Genetic and biomarker studies of human longevity

Deelen, J.

Chapter 6

Leukocyte telomere length associates with prospective mortality independent of immune-related parameters and known genetic markers

Joris Deelen, Marian Beekman, Veryan Codd, Stella Trompet, Linda Broer, Sara Hägg, Krista Fischer, Peter E Thijssen, H Eka D Suchiman, Iris Postmus, André G Uitterlinden, Albert Hofman, Anton J M de Craen, Andres Metspalu, Nancy L Pedersen, Cornelia M van Duijn, J Wouter Jukema, Jeanine J Houwing-Duistermaat, Nilesh J Samani and P Eline Slagboom

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Abstract

Human leukocyte telomere length (LTL) decreases with age and shorter LTL has previously been associated with increased prospective mortality. However, it is not clear whether LTL merely marks the health status of an individual by its association with parameters of immune function, for example, or whether telomere shortening also contributes causally to lifespan variation in humans. We measured LTL in 870 nonagenarian siblings (mean age 93 years), 1,580 of their offspring, and 725 spouses thereof (mean age 59 years) from the Leiden Longevity Study (LLS). We found that shorter LTL is associated with increased prospective mortality in middle (30-80 years; hazard ratio (HR) = 0.75, $P = 0.001$) and highly advanced age ($\geq$ 90 years; HR = 0.92, $P = 0.028$) and show that this association cannot be explained by the association of LTL with the immune-related markers insulin-like growth factor 1 to insulin-like growth factor binding protein 3 molar ratio, C-reactive protein, interleukin 6, cytomegalovirus serostatus, or white blood cell counts. We found no difference in LTL between the middle-aged LLS offspring and their spouses ($\beta = 0.006$, $P = 0.932$). Neither did we observe an association of LTL-associated genetic variants with mortality in a prospective meta-analysis of multiple cohorts (n = 8,165). We confirm LTL to be a marker of prospective mortality in middle and highly advanced age and additionally show that this association could not be explained by the association of LTL with various immune-related markers. Furthermore, the approaches performed here do not further support the hypothesis that LTL variation contributes to the genetic propensity for longevity.

Introduction

Telomeres are TTAGGG tandem repeat structures at the end of chromosomes that protect chromosomes from degradation and rearrangement [1]. In somatic cells, telomere length declines with every cell division and, accordingly, human leukocyte telomere length (LTL) decreases with age [2,3]. In addition, LTL differs between sexes, women have a longer LTL than men of the same age [4]. Shorter LTL has been associated with an increased risk of several age-related diseases, such as cardiovascular disease [5], hypertension [6], and cancer [7]. Likewise, several studies, although not all, have shown an association of shorter LTL with prospective mortality, mainly through infectious- and cardiovascular-related causes of death [8].

The shortening of telomeric DNA is mainly caused by incomplete DNA replication during the cell cycle S phase [9], but also by oxidative stress [10], which plays a role in the pathogenesis of viral infections [11]. An association of shorter LTL with increased prospective mortality is thus not necessarily explained by a causal effect of LTL on health conditions. The association of LTL with prospective mortality risk might be confounded by immune functions influencing prospective mortality, which may be investigated by immune-related markers. Previous studies showed that shorter LTL is associated with a decrease in serum levels of insulin-like growth factor 1 (IGF-1) [12,13], which is an important regulator of cell replication and, in addition, seems to play a role in the regulation of immunity and inflammation [14]. Furthermore, shorter LTL has been shown to associate with increased levels of the inflammatory markers C-reactive protein (CRP) and interleukin 6 (IL-6), although not in all studied populations [8]. During inflammation, IL-6 and other cytokines are secreted by T cells and macrophages and trigger the synthesis of CRP by the liver, ultimately resulting in clearance of necrotic and apoptotic cells. However, an increased level of IL-6 or CRP is not necessarily the result of increased inflammation [15,16].

Whether LTL is associated with familial longevity in middle age is not extensively studied. One study showed that offspring of Ashkenazi Jewish centenarians (n = 175) have a longer mean LTL as compared with controls from the general population (n = 93) [17]. Since the centenarians in this study (n = 86) and their offspring did not show a decline in LTL with age as observed in controls, the authors suggested that better LTL maintenance may be a feature in long-lived families. These interesting observations in a relatively small study warrant replication in larger populations.

