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Genome-wide linkage analysis for human longevity: Genetics of Healthy Aging Study

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Summary

Clear evidence exists for heritability of human longevity, and much interest is focused on identifying genes associated with longer lives. To identify such longevity alleles, we performed the largest genome-wide linkage scan thus far reported. Linkage analyses included 2118 nonagenarian Caucasian sibling pairs that have been enrolled in 15 study centers of 11 European countries as part of the Genetics of Healthy Aging (GEHA) project. In the joint linkage analyses, we observed four regions that show linkage with longevity; chromosome 14q11.2 (LOD = 3.47), chromosome 17q12-q22 (LOD = 2.95), chromosome 19p13.3-p13.11 (LOD = 3.76), and chromosome 19q13.11-q13.32 (LOD = 3.57). To fine map these regions linked to longevity, we performed association analysis using GWAS data in a subgroup of 1228 unrelated nonagenarian and 1907 geographically matched controls. Using a fixed-effect meta-analysis approach, rs4420638 at the *TOMM40/APOE/APOC1* gene locus showed significant association with longevity (P -value = 9.6×10^{-8}). By combined modeling of linkage and association, we showed that association of longevity with *APOEε4* and *APOEε2* alleles explain the linkage at 19q13.11-q13.32 with P -value = 0.02 and P -value = 1.0×10^{-5} , respectively. In the largest linkage scan thus far performed for human familial longevity, we confirm that the *APOE* locus is a longevity gene and that additional longevity loci may be identified at 14q11.2, 17q12-q22, and 19p13.3-p13.11. As the latter linkage results are not explained by common variants, we suggest that rare variants play an important role in human familial longevity.

Key words: *APOE* gene; association analysis; genome-wide linkage analysis; Human familial longevity; nonagenarian sibling pairs.

Introduction

Nonagenarians, centenarians, and their first degree family members have a life-long survival advantage (Perls *et al.*, 2002; Schoenmaker *et al.*, 2006) that can be attributed to a lower risk of coronary artery disease, cancer and type-2 diabetes (Terry *et al.*, 2003; Westendorp *et al.*, 2009). In middle age, members of long-lived families display

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characteristic of metabolic health such as low glucose levels and preserved insulin sensitivity (Roziing *et al.*, 2010; Slagboom *et al.*, 2011). The clustering of longevity in families suggests a heritable component (Gudmundsson *et al.*, 2000; Perls *et al.*, 2000) which has been estimated at approximately 25% in the general population (Skytthe *et al.*, 2003) with increasing importance at the highest ages (Hjelmborg *et al.*, 2006). However, the genetic basis of longevity, first clearly identified as a research priority by Schächter *et al.* (Schachter *et al.*, 1993), still remains to be elucidated.

Previously, it has been demonstrated that long-lived families carry as many GWAS-identified disease susceptibility alleles as the general population (Beekman *et al.*, 2010). It can therefore be hypothesized that long-lived families carry gene variants that promote healthy aging and protect from disease. The genomic location of such protective variants can be identified in a genome-wide linkage scan among long-lived siblings. In the past, by genotyping 400 micro-satellite markers in 137 long-lived sibships, Puca *et al.* (Puca *et al.*, 2001) detected linkage at 4q25 (LOD = 3.65). In a subsequent study, modest support was provided for this locus by a genome-wide linkage scan in 95 male sibling pairs concordant for healthy aging (Reed *et al.*, 2004). However, in a targeted study of 4q25 in 164 nonagenarian sibships no linkage was observed (Beekman *et al.*, 2006). By association of the positional candidate genes at 4q25, *MTP* was suggested to explain the linkage (Geesaman *et al.*, 2003), but this association could not be replicated in other studies (Bathum *et al.*, 2005; Nebel *et al.*, 2005; Beekman *et al.*, 2006). Extension of the original group of long-lived siblings as investigated by Puca *et al.* to 279 pairs also resulted in lack of evidence for linkage at 4q25, although novel linkage regions for longevity were observed at chromosome 3p24-22 and chromosome 9q31-24 (Boyden & Kunkel, 2010).

A number of studies have performed genome-wide association (GWA) analysis for longevity to uncover common genetic variation involved in this trait. A rather large association study from the Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium of 1836 individuals aged over 90 years and 1955 controls between 55 and 80 years did not reveal genome-wide significant loci (Newman *et al.*, 2010) and neither did the analyses of all-cause mortality and survival free of major disease in this cohort (Walter *et al.*, 2011). A smaller Dutch study of 403 nonagenarians and 1670 controls younger than 65 years identified the *APOE* gene as a mortality locus (Deelen *et al.*, 2011), which was confirmed in a German study of 763 long-lived individuals and 1085 younger controls (Nebel *et al.*, 2011) and a longitudinal study of 1606 Danes showed that the effect size of this association increases at the highest ages (Jacobsen *et al.*, 2010). Apparently, the influence of the common genetic variation on longevity is small which requires large meta-GWA studies for identification. Alternatively, rare genetic variants may play a more important role in longevity. As the previous linkage studies showed contradictory results potentially due to heterogeneity in the longevity phenotype, it is expected that longevity is influenced by many private rare variants.

