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GWAS of Longevity in CHARGE Consortium Confirms *APOE* and *FOXO3* Candidacy

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Background. The genetic contribution to longevity in humans has been estimated to range from 15% to 25%. Only two genes, *APOE* and *FOXO3*, have shown association with longevity in multiple independent studies.

Methods. We conducted a meta-analysis of genome-wide association studies including 6,036 longevity cases, age ≥90 years, and 3,757 controls that died between ages 55 and 80 years. We additionally attempted to replicate earlier identified single nucleotide polymorphism (SNP) associations with longevity.

Results. In our meta-analysis, we found suggestive evidence for the association of SNPs near *CADM2* (odds ratio [OR] = 0.81; p value = 9.66×10^{-7}) and *GRIK2* (odds ratio = 1.24; p value = 5.09×10^{-8}) with longevity. When attempting to replicate findings earlier identified in genome-wide association studies, only the *APOE* locus consistently replicated. In an additional look-up of the candidate gene *FOXO3*, we found that an earlier identified variant shows a highly significant association with longevity when including published data with our meta-analysis (odds ratio = 1.17; p value = 1.85×10^{-10}).

Conclusions. We did not identify new genome-wide significant associations with longevity and did not replicate earlier findings except for *APOE* and *FOXO3*. Our inability to find new associations with survival to ages ≥90 years because longevity represents multiple complex traits with heterogeneous genetic underpinnings, or alternatively, that longevity may be regulated by rare variants that are not captured by standard genome-wide genotyping and imputation of common variants.

Key Words: Longevity—GWAS—FOXO3—APOE.

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THERE is ample evidence that genetic factors are involved in extreme longevity both in humans and in other organisms. In model organisms, ranging from *Caenorhabditis elegans* to *Mus musculus*, mutations in the insulin/IGF-1 signaling (IIS) pathway have been shown to substantially increase lifespan (1–6). Other suggested mechanisms involve stress resistance as mediated by heat shock proteins (7,8), cellular senescence as mediated by telomere length (9) and inflammation/immune function (10). The genetic contribution to longevity in humans has been estimated to range from 15% to 25% (11–13).

Although many human candidate longevity genes have been investigated, only two genes have been widely replicated. The first consistent association reported was *APOE* (14). It was subsequently replicated in both candidate gene and genome-wide association studies (GWAS) with genome-wide significant evidence (15–20). The second longevity gene, identified in American male centenarians of Japanese descent and replicated in other candidate gene studies that included both men and women across ethnicities, was *FOXO3*, although this gene was not identified in GWAS (21–25).

Early GWAS have failed to identify novel longevity genes as reviewed by Murabito and coworkers (26). In 2010, the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium published a meta-analysis of 1,836 longevity (90+) cases and a comparison group of 1,955 individuals who died between 55 and 80 years of age (27). A total of 24 independent SNPs were identified, though none reached genome-wide significance. Finally, a recent GWAS by Deelen and coworkers identified one new longevity locus on 5q33.3 (28).

The key to success to GWAS has proven to be large sample sizes. Since the original CHARGE longevity GWAS (27) additional members of each cohort have reached or exceeded age of 90 years and additional studies have joined the consortium, permitting an expanded meta-analysis of longevity. Using the much expanded sample of 6,036 longevity cases and 3,757 controls and the same study design as the original CHARGE longevity study, we attempted to produce robust new associations and replicate earlier identified associations for longevity coming from several earlier studies.

Methods

Participants

The participants in our study were of European ancestry and included cohorts from the CHARGE Consortium (29). All cohorts periodically assess the vital status of their participants. Although some of the cohorts include multiple ethnic groups, only data from Caucasian participants were used. Informed written consent was obtained from all participants, and appropriate institutional approvals were obtained. A brief description of each population is given in the Supplementary Material.

Longevity Phenotype

Longevity was defined as reaching age ≥90 years. A more extreme longevity threshold of age 100 years was considered. However, the sample size was deemed insufficiently powered. Genotyped participants who died between the

ages of 55 and 80 years were used as the control group. The control group was limited to deceased participants to ensure that no control individuals could subsequently achieve longevity. The minimum age at death was set to match the minimum age at enrollment in the Rotterdam Study. The maximal age at death was set arbitrarily at the age of 80 years to include the majority of deaths and to exclude those participants who survived far beyond the average life expectancy for their respective birth cohort. Across the discovery cohorts, there were 6,036 participants who achieved longevity and the control group had 3,757 participants.

We did not have direct replication cohorts; however, we performed a look-up of our strongest associations in three independent studies with similar phenotypes, one of which is a published GWAS on longevity (30).

