CHAPTER 8

LDL cholesterol still a problem at old age? A Mendelian randomization study

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Abstract

Observational studies in older subjects have shown no or inverse associations between cholesterol levels and mortality. However, in old age plasma low density lipoprotein cholesterol (LDL-C) may not reflect the life-time level due to reverse causality and hence the risk may be underestimated. In the current study, we used an LDL genetic risk score (GRS) to overcome this problem. A weighted GRS was created using 37 single nucleotide polymorphisms associated with LDL-C levels. The LDL GRS was calculated in three Dutch cohorts, the Leiden Longevity Study (LLS) (n=3282), the Leiden 85-plus study (n=317), and the Rotterdam Study (n=4035). We assessed the association between the LDL GRS and LDL-C levels, chronological age, familial longevity, and mortality. In all age strata, individuals with high LDL GRS had higher LDL-C levels (p=0.012 to $p=8.5x10^{-15}$). The frequency of LDL increasing alleles decreased with increasing age (β=-0.027 (SE=0.01) per year, p=0.010). Moreover, individuals with a genetic predisposition for longevity had significantly lower LDL GRS compared to age-matched individuals of the general population (LLS nonagenarians versus >90 years: β=0.93 (SE=0.39), p=0.018, LLS offspring versus partners: β=0.23 (SE=0.10), p=0.019). In longitudinal analysis, high GRS was associated with increased all-cause mortality in individuals >90 years; with a 19% increased risk in individuals with the highest LDL GRS (p-trend=0.008). Results of the current study indicate that a genetic predisposition to high LDL-C levels contributes to mortality throughout life, also in the oldest old and a beneficial LDL genetic risk profile is associated with familial longevity.

Introduction

Observational studies including middle-aged individuals have shown a positive association between cardiovascular disease and cholesterol levels.[1;2] In addition, lowering cholesterol levels with statins reduces the risk of cardiovascular disease at all ages.[1] However, at older ages above 75 years, the contribution of high cholesterol as a cardiovascular risk factor is controversial. Mortality from disease in old age has been shown to be independent of total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels [3;4], whereas low total cholesterol levels have been associated with higher all-cause mortality in the oldest old.[3;5;6] At old age, LDL-C levels in plasma may not reflect life-time LDL-C level due to comorbidities.[3] This inverse health relation in old age raises the question whether lipid levels represent causal factors affecting cardiovascular/metabolic health at all ages. Fortunately, the use of genetic variants as an instrumental variable provides a possibility to investigate the associations free of biases such as reverse causality. In recent years, genome-wide association studies (GWAS) have identified several new genetic loci that are associated with lipoprotein levels. The largest GWAS meta-analysis, including 46 studies comprising more than 100,000 individuals, found 95 loci to be associated with cholesterol levels.[7]

Characteristics of lipid metabolism have further been linked to human lifespan regulation by association to familial longevity. For example, offspring of long-lived individuals have larger LDL particle sizes compared to their spouses or age- and lifestyle-matched controls.[8-10] Moreover, older people who carry the apolipoprotein E gene ε3/ε3 variant and have lower plasma levels of apoE, have a decreased mortality risk compared to carriers of the ε3/ε3 variant with high levels .[11]

In this study we created a genetic risk score (GRS), based on single nucleotide polymorphisms (SNPs) associated with LDL-C levels.[7] Using this GRS as instrumental variable, we evaluated the association between LDL-C and mortality in participants of three Dutch studies (figure 1). In addition, we assessed the association between the LDL GRS and familial longevity.

Methods

Study populations

To assess the associations between the LDL GRS and the various outcomes we made use of three Dutch cohort studies including 7634 participants, the Leiden Longevity

Study (LLS), the Leiden 85-plus study, and the Rotterdam Study. All cohorts had GWA data available and are briefly described here.

