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The role of lipid-related genes, aging-related processes, and environment in healthspan

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Summary

The inherent complexity of aging-related traits can temper progress in unraveling the genetic origins of healthspan. We focus on two generations in the Framingham Heart Study, the original (FHS) and offspring (FHSO) cohorts, to determine whether aging-related processes in changing environments can substantially impact the role of lipid-related genes discovered in candidate gene (the apolipoprotein E (*APOE*) e2/3/4 polymorphism) and genome-wide (the *APOB* rs1042034 (C/T)) studies, in regulation of total cholesterol (TC) and onset of cardiovascular disease (CVD). We demonstrate that the *APOE* e4 allele and *APOB* CC genotype can play detrimental, neutral, and protective sex-specific roles in the etiology of CVD at different ages and in different environments. We document antagonistic roles for the e4 allele in the onset of CVD characterized by detrimental effects at younger ages ($RR_{\leq 75\ years} = 1.49$, $P = 7.5 \times 10^{-4}$) and protective effects at older ages ($RR_{76+years} = 0.77$, $P = 0.044$) for FHS participants. We found that disregarding the role of aging erroneously nullifies the significant effects of the e4 allele in this sample (RR = 0.92, $P = 0.387$). The leading biogenetic pathways mediating genetic effects on CVD may be more relevant to lipid metabolism for *APOB* than *APOE*. Aging-related processes can modulate the strength of genetic associations with TC in the same individuals at different chronological ages. We found substantial differences in the effects of the same *APOE* and *APOB* alleles on CVD and TC across generations. The results suggest that aging-related processes in changing environments may play key roles in the genetics of healthspan. Detailed systemic integrative analyses may substantially advance the progress.

Key words: aging; biology of aging; disease; genetics; healthspan regulation; trade-offs.

Introduction

Recent increases in human life expectancy worldwide have raised serious concerns about potential expansions of morbidity and disability (Olshansky *et al.*, 2007; Sierra *et al.*, 2009). Discovering which genes regulate lifespan and health in late life could lead to major breakthroughs in extending healthspan.

The genetic susceptibility to the late life phenotypes is inherently complex due to the lack of a direct evolutionary program in their development. The lack of such a program implies that genes are connected with traits in late life through aging-related processes comprising a complex interplay of processes that accompany the declines in functioning of all systems in an aging organism and the concurrent development of diseases and related traits during the life course. These processes represent a superposition of intrinsic biological aging (senescence) and extrinsic environmental challenges; they contribute to intra- (age-related) and inter (heterogeneity) individual variability. They can be manifested at the phenotypic level, which is defined by sets of genes, as well as at the individual-gene level.

For example, decades of epidemiological studies provide solid evidence that the risks of most diseases in late-life change with age; e.g., the risks of developing coronary heart disease (CHD) at 50, 70, or 80 years are different (population estimates of incidence rates of major geriatric diseases in the U.S. can be seen in (Akushevich *et al.*, 2012)). Various characteristics of health, which can cause late-life diseases, can change over life course as well (e.g., lipid levels (Hershcopf *et al.*, 1982), glucose levels (Scheen, 2005), bone mineral density (Sheu *et al.*, 2011)). Prior studies also provided evidence of individual gene-level complexity such as, e.g., pleiotropy (Sivakumaran *et al.*, 2011), antagonistic pleiotropy (Schnebel & Grossfield, 1988; Williams & Day, 2003; Alexander *et al.*, 2007; Kulminski *et al.*, 2010), genetic trade-offs (van Heemst *et al.*, 2005; Kulminski *et al.*, 2011), differential effects at different ages (De Benedictis *et al.*, 1998; Ilveskoski *et al.*, 1999; Yashin *et al.*, 2001; Bergman *et al.*, 2007), and other modalities (reviewed in Martin, 2007).

Therefore, if we believe that a trait in late life can have genetic origin, we have to also keep in mind that: (i) the same genes causing that trait can change their role with individuals' aging; (ii) different genes can work at different chronological ages; and/or (iii) aging-related genetic effects can be altered by the environment, even in an antagonistic fashion.

Are aging-related processes a serious factor to consider in genetic association studies? Can they advance the progress in discovering mechanisms of genetic predisposition to healthspan? In this work, we convincingly demonstrate that aging-related processes in a changing environment can be critical for understanding the role of genes in traits in late life. We address this problem by considering the effects of polymorphisms from two lipid-related genes discovered in candidate gene (the apolipoprotein E (*APOE*) e2/3/4 polymorphism) and genome-wide (the *APOB* rs1042034 (C/T); Teslovich *et al.*, 2010) studies on age at onset of cardiovascular disease (CVD) and on regulation of total cholesterol (TC) across chronological ages and generations. We use data from the original

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Framingham Heart Study (FHS) and the FHS Offspring (FHSO) cohorts followed up for about 60 years.

Materials and methods

Study design and population

The original (FHS) and offspring (FHSO) cohorts were launched in 1948 and 1970, respectively (Splansky *et al.*, 2007; Govindaraju *et al.*, 2008; Cupples *et al.*, 2009). Briefly, the FHS includes $N = 5209$ respondents aged 28–62 years at baseline who have been biannually followed during about 60 years comprising 28 examinations. The FHSO respondents ($N = 5124$) aged 5–70 years at baseline were biological descendants ($N = 3514$), their spouses ($N = 1576$), and adopted offspring ($N = 34$) of the FHS participants who have been examined at seven visits. The FHS/FHSO participants have been followed for the onset of CVD through regular examinations at the FHS clinic, surveillance of hospital admissions, and death registries (Splansky *et al.*, 2007; Govindaraju *et al.*, 2008), currently through 2008. Biospecimens were mostly collected in the late 1980s and through 1990s from surviving participants (Myers *et al.*, 1996; Lahoz *et al.*, 2001; Cupples *et al.*, 2009). The procedure used for the *APOE* genotyping is described in Lahoz *et al.* (2001). The data include information on the *APOE* e2/3/4 polymorphism for 1258 FHS and 3924 FHSO participants. Genome-wide data are available for 1529 FHS and 3750 FHSO participants.