Genotyping and Imputation

As different genotyping platforms were used across studies, we imputed to ~2.5 million SNPs using the HapMap 22 CEU (Build 36) genotyped samples as a reference. Details on the study-specific quality control procedures for genotyping and imputation are found in Supplementary Table 1. Although genotyping between different studies was performed on different genotyping platforms, genotyping of cases and controls within studies was performed on the same genotyping platform in each study.

Statistical Analysis

We used logistic regression to test each SNP for association with longevity using an additive model adjusting for sex and principal components to adjust for population stratification. Fixed-effects inverse-variance meta-analysis was performed using METAL. The *p* values were corrected for genomic control. Associations with a *p* value $< 5 \times 10^{-8}$ were considered genome-wide significant, whereas associations with a *p* value $< 1 \times 10^{-5}$ were considered suggestive and taken forward to the look-up phase.

Detailed analysis of cohorts participating in the look-up phase and additional analyses can be found in the Supplementary Material. Conditional analysis of the *FOXO3* locus was performed using the GCTA tool which analyzes aggregate, not individual level data (31,32). We further attempted to replicate earlier identified SNP associations with longevity. Finally, we used gene networks available at www.genenetworks.nl as a bioinformatics resource to further investigate significant findings.

Results

General characteristics of discovery and look-up cohorts are found in Tables 1 and 2, respectively. Between 0.0% and 70.3%, varying per cohort, of those achieving longevity were still alive at the time that longevity status was ascertained. Among those who had died, the distribution of causes of death differed between longevity cases and the comparison group. While 1.0%–17.3% of those achieving longevity died of cancer, 10.1%–50.3% of deaths in the control group could be attributed to cancer.

None of the SNP-longevity associations reached the genome-wide significance threshold of 5×10^{-8} in the discovery phase (Supplementary Figure 1), although the strongest association was borderline significant for rs1416280 located 369 kb from *GRIK2* (odds ratio [OR] = 1.24; *p* value = 5.09×10^{-8}). In total, seven loci passed the threshold for suggestive association ($<1 \times 10^{-5}$) and were included in the look-up phase (Table 3). For look-up, we also included the most significant SNP in the current study from the known candidate longevity gene, *FOXO3* (*p* value = 8.56×10^{-5}). Forest plots of all eight SNPs can be found in Supplementary Figure 2. Although none of the SNPs reached Bonferroni adjusted significance in any of the look-up cohorts, consistent results considering direction of effect were found for *FOXO3* across discovery and look-up cohorts (Table 3). Association results for *CADM2* and, to a lesser degree, *GRIK2* were consistent across cohorts. In pathway analysis using the gene networks tool, both *CADM2* and *GRIK2* genes were found to be involved in neuronal pathways (Supplementary Table 2).

Of the 24 SNPs identified as suggestively associated in the original CHARGE longevity GWAS (27), the *p* value for only one of these (*GRIK2*; rs954551) improved in the current study (Supplementary Table 3). One additional region (*RGS7*) from the earlier study appeared among the strongest associations in the current study, but the association was not strengthened in the look-up phase of this study.

Next, we aimed to replicate the GWAS of Sebastiani and coworkers conducted on centenarians (Supplementary Table 4) (20). Two hundred and eighty-one SNPs were reported using four different genetic models (20). Six out of the 281 SNPs were reported to have a *p* value $<1 \times 10^{-5}$ in the additive model. We attempted to replicate these six SNPs, and only the SNP at the *APOE* locus replicated at the Bonferroni corrected significance level (OR = 1.20; *p* value = 4.8×10^{-4}). Similarly, of the four regions identified with linkage analysis of longevity sib-pairs (age ≥90 years) (33) we also replicated only the *APOE* locus (Supplementary Table 5).

Finally, in a candidate gene approach, we examined the original SNP reported for the *FOXO3* gene (rs2802292) in the current study and added our current study (each discovery cohort individually) to the published studies reporting on the same *FOXO3* SNP (Supplementary Table 6) (21–25,30). In the current study, rs2802292 reached a *p* value of .012 (OR = 1.09). When including the published studies, both candidate gene and GWAS, in the meta-analysis rs2802292 reached a *p* value of 1.85×10^{-10} (OR = 1.17). The Linkage Disequilibrium (LD) as measured by r^2 between this SNP and the top *FOXO3* SNP in the current study (rs10457180) was .64. In conditional analyses of the discovery phase