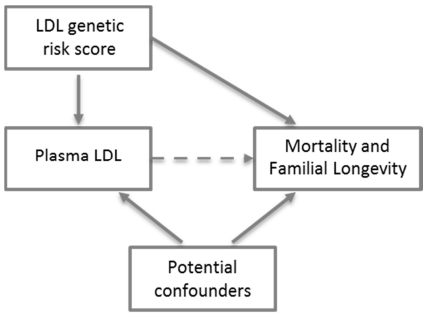

Figure 1. Graphical presentation of the research question in the current study

Leiden Longevity study

For the LLS, long-lived siblings of European descent were recruited together with their offspring and the spouses of the offspring (partners). 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.[12] In total, 931 long-lived siblings with a mean age of 94 years (range 89-104), 1671 offspring (mean age 61 years, range 39-81), and 744 partners (mean 60 years, range 36-79) were included. DNA from the participants of the LLS was extracted from samples at baseline using conventional methods. [13]

Leiden 85-plus study

Participants of the Leiden 85-plus study were inhabitants of Leiden, the Netherlands, who reached the age of 85 years between September 1, 1997, and September 1, 1999. There were no selection criteria on health, functioning or demographic characteristics. A total of 705 inhabitants reached the age of 85 years and a total of 599 individuals participated.[14] Individuals were visited at their place of residence and annual follow up visits were performed until death or age 90 years. Information about mortality was available until December 31, 2009. The date of death was obtained from the civic registry of Leiden.

Rotterdam Study

The Rotterdam Study is a population-based cohort study, including 7983 participants living in Ommoord, a district of Rotterdam, the Netherlands. All inhabitants aged 55 and over, were invited to participate in the study (n = 10275). The Rotterdam Study started in the early 1990s and periodical examinations were performed every three to five years. Analyses of this study are based on data from the third round of the

study which was performed between 1997-1999 (n=4035). The study was approved by the medical ethical committee of the Erasmus Medical Center and written informed consent was obtained from all participants.[15]

Lipoprotein levels

In offspring and partners from the LLS non-fasting venous blood samples were taken. Total and HDL cholesterol levels were determined using fully automated equipment (the Hitachi Modular or the Cobas Inergra 800 both from Roche, Almere, the Netherlands).

In the Leiden 85-plus study lipoprotein levels were obtained at the follow-up visit at age 90 years. Total cholesterol, triglyceride, and HDL cholesterol levels were analysed with fully automated computerized analyzers (Hitachi 747 and 911, Hitachi, Ltd, Tokyo, Japan).

In the Rotterdam Study, total cholesterol, HDL cholesterol, and triglyceride concentrations were measured from serum or plasma extracted from whole blood, using an automated enzymatic procedure (Boehringer Mannheim System).

In all three cohorts, LDL-C levels were calculated using the Friedewald equation.[16]

Genotyping

In the Leiden Longevity study genotyping was performed with Illumina Human660W-Quad and OmniExpress BeadChips (Illumina, San Diego, CA, USA). Individuals were removed if they showed a mismatch in gender or familial relatedness based on genotype and phenotype, leaving 931 nonagenarians, 1610 offspring and 741 partners for the analysis. In addition, SNPs which were not measured on both platforms and with a call rate <0.95, MAF <0.01 and $P_{HWE} < 10^{-4}$ were excluded, leaving 288635 (nonagenarians) and 298538 (offspring and partners) SNPs as input for the imputation. Imputation was performed separately for the LLS nonagenarians and LLS offspring and partners using IMPUTE2 with reference HapMap Phase I + II CEU release 22 (hg18/build36).

In the Leiden 85-plus study genotyping was performed with Illumina OmniExpress BeadChips (Illumina) in participants aged 90 years. Individuals were removed if they showed a mismatch in gender based on genotype and phenotype, leaving 317 individuals for the analysis. In addition, SNPs with a call rate <0.95, MAF <0.01 and $P_{HWE} <10^{-4}$ were excluded, leaving 603301 SNPs as input for the imputation. Imputation was performed using IMPUTE2 with reference HapMap Phase I + II CEU release 22 (hg18/build36).

In the Rotterdam Study genotyping was conducted using the Illumina Infinium II HumanHap 550K array among self-reported Caucasian individuals. Individuals were excluded if they had excess autosomal heterozygosity, mismatch between called and phenotypic gender, or recognized as being outlier with IBS clustering analysis. In addition, SNPs with a MAF ≤1%, P_{HWE} <10^{-5}, or call rate ≤90% were excluded, leaving 530683 SNPs. Imputation was performed using the maximum likelihood method implemented in MaCH (version 1.0.15) with reference to HapMap Phase I + II CEU release 22 (hg18/build36).