Analysis

We use data on longitudinally followed FHS and FHSO participants to comprehensively characterize the role of lipid-related genetic variants discovered in candidate gene (the *APOE* e2/3/4 polymorphism) and genome-wide (Teslovich *et al.*, 2010) studies, aging-related processes, human generations as a proxy for environmental changes, and TC as a proxy for lipids in the onset of CVD. First, we characterized the role of genetic markers in the onset of CVD. Then, we elucidated the role of lipids in genetic effects on CVD. Next, we addressed direct associations of the selected genetic markers with TC. These analyses were conducted using data from different FHS/FHSO examinations to ensure that the detected associations are: (i) not the results of a specific stochastic realization; (ii) robust to longitudinal attrition of the samples at risk of CVD; and (iii) not the result of disproportional (i.e., genotype-specific) survival selection of robust individuals until biospecimen collection. Finally, to better characterize the role of the aging-related processes in associations of the selected genetic markers with TC, these associations were evaluated in the same longitudinally followed individuals at different chronological ages.

Associations of genetic markers with ages at onset of CVD in genotyped subjects were characterized by Kaplan–Meier estimator and the Cox proportional hazard regression model at different FHS/FHSO examinations. The time variable in the analyses was 'age at onset of CVD (cases) or age at censoring (at death or the end of follow-up in 2008)'. Individuals who developed CVD prior to an examination in question were excluded from the respective analyses. Both the Kaplan–Meier estimator and the Cox regression model evaluate the probability of remaining free of CVD by each given age for each individual who was free of CVD at the start of the follow-up period. We used the robust sandwich estimator of variances in the Cox model to account for potential clustering (e.g., familial).

To address the role of lipid metabolism in genetic associations with CVD, we used two models. We evaluated individual and additive effects of TC and genetic markers on CVD in the same sample, i.e., with individuals missing TC measurements excluded, using the Cox regression model. Direct associations of the selected genetic markers with TC were evaluated using linear mixed effects regression models with unstructured covariance matrices parameterized in terms of variances and correlations to account for clustering. We used TC (log-base-10 transformed to adjust for deviations from normality) in the mixed effects model as a dependent variable.

The Cox and mixed effects models were adjusted for current age and sex, when applicable. These analyses were conducted separately for different examinations in the FHS/FHSO, using the data for all genotyped participants at each selected examination.

To characterize the role of the aging-related processes in genetic effects on TC, we focused on individuals who participated in all selected examinations, i.e., individuals who missed at least one examination were excluded from these analyses. We verified that inclusion of individuals who missed few examinations did not make qualitative difference. These analyses evaluated the associations in the same sample of individuals as they truly aged. We also evaluated the associations at different examinations. An advantage of the examination-specific estimates compared with modeling longitudinal changes is that the former approach is free of constraints on parameterization of longitudinal changes in TC.

Twenty-five SNPs discovered in the genome-wide association study by Teslovich *et al.* (2010) as associates of blood lipids were directly genotyped in the FHS. Of these, one single nucleotide polymorphism (SNP) was potentially important for our analyses, rs1042034 (chromosome two; nonsynonymous coding variant in the *APOB* gene) because it showed marginal significance with coronary artery disease and was associated with lipids (triglycerides and high-density lipoprotein cholesterol) in Teslovich *et al.* (2010), and had proportional hazards over age domain for different genotypes in the FHS cohort (examined in this study using the Kaplan–Meier empirical curves for the probability of staying free of CVD). This SNP was retained for the current analyses.

Although the phenotype-limited access data available for this study include information on TC and other lipids, the most comprehensive longitudinal measurements are available for TC only. Given our focus on the longitudinally followed population, only the TC has been retained for the current analyses, as a proxy for other lipids.

Blood lipids in early examinations of the FHS were measured without controlling for fast. In some FHS examinations, information on the fasting status of a limited number of individuals is available. We verified that fasting status makes no qualitative difference in our analyses [see Supplementary Information (SI), Table S1]. Accordingly, the entire genotyped samples, regardless of fasting status, were used in the analyses.

Basic characteristics of the genotyped study participants are given in Table S2 for each genotype of the *APOE* gene and rs1042034. Because the FHS cohort was followed for about 60 years, Table S2 indicates substantial differences in the proportions of diseased participants of the FHS and FHSO. The analyses were focused on carriers of the e4 (risk; e2/4, e3/4, and e4/4) and non-e4 (e2/2, e2/3, and e3/3) alleles (Kulminski *et al.*, 2011). Empirical screening suggested the major allele-dominant model for the rs1042034. Because both genetic variants were selected based on prior evidence, corrections for multiple comparison were not required. Statistical analyses were conducted using SAS (release 9.3, Cary, NC, USA).

Results

The APOE e2/3/4 polymorphism

The e4 allele and onset of CVD

Visual screening of the empirical age patterns of the probability of remaining free of CVD for men and women combined reveals qualitatively different roles of the e4 allele across ages, examinations, and the FHS/FHSO generations (Fig. 1). Specifically, in early examinations of the FHS cohort, the e4 allele shows antagonistic effects across ages favoring premature onset of CVD at younger ages and protecting against CVD at older ages (Fig. 1A) compared with the non-e4 genotypes. The antagonistic pattern in early examinations undergoes *smooth transition* to an entirely protective pattern for the e4 allele carriers in later examinations (Fig. 1B is representative of the later examinations). Contrary to the FHS, in the FHSO cohort, we observe an entirely detrimental pattern for the e4 allele carriers in early examinations (Fig. 1C). This pattern remains detrimental in later examinations and the detrimental effect tends to become more pronounced at older ages (Fig. 1D).