Table 1. General Characteristics of Discovery Cohorts

			Age at DNA Draw			Cause of Death (%)*			
		N	Mean (*SD*)	Women (%)	Alive (%)	CVD	Cancer	Other	Unknown
Rotterdam Study 1 (RS1)	90+ cases	899	82.3 (6.1)	79.0	23.6	31.4	7.9	60.7	0.0
	Comparison	1,192	66.5 (5.6)	41.3	0.0	32.2	40.1	27.7	0.0
Rotterdam Study 2 (RS2)	90+ cases	69	86.0 (6.2)	60.9	69.6	33.3	14.3	52.4	0.0
	Comparison	161	66.0 (3.3)	37.3	0.0	23.6	50.3	26.1	0.0
Study of Osteoporotic Fractures (SOF)†	90+ cases	1,720	72.7(5.8)	100.0	54.4	16.8	3.6	21.5	0.0
	Comparison	124	69.6 (3.4)	100.0	0.0	37.9	28.2	33.9	0.0
Cardiovascular Health Study (CHS)	90+ cases	791	77.7 (5.0)	62.3	41.7	51.5	17.3	30.6	0.6
	Comparison	560	69.5 (3.0)	53.2	0.0	33.6	39.3	27.0	0.2
Osteoporotic Fractures in Men Study (MrOS)‡	90+ cases	670	83.0 (3.4)	0.0	43.6	19.9	6.9	21.8	3.1
	Comparison	502	70.8 (3.4)	0.0	0.0	29.3	38.8	31.7	0.2
Framingham Heart Study (FHS)	90+ cases	320	86.7 (4.3)	66.9	0.0	24.1	11.6	51.9	12.5
	Comparison	484	65.4 (7.3)	34.3	0.0	24.6	43.8	25.2	6.4
Health and Retirement Study (HRS)	90+ cases	384	89.3 (3.1)	67.5	68.5	3.0	15.0	12.6	69.5
	Comparison	401	69.4 (6.2)	45.6	0.0	12.1	10.1	16.2	61.6
Age, Gene/Environment SusceptibilityReykjavik Study (AGES)	90+ cases	541	84.9 (2.8)	61.2	65.1	27.3	8.4	16.2	48.1
	Comparison	145	72.8 (3.0)	49.0	0.0	28.2	35.5	18.4	18.0
Religious Orders Study and Rush Memory and Aging Project (RADC)	90+ cases	468	85.9 (5.0)	75.2	40.0	0.0	0.0	0.0	100.0
	Comparison	78	70.2 (3.4)	48.7	0.0	0.0	0.0	0.0	100.0
Invecchiare nel Chianti (InCHIANTI)	90+ cases	101	92.8 (2.9)	72.3	70.3	8.9	3.0	7.9	80.2
	Comparison	75	72.8 (4.0)	36.0	0.0	6.7	20.0	4.0	69.3
Baltimore Longitudinal Study of Ageing (BLSA)	90+ cases	128	91.42 (3.96)	49.2	50.0	4.7	1.0	3.0	91.4
	Comparison	42	69.14 (6.25)	26.2	0.0	9.5	19.0	2.3	69.1

Notes: *In cases the % of all deaths is reported.
† In SOF, the vital status is not known for all individuals.
‡ In MrOS, the vital status is not known for all individuals.

Table 2. General Characteristics of Look-Up Cohorts

	NECS		LLFS	EU_longevity
	Cases (100+)	Comparison	Survival Analysis	Cases (90+); Comparison (<65)
N	801	914	4,567	5,409; 16,121
Age at DNA draw (median [range])	104 (95–119)	73 (54–90)	67 (25–110)	NA
Follow-up (median [range])	NA		3 (0–7)	NA
Women, %	72.0	56.0	55.0	NA
Alive, %	3.0	0.0	83.0	NA

Notes: LLFS = Long Life Family Study; NECS = New England Centenarian Study. No study specific information for the EU_longevity consortium was available. Causes of death were not available in the look-up cohorts.

cohorts, including both SNPs, only rs10457180 remained significant at nominal *p* value (OR = 0.94; *p* value = 5.53×10^{-4}).

Discussion

In this investigation of 6,036 longevity cases and 3,757 controls, we found suggestive evidence for the involvement of SNPs near *CADM2* and *GRIK2*. We further confirmed the associations of *APOE* and *FOXO3* with longevity.