Weighted genetic risk score

To create the LDL GRS we used the SNPs identified in the GWAS meta-analysis reported by Teslovich *et al.*.[7] We included all 37 SNPs associated with LDL-C levels (and possibly with total cholesterol, HDL cholesterol, and/or triglycerides).To build the LDL GRS, we first determined the number (or dosage in case of imputed SNPs) of unfavourable alleles for each individual, whereby the unfavourable allele was associated with higher LDL-C levels in the GWAS meta-analysis.[7] The number of unfavourable alleles was multiplied by the absolute effect size as published in the original paper.[7] Next, we calculated the GRS for each individual by summing the estimates (number of unfavourable alleles x absolute effect size) of all SNPs and divided it by the average of all effect sizes. In the final step the GRS was rescaled into a percentage of the maximum number of risk alleles (individuals GRS / maximum GRS score) x 100%. To use the GRS as a categorical variable, the GRS % was divided into three groups, using 47.5% and 52.5% as cut off values.

Statistical analysis

First, to assess the association between LDL GRS categories and LDL-C levels we combined the data of general population subjects (LLS partners, Leiden 85-plus study, and Rotterdam Study) and divided the individuals in age strata of 10 years. Data of LLS nonagenarians and offspring were excluded from this analysis since they have a genetic predisposition for longevity and to exclude possible familial effects. A general linear model was used adjusted for age, sex, and cohort. The explained variance in LDL-C levels by the LDL GRS was assessed by calculating the R^2 per cohort using a linear regression model.

Second, we assessed the cross-sectional association between the LDL GRS and chronological age. Individual level data from the LLS partners, Leiden 85-plus study, and the Rotterdam Study were combined to have a wide variation in age range. A general linear model was used adjusted for sex and cohort. Additional analyses were performed using only the individuals aged ≥ 50 years, and ≥70 years.

Differences in LDL GRS between LLS nonagenarians and individuals ≥90 years, and LLS partners and offspring were tested using a general linear model adjusted for age, sex, and, if necessary, familial relations.

Finally, the longitudinal association between LDL GRS categories and mortality in individuals ≥90 years was assessed using Poisson analysis to calculate incidence rate ratios. For this analysis, data of the LLS nonagenarians, Leiden 85-plus study and Rotterdam Study participants aged ≥90 years was used. Incidence rate ratios were adjusted for age, sex, and, if necessary, cohort and familial relations.

All statistical analyses were performed using IBM SPSS Statistics program for Windows (Version 20.0, USA) and Stata/SE version 12.1 for Windows. P-values ≤0.05 were considered statistically significant.

Results

The LDL GRS was calculated for 3282 participants (931 nonagenarians, 1610 offspring, and 741 partners) of the Leiden Longevity Study, 317 participants of the Leiden 85-plus study, and 4035 participants of the Rotterdam study. In table 1, the baseline characteristics are shown for the six age strata including the general population participants (LLS partners, Leiden 85-plus study, and Rotterdam Study), the LLS nonagenarians and offspring. Baseline characteristics per cohort are provided in supplementary table 1.

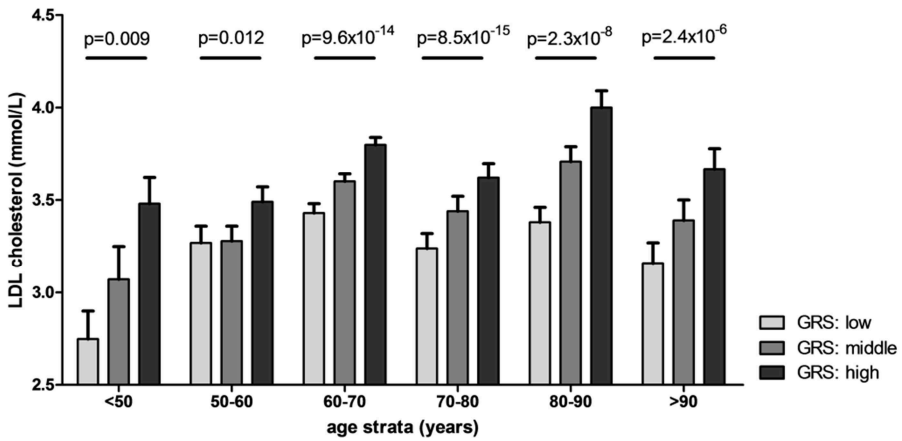

Figure 2: LDL cholesterol levels per GRS category in different age strata, using individual level data of LLS partners, Leiden 85-plus study, and the Rotterdam Study. Means and standard errors (SE) were assessed using a general linear model adjusted for age, sex, and cohort. P-values were assessed using the continuous values of the LDL GRS.

