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Association of the *FOXO3A* Locus with Extreme Longevity in a Southern Italian Centenarian Study

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Abstract

A number of potential candidate genes in a variety of biological pathways have been associated with longevity in model organisms. Many of these genes have human homologs and thus have the potential to provide insights into human longevity. Recently, several studies suggested that *FOXO3A* functions as a key bridge for various signaling pathways that influence aging and longevity. Interestingly, Willcox and colleagues identified several variants that displayed significant genotype–gender interaction in male human longevity. In particular, a nested case–control study was performed in an ethnic Japanese population in Hawaii, and five candidate longevity genes were chosen based on links to the insulin–insulin-like growth factor-1 (IGF-1) signaling pathway. In the Willcox study, the investigated genetic variations (rs2802292, rs2764264, and rs13217795) within the *FOXO3A* gene were significantly associated with longevity in male centenarians. We validated the association of *FOXO3A* polymorphisms with extreme longevity in males from the Southern Italian Centenarian Study. Particularly, rs2802288, a proxy of rs2802292, showed the best allelic association—minor allele frequency (MAF) = 0.49; $p = 0.003$; odds ratio (OR) = 1.51; 95% confidence interval (CI), 1.15–1.98). Furthermore, we undertook a meta-analysis to explore the significance of rs2802292 association with longevity by combining the association results of the current study and the findings coming from the Willcox et al. investigation. Our data point to a key role of *FOXO3A* in human longevity and confirm the feasibility of the identification of such genes with centenarian–controls studies. Moreover, we hypothesize the susceptibility to the longevity phenotype may well be the result of complex interactions involving genes and environmental factors but also gender.

Introduction

AGING IS A MULTIFACTORIAL AND COMPLEX process regulated by stochastic interactions (random damage to vital molecules), extrinsic interventions (such as diet and caloric restriction), and intrinsic/genetic alterations. A number of centenarian (people aged 100 years old or older) studies exploit the strong selection of favorable genotypes in exceptionally aged individuals to study candidate genes and to perform genome wide analyses. Replications of observed associations of genotypes with longevity, coupled with functional studies to define mechanisms whereby specific genotypes influence lifespan-associated phenotypes, are essential to delineate true positive genetic findings. Such findings could potentially lead to preventive and therapeutic interventions for several age-related diseases that cause significant morbidity and mortality among older people.

Numerous genes have been identified that are either positively or negatively selected in the centenarian population as a consequence of a demographic selection,[1–3] but no consistent replications have been observed in independent population, with the exception of *Apolipoprotein E* (*APOE*).[4,5] In addition, animal studies have provided insights into the types of genes that can be involved in the regulation of lifespan. The first longevity mutant to be identified was the *Caenorhabditis elegans* gene *PAX2* age-1.[6] This mutant encodes phosphatidylinositol-3-kinase (PI3K),[7] which has a key role in a signaling pathway that is homologous to the mammalian insulin–insulin-like growth factor-1 (IGF-1) pathway. Ge-

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netic studies in *C. elegans* have demonstrated that activation of the PIK3/Akt pathway by IGF-1 suppresses activity of the DAF-16 forkhead transcription factor, the nematode ortholog of mammalian FOXO proteins. FOXO transcription factors belong to the Forkhead family of proteins, a family characterized by a conserved DNA-binding domain (*Forkhead box*) for phosphorylation by the survival kinase Akt.[8–10] Particularly, in mammals the FOXO subgroup (FOXO1, FOXO3, FOXO4, and FOXO6) promotes the expression of numerous downstream genes that mediate stress resistance, innate immunity, metabolic processes, and toxin degradation.[11–15]

A moderate reduction in the intake of calories (also known as caloric restriction [CR]) is extremely effective in delaying age-related decline and increasing longevity in organisms ranging from yeast to mammals.[16–19] In this regard, Bonkowski and colleagues observed a striking difference in the response of growth hormone (GH) receptor/GH-binding protein knockout (GHRKO) and normal mice to an identical regimen of CR.[20] When male and female longevity data were analyzed separately, it became evident that the effects of CR on maximal longevity were sexually dimorphic in GHRKO mice but not in normal mice.