In this study, we performed genome-wide linkage analysis among 2118 European nonagenarian sibships of the Genetics of Healthy Aging (GEHA) Study (Franceschi *et al.*, 2007; Skytthe *et al.*, 2011).

This integrated European project was initiated in 2004 with the aim of identifying genes involved in healthy aging and longevity. The GEHA selection criterion of nonagenarian sibling pairs has been shown to result in families enriched for genetic influences on longevity by a smaller previous study of the same design (the Leiden Longevity Study) (Schoenmaker *et al.*, 2006). This study is characterized by a survival benefit in multiple generations, low prevalence of disease, and beneficial metabolic phenotypes, which is comparable to the observations reported for families of centenarian singletons such as the Longevity Study at Albert Einstein College of Medicine (Barzilai *et al.*, 2010). Sibships over 90 years of age and controls between 55 and 75 years of age have been recruited in 10 European countries and their genomic DNA has been isolated and genotyped centrally. This logistic achievement resulted in the largest linkage study for human longevity.

Results

In the Genetics of Healthy Aging Study (GEHA), nonagenarian sibling pairs have been recruited in 11 countries among 15 study centers (Skytthe *et al.*, 2011). Genotypings of 5734 SNPs (Illumina HumanLinkage12 set) were available after quality control in 4445 persons belonging to 2118 full sibships with a mean sibship size of 2.1 (Table 1).

To investigate potential substructure, identical-by-state (IBS) estimates for all pairs of individuals in the data set were computed using EIGENSTRAT (Price *et al.*, 2006). The first two resulting principal components (C1 and C2) were plotted against each other (Fig. 1), which gives a representation of the data in two dimensions. In the resulting scatter plot, each point represents an individual and each recruitment center is marked by its own symbol. After visual inspection of component 1 (C1) and component 2 (C2), C1 appeared to reflect the North-south localization and C2 dissociated the Fins from the rest of Europe. On the basis of this genetic heterogeneity among the study samples, the linkage analyses were performed in three geographical clusters: (i) Finland with $N_{\text{sibships}} = 150$; (ii) Northern Europe with $N_{\text{sibships}} = 1378$, consisting of Ukraine, Poland, Germany, Denmark, The Netherlands, United Kingdom, Belgium, and France; (iii) Southern Europe with $N_{\text{sibships}} = 590$, consisting of Italy and Greece.

We calculated nonparametric linkage scores over the whole genome for the three clusters combined (Fig. 2, blue line; Data S2). Besides the inflated LOD score probably due to linkage disequilibrium between markers at the beginning of chromosome 3, the combined linkage score for the GEHA study exceeded the genome-wide significant LOD score of 3 at chromosome 14q11.2 (8 cM), 19p13.3-p13.11 (36 cM), and 19q13.11-q13.32 (68 cM) (Fig. 3). For centers for which population-based mortality rates were available, i.e., Italy, UK, Denmark, France, Netherlands, Germany, and Finland, a weighted NPL statistic was computed. The weights are the products of the age- and sex-specific cumulative hazard for the two siblings (Houwing-Duistermaat *et al.*, 2009) that are based on country-specific mortality rates. In this weighted NPL analysis extremely old sibling pairs obtain more weight (Fig. 2, yellow line) and the LOD score at chromosome 17q12-q22 (82 cM) increased

Table 1 Number of sibships and sibship sizes for the GEHA sample sets

Cluster	Country	Center	Sibship size				Sibships	Mean age (years)	Male sibpairs	Female sibpairs
			2	3	4	5				
1	Finland	Tampere	124	24	1	1	150	92.6	11	102
2	Ukraine	Kiev	46	1	0	0	47	93.2	1	38
2	Poland	Warsaw	131	4	0	0	135	93.0	11	87
2	Germany	Kiel	90	4	0	0	94	92.8	12	53
2	Denmark	Odense	384	45	5	0	434	92.3	50	215
2	The Netherlands	Leiden	150	14	2	0	166	93.0	20	82
2	UK	Newcastle	98	1	0	0	99	92.8	7	55
2	UK	Belfast	52	4	1	0	57	92.9	4	40
2	Belgium	Louvain	76	1	0	0	77	92.9	13	40
2	France	Montpellier	238	27	3	1	269	93.3	30	157
3	Italy	Bologna	178	23	6	1	208	93.4	14	126
3	Italy	Rome	51	2	0	0	53	92.5	6	32
3	Italy	Calabria	181	7	1	0	189	93.0	24	79
3	Italy	Sassari	43	2	0	0	45	93.4	3	24
3	Greece	Athens	92	3	0	0	95	92.9	57	15
		Total	1934	162	19	3	2118	92.9	263	1145