LTL is a highly heritable trait [3,18]. Insights into the causal effects of LTL on human lifespan might be obtained by testing genetic variants influencing LTL for their association with prospective mortality. Recently, nine loci have been identified that influence LTL variation in Western populations [19,20]. These loci include the known telomere biology genes *CTC1*, *NAF1*, *OBFC1, RTEL1, TERC,* and *TERT*, explaining ~1% of the variance in LTL. In addition, genetic variation in two genes involved in telomere maintenance, *TERC* and *POT1*, was found to be associated with human longevity [17,21,22].

In this study, we explored the data of the Leiden Longevity Study (LLS) in which we measured LTL in nonagenarian siblings (n = 870), their offspring (n = 1,580) and the spouses thereof (n = 725; serving as controls). The survival benefit of LLS families is marked by a 30% decreased mortality risk in three generations, i.e., the parents of the nonagenarian siblings, their unselected additional siblings and their offspring, when compared with the general Dutch population [23]. In addition, the LLS offspring, who are considered "decelerated" or "healthy agers", have a lower prevalence of age-related diseases, such as type 2 diabetes, cardiovascular disease, and hypertension, as compared with their spouses, and show beneficial or "youthful" profiles for many metabolic parameters [24,25]. We first investigated whether the association of shorter LTL with increased prospective mortality, which is observed in both generations, could be explained by lymphocyte counts, serum CRP levels, serum IGF-1 to insulin-like growth factor binding protein 3 (IGFBP3) molar ratio (IGF-1/IGFBP3), serum IL-6, or the presence of cytomegalovirus (CMV) infection as immune-related markers. Next, we examined whether the LLS offspring have a longer LTL and a different association of LTL with age as compared with their spouses. Finally, we determined the effect of genetic variants associated with LTL on prospective mortality. We performed a prospective meta-analysis of multiple cohorts (n = 8,165) in which known LTL-associated single nucleotide polymorphisms (SNPs) were investigated separately and in combination as a genetic risk score (GRS).

Results

LTL and prospective mortality in two generations

The characteristics of the LLS nonagenarians, their offspring and the spouses thereof for demographic variables, LTL, immune-related markers, and mortality analysis are depicted in Tables 6.1, 6.2, and 6.3. We first analyzed the association between LTL and prospective mortality in the middle-aged and nonagenarian generations. We found that shorter LTL is associated with increased prospective all-cause mortality in the combined group of middle-aged LLS offspring and their spouses (30-80 years of age), i.e., per unit longer LTL there is a 25% decrease in mortality risk (hazard ratio (HR) = 0.75 (95% CI 0.64 – 0.88), P = 0.001). In addition, we observed a similar association in the LLS nonagenarians (≥ 90 years of age, HR = 0.92 (95% CI 0.86 – 0.99), P = 0.028). Since it has previously been reported that LTL declines with decreasing serum IGF-1 levels and increasing serum CRP levels, the effect of LTL on prospective mortality might be explained by the association of LTL with immune functions, as reflected by immune-related markers such as serum IGF-1/IGFBP3, serum CRP levels, serum IL-6 levels, presence of CMV infection and white blood cell (WBC) counts. We previously showed that long-lived family members from the LLS have a lower prevalence of CMV infection as compared to controls from the general population [26,27]. The levels of the other markers did not differ between long-lived family members and controls [28,29]. Most of these markers associated with LTL in the LLS (Table S6.1). Therefore,

Table 6.1 Characteristics of the Leiden Longevity Study samples used for the linear regression and prospective analysis of leukocyte telomere length.