Smooth changes in the empirical age patterns suggest underlying etiologic heterogeneity in the sample. This heterogeneity is characterized by qualitatively different risks of CVD at different ages. Specifically, one more homogeneous group is characterized by the development of CVD in early life with a pronounced detrimental role of the e4 allele. A characteristic feature of the other group is that the e4 allele can postpone CVD in these individuals to later life. Accordingly, the CVD risks in the entire FHS sample represent a concurrent superposition of the antagonistic risks characteristic of the more homogeneous groups. The existence of these groups can be verified by examining relative risks (RR) of premature onset of CVD in different FHS examinations for the entire sample and for the

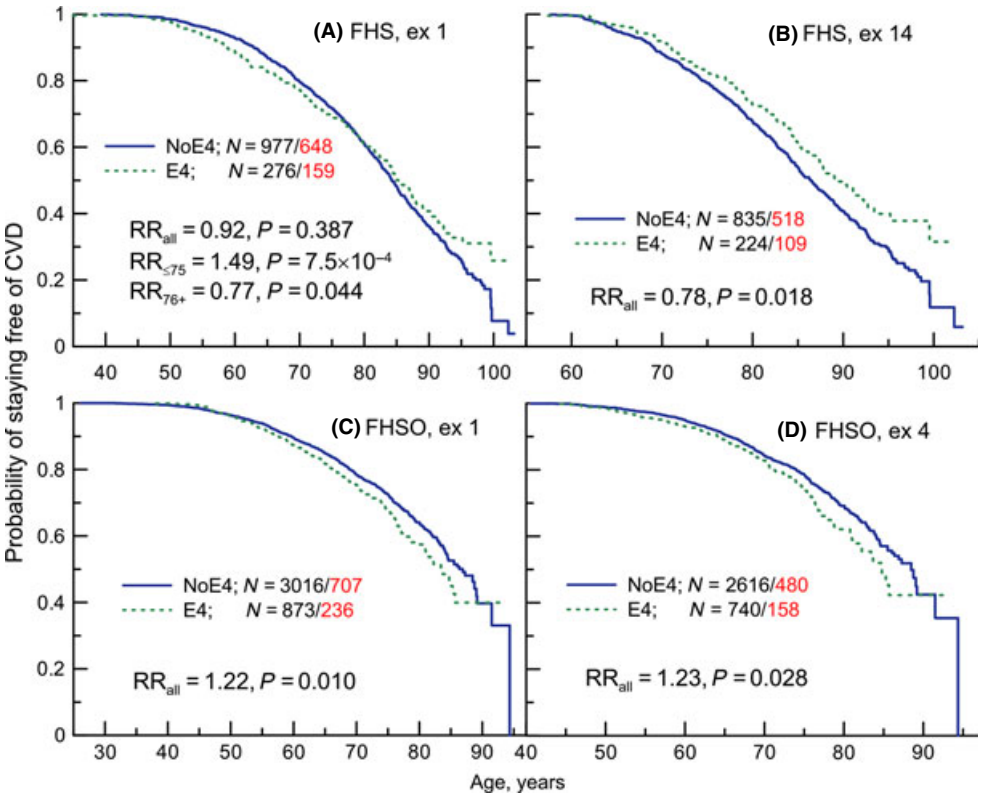

Fig. 1 Kaplan–Meier age patterns of the probability of staying free of CVD for carriers (E4) and noncarriers (NoE4) of the *APOE* e4 allele. (A) and (B) The FHS cohort, examinations (A) 1 and (B) 14. (C) and (D) The FHSO cohort, examinations (C) 1 and (D) 4. N = m/k denotes (m) the total number of carriers and (k) the number of CVD cases among them. Relative risks (RR) were evaluated using the Cox regression model (see Methods) for all subjects (all) and for more homogeneous 'younger' ($\leq$ 75) and 'older' (76 +) groups with antagonistic risks. The 'younger' group was defined as developing CVD in early life or being censored at younger ages (representatively, 75 years and younger at the end of follow-up in 2008). The 'older' group was defined as developing CVD in late life or being censored at older ages (representatively, 76 years and older at the end of follow-up in 2008). Censored individuals are not depicted.

more homogeneous 'younger' and 'older' groups (defined in Fig. 1 caption).

Figure 2A documents the existence of two phenotypic groups in the FHS cohort. In the younger group, the e4 carriers were at significantly higher risks of premature onset of CVD compared with the non-e4 carriers (Fig. 1A, $RR_{\leq 75}$, and Fig. 2A). These risks were significantly higher virtually regardless of examination despite the longitudinal attrition of the samples at risk of CVD; this group was virtually extinct after the 20th examination (Fig. 2A, filled bars). In contrast, in the older group, the e4 allele carriers were at consistently lower risks than the non-e4 carriers (Fig. 1A, RR_{76+}, and Fig. 2A). The RRs for this group were significant at most examinations.

Superposition of the antagonistic risks in these two groups nullifies the significant effect in the entire sample examined in early visits (Fig. 1A, RR_{all}, and Fig. 2A). At later examinations, when the contribution of the younger group diminishes due to its attrition, we can observe the protective effects of the e4 allele in the entire