FOXO3 is a known candidate gene for longevity and part of the well-characterized IIS pathway (6). Although originally identified in candidate gene studies, it did not earlier reach genome-wide significance (21–25). In this study, the strongest signal in the *FOXO3* gene was found for rs10457180 which is in LD (r^2 = .64) with the earlier identified SNP rs2802292. Pooling our data with that of independent studies from literature yielded a *p* value of 1.85×10^{-10}. Although we included all studies reporting on *FOXO3* association with longevity from the literature as well as contacted authors of earlier published GWAS for information on *FOXO3* in their study (19), publication bias, which is common in candidate gene studies, may be influencing the results of this analysis. In a conditional analysis of the discovery phase cohorts, only rs10457180 remained significant, which suggests that rs10457180 may be a better tagging SNP for the true causal variant. A point of notice is that *FOXO3* has 99% sequence homology in its exonic regions with its pseudogene (*ZNF286B*), which could complicate the calling of genetic variants for this gene (34,35). However, the investigated variants (rs2802292 and

Table 3. Association Results for Longevity: Discovery and Look-Up

						Discovery				EU_longevity		Centenarian (NECS)				Survival (LLFS)			
SNP	Chr	Gene	Distance (kb)	EA	EAF	*p* Value	OR	95% CI	Direction	effect	*p* Value	SNP	r^2*	OR	*p* Value	SNP	r^2*	1/HR	*p* Value
rs1416280	6	*GRIK2*	369	C	0.75	5.09×10^{-8}	1.24	1.15–1.34	+−++++++−+	+	.276	rs9377361	1.00	0.98	.833	rs9377361	1.00	1.00	.981
rs9841144	3	*CADM2*	−236	A	0.79	9.66×10^{-7}	0.81	0.74–0.88	−−−−−−−−+−	+	.825	rs9822731	0.96	1.03	.726	rs9822731	0.96	0.85	.053
rs4611001	1	*RGS7*	−28	A	0.97	1.84×10^{-6}	1.79	1.41–2.27	+?−++++++?+	−	.207	rs7536260	0.68	0.65	**.013**	rs75361849	1.00	1.07	.728
rs11023737	11	*SOX6*	−28	A	0.32	3.64×10^{-6}	0.83	0.77–0.90	−−−−−−−−−−	+	.684	rs2196961	0.27	1.03	.668	rs11023744	0.96	1.06	.397
rs11753077	6	*MBOAT1*	−76	T	0.64	7.51×10^{-6}	1.17	1.09–1.26	+++++++−+−+	+	.095	rs7763815	0.87	1.04	.572	Same	1.00	1.03	.633
rs10875746	12	*PFKM*	Intron	A	0.76	$7.8\,3\times10^{-6}$	1.20	1.11–1.30	+++++++++−+	+	.240	rs2228500	0.74	1.03	.796	rs2228500	0.74	0.93	.349
rs10007810	4	*LIMCH1*	Intron	A	0.23	8.80×10^{-6}	1.20	1.11–1.30	+−+++++++++	−	.979	Same	1.00	0.98	.841	Same	1.00	0.95	.521
rs10457180	6	*FOXO3*	Intron	A	0.70	8.56×10^{-5}	0.87	0.81–0.93	−−+−−−−+−−	−	**.023**	rs2153960	0.89	0.98	.822	rs2153960	0.89	0.84	**.021**

Notes: EA = effect allele; EAF = effect allele frequency; "+" = effect allele over-represented in cases; "−" = effect allele underrepresented in cases; "?" = not tested in this cohort. The *p* values < .05 in replication are represented in bold. EU_longevity: European consortium investigating the genetics of longevity with similar phenotype to discovery (cases: 90+; controls: < 65 y old); Centenarians (NECS, New England Centenarian Study): centenarian cases compared to younger controls; Survival (LLFS, Long Life Family Study): all-cause mortality Cox-regression analysis, 1/HR shown for direct comparison of the direction of the effect. In NECS and LLFS, imputation is not available. Study order for ***direction*** column: RS1, RS2, SOF, CHS, MrOS, FHS, HRS, AGES, RADC, InCHIANTI, BLSA.

*Linkage disequilibrium with discovery SNP.

rs10457180) are both intronic, which are not present in *ZNF286B*. Therefore, we believe our results are not affected by this phenomenon.

Although our look-up for both *CADM2* and *GRIK2* did not show consistent associations, these genes are interesting candidate genes for longevity. Both genes are involved in neuronal pathways, in particular in neuron cell–cell adhesion and regulation of glutamatergic synaptic transmission (www.genenetworks.nl). Neuronal pathways have been implicated earlier in all-cause mortality in a formal pathway analysis (36).

We did not replicate other earlier reported associations with longevity, except for *FOXO3* and *APOE*. Although many attempts to unravel the genetic contribution to the longevity phenotype have been undertaken, it has proven difficult to find robust associations (26). Genome-wide association studies have identified the genetic architecture of many complex traits with great success, even when the heritability of the trait is modest, as is the case for longevity. Yet the longevity phenotype remains elusive to uncovering new genetic associations.