	Offspring				Spouses				Nonagenarians			
	Men		Women		Men		Women		Men		Women	
	n	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
Age (years)	734	59.33 (6.51)	846	59.47 (6.52)	309	61.23 (7.40)	416	57.02 (6.85)	333	92.24 (2.72)	537	93.98 (2.34)
LTL (T/S ratio)*	734	1.43 (0.25)	846	1.48 (0.26)	309	1.42 (0.25)	416	1.49 (0.28)	333	1.25 (0.21)	537	1.29 (0.22)
IGF-1/IGFBP3 (molar ratio)	709	0.11 (0.02)	814	0.10 (0.02)	302	0.11 (0.02)	400	0.10 (0.02)	320	0.11 (0.02)	506	0.09 (0.02)
CRP (mg/L)	713	2.12 (2.94)	813	2.51 (3.73)	301	2.09 (2.83)	403	2.61 (3.56)	321	6.27 (10.39)	512	5.66 (10.00)
IL-6 (pg/ml)	667	0.60 (0.69)	758	0.54 (0.63)	280	0.57 (0.60)	384	0.57 (0.69)	NA	NA	NA	NA
Lymphocyte count (%)	695	27.74 (6.85)	797	30.07 (7.01)	301	28.39 (7.11)	392	30.37 (6.52)	317	21.11 (7.27)	492	21.98 (7.70)
Neutrophil count (%)	695	60.75 (7.74)	797	59.87 (7.59)	301	60.45 (8.14)	392	59.44 (7.18)	317	66.69 (8.31)	492	66.96 (8.91)
Monocyte count (%)	695	5.61 (1.34)	797	5.06 (1.29)	301	5.61 (1.32)	392	5.04 (1.31)	317	6.34 (1.79)	492	5.82 (1.63)
Eosinophil count (%)	695	2.77 (1.44)	797	2.35 (1.24)	301	2.77 (1.37)	392	2.48 (1.33)	317	3.12 (1.73)	492	2.65 (1.49)
Basophil count (%)	695	0.74 (0.35)	797	0.69 (0.31)	301	0.72 (0.35)	392	0.71 (0.34)	317	0.65 (0.36)	492	0.69 (0.39)

SD, standard deviation; *IGF-1*, insulin-like growth factor 1; *IGFBP3*, insulin-like growth factor binding protein 3; *CRP*, C-reactive protein; *IL-6*, interleukin 6. *A decrease in one T/S unit represents a decrease of 5,000 telomeric base pairs.

Table 6.2 Characteristics of the Leiden Longevity Study samples used for the linear regression and prospective analysis of leukocyte telomere length.

	Offspring				Spouses				Nonagenarians			
	Men		Women		Men		Women		Men		Women	
	n	Yes/no	*n*	Yes/no	*n*	Yes/no	*n*	Yes/no	*n*	Yes/no	*n*	Yes/no
CMV infection	713	276/437	815	414/401	301	155/146	407	212/195	326	178/148	514	322/192

CMV, cytomegalovirus.

we adjusted for IGF-1/IGFBP3, CRP, IL-6, CMV infection, and lymphocyte counts in the Cox proportional hazard model used for prospective analysis of mortality. This showed that the association of LTL with all-cause mortality in both generations of the LLS was independent from these immune-related markers (HR = 0.68 (95% CI 0.56 – 0.82), $P = 9.23 \times 10^{-5}$ (offspring and spouses) and HR = 0.90 (95% CI 0.84 – 0.97), P = 0.006 (nonagenarians), even though all markers showed an association (in one or both generations) with mortality (Table S6.2). Since both a low and/or high level of WBC counts and CRP could be detrimental, we also performed the analyses without individuals with low ($< 4 \times 10^9$, $n_{offspring/spouses} = 36$, $n_{nonagerarians} = 16$) and high ($> 10 \times 10^9$, $n_{offspring/spouses} = 96$, $n_{nonagerarians} = 58$) WBC counts and high CRP levels (> 30 mg/L, $n_{offspring/spouses} = 2$, $n_{nonagerarians} = 24$). However, these analyses provide similar results (data not shown). In addition, there was no interaction between LTL and immune-related markers. This indicates that the effect of LTL on prospective mortality could not be explained by its association with these immune-related markers.

LTL and familial longevity in middle age

Next, we compared LTL between the LLS offspring (n = 1,580) and their spouses (n = 725). We found no evidence for a difference in mean LTL between the groups considering age and gender as covariates in our linear regression model (β = 0.006 (95% CI -0.125 – 0.136), $P = 0.932$, Figure 6.1A). In addition, we found no evidence that the association of LTL with age is different among long-lived families, since the estimated decline of LTL (in T/S ratio units) per calendar year in the LLS offspring (β = -0.009) and their spouses (β = -0.006) was similar to other studies with participants of middle age [20] (Figure 6.1B). This indicates that LTL does not explain the propensity for familial longevity in middle age.

