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Evidence from case–control and longitudinal studies supports associations of genetic variation in *APOE*, *CETP*, and *IL6* with human longevity

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Abstract In this study, we investigated 102 single-nucleotide polymorphisms (SNPs) covering the common genetic variation in 16 genes recurrently regarded as candidates for human longevity: *APOE*; *ACE*; *CETP*; *HFE*; *IL6*; *IL6R*; *MTHFR*; *TGFB1*; *APOA4*; *APOC3*; *SIRT*s *1*, *3*, *6*; and *HSPA*s *1A*, *1L*, *14*. In a case–control study of 1,089 oldest-old (ages 92–93) and 736 middle-aged Danes, the minor allele

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frequency (MAF) of rs769449 (*APOE*) was significantly decreased in the oldest-old, while the MAF of rs9923854 (*CETP*) was significantly enriched. These effects were supported when investigating 1,613 oldest-old (ages 95–110) and 1,104 middle-aged Germans. rs769449 was in modest linkage equilibrium (R^2=0.55) with rs429358 of the APOE-ε4 haplotype and adjusting for rs429358 eliminated the association of rs769449, indicating that the association likely reflects the well-known effect of rs429358. Gene-based analysis confirmed the effects of variation in *APOE* and *CETP* and furthermore pointed to *HSPA14* as a longevity gene. In a longitudinal study with 11 years of follow-up on survival in the oldest-old Danes, only one SNP, rs2069827 (*IL6*), was borderline significantly associated with survival from age 92 (P-corrected=0.064). This advantageous effect of the minor allele was supported when investigating a Dutch longitudinal cohort (*N*=563) of oldest-old (age 85+). Since rs2069827 was located in a putative transcription factor binding site, quantitative RNA expression studies were conducted. However, no difference in *IL6* expression was observed between rs2069827 genotype groups. In conclusion, we here support and expand the evidence suggesting that genetic variation in *APOE*, *CETP*, and *IL6*, and possible *HSPA14*, is associated with human longevity.

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Introduction

Approximately 25% of the variation in human lifespan is thought to be caused by genetic variation (Herskind et al. 1996), a contribution considered to be minimal before age 60 years and most profound after age 85 years (Hjelmborg et al. 2006). Candidate genes for longevity encode proteins engaged in different biological processes including lipoprotein metabolism and inflammatory processes (Christensen et al. 2006).

In this study, we have set out to thoroughly investigate the common genetic variation within candidate genes previously reported to contain lifespan associated polymorphisms. Furthermore, we expand the study to include the genetic variation of genes belonging to the same functional groups. Of the 16 genes included in this study, only *SIRT1* and *3* have previously been investigated by a tagging single-nucleotide polymorphism (SNP) approach in a candidate association study (Flachsbart et al. 2006; Kuningas et al. 2007; Lescai et al. 2009).

We investigate the apolipoprotein E, C3, and A4 genes (*APOE*, *APOC3*, and *APOA4*), all of which are involved in lipoprotein metabolism, believed to pose an effect on longevity (Christensen et al. 2006). The APOE ε4 haplotype is by far the most validated genetic variation and has repeatedly been associated with human longevity (e.g., Asada et al. 1996; Bathum et al. 2006; Deelen et al. 2011; Gerdes et al. 2000; McKay et al. 2011; Nebel et al. 2011; Schachter et al. 1994), whereas one study has found association of rs2542052 in *APOC3* with longevity (Atzmon et al. 2006). The *APOE* polymorphism was recently reviewed in (Seripa et al. 2011). Also involved in lipoprotein metabolism is the cholesteryl ester transfer protein (cetp) for which rs5882 in *CETP* has been reported as a longevity SNP (Barzilai et al. 2003).

Genes encoding proteins engaged in immune regulatory processes are also key candidates for longevity (reviewed in Jylhava and Hurme 2010). Frequently discussed is the cytokine interleukin 6 (IL6) and its receptor alpha (IL6R); in *IL6*, a variable number of tandem repeat (VNTR) and a −174C/G polymorphism (rs1800795) have been described as associated with longevity (Capurso et al. 2007; Christiansen et al.

2004; Hurme et al. 2005; Rea et al. 2003). Another cytokine gene often suggested is the transforming growth factor beta 1 gene (*TGFβ1*), which is involved, among others, in regulation of cellular proliferation and differentiation. In *TGFβ1*, the +915 polymorphism (rs1800471) and a haplotype including rs1800471 have been reported to be of relevance for longevity (Carrieri et al. 2004).