It should be noted that susceptibility to many common diseases, as well as longevity, may well be the result of complex interactions involving genes, environmental factors, and, intriguingly, gender. In this scenario, *FOXO* is a key component of the insulin pathway, free radical production, and human longevity.

In the prospective population-based Leiden 85 plus Study (866 females and 379 males, aged 85 years or more), the effect of genetic variants in *FOXO1A* and *FOXO3A* were analyzed on metabolic profiles, fertility, fecundity, age-related diseases (i.e., diabetes, myocardial infarction, etc.), and mortality.[21] In detail, *FOXO3A* haplotypes showed no association with longevity and other investigated variables. One possible drawback of this finding is total absence of gender stratification. In fact, the hormonal environment as well as tissue-specific gene expression is known to differ significantly between genders in vertebrates. Hormones may differentially affect gene expression in somatic tissues, thus leading to the gender-specific susceptibility to a complex phenotype, such as longevity.[20] In this regard, Willcox et al.[22] performed a nested case–control study of five candidate longevity genes (*ADIPOQ, FOXO1A, FOXO3A, SIRT,* and *COQ7*) with links to the insulin–IGF signaling pathway (IIS). This nested case–control study was conducted in an ethnic Japanese population from the Island of Oahu, Hawaii, with 213 male longevity "cases" and 402 male "average-lived" controls. Three single-nucleotide polymorphisms (SNPs) were found to be associated with the "longevity" phenotype at the locus of the *FOXO3A* gene: rs2764264 ($p = 0.0002$), rs13217795 ($p = 0.0006$), and rs2802292 ($p < 0.0001$).[22] The aim of our study was to support the potential role of *FOXO3A* as a key component that influences longevity with a case–control study. We screened 480 long-living "cases" (281 males and 199 females) and 335 young controls (195 males, 140 females) from an isolated and homogeneous population of southern Italy (Southern Italian Centenarian Study, SICS) with an Illumina BeadChip 300K system, and we focused on the *FOXO3A* gene. As previously described, we hypothesized that gender might influence the association between *FOXO3A* and longevity; thus, we analyzed males and females separately. We applied a powerful method for correcting for stratification of our population. Last, we exploited a meta-analysis approach to pool Willcox's findings to find evidence for a statistical association for the SNP rs2802292.

Materials and Methods

Subject features

This case–control study was carried on as a part of the SICS. The SICS (2002) assembled a large cohort of 600 DNA samples of nonagenarians/centenarians (age range 90–109 years) and 800 DNA samples of young controls (age range 18–48 years) from a isolated region of southern Italy east of Naples with a high prevalence of longevity and health and characterized by a high level of endogamy.[23] All participants for the current study were well characterized; in fact, detailed phenotypic information about self-reported health history and cognitive and physical functions measured through Blessed memory tests and Barthel scores of activity of daily living; demographics and exposure to common risk factors such as alcohol and smoking were also collected. For the aim of the present investigation, 281 long-lived men (age range 90–108 years) and 195 controls (age range 18–48 years) were genotyped (Table 1).

The subjects were enrolled by an Italian subsidiary (Associazione Longevità) of Elixir Pharmaceuticals (former Centagenetix Inc). All subjects donated blood samples for DNA study and gave written informed consent to the study. The study was conducted in accordance with the ethical principles that have their origins in the Declaration of Helsinki.