Prospective meta-analysis of LTL-associated genetic variants

To determine whether the genetic component of LTL contributes to prospective mortality, we investigated whether the lead SNPs from the 7 loci that showed association with LTL variation ($P < 5 \times 10^{-8}$) in the largest genome-wide association study (GWAS) up to now [20], as well as a GRS based on these SNPs, also associate with prospective mortality. To this end, we performed a prospective meta-analysis of mortality in 8,165 individuals above 75 years from 6 different cohorts, of whom 3,893 had died (Table S6.3). This analysis showed no association of the LTL SNPs, nor of the GRS, with all-cause, cardiovascular or cancer mortality after Bonferroni correction to adjust for multiple testing ($P_{adjusted} > 0.0056$, Tables 6.4, S6.4, and S6.5), although we had an 80% power (α = 0.05) to detect HR's below 0.91.

Discussion

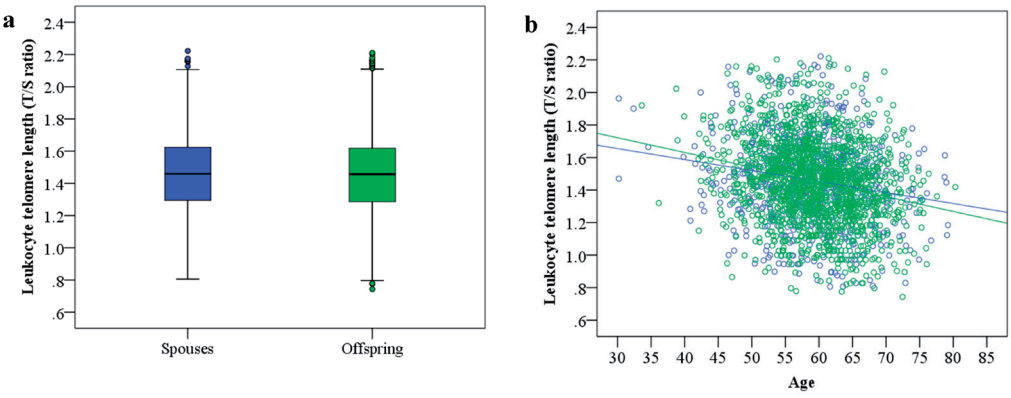
To examine the association between telomeres and human lifespan we studied LTL in nonagenarians and their middle-aged offspring from the LLS for association with prospective mortality and familial longevity in middle age. Interestingly, carriers of long telomeres, as compared to those with shorter

Table 6.3 Characteristics of the Leiden Longevity Study samples used for the prospective analysis of leukocyte telomere length.

	n	n_{deaths}	Mean age (SD)	Age range	Men/women	Mean follow-up time (SD)
LLS offspring + spouses	2,294	106	59.18 (6.78)	30 - 80	1,037/1,257	7.56 (0.95)
LLS nonagenarians	870	751	93.31 (2.63)	89 - 103	333/537	7.57 (0.84)

SD, standard deviation; *LLS*, Leiden Longevity Study.

Figure 6.1 Characteristics of leukocyte telomere length (LTL) in the Leiden Longevity Study (LLS) offspring and their spouses. Mean LTL (A) and the age-related decline of LTL (B) in the LLS offspring (n = 1580, green) and their spouses (n = 725, blue).



telomeres, have a clear survival benefit, which is independent of immune-related markers associated with LTL. We found no association of LTL with familial longevity in middle age. Neither did we observe an association of LTL-associated genetic variants with mortality in a prospective meta-analysis of multiple cohorts (n = 8,165). This confirms the study of monozygotic twins, in which LTL predicts prospective mortality in the absence of genetic differences between the twins [30].

The observed association of shorter LTL with increased prospective mortality in two generations of the LLS is in accordance with previous studies [8]. However, it is unclear what biological phenomenon telomere length in blood reflects. Shorter LTL has previously been associated with decreased serum levels of IGF-1 [12,13] and increased levels of CRP and IL-6 [8], which are known markers of inflammation [31,32]. Hence, the established association between LTL and prospective mortality might be explained by confounding factors such as immune functions. These factors could on the one hand associate with LTL, by affecting replication of specific cell populations, and on the other hand with prospective mortality, reflecting the health status of an individual. To test this hypothesis, we investigated several immune-related markers, namely serum IGF-1/IGFBP3, which is a marker for the amount of biologically active IGF-1, serum CRP levels, serum IL-6 levels, WBC counts, and seropositivity for CMV

Table 6.4 Association between leukocyte telomere length-associated genetic variants and all-cause mortality.