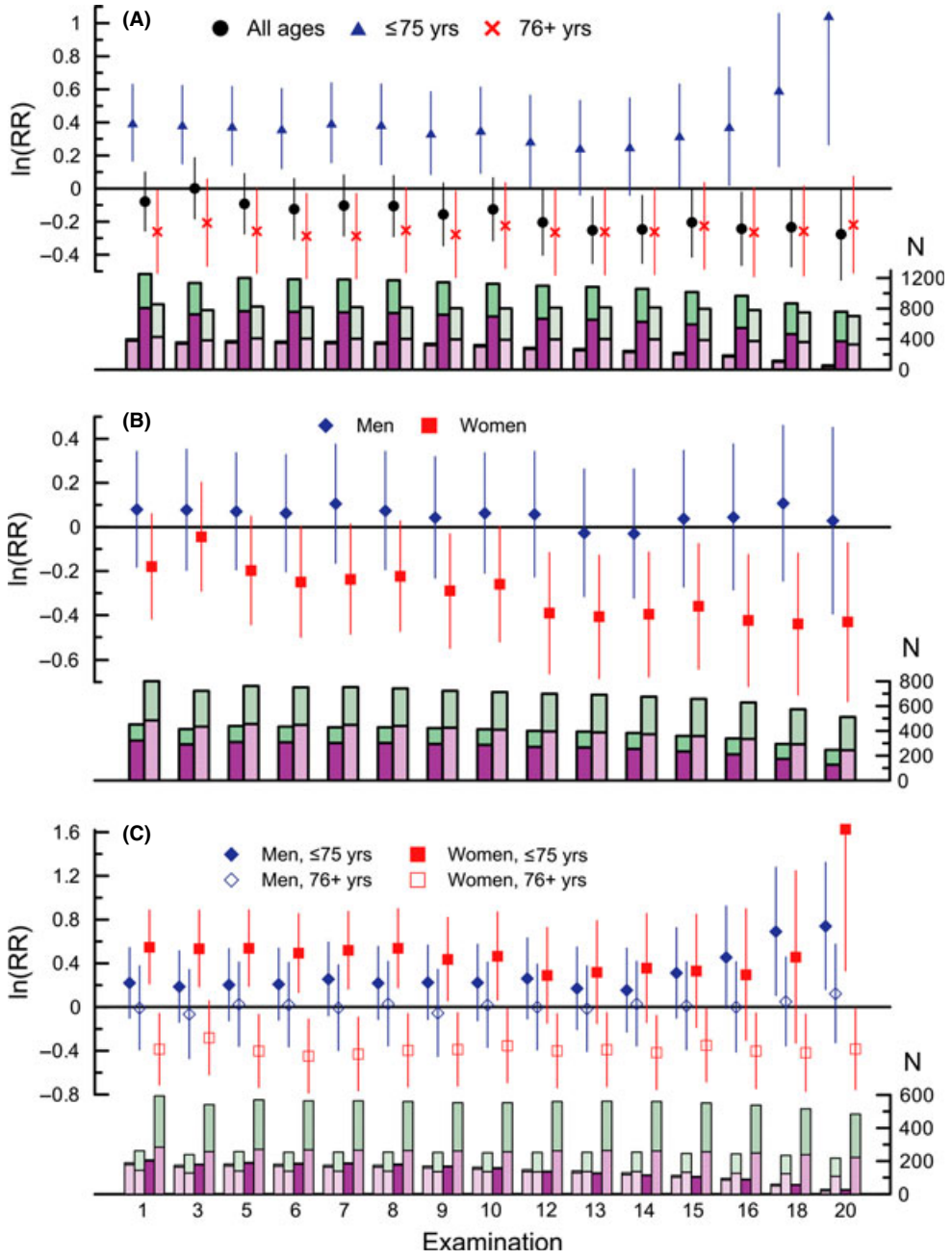

Fig. 2 Natural log-transformed relative risks (RR) of premature onset of CVD in the *APOE* e4 allele carriers compared with the non-e4 carriers at different examinations in the FHS cohort stratified: (A) into younger or older group (defined in Fig. 1 caption), (B) by sex, and (C) into younger or older group and by sex. Thin bars show 95% confidence intervals (CIs) (negative direction at examination 20 is shown for the sake of better resolution). Filled bars depict sample sizes of subjects with (lower part, shown by magenta) and without (upper part, shown by light green) CVD.

sample (Figs 1B and 2A). Reducing the age-at-onset cutoff from 75 years (see Fig. 1 caption) to younger ages made no qualitative difference.

Analysis of the FHSO cohort confirmed the empirical finding (Fig. 1C,D) that the detrimental effects of the e4 allele on onset of CVD were largely manifested at older ages (see SI, Fig. S1A).

Sexual dimorphism

Given the potential sexual dimorphism of the *APOE* gene (Kolovou *et al.*, 2009), we further examined the empirical age patterns for each sex. This analysis revealed that the observed associations in the FHS and FHSO cohorts were largely attributed to women. These findings are quantified in Fig. 2B,C (the FHS) and Fig. S1B (the FHSO). Figure 2B shows that the pattern of the RRs across examinations of the FHS cohort for women resembles that for men and women combined (Fig. 2A, dotted symbols) in the sample with age-specific heterogeneity disregarded, although for women the associations are more significant at later examinations. Male e4-allele carriers in the heterogeneous sample were seemingly not at risk (Fig. 2B). Female e4-allele carriers in the FHSO were at significant risk of premature onset of CVD in the first six of seven FHSO examinations (see SI, Fig. S1B). Male e4 carriers in the FHSO can potentially be at risk of premature onset of CVD although this effect (nonsignificant) was limited to the first three examinations.

Figure 2C shows that younger FHS men and women carrying the e4 allele were at consistently higher (not significant for men) risk of premature CVD compared with the non-e4 carriers. Older FHS women carrying the e4 allele were at significantly lower risk of premature CVD compared with the non-e4 carriers at virtually all examinations. Men in this group showed no e4-specific onset of CVD.

Analysis of the FHSO cohort showed that the detrimental effect of the e4 allele on onset of CVD was primarily attributed to older women (Fig. S1C).

Lipids, the e4 allele, and onset of CVD

Because TC was not reported for all genotyped subjects, all those with missing *APOE* and TC information were excluded from these analyses. This resulted in substantially smaller sample sizes particularly in the FHS. To increase power, we aggregated samples of different sexes in each cohort with evidence of the same-type association of the e4 allele with the onset of CVD because they likely shared the same underlying mechanisms (the number of subjects in the analyses is given in each respective figure):

- Group A: The FHS men and women from the younger group ($\leq$ 75 years) with detrimental effect of the e4 allele (Fig. 2A);
- Group B: The FHS women from the older group (76+ years) with protective effect of the e4 allele (Fig. 2C);
- Group C: The FHSO women from the older group (70+ years) with detrimental effect of the e4 allele (Fig. S1C);
- Group D: The FHS men from the older group (76+ years) with neutral role of the e4 allele (Fig. 2C);
- Group E: The FHSO men and women from the younger group (< 70 years) with no significant role of the e4 allele (Fig. S1A);
- Group F: The FHSO men from the older group (70+ years) with neutral role of the e4 allele (Fig. S1C).

The results of the analyses of the connections among CVD, e4 allele, and TC (summary is sketched in Fig. 3) showed that TC virtually did not mediate the significant associations of the e4 allele with the onset of CVD in Groups A–C (see SI, Fig. S2). Nor was TC itself consistently associated with the onset of CVD in these groups (see SI, Fig. S2). In contrast, TC was typically associated with the onset of CVD in Groups D–F in which the e4 allele was neutral for the onset of CVD (see SI, Fig. S3). The lack of, or presence of, associations of TC with the onset of CVD in Groups A–E was not explained by sample size differences (see SI, Figs. S2 and S3).