Table 1. Baseline characteristics

	Age strata (years)						Leiden Longevity study	
	<50	50-60	60-70	70-80	80-90	>90	Offspring	Nonagenarians
N	88	327	1859	1739	655	425	1610	931
Age (years)	45.8 (4.3)	55.7 (2.6)	65.7 (2.5)	74.7 (2.9)	83.9 (2.7)	90.8 (2.1)	59.4 (6.5)	93.4 (2.7)
Male (%)	25 (28.4)	108 (33.0)	834 (44.9)	785 (45.1)	199 (30.4)	113 (26.6)	747 (46.4)	350 (37.6)
Body mass index (kg/m^2)	24.5 (3.3)	25.8 (3.8)	26.7 (3.9)	26.9 (3.9)	26.6 (4.0)	25.8 (4.4)	25.3 (3.6)	25.5 (3.6)
Total cholesterol (mmol/L)	5.33 (1.02)	5.67 (1.02)	5.85 (1.00)	5.77 (0.97)	5.77 (1.09)	5.22 (1.04)	5.59 (1.12)	NA
LDL cholesterol (mmol/L)	3.12 (0.83)	3.36 (0.86)	3.73 (0.91)	3.69 (0.87)	3.67 (1.00)	3.25 (0.89)	3.32 (0.93)	NA
HDL cholesterol (mmol/L)	1.46 (0.44)	1.45 (0.47)	1.38 (0.38)	1.38 (0.40)	1.43 (0.44)	1.36 (0.42)	1.45 (0.44)	NA
Triglycerides (mmol/L)	1.61 (1.11)	1.75 (1.05)	1.67 (0.88)	1.55 (0.80)	1.50 (0.70)	1.41 (0.84)	1.76 (1.03)	NA
Cohort (%)								
LLS Partners	88 (100)	327 (100)	284 (15.3)	42 (2.4)	0 (0)	0 (0)		
Leiden 85-plus study	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	317 (74.6)		
Rotterdam Study	0 (0)	0 (0)	1575 (84.7)	1697 (97.6)	655 (100)	108 (25.4)		

Continuous variables are presented as means and standard deviations, categorical variables are presented as numbers and percentages.

Table 2. Association between LDL genetic risk score categories and mortality in participants aged ≥90 years

	Genetic risk score category								
	Low		Middle		High		IRR (95% CI)		
	Deaths	PY (x1000)	Deaths	PY (x1000)	Deaths	PY (x1000)	2 vs 1	3 vs 1	P-trend
Leiden 85-plus study	89	0.805	82	0.755	97	0.785	0.98 (0.72-1.32)	1.11 (0.84-1.49)	0.445
Rotterdam Study	31	0.122	37	0.160	25	0.111	0.95 (0.55-1.66)	0.98 (0.54-1.78)	0.962
LLS Nonagenarians	294	1.309	251	1.039	199	0.738	1.07 (0.90-1.27)	1.25 (1.04-1.49)	0.010
Combined	414	2.235	369	1.950	321	1.634	1.04 (0.91-1.18)	1.19 (1.05-1.34)	0.008

Incidence rates and incidence rate ratios are shown with their 95% confidence interval. Incidence rate ratios were assessed using Poisson analysis, adjusted for age, sex, cohort, and were necessary familial relations.
Abbreviations: PY, person years; IR, incidence rate; IRR, incidence rate ratio; CI, confidence interval; vs, versus; LLS, Leiden Longevity Study.

In each age stratum there was a linear association between the LDL GRS and LDL-C levels (figure 2), with individuals in the highest LDL GRS group having the highest LDL-C level (p=0.012 to $p=8.5x10^{-15}$). Associations between the LDL GRS and LDL-C levels in the separate cohorts are provided in supplementary figure 1, showing a linear association in each cohort. The LDL GRS explained 7.1% of the variance in LDL-C levels in the LLS offspring, 4.4% in the LLS partners, 6.0% in the Leiden 85-plus study, and 3.8% in the total Rotterdam Study.

The next step was to assess the association between the LDL GRS and chronological age. Figure 3 shows the cross-sectional relation between the LDL GRS and age. With increasing age, the LDL GRS decreased. Regression analysis showed a significant association between LDL GRS and chronological age (β=-0.27 (SE=0.1) per 10-year increase in age, p=0.010, adjusted for sex and cohort). Additional separate analyses were performed in participants aged ≥50 years and in participants aged ≥70 years. In both age groups the significant association between the LDL GRS and age remained when excluding the younger participants (≥50 years: β=-0.26 (SE=0.11) per 10-year increase in age, p=0.015; ≥70 years: β=-0.52 (SE=0.18) per 10-years increase in age, p=0.005).

