenotypes remains to be elucidated, although several studies have already implicated a role for klotho in the

**TABLE 4. Multivariate Regression Analysis for Cardiovascular Events**

<table>
<thead>
<tr>
<th>Event</th>
<th>P_{mult}†</th>
<th>Genotype</th>
<th>Beta‡</th>
<th>SE</th>
<th>P_{β}</th>
<th>OR/RR§</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke</td>
<td>0.009</td>
<td>FF</td>
<td>1.77</td>
<td>0.82</td>
<td>0.031</td>
<td>5.88</td>
<td>1.18 to 29.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>3.42</td>
<td>1.27</td>
<td>0.007</td>
<td>30.65</td>
<td>2.55 to 368.00</td>
</tr>
<tr>
<td>Mortality</td>
<td>0.010</td>
<td>FF</td>
<td>0.77</td>
<td>0.31</td>
<td>0.012</td>
<td>2.15</td>
<td>1.18 to 3.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W</td>
<td>1.50</td>
<td>0.62</td>
<td>0.015</td>
<td>4.49</td>
<td>1.35 to 14.97</td>
</tr>
</tbody>
</table>

*Model is adjusted for age, sex, body mass index, LDL-C, HDL-C, triglycerides, SBP, DBP, genotype status.
†P_{mult} refers to the overall significance of the KL-VS allele (see Materials and Methods).
‡Homozygous wild-type (FF) and KL-VS (VV) individuals are compared with heterozygous individuals (FV); §mortality data are prospective and so RRs are calculated.

**Figure 4.** Mortality analysis of Ashkenazi probands stratified by KL-VS genotype. P value indicates overall significance of the KL-VS allele as determined by the χ² test.
renin-angiotensin system,\textsuperscript{10,21,22} as well as in the NO production pathway.\textsuperscript{2,3}

Analysis of the history of cardiovascular events revealed an association between KL-VS genotype and stroke. Although this result may be anticipated on the basis of the effect of klotho on SBP, a known risk factor for stroke,\textsuperscript{23} in a multivariate model adjusting for blood pressure, the KL-VS was still a strong predictor of stroke. Wild-type individuals had a 5.88-fold (95% CI, 1.18 to 29.41) and homozygous KL-VS individuals a 30.65-fold (95% CI, 2.55 to 368.00) increased OR for stroke, indicating an effect of the KL-VS allele on stroke independent of its effect on blood pressure. A possible explanation for this result stems from the observation that klotho deficiency in mice increases the levels of PAI-1.\textsuperscript{11} Increased PAI-1 serum levels have been associated with increased risk for stroke,\textsuperscript{24} thus providing an alternate pathway through which klotho may act.

Although previous analyses of the effect of the KL-VS allele on longevity have been limited to cross-sectional data, the current study incorporates prospective mortality data generated in individuals \( \geq 95 \) years of age. As observed in the cross-sectional analyses of CVD risk factors and events, heterozygous individuals had a marked advantage. Wild-type individuals have an increased RR of 2.15 (95% CI, 1.18 to 3.91) and homozygous KL-VS individuals a 4.49-fold increased RR (95% CI, 1.35 to 14.97) for mortality relative to heterozygous individuals, after adjusting for potential confounders. These data strongly support a role for the KL-VS allele in longevity.

The consistent observation of opposing effects of one copy of the KL-VS allele versus carrying two copies offers a possible explanation for the relatively high frequency of the KL-VS allele in the general population (frequency 0.16). This model of “balancing selection” has been observed for the hemoglobin S allele in sickle cell anemia\textsuperscript{25} and for the KL-VS allele in longevity.

Conclusions

In the current study, we presented strong evidence confirming the role of a common variant of KLOTHO, the KL-VS allele, in human longevity. Cross-sectional and prospective studies confirm a heterozygous advantage for survival at later ages in conjunction with a marked homozygous KL-VS disadvantage. This genetic model held true for cardiovascular risk factors HDL-C and SBP, associated previously with KLOTHO, as well as a history of stroke. Collectively, these data implicate an important role for KLOTHO in the etiology of CVD and, ultimately, mortality.

Acknowledgments

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