The e4 allele was consistently and mostly significantly associated with increasing levels of TC in Groups A and C in which this allele showed a detrimental effect on the onset of CVD (see SI, Fig. S4).

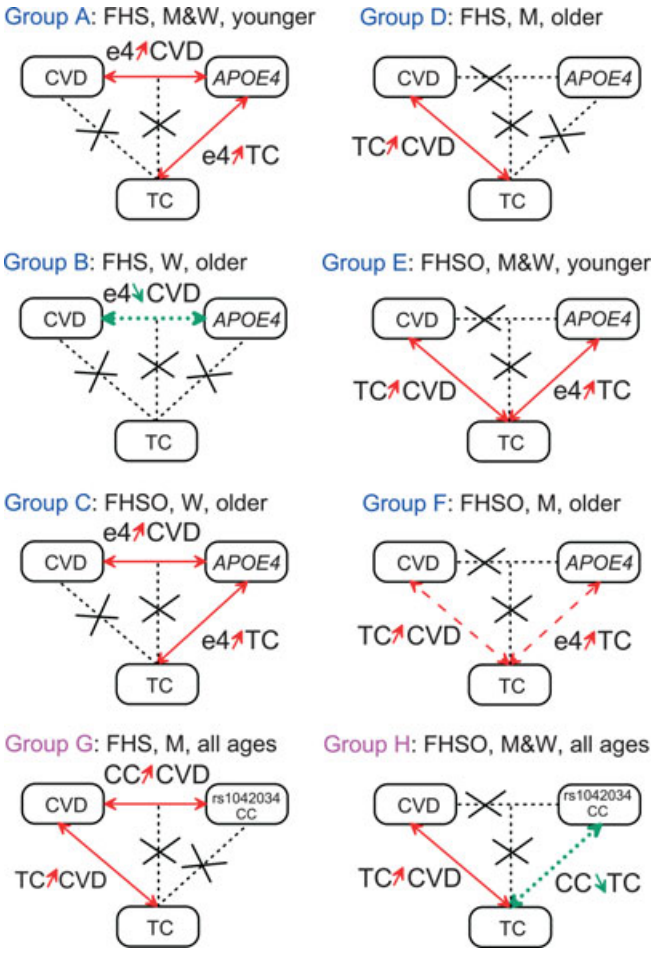

Fig. 3 Schematic representation of the connections among (Groups A–F) *APOE* e4 allele, (Groups G–H) rs1042034 CC genotype, CVD, and total cholesterol (TC). Black dashed lines show nonsignificant associations. Solid red (dotted green) lines show detrimental (protective) effects. Long-dashed red line: the associations are largely limited to the first FHSO examinations due to attrition of the sample for men at the later examinations. M = men, W = women. FHS: 'younger' ('older') means individuals from the younger (older) group as defined in Fig. 1. FHSO: 'younger' ('older') means individuals from the younger (older) group as defined in Fig. 1 for the FHS but for cutoff at 70 years (see also Fig. S1).

No consistently significant associations of the e4 allele with TC were seen in Group B (protective role of the e4). In groups D–F (neutral e4), we observe no (Group D), highly significant (Group E), and marginally significant (Group F) associations of the e4 with TC. Marginal significance in Group F is likely attributed to its smaller sample size (see SI, Fig. S4B). The lack of, or presence of, associations of TC with the e4 allele in the other groups was not sensitive to the sample size differences.

Aging-related processes, the e4 allele, and TC

The analyses of the associations of the e4 allele with TC (see SI, Fig. S4) reveal two important peculiarities. First, in the FHS (see SI, Fig. S4A), we observed the same direction of the effects of the e4 allele in all groups (i.e., A, B, and D) in later examinations. Second, in the FHSO (see SI, Fig. S4B), we observed a gradual decline of the effect size through examinations in all groups (i.e., C, E, and F). Given no obvious role of the sample size, these dynamics might be attributed to aging-related processes in these cohorts.

To examine the role of aging-related processes in the associations of the e4 allele with TC, we aggregated all groups in the FHS and selected all individuals who participated in seven examinations (i.e., 8, 9, 10, 13, 14, 15, and 20) with available measurements of TC ($N = 802$). We did the same for the FHSO and selected individuals who participated in examinations one through seven ($N = 2252$). Figure 4 convincingly demonstrates that the effect of the e4 allele can change with chronological age. Most striking is that the effect changes in opposite directions in the FHS and FHSO cohorts, i.e., it strengthens in the FHS and diminishes in the FHSO at the same ages. The observed opposite trends cannot be explained by age differences (Fig. 4) or by the use of anticholesterol medications (Figure S5). Figure 4 also shows significant overall declines of mean TC levels at old ages regardless of the presence of the e4 allele in both the FHS and FHSO samples (Fig. 4; see bars and nonoverlapping confidence intervals across examinations). These patterns imply that the observed opposite trends in the effects (i.e., betas) cannot be explained by nonspecific aging-related changes in individuals in these cohorts; they pinpoint the differential roles of the e4- and non-e4 alleles in aging individuals.