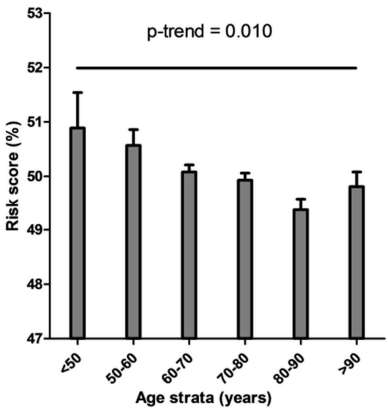

Figure 3: Mean percentage of LDL GRS for each age category, including LLS partners, participants of the Leiden 85-plus study, and the Rotterdam Study. The LDL GRS is plotted as mean percentage with standard error. P-value calculated for the association between age and LDL GRS, adjusted for sex and cohort.

Next, we investigated if the LDL GRS was associated with familial longevity. For this purpose, we compared the mean LDL GRS in the LLS nonagenarians versus the individuals aged ≥90 years from the Leiden 85-plus study and the Rotterdam Study (figure 4A), and in the LLS offspring versus the LLS partners (figure 4B). The mean

GRS was significantly lower for individuals with a predisposition for longevity compared to the individuals from the general population within the same age range. LLS nonagenarians had a mean LDL GRS of 48.8% (SD=0.2) compared to a mean LDL GRS of 49.7% (SE=0.3) for the individuals aged ≥90 years from the general population ($p_{difference}$=0.018). LLS offspring had a mean LDL GRS of 50.0% (SE=0.1), while the partners had a mean LDL GRS of 50.7% (SE=0.2) ($p_{difference}$=0.019).

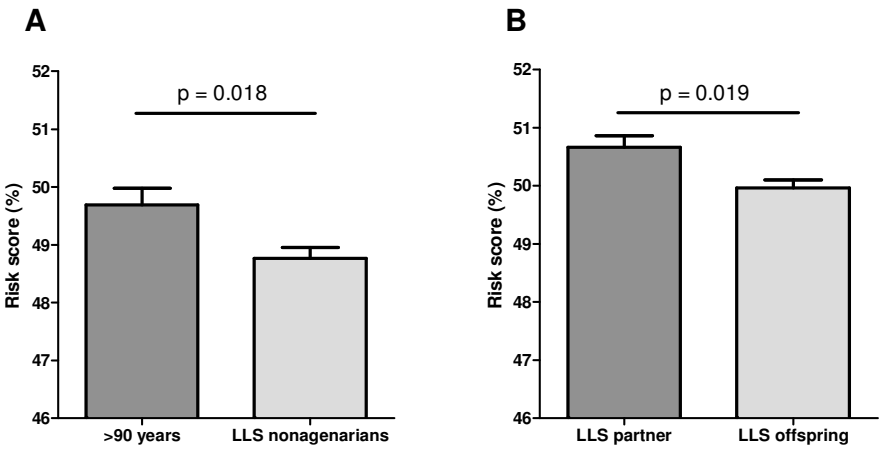

Figure 4: Mean percentage of LDL GRS in participants of the general population (dark grey) and genetically enriched for longevity (light grey). A: Subjects aged ≥90 years from the Leiden 85-plus study and Rotterdam Study versus Leiden Longevity study (LLS) nonagenarians; B: LLS partners versus offspring. GRS plotted as mean with standard error, adjusted for age, sex, and familial relations.

Finally, we investigated the association between the LDL GRS and mortality in the elderly. For this purpose, we used the data of the LLS nonagenarians and of participants aged ≥ 90 years from the Leiden 85-plus study and the Rotterdam Study. The combined analysis of the three studies showed a significant association between the LDL GRS and increased all-cause mortality (table 2). Individuals in the middle LDL GRS group had 4% increased mortality risk (IRR 1.04, 95% CI: 0.90-1.19), and individuals in the highest group had 19% increased mortality risk (IRR 1.19, 95% CI: 1.02-1.37), compared to individuals in the lowest LDL GRS group (p-trend=0.008). Analyses in the individual studies showed a significant association between LDL GRS and mortality in the LLS nonagenarians (p=0.010), with 25% increased mortality risk (95% CI: 1.04-1.49) for individuals in the highest LDL GRS group, compared to individuals in the lowest group. Within the Leiden 85-plus study and the Rotterdam Study, the LDL GRS was not significantly associated with mortality (p=0.445 and p=0.962, respectively).