Genotyping by the Illumina BeadChip system

Genotyping was carried out using the Infinium II Assay-HumanHap BeadChip 317K-duo system (Illumina) using standard protocols. In fact, Illumina's Infinium II Whole-Genome Genotyping Assay is designed to interrogate a large number of SNPs at unlimited levels of loci multiplexing. The HumanHap 317-Duo workflow can be divided in three main

Table 1. Age (Years) Distribution by Gender and by Centenarian/Control Phenotype

	Males			*Females*		
Phenotype	n	*Mean ± SD*	*Min–Max*	n	*Mean ± SD*	*Min–Max*
Centenarians	281	94.96 ± 2.97	90–108	199	98.05 ± 3.25	90–109
Controls	195	33.53 ± 7.33	18–48	140	31.39 ± 7.31	18–48

n, number of individuals; SD, standard deviation; Min, minimum age; M, Maximum age.

segments: (1) sample preparation; (2) sample fragmentation and hybridization; (3) extension, staining, and scanning.

The BeadChps were imaged using a two-color confocal laser system with 0.8-μm resolution. The bead intensities were extracted, and genotypes were calculated using an Illumina-supplied cluster file, which is based on a set of reference samples. The normalization algorithm adjusts for nominal offset, cross-talk, and intensity variations observed in the two-color channels. The data for each BeadChip were self-normalized using information contained within the array. This normalization algorithm removes outliers, adjusts for channel-dependent background and global intensity differences, and also scales the data. The X and Y color channels undergo an affine coordinate transformation to make the data appear as canonical as possible, with the homozygotes lying along the transformed x and y axes. All genotypes were evaluated using a quantitative quality score called GenCall score. A GenCall score ranges from 0 to 1 and reflects the proximity within a cluster plot of the intensities of that genotype to the centroid of the nearest cluster.

Statistical analysis

Quality control. Individuals showing a genotyping rate <93% and SNPs with a genotyping rate <95% were excluded from the dataset. We screened for contaminations and evaluated relationships among individuals using identity by descent (IBD) estimations. Samples showing extreme heterozygosis and related individuals were removed. X chromosome data were used to check for the discordances in terms of gender assignment. SNPs with a minor allele frequency (MAF) < 0.01 and markers deviating from the Hardy–Weinberg equilibrium (HWE) in the control population (p-HWD < 0.001) were excluded from the analysis.

Population structure. To assess the absence of population stratification, we applied the principal component analysis (PCA) approach, a method that can capture subtle and extensive variations due to ethnical, experimental, and technical features.[24] All autosomal SNPs and individuals that passed the quality control filters (299,772 SNPs, 258 centenarians, 178 controls) were used as input to the EigenSoft 2.0[24] software using the default parameters, except for the number of outliers removal iterations, which was set to 0 to obtain estimates for all subjects.

Imputation. To impute SNPs from multimarker tags, we used Impute v0.5.0.[25]

Association tests. We evaluated the association of markers with longevity phenotype by means of allelic association tests (1 degree of freedom [df]) and applied the Pearson χ^2 test (2 df) and Fisher exact test (if cell counts were <5) to compare genotype frequencies between cases and controls. We also performed logistic regression under an additive model and logistic regression comparing homozygous minor versus homozygous major alleles. Correction for population stratification was performed using logistic regression under an additive model adjusting along the top four statistical significant principal components. Allele frequencies, genotype counts, odd ratios (OR), and 95% confidence intervals (95% CI) were also estimated for each SNP.

Meta-analysis. We used the OR and 95% CI for homozygous minor versus homozygous major alleles from the current analysis and from Willcox et al. study to calculate the natural logarithms of the OR (Log-OR) and its standard error (SE). The Log-OR estimates were combined to obtain a summary OR following fixed[26] and Der Simonian and Laird random effects[27] models using inverse variance calculations. Between-dataset heterogeneity was identified using the I^2 metric for inconsistency,[28] and its statistical significance was assessed by means of the χ^2 distributed Q statistic [29] (the number of df is given by $k - 1$, where k is the number of analyzed datasets). All calculations have been performed using R v2.7.1 software.