We also explored the heat shock protein (hsp) encoding genes *HSPA1A*, *1L*, and *14*, all playing a role in folding of newly translated proteins and in stabilization of proteins against aggregation, processes reported to decrease during aging (Singh et al. 2007). The A/C-110 polymorphism in *HSP1A* and T2437C in *HSP1L* have been described to be independently associated with longevity (Altomare et al. 2003; Ross et al. 2003; Singh et al. 2010), as well as in separate haplotypes (Li et al. 2009a; Singh et al. 2010).

The sirtuins 1, 3, and 6 probably function as intracellular regulatory proteins affecting for example IGF1/insulin signaling and DNA repair, although their roles are not entirely clear (Olmos et al. 2011). In *SIRT3*, a *G477T* polymorphism (Rose et al. 2003) and a VNTR polymorphism (Bellizzi et al. 2005) have been put forward as longevity polymorphisms.

Finally, we examined three genes not belonging to any of these biological processes but still considered reliable candidates in previous studies: the highly discussed candidate gene angiotensin I-converting enzyme (*ACE*), for which the I/D insertion/deletion (tagged by rs4343 (Abdollahi et al. 2008)) has been reported by several authors to be associated with longevity (Arias-Vasquez et al. 2005; Cellini et al. 2005; Frederiksen et al. 2003; Luft 1999; Novelli et al. 2008; Schachter et al. 1994; Zajc et al. 2012); the iron absorption regulatory protein hfe, for which the Cys282Tyr variation (rs1800562) was found to decrease with age (Bathum et al. 2001; Lio et al. 2002); and, finally, *MTHFR*, which is of importance for the essential remethylation of homocysteine to methionine, where the C677T (rs1801133) polymorphism has been suggested as associated with longevity (Stessman et al. 2005; Todesco et al. 1999).

A considerable number of the associations mentioned above could not be replicated in subsequent studies. These studies include rs2542052 in *APOC3* (Novelli et al. 2008), rs5882 in *CETP* (Cellini et al. 2005; Novelli et al. 2008), I/D insertion/deletion in *ACE* (Agerholm-Larsen et al. 1997; Bladbjerg et al. 1999; Blanche et al. 2001; Yang et al. 2009), rs1800562 in *HFE* (Carru et al. 2003; Coppin et al. 2003), and rs180113 in *MTHFR* (Bladbjerg et al. 1999; Khabour et al. 2009; Hessner et al. 2001; Brattstrom et al. 1998) and−176C/G in *IL6* (Pes et al. 2004; Wang et al. 2001). Furthermore, a few meta-analyses or pooled analyses have been published; for *APOE* ε4 and the *ACE* I/D, insertion/deletion association was supported (McKay et al. 2011; Zajc et al. 2012), while for the *IL6* −176C/G polymorphism, a North/South European gradient of association was suggested (Di Bona et al. 2009). In any case, the involvement in longevity of the majority of these specific polymorphisms still remains uncertain.

The majority of candidate gene studies published to date have investigated only very few polymorphisms in each gene. To more thoroughly examine as much of the known variation in each of these genes as possible, we here apply a tagging SNP approach using a total of 102 tagging SNPs covering the 16 gene regions. With the exception of *SIRT 1* and *3* (Flachsbart et al. 2006; Kuningas et al. 2007; Lescai et al. 2009), such an approach has, to our knowledge, not been applied before in a candidate gene study. Moreover, preceding studies have generally used a case–control study design, thus raising concerns of bias introduced by differences in characteristics of cases and controls, for example cohort effects. Here we apply a case–control approach using two cohorts of 1,089 oldest-old (age 92–93 years) and 736 middle-aged (age 46–67 years) Danish individuals, as well as a longitudinal approach with 11 years of follow-up survival data of the 1,089 oldest-old. Finally, we include replication data from Dutch and German study populations, as well as exploration of the initial findings from the Danish case–control study in the Danish longitudinal data and vice versa. Thus, the study presented here offers a good opportunity to confirm the putative association of variation in these major candidate genes with longevity.

Materials and methods

Subjects

Discovery cohorts

Detailed characteristics for the discovery cohorts were previously described in (Soerensen et al. 2010). In

short, the study population of oldest-old were 1,200 participants from The Danish 1905 Birth Cohort Study (Nybo et al. 2001), while the 800 younger controls were singleton participants from the Study of Middle Aged Danish Twins (Skytthe et al. 2002). The survival status information was retrieved from the Danish Central Population Register (Pedersen et al. 2006), and the vital status was followed until death or January 1, 2010. After data cleaning, the final sample size was 1,089 oldest-old and 736 middle-aged individuals. Permission to collect blood samples and usage of register-based information was granted by The Danish National Committee on Biomedical Research Ethics.