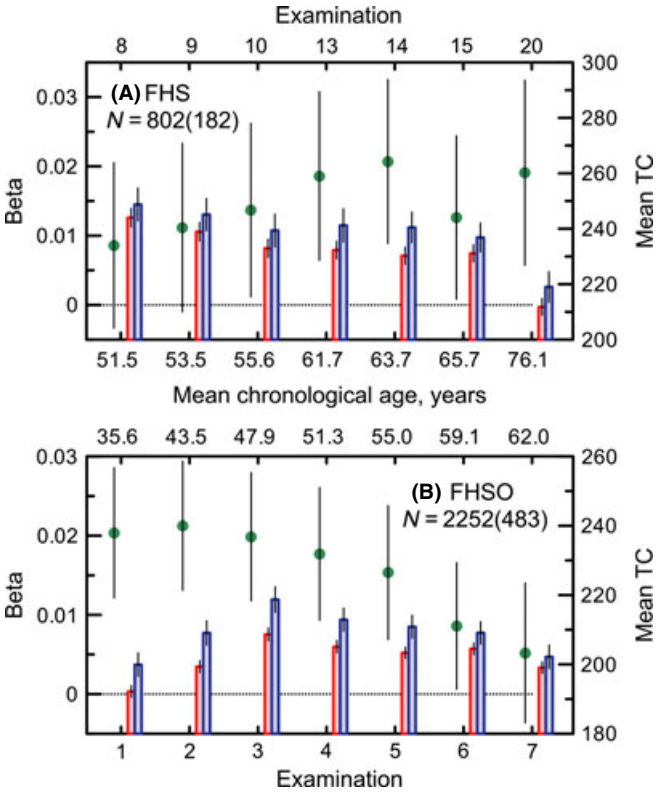

Fig. 4 Associations of total cholesterol (log-base-10 transformed) with *APOE* in individuals who participated in each of the seven (A) FHS and (B) FHSO examinations (shown on outer x-axes). Inner x-axes show mean chronological age in each longitudinally followed sample. Linear regression coefficient betas (filled green dots) show the effect sizes and directions for the e4 allele vs. the non-e4 allele carriers. Dotted line shows zero level (no effect) for beta. Red back-slashed (blue forward slashed) bars depict mean levels of total cholesterol (TC; mg/100 mL) for the non-e4 (e4) allele carriers. N shows the entire (e4-allele carrier) sample size. Thin bars show 95% CIs.

The rs1042034 SNP of the *APOB* gene

Analysis of the empirical Kaplan–Meier patterns showed that the rs1042034 minor allele homozygous genotype (CC) increased the risks of premature onset of CVD in the FHS cohort (Fig. 5 shows a representative pattern at the first examination; at subsequent examinations, the patterns are qualitatively the same as evidenced by the estimates of relative risks in Fig. S6A). A highly significant (RR = 2.18, $P = 4.5 \times 10^{-5}$) detrimental effect was attributed to men (Fig. 5B). This effect was pronounced regardless of the FHS examination and despite the attrition of this sample (see SI, Fig. S6A). The CC genotype in the FHSO cohort can be protective against premature onset of CVD although this effect was not significant due to the small number of CVD cases (see SI, Fig. S6B).

TC did not play a noticeable role as a mediator of the association of the CC genotype with the onset of CVD in either the FHS or the FHSO despite the highly significant association of TC with the onset of CVD in the combined sample of men and women (see SI, Fig. S7 and Fig. 3). The CC genotype did not show a consistent association with TC in the FHS, but it was associated with a lowering of the TC levels in the FHSO, although this effect diminished at later examinations (see SI, Fig. S8). No consistent sexual dimorphism was observed in these associations (see SI, Fig. S8). Similarly, as in the case of *APOE* (Fig. 4), the decline in the effect size in Fig. S8B may be attributed to differential roles of the rs1042034 genotypes in aging individuals (Fig. 6).

Discussion

The analyses conducted using data from different examinations of two generations of the FHS participants document the puzzling complexity of the role of lipid-related genes, the *APOE* e2/3/4 polymorphism, and the *APOB* rs1042034 (C/T) SNP, aging-related processes, environment, and lipids in the onset of CVD, even in the same FHS population.

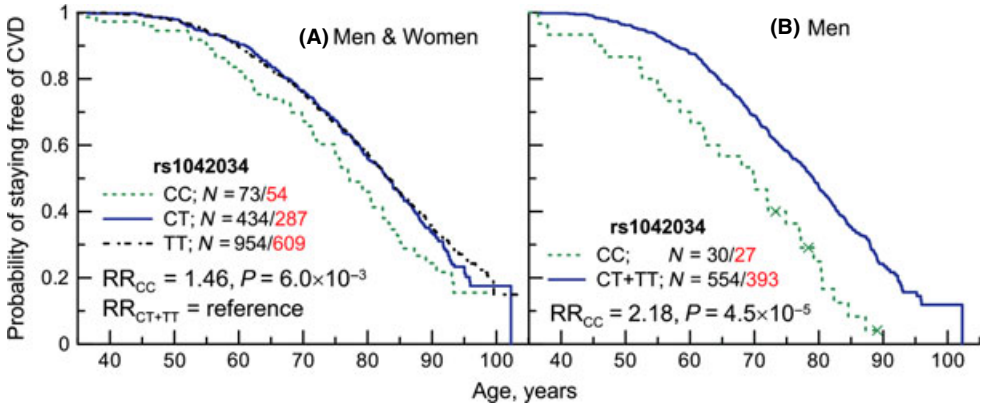

Fig. 5 Kaplan–Meier age patterns of probability of staying free of CVD for carriers of different genotypes of the rs1042034 SNP in the FHS cohort during the entire follow-up (since the baseline). (A) Men and women combined. (B) Men only. N = m/k denotes (m) the total number of carriers and (k) the number of CVD cases among them. Relative risks (RR) were evaluated using the Cox regression model (see Methods) for minor allele homozygotes (CC) compared with the major allele carriers (CT + TT) because no differences among CT and TT carriers were seen. Censored individuals are shown for minor allele homozygous men.

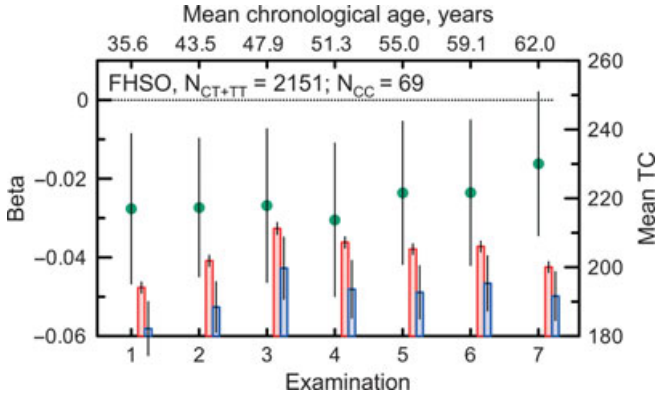

Fig. 6 Associations of total cholesterol (log-base-10 transformed) with rs1042034 in individuals who participated in each of the first seven FHSO examinations. Linear regression coefficient betas (filled green dots) show the effect sizes and directions for the minor allele homozygous genotype (CC) vs. major allele (CT+TT). Dotted line shows zero level (no effect) for beta. Red back-slashed (blue forward slashed) bars depict mean levels of total cholesterol (TC; mg/100 mL) for the major allele carriers (minor allele homozygotes). Upper axis shows mean chronological age in this sample. N shows the sample size for the selected genotypes. Thin bars show 95% CIs. Results for the FHS cohort were not presented because the CC genotype was not consistently associated with TC in the FHS cohort (see SI, Fig. S8).