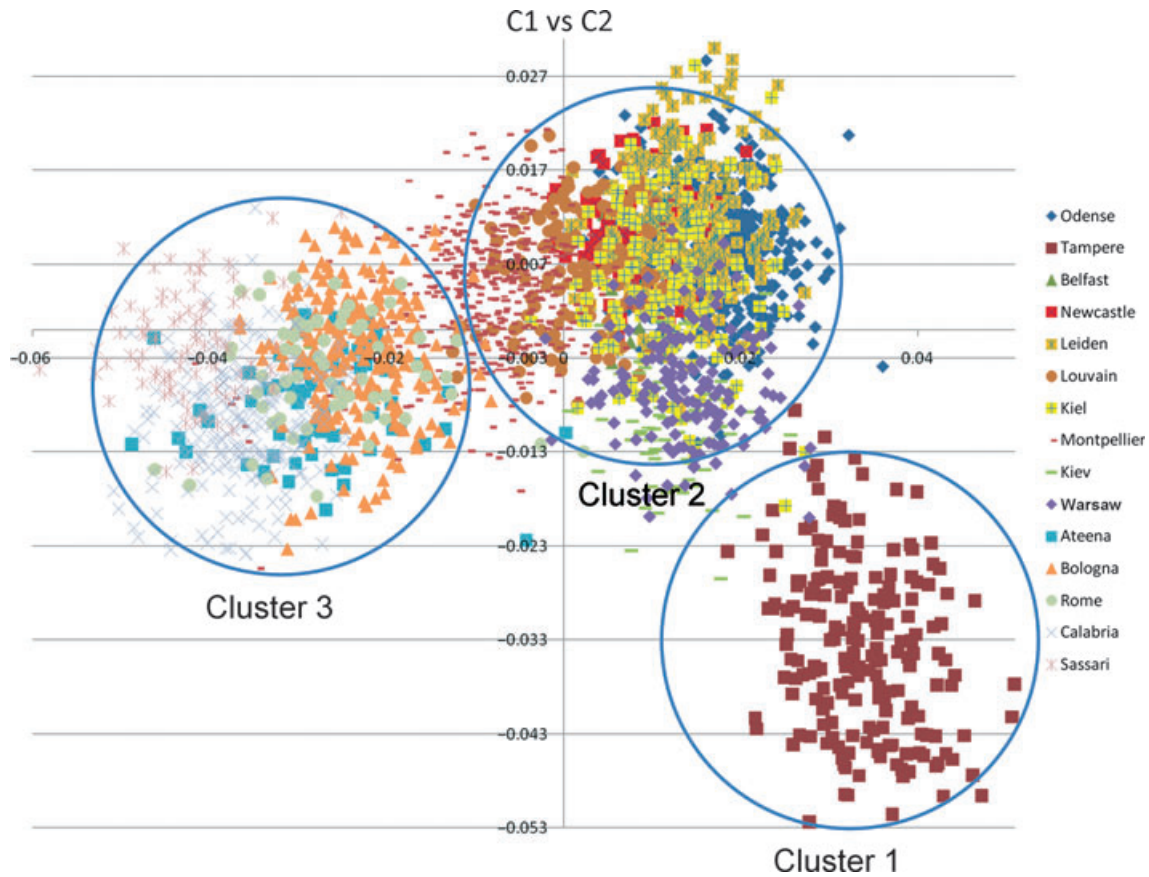


Fig. 1 Population structure among GEHA study and the grouping of countries into three clusters. Cluster 1: Finland; Cluster 2: Northern Europe consisting of Ukraine, Poland, Germany, Denmark, The Netherlands, United Kingdom, Belgium, and France; Cluster 3: Southern Europe consisting of Italy and Greece.

from 2.95 to 3.71 (Fig. 3; Table 2). This increase has been observed in each of the three clusters Northern Europe, Southern Europe, and Finland. The northern European cluster contributes most to the

linkage at 14q11.2 (8 cM), 19p13.3-p13.11 (36 cM), and 19q13.11-q13.32 (68 cM), whereas all three clusters contribute to linkage at 17q12-q22 (Fig. S1).

Fig. 2 Genome-wide linkage graphs of among 2118 sibships of the GEHA study. The blue line displays the NPL LOD scores and the yellow line the age-weighted LOD scores.