Study populations for replication of initial findings

For replication of the initial observations from the Danish case–control study, two German samples of 1,613 unrelated long-lived individuals (age 95–110) and 1,140 middle-aged controls (mean age 67.2) were used. The participants were identified through local registry offices and from the Federal Administrative Office. They were recruited from the different geographic regions of Germany and were all of German ancestry (Nebel et al. 2005). Approval was received from the Ethics Committee of the University Hospital Schleswig-Holstein.

For replication of initial findings from the Danish longitudinal study, the Dutch Leiden 85-plus Study was used. This prospective cohort was initiated in 1997, when all 85-year-old inhabitants of the city Leiden in the Netherlands were invited to participate. The cohort consists of 563 participants, all Caucasians and members of the 1912–1914 birth cohort recruited from 1997 to 1999 (Bootsma-van der Wiel et al. 2002). The Medical Ethical Committee of the Leiden University Medical Centre approved the study, and informed consent was obtained from all participants or, in case of severe cognitive impairment, from their guardian.

Selection of candidate genes and SNPs

The 16 genes were chosen as part of a large candidate gene study based on comprehensive literature/data base searches for candidate longevity genes, candidate longevity SNPs, and tagging SNPs. The details of this selection are described in the Supplementary Material (see Online Resource 1). The chromosomal regions investigated are the gene regions plus 5,000 kb upstream and 1,000 kb downstream. Tagging SNPs were identified via HapMap consortium database (http://hapmap.ncbi.nlm.nih.gov/index.html.en, CEU cohort) and analyzed using the HaploView software (http://www.broadinstitute.org/haploview/haploview; Barrett et al. 2005). Finally, for optimal genotyping, SNPs known to perform poorly on the Illumina GoldenGate genotyping platform were excluded.

Genotyping

DNA was isolated from blood spot cards using the QIAamp DNA Mini and Micro Kits (Qiagen, Germany); genotyping was performed using Illumina GoldenGate technology (Illumina Inc, San Diego, CA, USA), and data clean up, data verification, and reproducibility checks were conducted as previously described (Soerensen et al. 2010). In total, 16 SNPs were excluded, leading to 102 SNPs for data analysis. Genotyping in the German and Dutch study populations was performed using Sequenom MassARRAY iPLEX®Gold technology (Sequenom®, Inc., San Diego, CA, USA); see the Supplementary Material (Online Resource 1) for details.

qPCR experiments of *IL6* expression in whole blood samples

Whole blood samples were collected, stabilized, and transported in PAXgene Blood RNA Tubes (Qiagen) and stored at −80°C. RNA was isolated using the PAXgene Blood RNA Kit (Qiagen), and the integrity and concentration of RNA were determined by the RNA 6000 Nano Kit and a Bioanalyser 2100 (Agilent Technologies, USA). Reverse transcription was performed by the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) taking maximum 2 μg of purified RNA in a total volume of 20 μl. *IL-6* expression was determined by qPCR using TaqMan Gene Expression Assays (Applied biotechnology) in duplex reactions of FAM-labeled *IL6* primers (Hs00985637_m) and VIC-MGB-labeled *ACTB* (beta actin) endogenous control (limited) primers (4326315E). First validation experiments were carried out checking for equal efficiency of the primers in single and duplex reactions with varying cDNA dilutions and by

subsequently determining the dynamic range by standard curves. Consequently, 20 μl reactions including 1 μl of each primer and 4 μl cDNA samples diluted 1:10 were made in triplicates applying the standard protocol of the StepOnePlus real-time PCR system (Applied biotechnology). Thresholds established in the validation experiments were applied, the real-time curves were confirmed, and in case of C_t values off range, such samples were excluded.

Statistical analysis

Single-SNP case–control comparisons of allele frequencies (CCA) and genotype frequencies (CCG), as well as case–control comparisons of haplotype frequencies (CCH) were performed for the Danish cohorts using the Plink statistical software (http://pngu.mgh.harvard.edu/purcell/plink; Purcell et al. 2007). CCH was analyzed by regression analysis adjusting for sex. Haplotypes were defined by a sliding window of three SNPs (only considering haplotypes with frequency >5%). In sex-stratified CCG analysis, first an additive model (Cochran–Armitage test) and secondly recessive and dominant models were applied. To adjust CCG for sex, when analyzing both genders combined, CCG was analyzed using a generalized linear model in the R software (http://www.r-project.org). Again additive, recessive, and dominant models were applied. The German replication data were analyzed in the same way for CCAs and CCGs. Finally, gene-based analysis was conducted in Plink by set-based analyses for each gene, calculating a mean of the single-SNP *P* values taking into account linkage disequilibrium (LD) between the SNPs. The P-set value was corrected for multiple testing for the number of SNPs in each gene test by permutation (as described below).