SNP/GRS	*n*	n_{deaths}	HR	SE	95% CI	*P*
rs11125529	8,165	3,893	1.01	0.04	0.94 - 1.08	0.863
rs10936599	8,165	3,893	1.00	0.03	0.95 - 1.06	0.966
rs7675998	8,165	3,893	0.99	0.03	0.94 - 1.05	0.728
rs2736100	8,165	3,893	1.03	0.02	0.99 - 1.08	0.159
rs9420907	8,165	3,893	1.01	0.03	0.94 - 1.08	0.823
rs8105767	8,165	3,893	0.97	0.03	0.92 - 1.02	0.226
rs755017	8,165	3,893	0.92	0.03	0.86 - 0.98	0.009
Unweighted GRS	8,165	3,893	1.00	0.01	0.98 - 1.02	0.985
Weigthed GRS	8,165	3,893	0.97	0.02	0.93 - 1.01	0.133

SNP, single nucleotide polymorphism; *GRS,* genetic risk score; *HR*, hazard ratio; *SE*, standard error; *95% CI*, 95% confidence interval.

infection. Whereas most of these markers were indeed associated with LTL and showed an individual effect on prospective mortality, shorter LTL remained independently associated with prospective mortality in two generations. We should note that we did not have data available regarding other relevant immune-related markers such as erythrocyte sedimentation rate and fibrinogen. The associations of LTL with prospective mortality could also be confounded by non-cell-autonomous senescence as a consequence of viral infection [33]. Another explanation for the association between LTL and prospective mortality might be found in the association of LTL with metabolic parameters associated with cardiovascular disease risk and/or mortality, such as fasting insulin and homocysteine [8]. Whether these markers explain LTL-related prospective mortality still needs to be determined.

Previous studies have shown that telomere length dynamics is age-related, i.e., the rate of LTL shortening during adulthood is much slower than during early life [34]. However, the age range of our samples within the middle-aged and highly advanced aged groups is relatively small and all our individuals are in adulthood (above 30 years of age). Hence, our finding that shorter LTL is associated with increased mortality is less likely to be confounded by this phenomenon.

Our finding that LTL is not associated with familial longevity in middle age is in contradiction to the observations in long-lived Ashkenazi Jewish families [17]. This discrepancy could be explained by natural variation, such as differences in the demography or age of the samples (mean age 68 and 72 years (among Ashkenazi offspring and controls, respectively) versus 59 years (LLS offspring and spouses)) or by differences in the selection criteria which may have an effect on the genetic component of the longevity trait (offspring of centenarians (mean age 97 years) versus offspring of nonagenarian siblings (mean age 94 years)). Another possible explanation is the small sample size of the study of Atzmon and colleagues ($n_{offspring}$ = 175 and $n_{controls}$

= 93) in comparison to the current study ($n_{offspring}$ = 1,580 and $n_{spouses}$ = 725), which may have led to a non-random selection of individuals from the population, resulting in a false positive association.

The GRS composed of the 7 genetic variants associated with LTL variation in the largest GWAS reported so far is associated with coronary artery disease risk [20], but does not associate with prospective mortality in our study of 8,165 individuals of whom 3,893 died during follow-up. Since the 7 genetic variants only explain ~1% of the variation in LTL [20], the sample size of the current study might be insufficient to detect their effect on prospective mortality. Hence, on the basis of our data we cannot exclude a causative role for genetic variants in LTL related genes in prospective mortality.

Critical telomere length in tissues may be causally involved in lifespan regulation and our results further highlight the role of telomere length in blood as marker for prospective mortality. The lack of association of LTL with familial longevity in middle age and of the LTL-associated genetic variants with prospective mortality provides thus far no support for LTL causally contributing to lifespan variation in humans. However, LTL does reflect environmental effects, as demonstrated by the observation that there is a correlation in LTL between spouses [18]. Assortative mating may thus have obscured a difference between the LLS offspring and their spouses. However, the LLS offspring do have a more "youthful" metabolic profile and a lower prevalence of age-related diseases compared with their spouses, whereas LTL is not different between the groups. This indicates that LTL seems to associate with mortality independent of the familial trait that influences the metabolic health in these families in middle age. LTL meets three of the four criteria we proposed for a biomarker of healthy aging in a recent review [35], i.e., LTL associates with chronological age and with morbidity and mortality in prospective studies. However, LTL cannot be used to discriminate individuals in middle age according to their genetic propensity for longevity. Other potential biomarkers of healthy aging, such as fasting glucose and free triiodothyronine, did meet all criteria in studies of various human cohorts.