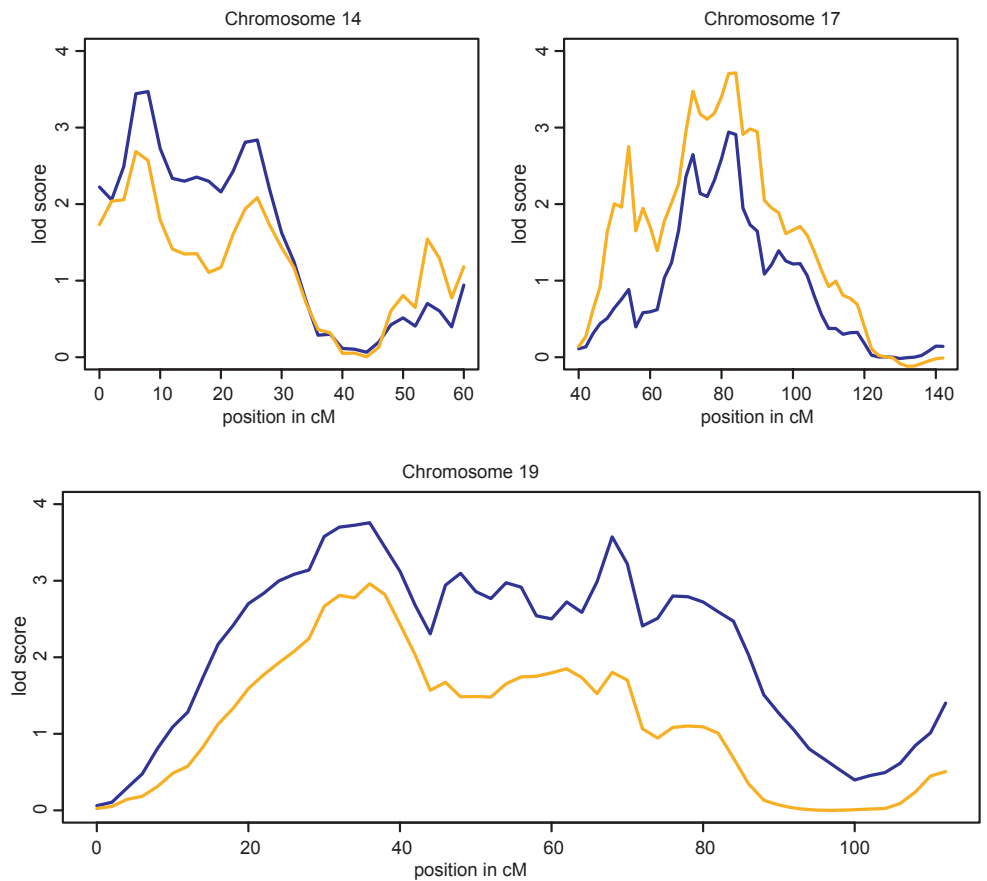
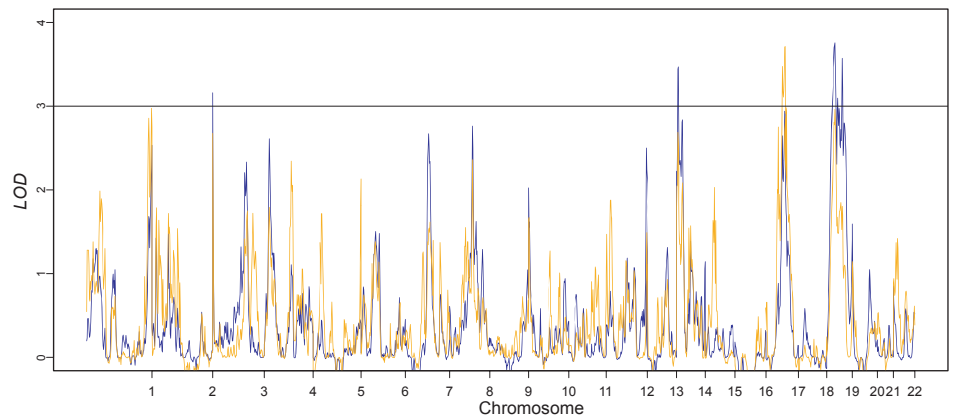


Fig. 3 Chromosomal regions linked to longevity with LOD score above 3. The blue line displays the NPL LOD scores and the yellow line the age-weighted LOD scores.

As gender-specific effects play a role in human longevity, linkage analysis was also performed in male-only ($N_{\text{pairs}} = 263$) and female-only ($N_{\text{pairs}} = 1145$) sibling pairs. Although NPL analysis did not reveal any linkage for male-only pairs, significant linkage (Weighted-NPL 3.61) was observed at 8p11.21-q13.1 (70 cM) among the male-only pairs in the age-weighted analysis (Fig. 4, lower panel; Table 3). Among the female-only sibpairs (Fig. 4, upper panel) significant linkage was observed at chromosome 15q12-q14 (22 cM) (Weighted-NPL 3.16) and at 19q13.33-q13.41 (80 cM) (NPL = 4.97) (Table 3). Further investigation of these

linkage areas revealed that the Northern Europe cluster and the Finland cluster provide evidence for the 8p11.21-q13.1 and 15q12-q14 loci, and that the 19q13.33-q13.41 locus seems only to link with female longevity in the Northern Europe cluster (Fig. S2).

When we consider the genes in the 1-LOD drop intervals of the linkage analysis as candidate genes for longevity, we have a list of 1151 unique position candidate genes (Data S3) for which we have performed gene set enrichment analysis (Table S3, Data S4). The 1-LOD drop intervals were investigated for

Table 2 Four linkage regions with LOD score above 3 in combined GEHA samples for NPL and age-weighted linkage analyses

	14q11.2	17q12-q22	19p13.3-p13.11	19q13.11-q13.32
1LOD-drop interval	0–12 cM [†]	68–95 cM [†]	20–42 cM [†]	58–85 cM [†]
Start SNP	Rs10484218	Rs2429990	Rs432001	Rs7250748
End SNP	Rs977870	Rs12947910	Rs919333	Rs10403760
NPL	3.47	2.95	3.76	3.57
NPL_subsets [‡]	3.15	3.26	3.85	3.80
Weighted_NPL_subsets [§]	2.69	3.71	2.96	1.85

[†]According to genome build 35.

[‡]Population mortality rates were available in: Cluster 1: Finland; Cluster 2: Denmark, UK, The Netherlands, France; Cluster 3: Italy.