LD estimates for the *APOE* SNPs were obtained using the Plink software, and the LD data were used in the HaploView software (http://www.broadinstitute.org/haploview/haploview; Barrett et al. 2005) for generation of an *APOE* LD plot.

Two survival variables were applied in the Danish longitudinal study. First, we wanted to explore the genetic contribution to surviving for a short period versus a longer period of time during the ninth decade of life. The distribution of survival times was right skewed (data not shown); hence, a cutoff with respect to time of death was put at December 31, 2000, when about half of the males and one third of the females in the cohort had died. Accordingly, the early/late death variable was defined as early=time of death between 1998 (date at intake) and December 31, 2000 (i.e., individuals living to maximum age 95) and late=time of death after January 1, 2001 (i.e., individuals surviving to age 95+). Fourteen of the 1089 oldest-old individuals were alive by January 1st 2010 (the recent update). Their remaining life expectancies were imputed using www.mortality.org holding cohort mortality data for the Danish population (based on data from the Statistics Denmark). As a second survival variable, we employed the number-of-days-lived variable defined as the exact number of days lived from date of intake in 1998 to either death or the imputed date. The assumptions of normal distribution and equal variance were tested in the STATA 11.1 statistical program (Stata Corporation, College Station, TX, USA) by Shapiro–Wilk and Breusch–Pagan/Cook–Weisberg tests, and since the variable did not demonstrate normal distribution, it was transformed to the square root for a better fit. Association analysis was conducted by linear (number-of-days-lived variable) or logistic (early/late death variable) regression in Plink, adjusting for sex and age or stratifying by sex, while adjusting for age and applying additive, recessive, and dominant models.

In the replication study, survival analysis using genotype data from the Leiden 85-plus Study was performed in Stata 11.1 by applying a sex-adjusted Cox proportional hazard model. Kaplan–Meier plots for the Dutch and the Danish cohorts were generated in Stata 11.1.

For all analyses, a nominal significance level was set to 0.05. Correction for multiple testing was performed by the permutation approach (applying max(*T*) permutation mode set at 10,000 permutations) for analyses carried out in the Plink and R software.

Data on *IL6* expression measurements was analyzed in the STATA 11.1 statistical program by linear regression of ΔC_t and rs2069827 genotype data, adjusting for age at blood sampling (the latter was initially observed to be a confounder). Assumptions of normal distribution and equal variance were tested for ΔCt by Shapiro–Wilk and Breusch–Pagan/Cook–Weisberg tests. A box plot of mean ΔCt by rs2069827 genotype groups was generated in STATA 11.1.

Results

The 102 genotyped SNPs in the 16 candidate genes are listed in Supplementary Table 1 (Online Resource 2), whereas the Danish discovery cohorts and German and Dutch replication populations are described in Table 1.

Case–control study of middle-aged and oldest-old Danes

Comparison of allele frequencies between middle-aged and oldest-old Danes showed more significant findings than expected by chance (see Supplementary Table 2, Online Resource 3) and left two out of 102 SNPs significant after correction for multiple testing. The minor allele frequency of rs9923854 (*CETP*) was enriched in the 92–93 year olds, implying that this allele is advantageous for survival from middle-age to old age (OR >1). In contrast, rs769449 (*APOE*) displayed a decreased prevalence of the minor allele, thus indicating a disadvantageous effect on survival (OR <1). Analysis of genotype frequencies supported these findings. Haplotype case–control comparisons identified three haplotypes significantly associated with longevity after correction for multiple testing; all three included either rs9923854 (*CETP*) or rs769449 (*APOE*) and demonstrated effects in line with the single-SNP analysis. The data are summarized in Table 2.

As the association of SNPs with longevity has previously been suggested to differ between genders (Candore et al. 2006; Franceschi et al. 2000; Li et al. 2009b; Soerensen et al. 2010), the analyses were repeated while stratifying by gender. The sex-specific analyses of rs9923854 (*CETP*) and rs769449 (*APOE*) were in complete agreement with the result of the combined group, although rs769449 (*APOE*) did not pass correction for multiple testing (see Supplementary Table 3, Online Resource 4).