LTL could reflect the compartment of vital haematopoietic stem cells (HSCs) in individuals. Leukocytes consist of different subsets of cells, namely lymphocytes, monocytes and granulocytes (neutophils, basophils and eosinophils), which all originate from the HSC. Telomere length differs between leukocyte subsets [36]. However, since there is synchrony between the different subsets, an individual's LTL likely reflects the telomere length of the HSCs [37]. In this study, we show that LTL is associated with several leukocyte subset counts, namely lymphocyte, neutrophil and basophil counts (Table 6.1), which indicates that mean LTL is influenced by the frequency of the different leukocyte subsets. However, when we adjusted the prospective analysis of mortality for these counts, the effect of LTL on prospective mortality remained unchanged, indicating that the proportion of LTL variation caused by the frequency of the different leukocyte subsets does not influence prospective mortality. Nevertheless, this leaves the possibility open that LTL reflects the available HSC population.

Telomere dysfunction was found to be determined by the frequency of critically short telomeres. A recent study showed that the rate of increase in the frequency of these critically short telomeres and not the rate of telomere length shortening determines longevity in mice [38]. Since in the current study we only determined the mean LTL of an individual, we could not discriminate between individuals according to the frequency of dysfunctional telomeres. It would therefore be interesting to use quantitative fluorescence *in situ* hybridization, a method that is able to quantify critically short telomeres in subsets of cells [39], to determine the influence of the frequency of dysfunctional telomeres on longevity and prospective mortality in humans.

In conclusion, we confirmed LTL to be a marker of prospective mortality in middle and highly advanced age and additionally show that this association could not be explained by the association of LTL with the immune-related markers IGF-1/IGFBP3, CRP, IL-6, CMV serostatus, or WBC counts or by the currently known genetic variants contributing to LTL variation. Furthermore, we have shown that LTL is not associated with familial longevity in middle age. Hence, the approaches followed here do not further support the hypothesis that LTL contributes to the genetic propensity for longevity. Further studies need to be performed to determine which other environmental or novel genetic effects could underlie the association of LTL with prospective mortality.

Material and methods

Study populations

Leiden Longevity Study

For the LLS, long-lived siblings of European descent were recruited together with their offspring and the spouses of the offspring. Families were included if at least two long-lived siblings were alive and fulfilled the age criterion of 89 years or older for men and 91 years or older for women, representing less than 0.5% of the Dutch population in 2001 [23]. In total, 944 long-lived proband siblings with a mean age of 94 years (range, 89-104), 1,671 offspring (61 years, 39-81), and 744 spouses thereof (60 years, 36-79) were included. DNA from the LLS was extracted from samples at baseline using conventional methods [40] and genotyping was performed with Illumina Human660W-Quad and OmniExpress BeadChips (Illumina, San Diego, CA, USA). Imputation was performed using IMPUTE2 with reference HapMap Phase I + II CEU release 22 (hg18/build36).

A description of the cohorts used for the prospective meta-analysis of LTL-associated genetic variants is provided in the Supplementary Information.

Measurement of leukocyte telomere length

Mean LTL was measured as a ratio (T/S) of telomere repeat length (T) to the copy number (S) of the single-copy gene *36B4*, as previously described [20]. The inter- and intra-run coefficients of variation were 2.73% and 2.73% for the LLS nonagenarians and 3.74% and 2.85% for the LLS offspring and spouses, respectively. LTL was obtained

in 3,194 samples from the LLS, of which 19 were removed due to a deviation from the mean > 3 SD, leaving 3,175 samples for the analysis (Tables 6.1, 6.2, and 6.3). If we consider an LTL attrition rate of 30 telomeric base pairs per year and a decline of 0.006 T/S units per year [20], a decrease of one T/S unit reflects a decrease of 5,000 telomeric base pairs in our study. This LTL attrition rate is based on several studies that have used DNA blotting to measure LTL [20]. A recent review indicated that the LTL attrition rate is most likely somewhere in the range of 20-30 base pairs/year [41], so, the estimated telomeric base pairs representing one T/S ratio may vary between 3,333 and 5,000. In the analyses of LTL, one LTL unit represents 1 kb telomeric base pairs.