[§]weighted with ages of the siblings.

association with longevity by comparison of unrelated nonagenarians to geographically matched younger controls. Of each sibpair of the centers that provided the largest contribution to the linkage results [i.e., Netherlands, Denmark, France, Italy (Bologna), and UK (Newcastle)] (Table S1), the eldest sibs were taken as cases and as controls for France and Bologna, GEHA controls were taken. As controls for UK, Netherlands and Denmark already genotyped available younger population controls were used. We performed a meta-analysis for 57 607 imputed SNPs in the seven

candidate regions with longevity. We tested for differences in genotype frequencies between cases and geographically matched controls using a trend test, i.e., assuming additive genetic effects. The test statistic was adjusted for uncertainty in imputations and for the inflation factor before computing the p-values. We used a fixed-effect meta-analysis approach to combine the results across centers. Finally, we used a Bonferroni correction based on the number of SNPs within the region to correct for multiple testing.

No significant association (P -value $< 8.7 \times 10^{-7}$) was observed at the loci at chromosome 14q11.2, 17q12-q22, and 19p13.3-p13.11, and neither at the sex-specific loci. At 19q13.11-q13.32 locus, rs4420638 at the TOMM40/APOE/APOC1 gene locus showed significant association with longevity (P -value = 9.6×10^{-8}). As the linkage plots per center show that especially the sample from Denmark provides the statistical evidence for an increased LOD score at this position (Fig. S3), we tested in the Danish data set with the software LAMP (Burdick *et al.*, 2006) for residual linkage at the 19q13.11-q13.21 locus if the *APOE* ϵ 2: ϵ 3: ϵ 4 rs7412 and rs429358 genotypes were taken into account. It appeared that the *APOE* ϵ 4 allele, although with moderate significance, contributed to the linkage peak (P -value = 0.02). Remarkably, with a much higher significance the *APOE* ϵ 2 allele also explained the linkage peak (P -value = 1.0×10^{-5}).

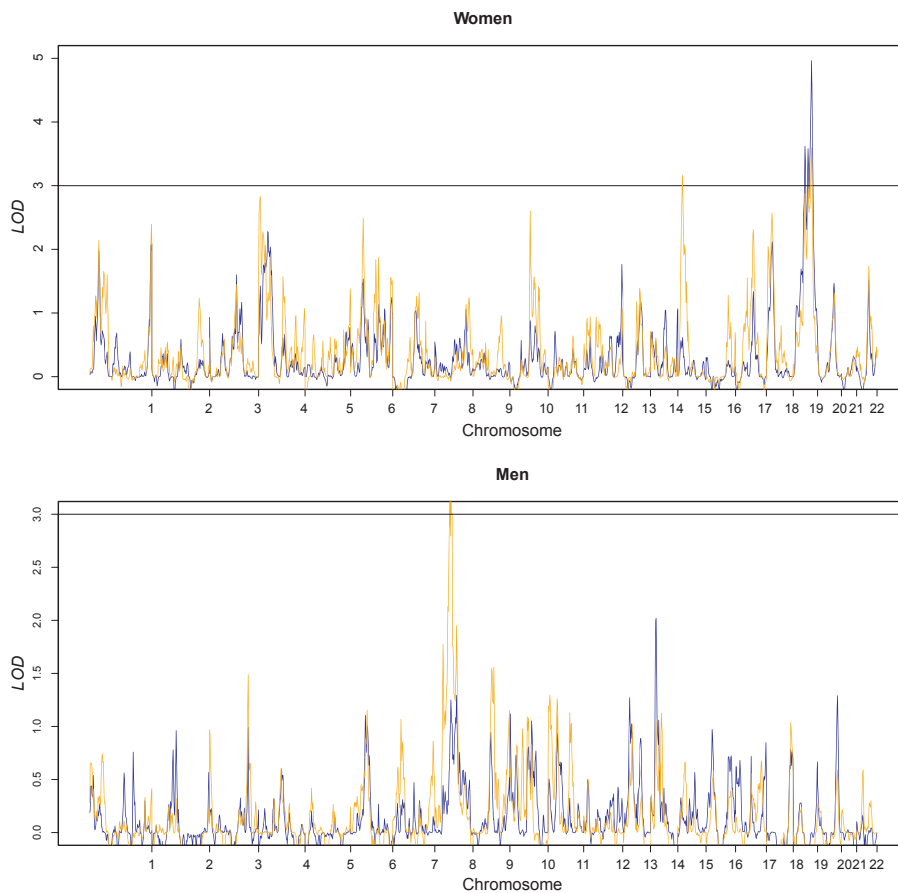


Fig. 4 Genome-wide linkage graphs of among 1145 female-only sibships and 263 male-only sibships of the GEHA study. The blue line displays the NPL LOD scores and the yellow line the age-weighted LOD scores.

Table 3 Sex-specific linkage regions with LOD score above 3 in combined GEHA samples for NPL and age-weighted linkage analyses

Sex	8p11.21-q13.1	15q12-q14	19q13.33-q13.41
	Men	Women	Women
1LOD-drop interval	64–82 cM [†]	14–28 cM [†]	66–90 cM [†]
Start SNP	Rs801100	Rs1871009	Rs1236093
End SNP	Rs4368961	Rs580839	Rs1661965
NPL	1.14	0.62	4.97
NPL_subsets [‡]	1.96	1.44	3.92
Weighted_NPL_subsets [§]	3.61	3.16	3.60

[†]According to genome build 35.

[‡]Population mortality rates were available in: Cluster 1: Finland; Cluster 2: Denmark, UK, The Netherlands, France; Cluster 3: Italy.

[§]Weighted with ages of the siblings.