Gene-based analysis confirmed the association of variations in *CETP* and *APOE* with longevity (see Table 3). *HSPA14* and *SIRT6* also showed significance in the gene-based analysis, but the gene-based *P* value for *SIRT6* reflected the association of only a single SNP, since the remaining *SIRT6* SNPs were insignificant at the single-marker level (data not shown).

Using already available data on the well-known ApoE ε2/3/4 polymorphism (Bathum et al. 2006; Caliebe et al. 2010; Jacobsen et al. 2010) from 1,270 of the participants, we found that rs769449 (*APOE*) was in moderate LD with the ApoE ε4 defining SNP rs429358 (R^2= 0.55). An LD plot for the *APOE* SNPs genotyped in this study and the two ApoE ε SNPs rs429358 and rs7412 is shown in Supplementary Figure 1 (Online Resource 5). In order to determine whether the effect of rs769449 was independent of APOE ε, logistic regression was performed for case–control status and rs769449 genotype data adjusting for gender and for either rs429358 or rs7412 genotype data. Using the 1,270 individuals with available information, the initially significant association (P=0.043) remained unchanged after adjusting for

Table 1 Characteristics of the Danish discovery cohorts and the German and Dutch study populations

The Danish discovery cohorts	MADT		1905
Number of individuals	736		1,089
Average age in years at intake (range)	50.6 (46.0–55.0)		93.2 (92.2–93.8)
Males/females (%) at intake	50.4:49.6		28.7:71.3
Mean follow-up time (SD)	–		3.53 (2.5)
Follow-up time (years)	–		11.4
	German case–control study		Dutch longitudinal study
Study populations for replication of initial findings	Controls	LLI	Leiden 85-plus Study
Number of individuals	1,104	1,613	563
Average age at intake (SD)	67.2 (4.07)	98.4 (2.7)	85.0 (0)
Males/females (%) at intake	25.6:74.3	26.8:73.2	33.4:66.6
Mean follow-up time (SD)	–	–	5.49 (3.0)
Follow-up time (years)	–	–	10.4

MADT the Study of Middle Aged Danish Twins, *1905* the Danish 1905 birth cohort, *LLI* long-lived individuals, *SD* standard deviation

Table 2 SNPs significantly associated with longevity in the case–control comparison of middle-aged and oldest-old Danish cohorts

Allele and genotype case–control comparison										
Gene	SNP	Minor/ Major allele	Chr./ position	Position in gene	MAF MADT/1905	PCCA uncorrected	PCCA corrected	OR (95% CI)	PCCG uncorrected	Change in frequency of common homozygotes from middle aged to old aged
APOE	rs769449	A/G	19/50101842	Intronic	0.166/0.123	0.0002	**0.018**	0.700 (0.579–0.846)	0.091	↑
CETP	rs9923854	C/A	16/55574503	Intronic	0.091/0.164	4.42×10^{-10}	$\mathbf{1.0\times10^{-4}}$	1.948 (1.576–2.409)	0.001	↓
Haplotype case–control comparison										
CHR	Gene	SNP1	SNP2	SNP3	Haplotype	Freq MADT	Freq 1905	OR	PCCH uncorrected	PCCH corrected
19	APOE	rs405509	rs440446	rs769449	AGA	0.141	0.095	0.65	9.68×10^{-5}	0.0208
16	CETP	rs5882	rs9923854	rs1800777	GCG	0.083	0.128	1.70	2.35×10^{-5}	0.0038
		rs1800774	rs5882	rs9923854	GGC	0.084	0.129	1.69	2.11×10^{-5}	0.0032

PCCA or PCCG values being significant after correction of multiple testing is shown in bold

Chr chromosome, *MAF* minor allele frequency, *MADT* the Study of Middle Aged Danish Twins, *1905* the 1905 cohort, *PCCA* allelic case–control comparison *P* value, *OR* odds ratio, *95% CI* 95% confidence interval, *PCCG* genotypic case–control comparison *P* value, *Freq* frequency, *PCCH* haplotypic case-control comparison *P* value