Haplotype analyses. We used HaploView 4.0[30] for blocks definition, haplotypes estimation, and association tests. We defined haplotype blocks using the Four Gamete Rule, a variant on the algorithm described in the Wang study,[31] and we estimated haplotypes using the default accelerated expectation-maximization (EM) algorithm.[30] Differences in haplotype frequencies between centenarians and controls were tested by means of χ^2 tests; haplotypes with a frequency <0.01 were excluded from the analysis.

Covariate analyses. Associations between clinical risk factors and cognitive variables with genotypes were tested among nonagenarians by means of logistic and linear regression using R v2.7.1 software (http://www.R-project.org). All statistical analyses were performed using PLINK v1.04[32] software except when specified.

Results

We recruited and phenotyped 281 male longevity "cases" (mean age 95 years) and 195 male "controls" (mean age 33 years) as part of the SICS. Subsequently, we genotyped these subjects (Table 1) using the Illumina 300k SNPs mapping BeadChip. After a preliminary phase of quality control (see Methods section), we looked for evidence of population heterogeneity on a set of 258 males centenarians (cases) and 178 males controls by applying PCA as implemented in the EigenSoft 2.0 package.[24] The estimation of the over-dispersion of trend test statistics (λ) was 1.05, decreasing to 1.027 after correction for the first four significant principal components. These results indicates a small or null confounding effect of population structure on our association results (Fig. 1)

To test for associations between SNPs and the longevity phenotype, we compared allele and genotype frequencies between cases and controls by means of allelic and genotypic association tests (Table 2, Supplemental Table 1). Results from all analyses were very similar for significantly associated SNPs whether or not adjustments for population structure were applied (Supplemental Table 2). None of the analyzed SNPs showed deviations from HWE (p-HWD < 0.05) or significant differences in missing data fractions between cases and controls ($p < 0.05$). All SNPs showed a genotyping call rate >98.8%.

We also performed logistic regression analyses assuming an additive model and by comparing homozygous minor versus homozygous major alleles on a set of SNPs located at the *FOXO3A* locus (Table 2). To confirm the null influence

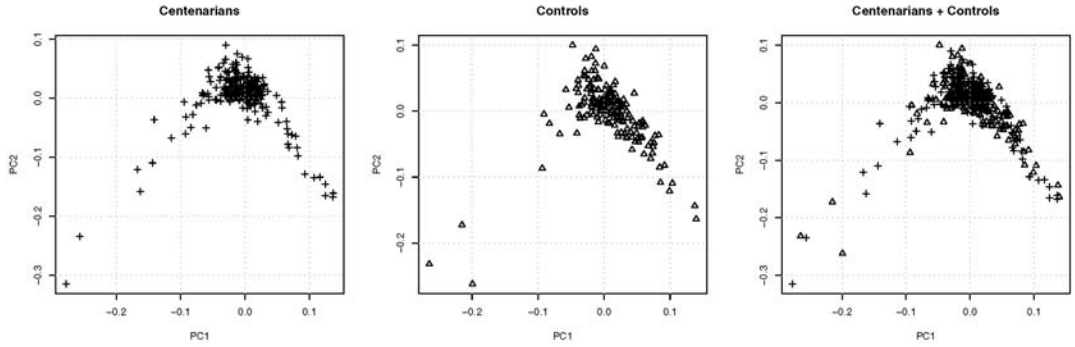

FIG. 1. Population structure of centenarians (+) and control subjects (Δ). Each scatterplot shows the first two principal components that were estimated using genotype data for more than 300K SNPs in centenarians and control subjects using the program EigenSoft 2.0. The two populations are ethnically identical, as shown in the rightmost scatter plot.

of population structure on our results, we also performed association tests correcting along the top four principal components by means of logistic regression assuming an additive effect of allele dosage on the same SNP set. We first focused on rs2802288, showing linkage disequilibrium (LD) with rs2802292 ($r^2 = 1$) in the HapMap CEU data panel.[33] Particularly, allelic association tests highlighted significant association for rs2802288 in males (MAF = 0.49; $p = 0.003$; OR = 1.51; 95% CI, 1.15–1.98). Moreover, this association was also confirmed by logistic regression under an additive model ($p = 0.0026$; OR = 1.53; 95% CI, 1.16–2.03) and by comparing homozygous minor versus homozygous major alleles ($p = 0.0023$; OR = 2.44; 95% CI, 1.37–4.34) (Table 2).