As with any chosen methodology GWAS has its pitfalls. The most obvious pitfall of a GWAS is the low *p* value of significance required to call a variant significantly associated. There are many potential sources of Type I and II errors in GWAS, including case–control misclassification, nongenetic covariates, and population stratification, which can only partly be accounted for in the design of the study (37). We have used the standard approach of considering *p* values $< 5 \times 10^{-8}$ as significant to prevent false positive findings. As a result, we accept the presence of false negative findings. Recently, we have shown that the number of false positive findings increases exponentially when higher *p* values are used in GWAS setting, further justifying the use of a stringent cut-off (38). We do not believe that case–control misclassification is a big issue in our study as we have very clear cut-offs for being included as a case (≥90 years) or control (deceased between 55 and 80 years). The potential influence of nongenetic covariates is more difficult to ascertain. However, these are assumed to be independent of genotypes and are therefore unlikely to be confounders. In addition, it has been shown that the power of meta-analysis is not optimal for SNPs with small to modest effect sizes (39). However, it is common practice in GWAS to perform large-scale meta-analyses as the sample size required to detect variants at such low *p* values is not sufficient in any one study. Increasing the sample size of the current study may help identify new variants.

There are several potential explanations for why longevity has proven difficult to dissect genetically. First, the longevity phenotype simply could be too complex and heterogeneous for successful identification of longevity genes. Achieving longevity may require a great number of "protective" genes all with small effects (20). Although in highly heritable traits, like height, genes with very small effects have been uncovered, in a trait like longevity it may be challenging to identify individual genetic variants given the numbers of available genotyped individuals reaching longevity. In addition, the complexity of the longevity phenotype is made more challenging due to its interaction with a number of age-related diseases, that is, cancer, cardiovascular disease, and dementia, all of which have independent genetic risk variants. Although it has been shown that currently identified variants for age-related diseases do not strongly associate with longevity (40), suggesting that there are longevity-specific variants remaining to be discovered, this genetic and phenotypic heterogeneity of longevity makes it very challenging to identify genetic variants for longevity.

Using biomarkers that explain (a part of) longevity might be a more fruitful pursuit for finding associations with longevity itself. Unfortunately, no good biomarkers of aging currently exist, although many have been proposed (41). Telomere length, a marker of senescence, could be an interesting biomarker of aging and, GWAS have already proven successful in identifying genes for this trait (42). An alternative approach could be the development of novel phenotypes like the healthy aging index, which incorporates information across physiologic systems and predicts mortality better than age itself (43).

The second potential reason for not finding any genome-wide significant associations with the longevity phenotype is that GWAS targets common variants. As longevity in the general population is quite rare, but common in specific long-lived families, it could be that rare variants, rather than common variants are involved in the longevity phenotype (44). Such variants are not likely to be picked up in GWAS, but may be uncovered with rare variant association analysis, which requires sequencing technology (45).

Another avenue that deserves consideration for future studies is the identification of joint genetic effects (epistasis) (46). Epistasis has been demonstrated to be an important contributor to age-related diseases such as cardiovascular disease (47). Methods for identifying epistasis are improving and becoming feasible for genome-wide studies (48).

Finally, it is possible that epigenetic changes could influence the expression of genetic variations leading to longevity. Epigenetic modifications such as DNA methylation and histone modification are essential for development and differentiation, but can also arise later in life (49). Age-related changes in DNA methylation have been implicated in senescence and longevity (50). Such changes may be a major contributor to longevity that might make it difficult to find associations with common genetic variants. A known phenomenon in longevity is that the majority of individuals reaching longevity are female. Whether this has a genetic or epigenetic basis is unknown and should be investigated in future studies.

A limitation of our phenotype is our definition of longevity, namely, living to age ≥90 years may not be "extreme" enough as a case definition. We considered more extreme

longevity cut-offs, including centenarian status; however, we found that the sample size for such currently was not enough to have sufficient power to detect association in GWAS. Similarly, the definition of dying before 80 years might be too liberal for the definition of controls since they survived far beyond the average life expectancy for their respective birth cohort. Furthermore, more extreme cut-offs would have resulted in a too small sample size for GWAS.

In conclusion, we confirmed the association of *FOXO3* and *APOE* with longevity, but did not find any new associations, aside from the suggestive evidence for *CADM2* and *GRIK2*. Notably, variants in neuronal pathway genes were confirmed to play a role in longevity. Future genetic studies should consider: (i) using more extreme age definitions for longevity, (ii) performing rare variant association analyses, (iii) analyzing epistasis, (iv) exploring epigenetic changes associated with longevity, (v) sex-specific analysis, and (vi) increasing the sample sizes of both cases and controls.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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