Table 3 Danish case–control study; gene-based test of PCCAs

Gene	No. of SNPs genotyped	Single-SNP-based test (PCCA<0.05)			Gene-set-based test
		SNP	PCCA	OR	P-set
CETP	20	rs1800777	0.020	1.517	1×10^{-5}
		rs289714	0.019	1.233	
		rs4784744	0.027	0.856	
		rs5883	0.022	1.385	
		rs9923854	4.42×10^{-10}	1.948	
		rs9930761	0.004	1.460	
APOE	3	rs405509	0.025	0.859	0.0016
		rs769449	0.0002	0.700	
HSPA14	6	rs12770830	0.011	1.346	0.0019
		rs17268499	0.004	1.292	
		rs7905174	0.005	1.236	
		rs9787671	0.001	1.343	
SIRT6	3	rs107251	0.001	1.348	0.0043

PCCA allelic case–control comparison *P* value, *OR* odds ratio, *P-set* set/gene-based test

rs7412 (P=0.037), while adjusting for rs429358 completely eliminated the effect of rs769449 (P=0.96).

Replication studies in a German case–control population and in the Danish longitudinal data

When analyzing the *APOE* and *CETP* SNPs in a German population of 1,613 oldest-old and 1,104 middle-aged individuals, rs769449 (*APOE*) was supported at the allelic level, whereas rs9923854 (*CETP*) showed borderline significance at the allelic level (95% CI (OR)=0.960–1.358), and was supported at the genotype level (see Table 4). In order to investigate whether the two SNPs also affected survival *during* old age, regression analysis on survival data on the oldest-old Danes (see below) was performed. We found no effect of rs769449 (*APOE*), while rs9923854 (*CETP*) showed nominal significance in females separately, with an estimate pointing in the same direction as observed in the case–control comparison (the data are summarized in Table 4).

Longitudinal study in the cohort of oldest-old Danes

In order to evaluate the association of the 102 SNPs with survival at advanced ages and to estimate their quantitative effects, regression analyses of the survival variables using 11 years of follow-up survival data on the oldest-old Danes were performed. After adjusting for multiple testing, only rs2069827 (*IL6*) obtained a corrected *P* value below 0.10 for the Number_of_days_lived variable: *P*-uncorrected=0.0007, *P*-corrected=0.064, and only when applying a dominant model. In the gene-based analysis, *IL6* showed a P-set value of 0.0068, yet this estimate reflected only rs2069827, since the other *IL6* SNP (rs12700386) was insignificant in the single-marker analysis (data not shown). None of the remaining 15 genes showed P-set values below 0.05 (which was based on more than one significant SNP) confirming the lack of association between variations in any other genes than *IL6*.

From the regression analysis, the quantitative effect of carrying at least one rs2069827 A allele was estimated to be 0.77 and 0.74 additional years of survival for AA/AC males and females, respectively, compared to a mean survival time of 2.91 and 3.74 years for rs2069827-CC males and females, respectively, hence indicating a considerable effect.

Replication studies in a Dutch prospective cohort and in the Danish case–control data

Investigation of this single borderline significant *IL6* SNP in the Dutch replication cohort using Cox regression supported an effect on late life survival; HR=0.74,

Table 4 Replication of initial findings from the Danish case–control study in a German case–control population and in the Danish longitudinal study

		Danish case–control study			German case–control study				
Gene	SNP	MAF controls/cases	PCCA uncorrected	OR (95% CI)	MAF controls/cases	PCCA uncorrected	OR (95% CI)	PCCG uncorrected	Concordance between studies
APOE	rs769449	0.166/0.123	0.0002	0.700 (0.579–0.846)	0.119/0.055	5.20×10^{-17}	0.430 (0.351–0–526)	0.091	Yes
CETP	rs9923854	0.091/0.164	4.42×10^{-10}	1.948 (1.576–2.409)	0.106/0.119	0.133	1.142 (0.960–1.358)	0.001[b]	Yes
		Danish case–control study			Danish longitudinal study				
Gene	SNP	PCCA uncorrected	OR (95% CI)		*P* value		Concordance between studies		
APOE	rs769449	0.0002	0.700 (0.579–0.846)		>0.05		–		
CETP	rs9923854	4.42×10^{-10}	1.948 (1.576–2.409)		>0.05[a]		Yes		

PCCA allelic case–control comparison *P* value, *OR* odds ratio, *95% CI* 95% confidence interval, *PCCG* genotypic case–control comparison *P* value

[a] For females: OR=1.380 (95% CI=1.002–1.899), *P*=0.048 (early_late variable), and β-coefficient=2.114 (95% CI=0.089–4.140), *P*=0.041 (Number_of_days_lived variable)

[b] OR (heterozygotes)=1.32, 95% CI=1.11–1.58, OR (rare homozygotes)=1.76, 95% CI=1.47–2.11