Successively, we imputed missing SNPs by means of Impute[25] software to represent the 6q21 region better. Interestingly, the imputed rs2802292 (MAF = 0.47) that was previously indicated by Willcox et al.[22] as the best association, showed $r^2 = 0.79$ with rs2802288 (MAF = 0.49), similar to that observed in the HapMap CEU panel.[33] In fact, our imputation analysis confirmed that rs2802292, unrepresented on the BeadChip, was responsible for the strongest association effect in this region (MAF = 0.468; $p = 0.0028$; OR, 1.53; CI 95%, 1.16–2.03, allelic association test). Additionally, we confirmed this significant association by logistic regression under additive model ($p = 0.0022$; OR, 1.57; CI 95%, 1.17–2.10) and by comparing homozygous minor versus homozygous major alleles ($p = 0.0019$; OR, 2.58; CI 95%, 1.42–4.70) (Table 2).

To evaluate a possible joint effect of the *FOXO3A* gene and the environmental exposure (such as smoke, etc.) or *FOXO3A* and the nongenetic attributes (such as clinical-cognitive variables), we performed a covariates analysis. No significant association of genotypes with cognitive variables (Barthel score, cognitive score, anxiety, depression) or clinical phenotypes (diabetes mellitus, glaucoma, high blood pressure, kidney diseases, osteoporosis, smoking history, cancer) was detected. As a result, we could hypothesize that *FOXO3A* has an effect on longevity independently of the tested variables. Subsequently, we generated a LD plot with the software HaploView v4.0.[30] LD blocks are delineated by black lines in Fig. 2 and defined using the Four Gamete Rule, a variant on the algorithm described in previously published study,[31] as implemented in HaploView software.[34]

Using this analysis, multimarker association tests showed a slightly significant association ($p = 0.03$) for a 24kb haplotype (Fig. 2, block 1; Table 3) comprising three SNPs carrying the minor allele (A) for rs2802288, and defining boundaries of a locus affected by a potential causative mutation for the extreme longevity phenotype. As can be observed in Fig. 2, the LD plot defines a locus that includes the 5′-untranslated regions and the first coding region of the investigated gene. Subsequently, to confirm the gender-specificity of the *FOXO3A* association, we also studied 199 female longevity "cases" (mean age 98 years) and 140 female "controls" (mean age 31 years) drawn from the same population (88% power to detect the association). No differences in terms of allele frequencies between centenarians and controls were detected for rs2802288 in female individuals (176 centenarians and 113 controls that pass the quality control filtering criteria) (MAF = 0.48; $p = 0.3975$; OR = 0.86; 95% CI, 0.62–1.21) (Table 2). The absence of association in the female gender has been further confirmed by logistic regression assuming an additive model ($p = 0.4034$; OR = 0.87; 95% CI, 0.62–1.21) and by comparing homozygous minor *versus* homozygous major alleles ($p = 0.4048$; OR = 0.75; 95% CI, 0.39–1.47) (Table 2).

Furthermore, we undertook a meta-analysis to explore the significance of the rs2802292 association with longevity by combining the association results of the current study and the findings coming from the Willcox et al. investigation. We combined the logarithm of the OR estimates and 95% CI for homozygous minor versus homozygous major alleles from the current analysis (OR = 2.44; 95% CI, 1.37–4.34) and from the Willcox et al. study (OR = 2.75; 95% CI, 1.51–5.02) to obtain a summary OR using fixed[26] and Der Simonian and Laird random effects models.[27] Fixed effects models assume that the effect of a risk allele has the same value in each dataset whereas random effects models assume that the risk allele effects for each study vary around some overall average effect.[35] The inconsistency metric I^2,[28] was 0%, while the Q statistic[29] was not statistically significant ($p = 0.88$), indicating a null between-study heterogeneity evidence. Because no evidence of between-study heterogeneity has been proven, fixed effects[26] and Der Simonian and Laird random effects[27] coincide, generating identical summary point estimates (OR = 2.66; 95% CI, 1.74–4.07; $p = 0.0001$) (Fig. 3).