Discussion

To identify chromosomal regions involved in longevity, we performed the largest linkage analysis thus far reported. This analysis included 2118 nonagenarian sibling pairs from 11 European countries enrolled as part of the Genetics of Healthy Aging (GEHA) project. We identified four regions linked to human longevity at 14q11.2, 17q12-q22, 19p13.3-p13.11, and 19q13.11-q13.32. In addition to these loci, we identified three loci that were linked to longevity in a sex-specific manner: 8p11.21-q13.1 (men), 15q12-q14 (women), and 19q13.33-q13.41 (women). In these seven linked regions, we performed association analysis using GWAS data that was available in a subset of the cohorts, and we showed that genetic variation at the *APOE* gene locus was significantly associated with longevity. The absence of the *APOEε4* allele among the nonagenarians is obvious (Deelen *et al.*, 2011) and contributed to the linkage, suggesting that *APOE* is a mortality gene. However, the linkage was mainly explained by the shared presence of the *APOEε2* allele, indicating that the *APOE* gene indeed is a longevity gene.

Affected sibpair analysis is a powerful tool to map strong dominant as well as recessive gene effects, but to map rare genetic variation with relatively small effect a group of at least 600 sibling pairs with one sibling over age 95 years should be examined (Tan *et al.*, 2004). As the GEHA project yielded an impressive number of 2118 genotyped nonagenarian sibling pairs to investigate for linkage (Skytthe *et al.*, 2011), of which 899 sibling pairs contained one sib aged over 95 years of age, this linkage study has sufficient power to detect the loci with relatively small effects.

Previous studies for longevity loci detected linkage at 4q25 (Puca *et al.*, 2001), 3p24-22, 9q31-34, 12q24 (Boyden & Kunkel, 2010), 6p12.1, 7q11.21, 14q22.1 (Edwards *et al.*, 2011). None of these loci showed linkage in the GEHA project. The largest of the previous linkage studies examined 279 long-lived sibling pairs, which is considered a relatively small study and therefore the previous linkage results should be considered with caution, given the complexity of human longevity (Tan *et al.*, 2004). Otherwise, the effect of previously detected loci on longevity could be private for a specific genetic background or specific environment, or even private for a small number of families.

In the chromosomal region linked to familial longevity at 19q13.11-q13.32, we found rs4420638 to be significantly associated with familial longevity. This SNP tags the LD block harboring the *TOMM40*, *APOE*, and *APOC1* genes. It is known that the *APOE* gene exhibit three isoforms that are caused by two functional SNPs rs7412 (*APOEε2*) and rs429358 (*APOEε4*) which have been associated with longevity (Schachter *et al.*, 1994). The *APOEε4* allele frequency in GEHA nonagenarian sibling cases was 6.8%, which was significantly lower than the allele frequency of 12.7% among the geographically matched controls. *APOEε4* allele carriers have about 50% lower chance to become a nonagenarian than non-*APOEε4* allele carriers (OR = 0.48, 95% CI = 0.42–0.55). Recently, this gene region has been explored for other functional variation and multiple cis-elements are found to influence both *APOE* and *TOMM40* promoter activity and a complex transcriptional regulatory structure has been suggested modulates regional expression (Bekris *et al.*, 2012). Hence, future research should consider exploring these other functional variants for their role in familial longevity.

Genetic association analysis as an approach is suitable to detect the effect on a trait of common genetic variation (minor allele frequency larger than 1%). We did not observe significant associations in the families that contributed to linkage of common variants in the linked regions with longevity which can be explained by the possibility that the positive linkage signals may be based on allelic and/or locus heterogeneity, i.e., different genetic variants in the same gene or gene region contributed to the trait in different families. The less likely explanation is that the power, which was large enough for detecting linkage, may be too small to detect genetic association among 57K SNPs in the linkage regions in 1228 cases. Thus, common variants may not contribute to the longevity phenotype and by applying next generation sequencing analyses of the nonagenarians contributing to linkage rare, private genetic variation in the linkage regions may be found.

This genome-wide linkage scan did not provide substantial evidence for linkage to *FOXO3A* on chromosome 6, of which the T allele of rs2802295 predisposes to survive into old age (reviewed in Chung *et al.*, 2010). This may be due to the relatively small effect sizes conferred by SNPs at that locus or it could be that the nonagenarian sibling design is underpowered to detect the association of *FOXO3A* with longevity because its effect seems the strongest at ages above 95, in which age range the GEHA study has limited numbers of participants. Besides the NPL analyses we performed linkage analyses in which the contribution of the sibling pairs has been weighted for the age of the eldest sibling. Thus, when a locus is involved in survival after an extremely old age, as is suggested for the *FOXO3A* locus, linkage signals would increase in this weighted analyses. Despite the use of these analysis tools, we did not observe any LOD score above LOD score of 1 at the *FOXO3A* locus. We therefore conclude that genetic variation at the *FOXO3A* gene might not contribute to longevity in the GEHA Study.

Previously, it has been suggested that genetic variation in the *FOXO1* gene is specifically contributing to human female longevity (reviewed in Chung *et al.*, 2010). However, at chromosome 13q14.11 harboring the *FOXO1* gene we found no evidence for linkage with female longevity (LOD < 0.05) and at the gene position

of *FOXO1* we found no evidence for association in the females-only meta-analysis (P -values >0.042) in the GEHA Study. Potentially, the effect of this locus is not only influenced by gender but also by genetic background.