95% CI=0.576–0.951, *P*=0.019. The Kaplan–Meier survival plots for the Danish and Dutch cohorts are shown in Fig. 1. When inspecting the Danish case–control data, no association was observed for rs2069827 (*IL6*).

qPCR investigation of *IL6* expression

The http://manticore.niehs.nih.gov/snpfunc.htm data base on SNP function prediction locates rs2069827 to a putative transcription factor binding site in the 5′ untranslated region of *IL6*. Hence, the level of *IL6* mRNA was analyzed by qPCR using 198 representative samples from the middle-aged cohort, for which RNA was available. No significant difference in mRNA levels was observed between genotype groups for rs2069827 (*P* value=0.532). The average ΔC_t values in each rs2069827 group are depicted in Fig. 2, clearly illustrating no difference between heterozygotes and common homozygotes.

Discussion

Several candidate genes have been explored for their part in human longevity. The majority of the previous studies on the genes investigated here have focused on one or a few variations and have often been followed by contradictory replication results. Interestingly, in this study we did include three of the specific SNPs reported previously (rs4343 (*ACE*), rs5882 (*CETP*), and rs1801133 (*MTHFR*)). Retrospectively inspecting these SNPs in the data shows that only rs4343 (*ACE*) was nominally significantly associated with longevity (*P*<0.05) in concordance with the initial publications, while no effect of the other two SNPs could be noted. Thus, these results emphasize that such a single-marker candidate approach might point to a specific polymorphism, yet it does not enable conclusions on the possible relevance of variation in the gene region as such.

To facilitate the confirmation of the relevance of a number of well-known candidate genes for longevity, we thus extended the examination of these genes to cover the majority of the common variation in the regions by using a total of 102 tagging SNPs. Applying the case–control approach, two SNPs, rs769449 (*APOE*) and rs9923854 (*CETP*), were found to be associated with longevity. The effect of these was supported by haplotype and gene-based analyses and

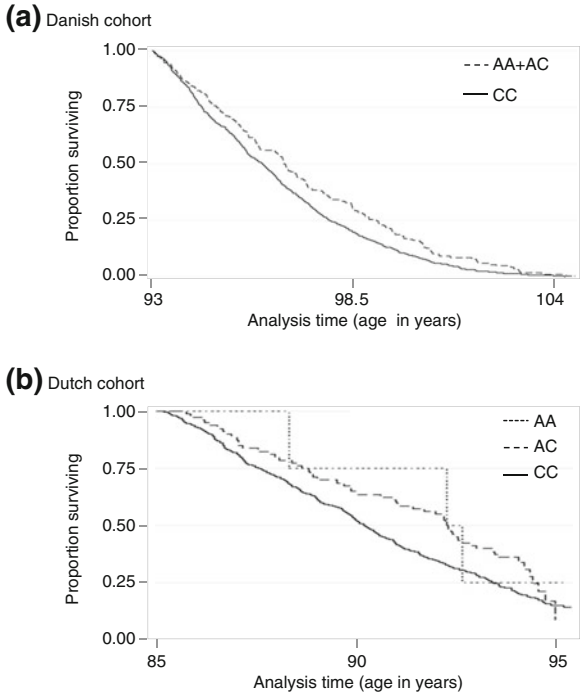

Fig. 1 Kaplan–Meier survival estimates by rs2069827 (*IL6*) genotype groups in the **a** Danish and **b** Dutch prospective cohorts

was supported in the German replication population, hence pointing to importance of genes involved in lipoprotein metabolism for longevity.

The effect of *APOE* rs769449 did, however, most likely reflect the well-known effect of the *APOE* ε4 haplotype defining rs429358 and was not evident in advanced age survival. The *APOE* ε4 haplotype has in our cohorts not only been reported to pose a negative effect on survival from middle age to old age (Gerdes et al. 2000; Tan et al. 2004) but also on survival during old age (Bathum et al. 2006; Deelen et al. 2011; Jacobsen et al. 2010). Moreover, in the cohort individuals used in the present paper for investigating the dependency between APOE ε and rs769449, rs429358 alone also shows a modest effect on survival during old age (unpublished data). Yet, such an effect was not seen for rs769449, indicating that rs769449 does not reflect all the effects of rs429358. In contrast, rs9923854 (*CETP*) did show nominal effects on survival during old age, yet only in females, indicating that the effect of this SNP might become gender specific toward the most late part of life.