Table 2. *FOXO3A* Locus in Long-Living Individuals and Controls

		MAF			*Allelic*		*Additive*		*Homozygous*	
SNP	*Gender*	*Overall*	*Cases*	*Controls*	*OR (95% CI)*	*p*	*OR (95% CI)*	*p*	*OR (95% CI)*	*p*
rs9486902	M	0.2	0.22	0.18	1.27(0.90–1.80)	0.1659	1.30 (0.91–1.85)	0.1524	0.92 (0.30–2.80)	0.8786
	F	0.23	0.21	0.27	0.69 (0.47–1.02)	0.0634	0.67 (0.45–1.01)	0.0553	0.52 (0.16–1.70)	0.2800
rs2802288	M	0.49	0.53	0.43	1.51 (1.15–1.98)	0.003	1.53 (1.16–2.03)	0.0026	2.44 (1.38–4.34)	0.0023
	F	0.48	0.47	0.5	0.86 (0.62–1.21)	0.3975	0.87 (0.62–1.21)	0.4034	0.75 (0.39–1.47)	0.4048
rs10499051	M	0.12	0.14	0.1	1.57 (1.02–2.41)	0.04	1.56 (1.01–2.41)	0.0409	NA	0.9982
	F	0.12	0.11	0.12	0.95 (0.57–1.60)	0.8497	0.95 (0.56–1.61)	0.8474	0.32 (0.03–3.62)	0.3598
rs2802292[a,b]	M	0.47	0.51	0.41	1.53 (1.16–2.03)	0.0028	1.57 (1.17–2.10)	0.0022	2.58 (1.42–4.70)	0.0019
	F	NA	NA	NA	NA	NA	NA	NA	NA	NA
rs13220810	M	0.2	0.18	0.22	0.79 (0.563–1.11)	0.1682	0.79 (0.56–1.10)	0.1653	0.80 (0.29–2.20)	0.6619
	F	0.22	0.23	0.2	1.17 (0.78–1.77)	0.4482	1.17 (0.78–1.78)	0.4471	1.12 (0.35–3.57)	0.8441
rs2764264[b]	M	0.4	0.43	0.36	1.37 (1.04–1.81)	0.0267	1.40 (1.05–1.86)	0.0227	1.99 (1.07–3.70)	0.0285
	F	0.41	0.4	0.42	0.93 (0.66–1.30)	0.6575	0.92 (0.65–1.31)	0.6553	1.03 (0.49–2.18)	0.9312
rs7341233	M	0.17	0.17	0.15	1.16 (0.80–1.67)	0.4364	1.15 (0.80–1.64)	0.4512	2.14 (0.67–6.79)	0.1963
	F	0.14	0.16	0.12	1.51 (0.92–2.48)	0.1047	1.51 (0.92–2.49)	0.1070	3.61 (0.41–31.41)	0.2454
rs17598747	M	0.15	0.15	0.15	1.05 (0.71–1.53)	0.8172	1.04 (0.72–1.50)	0.8237	1.72 (0.53–5.60)	0.3683