Discussion

In old age, the importance of high LDL-C levels as risk factor for mortality is unclear since observational studies have shown no or inverse associations. Due to confounding or reversed causality, the plasma LDL-C levels may not reflect the life-time level. To overcome the potential influences of reverse causality and confounding we used in the current study the LDL GRS as an instrumental variable. The LDL GRS was strongly associated with LDL-C levels and the number of LDL increasing alleles decreased with increasing age. Furthermore, individuals with a genetic predisposition for longevity had a lower LDL GRS compared to age-matched controls. Finally, we showed that the LDL GRS was associated with all-cause mortality above 90 years in the pooled analysis of three independent populations, although this effect was mainly driven by one study. All these results indicate that a genetic predisposition to high LDL-C levels contributes to mortality throughout life, also in the oldest old, and a beneficial LDL genetic profile is associated with familial longevity.

Observational studies have repeatedly shown a positive association between high cholesterol levels and increased mortality risks.[17] However, it is unclear whether this positive association remains in the elderly. Several studies in people aged 80 years and over showed an association between low total cholesterol levels and increased mortality.[5] Previously reported analysis of the Leiden 85-plus study did not observe any association between high LDL-C levels and mortality, and high total cholesterol levels were associated with longevity.[3;4] Cholesterol levels of elderly aged 85 years and over might not reflect their life-time cholesterol level, due to reverse causality and possible selective survival.[3] Our results using the LDL GRS as an instrumental variable indicate that the results from observational studies in elderly using plasma cholesterol as a reflection of risk were probably biased.

The observed association between the LDL GRS and mortality was only significant in the LLS siblings and the combined analysis. This might be explained by the lower number of individuals aged 90 years and over in the Leiden 85-plus and Rotterdam study compared to the LLS siblings. We did observe that the level of LDL GRS decreased by increasing age, this was however not reflected in all prospective studies. A similar phenomenon was observed earlier for the *APOE* gene. A lower frequency of the APOE ε4 allele with increasing age was reported already in 1988.[18] However, associations between the *APOE* gene and mortality were reported since 1994 in large studies. [19;20] Nowadays, the association between genetic variation in

the *APOE* gene and longevity has repeatedly been validated in large prospective studies with sufficient statistical power. [20;21]

Genetic risk scores based on SNPs associated with cholesterol levels have been used previously. Within the CARDIoGRAM consortium, including more than 53146 myocardial infarction cases and controls, the association between cholesterol levels and the risk of myocardial infarction (MI) was compared to the association between GRS and the risk of MI.[22] An increase in both plasma LDL-C levels as well as LDL-C conferred by the GRS was associated with an increased risk of MI. Increased HDL cholesterol was associated with a decreased risk for MI, although the HDL GRS was not associated with the risk for MI, indicating that HDL cholesterol is not a causal risk factor for MI.[22] Recently, a GRS based on LDL-C SNPs was tested in two British prospective studies, including middle-aged men and women.[23] Participants in the top quintile of the genetic score distribution tended to have a 36-49% increased risk of having a high CVD risk, determined by the Framingham 10-year CVD risk more than 20%, compared to individuals in the lowest quintile. Our study shows that an association with mortality is still present at old age.

In the current study we observed a difference in LDL GRS between offspring of nonagenarians and their spouses and between LLS nonagenarians and individuals aged ≥90 years. Individuals with a genetic predisposition for longevity had a lower LDL GRS, indicating the beneficial effects of low LDL cholesterol levels. This finding is the first difference in genetic risk scores observed between the LLS offspring and partners. Previous studies found a lower prevalence of diabetes mellitus, hypertension, and myocardial infarction in LLS offspring compared to their partners.[24] Furthermore, the offspring had a more beneficial metabolic profile.[25] A GRS based on diabetes risk alleles has previously been tested in the LLS partners and offspring, and despite the better glucose tolerance of the offspring, this was not associated with differences in GRS.[25]

To summarize, previous observational studies including older individuals have shown no or inverse associations between cholesterol levels and mortality, suggesting that the causal relation between LDL and (cardiovascular) disease is absent at old age. Results of the current study indicate that a genetic predisposition to high LDL-C contributes to mortality throughout life, also in the oldest old and a beneficial LDL genetic risk profile is associated with familial longevity.

Supplementary material

Supplementary material is available upon request.

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