When we investigated the cluster-specific linkage results, we observed that Northern Europe cluster contributes to all linkage loci, the Southern Europe cluster only contributes to the 17q12-q22 and 19p13.3-p13.11 loci and the Finland cluster only contributes to the 14q11.2 and 17q12-q22 loci (Fig. S3). The Southern Europe cluster and the Finland cluster did not obtain LOD scores above 3 on their own and the Northern Europe cluster obtained LOD scores above 3 only at chromosome 19 at 20, 68, and 82 cM. This illustrates that probably, in addition to the sample size of the cluster, the environment and genetic background play a role in the complex phenotype of human familial longevity.

Besides linkage at the *APOE* locus, we detected linkage at 14q11.2, 17q12-q22, and 19p13.3-p13.11 considering the total GEHA study. These chromosomal regions linked to human longevity cover many genes that are potential candidate genes involved in neurodegenerative disease, auto-immune disease, cardiovascular disease, and cancer. In the NCBI GWAS catalog (<http://www.genome.gov/gwa-studies/>) genome-wide association has been reported at the 14q locus for the T-cell receptor alpha with narcolepsy. At the 17q12-q22 locus, genome-wide association has been reported for *MAPT* and *NSF* with Parkinson's disease, for *CA10* with age of onset of menarche, and for *STAT3* with Multiple Sclerosis and Crohn's disease. At the chromosome 19p13.3-p13.11 locus, genome-wide association has been reported for *TYK2* and *ICAM3* with Crohn's disease, for *LDLR* with myocardial infarction, for *SMARCA4* with coronary heart disease, and for *ABHD8* and *ANKLE1* with breast cancer.

In conclusion, we identified four chromosomal regions linked to human familial longevity and we are the first identifying genetic linkage with familial longevity at the *APOE* gene locus. As genetic variation at the *APOE* gene explains the linkage, we confirm that the *APOE* locus is indeed a longevity gene. Identification of additional longevity genes in the linkage regions will provide biological insight into the pathways underlying human familial longevity, metabolic health, and the resistance to age-related disease.

Experimental procedures

Study subjects

The individuals investigated in this study participate in Genetics of Healthy Aging (GEHA) Study (Skytthe *et al.*, 2011). Families participating in the GEHA study have at least two siblings meeting four inclusion criteria: (i) participants are at least 90 years old, (ii) participants have at least one living brother or sister who fulfills the first criterion and is willing to participate, (iii) the nonagenarian sibship has an identical mother and father, and (iv) the parents of the nonagenarian sibship are European and Caucasian. In total, 2249 sibships have been recruited.

In accordance with the Declaration of Helsinki, written informed consent was obtained from all participants prior to entering the study. Good clinical practice guidelines were maintained. The study

protocol was approved by the local medical ethical committees of the 11 participating countries before the start of the study.

DNA isolation

The National Institute for Health and Welfare (THL, Helsinki), extracted high molecular DNA from 7 ml of whole blood using automated DNA purification, Autopure LS (Qiagen), based on Puregene salting out methodology. DNA concentrations were adjusted to 50 ng/ μ L, verified using PicoGreen dsDNA Quantitation kit (Molecular Probes, Life Technologies, Paisley, UK). The samples were then subjected to quality control by ABI 3730 DNA analyzer (Applied Biosystems, Life Technologies) using two sex-specific and four autosomal microsatellite markers.

Genotyping for linkage analysis

The Centre National de Génotypage (CNG, Paris) performed genotyping of 6090 SNPs in the nonagenarian siblings of the 2249 sibships recruited in the GEHA project with the Illumina HumanLinkage-12 Genotyping BeadChip. Genotyping call rate was larger than 95% per sample. We observed 19 duplicates that are either MZ twins or duplicate samples of which two were found in two different families. Five full sibs were identified as unrelated and eight sample pairs that are supposed to be unrelated were identified as potential half-sibs. Of all sibships, 35 appeared to be composed of half-sibs. Finally, two sample pairs that are supposed to be unrelated were found full sibs. After removing the samples with unexpected relations, we obtained reliable genotyping for 4445 individuals from 2118 sibships (Table 1). As the recruitment of the GEHA study has been a major enterprise, it turned out in a later phase of the project that some cases were aged below 90 years of age at the time of interview (Table S2 and Fig. S4).

We applied the EIGENSTRAT program (Price *et al.*, 2006) with default parameters to infer axes of variation with the 6090 SNPs per individual to identify genetic population stratification.

Affected sibpair analysis

Unlikely recombinants were detected using Merlin-0.10.2 (Abecasis *et al.*, 2002) and erroneous genotypes were removed with Pedwipe. In addition, Pearson's r^2 was estimated using the total data set. For the nonparametric (NPL) analyses, 275 SNPs were removed to obtain a genome-wide SNP set of 5734 SNPs with pairwise r^2 smaller than 0.4. MERLIN-0.10.2 (Abecasis *et al.*, 2002) was used to estimate the information content over the genome (Data S1) and to estimate IBD probabilities in the sibling pairs without parents.