	F	0.12	0.13	0.1	1.44 (0.84–2.46)	0.1825	1.43 (0.84–2.43)	0.1911	2.79 (0.31–25.35)	0.3626
rs9285397	M	0.22	0.24	0.2	1.29 (0.93–1.80)	0.1274	1.34 (0.94–1.90)	0.1054	1.76 (0.53–5.88)	0.3561
	F	0.26	0.24	0.31	0.71 (0.49–1.03)	0.0699	0.69 (0.47–1.02)	0.0637	0.71 (0.26–1.99)	0.5190
rs12202209	M	0.15	0.15	0.15	1.07 (0.73–1.56)	0.739	1.06 (0.74–1.53)	0.7479	1.74 (0.53–5.60)	0.359
	F	0.12	0.13	0.1	1.44 (0.84–2.46)	0.1825	1.43 (0.84–2.43)	0.1911	2.79 (0.31–25.35)	0.3626
rs9480866	M	0.21	0.22	0.19	1.24 (0.87 1.75)	0.206	1.27 (0.89–1.80)	0.1881	1.71 (0.52–5.71)	0.3781
	F	0.24	0.21	0.29	0.67 (0.46–0.99)	0.0412	0.65 (0.44–0.97)	0.0370	0.51 (0.17–1.53)	0.2279
rs12207868	M	0.15	0.15	0.15	1.06 (0.72 1.54)	0.7751	1.05 (0.73–1.52)	0.7829	1.72 (0.53–5.60)	0.3683
	F	0.12	0.13	0.1	1.43 (0.84–2.44)	0.1905	1.42 (0.83–2.41)	0.1992	2.77 (0.30–25.16)	0.3662
rs13217795[a,b]	M	0.35	0.38	0.3	1.42 (1.06–1.90)	0.0176	1.44 (1.07–1.945)	0.0159	2.30 (1.14–4.64)	0.0196
	F	NA	NA	NA	NA	NA	NA	NA	NA	NA
rs2153960	M	0.38	0.41	0.34	1.31 (0.99 1.74)	0.0578	1.33 (1–1.78)	0.0521	1.87 (0.99–3.53)	0.0542
	F	0.4	0.38	0.42	0.83 (0.59–1.18)	0.3009	0.83 (0.59–1.18)	0.2979	0.83 (0.39–1.73)	0.6107
rs3800229	M	0.36	0.38	0.33	1.25 (0.94 1.67)	0.1187	1.26 (0.94–1.68)	0.1162	1.65 (0.87–3.12)	0.1222
	F	0.38	0.36	0.41	0.81 (0.57–1.14)	0.2285	0.81 (0.57–1.15)	0.2310	0.75 (0.36–1.58)	0.4478
rs1935949	M	0.36	0.38	0.33	1.25 (0.94 1.67)	0.118	1.26 (0.95–1.68)	0.1143	1.65 (0.87–3.12)	0.1222
	F	0.38	0.36	0.41	0.81 (0.57–1.14)	0.2200	0.81 (0.57–1.14)	0.2222	0.77 (0.37–1.62)	0.4911