Measurement of immune-related parameters

In the LLS all standard serum measurements were performed using fully automated equipment. WBC counts were measured using the Sysmex XE-2100 (TOA Medical Electronics, Kobe, Japan). IGF-1 and IGFBP3 were measured using the Immulite 2500 (DPC, Los Angeles, CA, USA) [28] and high-sensitivity CRP was measured using the Hitachi Modular P800 (Roche, Almere, the Netherlands) [29]. Since the CRP levels were not normally distributed the log transformed values were used for analysis. IL-6 was measured with the PeliKine Compact human IL-6 ELISA kit (Sanquin Reagents, Amsterdam, the Netherlands) [42]. For calculation of IGF-1/IGFBP3 we used the following formula:

*IGF-1/IGFBP3 = IGF-1 (ng/ml)*0.130 / IGFBP3 (ng/ml)*0.036*

For all serum parameters, measurements with a deviation from the mean > 3 SD were removed. CMV serostatus was determined on blinded samples using the CMV-IgG-ELISA PKS assay (Medac GmbH, Wedel, Germany) [26]. The characteristics of the measured parameters are depicted in Tables 6.1 and 6.2.

Statistical analysis

Prospective analysis

Prospective analysis of LTL and LTL-associated genetic variants was performed using a Cox proportional hazards model adjusted for age, gender, population stratification, and study specific covariates. The number of individuals and (cause-specific) deaths for every cohort, as well as the follow-up times, are depicted in Tables 6.3 and S6.3. To determine whether the association of LTL with mortality was independent of immune-related markers, we fitted a model with and without adjustment for immune-related markers and determined whether the association of LTL with mortality remained ($P < 0.05$).

Association of LTL with immune-related markers

To determine the association of LTL with serum parameters in the LLS, we performed linear regression, adjusted for age, gender, and familial relationships, using the following model in STATA/SE 11.2 (StataCorp LP, College Station, TX, USA).

*T/S ratio ~ β0 + β1*age+ β2*gender + β3*(age*gender) + β4*group + β5*immune-related marker*

age was coded in years, *gender* was coded as 1 (male) or 2 (female), and *group* was coded as 0 (LLS spouse) or 1 (LLS offspring). Robust standard errors were used to account for sibship relations.

We assumed a linear association between LTL and the different immune-related markers since the augmented partial residual plots of the markers showed no visual deviation from linearity.

Association of LTL with familial longevity in middle age

To determine the association of LTL with familial longevity in middle age in the LLS offspring (n = 1,580) and their spouses (n = 725), linear regression, adjusted for age, gender, and familial relationships, was performed using the following model in STATA/SE 11.2 (StataCorp LP):

*T/S ratio ~ β0 + β1*age+ β2*gender + β3*(age*gender) + β4*group*

age was coded in years, *gender* was coded as 1 (male) or 2 (female), and *group* was coded as 0 (LLS spouse) or 1 (LLS offspring). Robust standard errors were used to account for sibship relations.

Genetic risk score

To determine the joint effect of LTL-associated genetic variants on all-cause, cardiovascular, and cancer mortality, we created a GRS using a previously described approach [20,43]. The unweighted GRS of a subject was defined as the combined number of risk alleles associated with shorter LTL in a previous GWAS [20]. For the weighted GRS, the β for each SNP in this GWAS was added as weight and the total score was divided by the sum of all weights.

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Supplementary Information

The supplementary information belonging to this chapter can be found at: http://ije.oxfordjournals.org/content/early/2014/01/14/ije.dyt267/suppl/DC1.

Table S6.1 Results regression analysis LTL and immune-related markers.

Table S6.2 Association between LTL and mortality adjusted for immune-related markers.

Table S6.3 Characteristics of the samples used for the prospective analysis of LTL-associated genetic variants.

Table S6.4 Association between LTL-associated genetic variants and cardiovascular mortality.

Table S6.5 Association between LTL-associated genetic variants and cancer mortality.