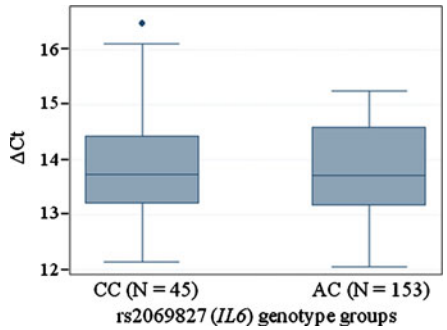

Fig. 2 Mean age-adjusted ΔC_t values by rs2069827 (*IL6*) genotype groups

Apoe is involved in lipoprotein metabolism via its binding to low-density lipoprotein (LDL) receptors on the surface of for instance liver cells, hereby mediating endocytosis and consequently removal of LDL from the circulation. The isoforms of APOE (E2, E3, and E4) have been shown to interact differently with diverse LDL receptors, leading to differences in LDL levels, APOE4 being associated with the highest LDL levels. Moreover, APOE4 possibly also influences oxidative stress and inflammatory states. These mechanisms are probably the basis of the increased risk of premature atherosclerosis, coronary heart disease, Alzheimer's disease, and mortality repeatedly found in *APOE* ε4 carriers (Chung et al. 2010; Dietrich et al. 2005; Jofre-Monseny et al. 2007; Vitek et al. 2009). The association of rs769449 (*APOE*) to longevity identified here is probably a proxy for these functional effects.

Cetp has opposing effects on lipoprotein metabolism. On one hand, it increases LDL and very-low-density lipoproteins (VLDLs) levels by transferring cholesterol esters from high-density lipoproteins (HDLs) to LDLs/VLDLs. On the other hand, it increases removal of lipoproteins from the circulation as part of transferring triglycerides from VLDLs/LDLs to HDLs (reviewed in Weber et al. 2010). Hence, one might expect the rare allele of rs9923854 (*CETP*) to contribute to decreased cholesterol ester transfer activity and/or increased triglyceride transfer activity, although the role could well be much more complex. This is underlined by the difference in success observed in clinical studies of cetp inhibitors for prevention of atherosclerosis (Weber et al. 2010).

Besides the two single-marker associations, a test at the level of the entire gene indicated a positive effect of the rare variants of *HSPA14* on survival from middle-aged to old-aged. *HSPA14* belongs to the same functional group as *HSPA1A* and *HSPA1L* (70 kDa heat shock proteins (HSP70s)) previously reported as associated with human longevity (Altomare et al.

2003; Ross et al. 2003; Singh et al. 2010). Hsp14 (also named hsp70L1) is a rather recently identified hsp70 protein (Wan et al. 2004) and is known to be located in ribosomes, probably playing a role in the folding of newly translated proteins (Otto et al. 2005). Accordingly, the rare alleles of the *HSPA14* SNPs reported here could be hypothesized to enhance such activity and, thus, avoid accumulation of newly translated proteins during aging. However, hsp14 also enhances cellular immunity in dendrites, e.g., stimulating secretion of for instance IL12 and TNFα, and has therefore been suggested as an adjuvant for the treatment of cancer and infectious disease (Wu et al. 2005). An enhancement of such effects could intuitively be considered to pose a positive effect on longevity, although the connection between immune regulatory processes and longevity is, as explained below, probably not that straightforward.

When investigating survival *during* old age in the longitudinal study, we found rs2069827 (*IL6*) as the only SNP posing an effect, an effect which was confirmed in the Dutch replication cohort. The relation of immune regulatory genes to longevity is in general based on the idea of a balance between pro- and anti-inflammatory processes, since aging is accompanied by both a decline in several immune functions (immunosenescence) and an increase in a chronic low-grade inflammation state (inflammaging). The genetic polymorphisms associated with longevity are presumed to increase the ability to cope with inflammaging (Jylhava and Hurme 2010). Therefore, one might expect the rare allele of rs2069827 (*IL6*) to hold a low pro-inflammatory activity. Such low activity could presumably be caused by low IL6 expression, yet in our qPCR study we observed no difference in IL6 mRNA levels between rs2069827 genotype groups. Hence, the impact of this SNP does not appear to be mediated through changes in *IL6* expression. Finally, rs2069827 may simply be a proxy for a causal variant not genotyped in the present study.

In conclusion, we here add data to the proposed roles of 16 frequently discussed candidate genes, indicating that variation in *APOE* and *CETP*, and possible *HSPA14*, is of importance in survival from middle age to old age, whereas genetic variation in *IL6* seems to affect survival during late life.

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