The role of genes in onset of CVD

Our analyses convincingly documented sex-specific antagonistic roles of the *APOE* e4 allele in the onset of CVD at different ages. Specifically, the e4 allele was associated with premature onset of CVD compared with the non-e4 genotypes in younger individuals, primarily in women, in the parental (FHS) generation whereas older women carrying the e4 allele in the same generation lived longer lives without CVD. We showed that the observed protective effect of the e4 allele was specific to the parental but not to the offspring (FHSO) generation.

The analyses of the FHSO generation documented a detrimental role of the e4 allele in the onset of CVD which was primarily attributed to older women. These results suggest that the detrimental effect of the e4 allele can shift from younger ages in the parental generation to older ages in the offspring generation.

We also reported on a sex-specific differential mode of action of the rs1042034 minor allele homozygous genotype (CC) in the FHS and the FHSO generations. Specifically, the analyses revealed that men carrying the CC genotype were at highly significant risk of premature onset of CVD compared with men carrying the major allele in the FHS (e.g., RR = 2.18, $P = 4.5 \times 10^{-5}$ when followed from baseline; Fig. 5B) but not in the FHSO.

These findings are robust to longitudinal attrition of the samples at risk of CVD. The latter ensures that we are dealing with real phenomena highlighting the presence of hidden heterogeneity in aging-related diseases for carriers of the same alleles. Detecting the presence of more homogenous groups with gene-specific etiology reinforces the importance of moving toward personalized gene-based medicine.

Sexual dimorphism in the effect of genes involved in lipid metabolism, particularly on CHD, is well documented in the literature and typically attributed to differential hormonal and insulin regulation in men and women (Kolovou *et al.*, 2009). The main studies summarized in two meta-analyses (Wilson *et al.*, 1996; Song *et al.*, 2004) typically characterized the e4 allele as a risk allele for CHD. Some of these studies, however, reported on cardio-protective effects of the e4 allele (Utermann *et al.*, 1984; Song *et al.*, 2004). In relation to age, the role of the e4 allele in CVD has not been extensively studied (Minihane *et al.*, 2007); some studies, however, highlight attenuating effects of this genotype with age (Jarvik *et al.*, 1994; Kuusisto *et al.*, 1995; Ilveskoski *et al.*, 1999). Qualitatively, these results are corroborated by the finding of a lack of association of this allele with frailty which develops in older individuals, often after CVD-related illnesses (Rockwood *et al.*, 2008).

The role of genes and lipid metabolism in onset of CVD

To gain insights on potential mechanisms connecting genes involved in lipid metabolism with the onset of CVD, we examined the role of TC in the qualitatively different, detected phenotypic groups (Fig. 3). This analysis showed that TC did not mediate the

associations of the 'risk' genotypes (i.e., the e4 allele or the rs1042034 CC genotype) with the onset of CVD in any of the eight groups regardless of whether or not these genotypes were directly associated with TC in these groups (Fig. 3, vertical lines).

Analysis of the direct association of TC with the onset of CVD highlighted the qualitative differences of the roles of the e4 allele and the CC genotype. Specifically, TC was not associated with the onset of CVD in groups with significant roles of the e4 allele (Fig. 3, Groups A–C), whereas it was in the groups with a neutral role of this allele (Fig. 3, Groups D–F). These connections held regardless of whether or not the e4 allele was directly associated with TC in these groups. TC was, however, associated with the onset of CVD in groups with significant and neutral roles of the CC genotype (Fig. 3, Groups G–H).

Analysis of the role of TC suggested that the e4 allele and the CC genotype can contribute to the development of CVD through qualitatively different biogenetic mechanisms (Fig. 3). These differences reflect the multifactorial nature of gene actions on traits in late life, such as CVD. In relation to the *APOE* gene, the analyses suggested four qualitatively different etiologic pathways.

First, younger men and women in the FHS (Group A) and older women in the FHSO (Group C) likely developed CVD through the same mechanism. Because the e4 allele can contribute to increasing TC levels and to the risk of CVD, but TC was not associated with CVD, the impact of the e4 allele in these groups was likely lipid independent (Minihane *et al.*, 2007). Potential lipid-independent mechanisms include oxidative stress and/or inflammation (Jofre-Monseny *et al.*, 2008). The role of this mechanism might be strengthened through generations and be shifted from younger to older ages.

Second, men and younger women in the FHSO (Groups E–F) can develop CVD through lipid metabolism because TC was associated with CVD in these groups. Given that the e4 allele was associated with TC, it might be erroneously concluded that this allele was associated with CVD through lipid metabolism. The lack of direct association of the e4 allele with CVD ensures that this is not the case. This is because risk factors (also called intermediate phenotypes or endophenotypes (Yashin *et al.*, 2010)) for aging-related diseases are themselves complex phenotypes with different genetic pathways involved in their regulation. Accordingly, the e4 allele can impact one of multiple mechanisms (e.g., cholesterol efflux from macrophage) whereas the other mechanism(s) (e.g., regulating homeostasis of lipids) can cause CVD. Thus, the detection of genetic associations with endophenotypes might not be sufficient for inferring the roles of the respective genes in downstream aging-related diseases. Of importance is that this mechanism was not manifest in the parental generation.

Third, the characteristic mechanism for developing CVD among older men in the FHS (Group D) was likely related to lipid metabolism with no apparent role for the e4 allele.

Fourth, most interestingly, the mechanism related to lipid metabolism either was not working in the FHS older women (Group B) or they were robust to its dysfunction. These women can have a unique *APOE* e4-relevant cardio-protective mechanism through, for example, regulation of C-reactive protein levels (Rontu *et al.*, 2006). Current analyses do not provide evidence that such protective mechanisms were present in the FHSO generation.