Next, proportion of alleles shared identical by descent was estimated in Merlin (Abecasis *et al.*, 2002) allowing for center-specific allele frequencies. Nonparametric linkage analysis (NPL) (Kruglyak & Lander, 1996) was performed using a score test statistic for affected sibling pairs (Callegaro *et al.*, 2009) that was computed at a grid of 2 cM. NPL statistics per cluster were obtained by summing the NPL statistics per involved center. This statistic compares the actual proportion of alleles shared identical by descent by a sibling pair with

its expectation of 0.5 under Mendelian segregation. The variance of the statistic was obtained via simulation in Merlin. By simulation, the variance is adjusted for incomplete marker informativeness and correlation of sibling pairs within families. The statistic assumes that there is no linkage disequilibrium between the markers. When this assumption is violated, the peaks will be inflated.

For centers for which population-based mortality rates were available, i.e., Italy, United Kingdom, Denmark, France, Netherlands, Germany, and Finland, a weighted NPL statistic was computed. The weights are the products of the age- and sex-specific cumulative hazard for the two siblings (Houwing-Duistermaat *et al.*, 2009). These cumulative hazards are based on country-specific mortality rates. In this analysis, extremely old sibling pairs obtain more weight in the analysis.

Subjects for association analysis within linked regions

To fine map the 1-LOD drop linkage regions, association analysis was performed in the largest centers and those most contributing to the linkage results. From each pair the eldest siblings from the United Kingdom, Netherlands, Denmark, France, and Bologna, and GEHA controls from France and Bologna were genotyped. As controls for United Kingdom, Netherlands, and Denmark available geographically matched control data sets were used: TwinUK for UK nonagenarians (Perola *et al.*, 2007), controls from the Leiden Longevity Study (LLS) for Dutch nonagenarians (Beekman *et al.*, 2010), and Danish GOYA controls (Paternoster *et al.*, 2011) for Danish nonagenarians. The characteristics of the samples are displayed in table S1.

Genotyping for association analysis within linked regions

The GEHA nonagenarians from the UK, Netherlands, Denmark, and France (CNG, Paris, France) and the LLS controls (genotyping facility Erasmus MC, Rotterdam, The Netherlands) have been genotyped using Illumina Infinium HD Human660W-Quad BeadChips, the GEHA nonagenarians from Bologna, their controls and the France controls have been genotyped using Illumina HumanOmniExpress BeadChips (genotyping facility Erasmus MC, Rotterdam, The Netherlands) and the Danish controls (GOYA) and UK controls (TwinsUK) have been genotyped using Illumina Infinium HD Human610-Quad BeadChips. Autosomal SNPs were included in analysis if they had less than 5% missing data, Hardy-Weinberg P -values in cases and controls greater than 1×10^{-4} and minor allele frequency (MAF) was larger than 1% (if n_{cases} and $n_{\text{controls}} \geq 200$) or 5%. SNPs that passed this quality control in both cases and controls were used as input for imputation to HAPMAP2 release 22 using IMPUTE2. SNPs with a R_2T value lower than 40 and a MAF lower than 1% (if n_{cases} and $n_{\text{controls}} \geq 200$) or 5% were excluded from analyses (Uh *et al.*, 2011).

Association analysis within linked regions

Association analysis has been performed using CC-assoc applying score test (Uh *et al.*, 2011). For meta-analysis, a fixed-effect

approach was used and adjustment for population stratification per study was performed by multiplying the variances with the genomic inflation factor (λ), which measures overdispersion of test statistics from association tests. Scores and adjusted variances of the five countries were combined to obtain a single metastatistic. We tested 57 607 SNPs within the 1-LOD drop intervals and according to Bonferroni adjustment for multiple testing, P -values below 8.7×10^{-7} were considered genome-wide significant.

Combined linkage and association at APOE ϵ 2 ϵ 3 ϵ 4 locus

To investigate whether the linkage identified can be explained by the APOE ϵ 2 ϵ 3 ϵ 4 polymorphisms, we performed linkage and association modeling in pedigrees (LAMP) (Li *et al.*, 2005) in the Danish sample in which rs7412 and rs429358 have been genotyped by TaqMan using standard protocol of the supplier.

Gene set enrichment analyses

For each linkage region, the 1-LOD drop intervals have been determined. Next, the genes located in these 1-LOD drop intervals have been listed and tested for pathway enrichment using default settings in DAVID (<http://david.abcc.ncifcrf.gov/>).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Linkage regions in the three geographically clusters at chromosome 14 (upper left panel), chromosome 17 (upper right panel) and chromosome 19 (lower panel).

Fig. S2 Sex-specific linkage regions in the three geographically clusters. Light blue line represents Northern Europe cluster, orange line represents Southern Europe cluster, purple line represents Finland.

Fig. S3 Linkage at chromosome 19 for the three largest centers from the Northern European cluster.

Fig. S4 Histogram of ages in the GEHA study.

Table S1 Nonagenarian cases and controls for association analyses in candidate longevity regions.

Table S2 Mean age and age range of GEHA nonagenarian sibling cases.

Table S3 Gene Ontology term enrichment among the genes resided in regions linked to longevity.

Data S1 Information content.

Data S2 Genome wide linkage results.

Data S3 Positional candidate genes.

Data S4 Gene set enrichment results from DAVID.