No etiologic pathway discussed above suggests a leading role for the e4 allele in CVD through lipid metabolism (Minihane *et al.*, 2007).

In relation to the *APOB* gene, the lack of a direct association of the CC (rs1042034) homozygote with TC in the FHS and its presence in the FHSO might explain the association of the CC homozygote with CVD in the FHS and its absence in the FHSO. This is because the CC homozygote is associated with decreasing levels of cholesterol (TC in this study and triglycerides in Teslovich *et al.* 2010). Accordingly, the lack of an association of the CC homozygote with TC implies less favorable genetic profiles for endophenotypes that can favor development of CVD. The presence of such an association implies more favorable genetic profiles for endophenotypes that can protect against developing CVD. Therefore, the rs1042034 CC genotype can contribute to CVD through lipid metabolism.

Aging-related processes can directly modulate the role of genes in lipid metabolism

To address this issue, we examined the strength of the association of the *APOE* e4 allele and the rs1042034 CC genotype with TC in the same individuals at different chronological ages. These analyses revealed opposite changes in strength of the effect of the e4 allele on TC in the parental (the impact of the e4 allele on TC strengthened) and offspring (the impact of the e4 allele on TC diminished) generations even in the same range of chronological ages (Fig. 4). This observation reinforced the role of the aging-related processes in a changing environment as an essential component in genetic predisposition to complex diseases in late life. A qualitatively similar trend of declining strength of association with TC was observed for the CC genotype in the FHSO (in the FHS this association was not significant). The results on the diminishing strengths of genetic associations with chronological age in the FHSO cohort were in line with the observations of declining differences in TC levels in the e3/4 and e3/3 genotypes in the National Heart, Lung, and Blood Institute (NHLBI) Twin Study which has been attributed to aging (Jarvik *et al.*, 1997).

Are the differences in the observed genetic effects in the FHS and the FHSO cohorts due to intergenerational changes or selection of robust survivors?

The answer to this question is challenging because the genotyped individuals represent selected subpopulations of the most robust survivors in each cohort (see Methods). Differences in survival selection limit inferences based on comparing individuals of the same chronological age in the FHS and the FHSO. However, because the lipid-related genes are directly associated with diet and lifestyle (Eichner *et al.*, 2002; Ordovas, 2007) and because these factors experienced dramatic changes across generations (Vijg & Suh, 2005), intergenerational changes should be expected (Boomsma *et al.*, 1996).

Aging-related processes in changing environment are the key to understanding weak genetic effects on complex traits in late life

This study provides substantive arguments about the crucial role of aging-related processes in genetic predispositions to traits in late life, supporting the call for better understanding of the origin of weak genetic signals (Gibson, 2009; Eichler *et al.*, 2010; MacRae & Vasan, 2011).

For example, it is often argued that detection of alleles with small effects on a phenotype in question requires large samples. Conventional analysis of the FHS cohort can indeed suggest that because the effect of the *APOE* e4 allele on CVD is tiny (RR = 0.92, $P = 0.387$) its detection requires larger samples. Detailed analyses show, however, that the effect is weak because the e4 allele can play antagonistic roles at different ages (i.e., $RR_{\leq 75} = 1.49$, $P = 7.5 \times 10^{-4}$ and $RR_{76+} = 0.77$, $P = 0.044$; see also Figs 1–2). These antagonistic roles are likely due to different etiologic mechanisms in developing CVD (Fig. 3). Increasing the sample size by pooling data from the FHS and the FHSO cohorts does not help at all (RR = 1.08; $P = 0.217$) because of the complex roles of genes in traits in late life. This explicit example demonstrates that '… increasing the size of human disease cohorts is likely only to scale the heterogeneity in parallel' (MacRae & Vasan, 2011) with no obvious chance to improve our understanding.

Concluding remarks

The results of our analyses suggest that aging-related processes in changing environments can be key players in genetic susceptibility to healthspan. Identifying more homogenous groups of individuals at risk with substantially different etiologic pathways may open a realistic avenue for individualized gene-based medicine. More detailed analyses of existing genotype data using systemic integrative approaches will substantially advance the progress in the field.

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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 Natural log-transformed relative risks (RR) of premature onset of CVD (diseases of heart and stroke) in the *APOE* e4 allele carriers compared with the non-e4 carriers at different examinations in the FHSO cohort stratified: (A) into younger (< 70) or older (70 +) group (defined next), (B) by sex, and (C) into younger or older group and by sex.

Fig. S2 Natural log-transformed relative risks (RR) of premature onset of CVD (diseases of heart and stroke) in the *APOE* e4 allele carriers compared with the non-e4 carriers at different examinations in the (A) FHS and (B) FHSO cohorts.

Fig. S3 Natural log-transformed relative risks (RR) of premature onset of CVD (diseases of heart and stroke) in the *APOE* e4 allele carriers compared with the non-e4 carriers at different examinations in the (A) FHS and (B) FHSO cohorts.

Fig. S4 Associations of total cholesterol (log-base-10 transformed) with *APOE* in participants of different examinations in the (A) FHS and (B) FHSO cohorts.

Fig. S5 Association of total cholesterol (log-base-10 transformed) with *APOE* in individuals who participated in each of the seven (A) FHS and (B) FHSO examinations (shown on outer *x*-axes).

Fig. S6 Natural log-transformed relative risks (RR) of premature onset of CVD (diseases of heart and stroke) in carriers of the minor allele homozygous genotype (CC) of rs1042034 compared with the major allele (CT + TT) at different examinations in the (A) FHS and (B) FHSO cohorts.

Fig. S7 Natural log-transformed relative risks (RR) of premature onset of CVD (diseases of heart and stroke) in carriers of the minor allele homozygous genotype (CC) of rs1042034 compared with the major allele (CT + TT) at different examinations in the (A) FHS and (B) FHSO cohorts.

Fig. S8 Associations of total cholesterol (log-base-10 transformed) with rs1042034 in participants of different examinations in the (A) FHS and (B) FHSO cohorts.

Table S1 Association of total cholesterol with the selected genetic markers and age at onset of CVD in participants of the FHS examination 10 and the FHSO examination 4 stratified by fasting status.

Table S2 Basic characteristics of the genotyped FHS/FHSO participants.