[a]Imputed SNPs.
[b]SNPs explored by Willcox et al.
Gender, Gender-specific analysis (M, males;F, females); MAF overall, minor allele frequency based on whole sample; MAF cases, minor allele frequency in centenarians; MAF controls, minor allele frequency in the control population; OR, odds ratio; 95% CI, 95% confidence intervals; *p*, *p* value, respectively, for allelic association test (allelic), logistic regression under additive model (additive), and logistic regression comparing homozygous minor (mm) versus homozygous major alleles (MM) (homozygous); NA, not available.

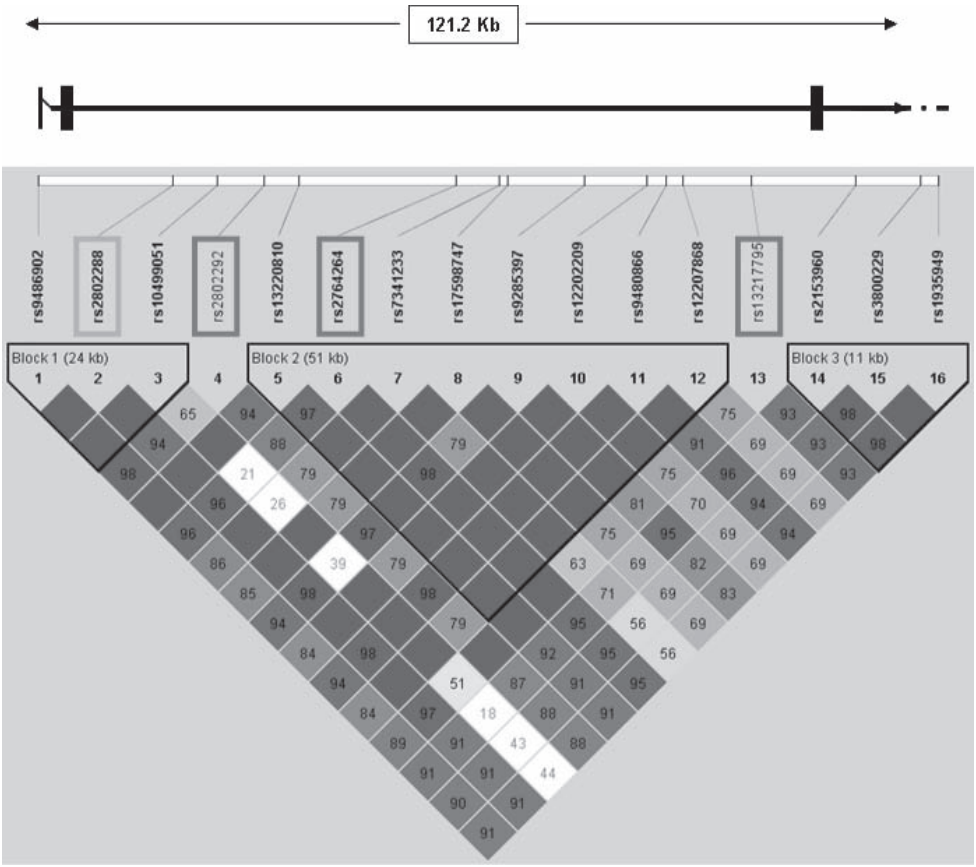

FIG. 2. Pattern of LD of *FOXO3A* region displayed using D′ with HaploView. LD displays were generated using the D′ color scheme. The different shades of gray indicate different D′ values ($0 < D' < 1$). Haplotype blocks were generated using the Four Gamete Rule as implemented in HaploView software. Boxed in light gray is rs2802288, boxed in dark gray are the SNPs explored by Willcox et al.

Discussion

Research into aging has been rejuvenated by the recent discovery of SNPs in candidate genes that can extend the lifespan of laboratory model organisms. Thus, these polymorphisms can also delay, ameliorate, or even abolish the impact of many aging-related diseases, including cardiovascular disease, neurodegeneration, and cancer. *FOXO* transcription factors have been implicated in regulating different cellular functions, such as differentiation, metabolism, proliferation, and survival.[11,36,37]

In this respect, in the Leiden Prospective Study the overall and individual haplotype frequencies were not different between the elderly and young control group for the *FOXO3A* locus.[21] However, possible limitations of the study are in the absence of gender stratification. In fact, due to the profound effect of this pathway on the reproductive/hormonal system, which differs between males and females, it

Table 3. Haplotype Association Test of Block 1 Using HaploView

Block 1	*Gender*	*Frequency*	*Frequency for cases*	*Frequency for controls*	p *value*
CGA	M	0.51	0.48	0.57	0.003
	F	0.51	0.53	0.49	0.3683
TAA	M	0.2	0.21	0.17	0.1588
	F	0.23	0.21	0.27	0.0634
CAA	M	0.17	0.18	0.16	0.4968
	F	0.13	0.15	0.11	0.2556
CAG	M	0.12	0.14	0.09	0.0381
	F	0.12	0.11	0.12	0.8689

Block 1, haplotypes in block 1, Gender, gender-specific analysis (M = males, F = females); Frequency, haplytype frequency based on whole sample; Frequency for cases, haplotype frequency in centenarians, Frequency for controls, haplotype frequency in the control population; *p*-vaule, haplotypic association test *p*-value.

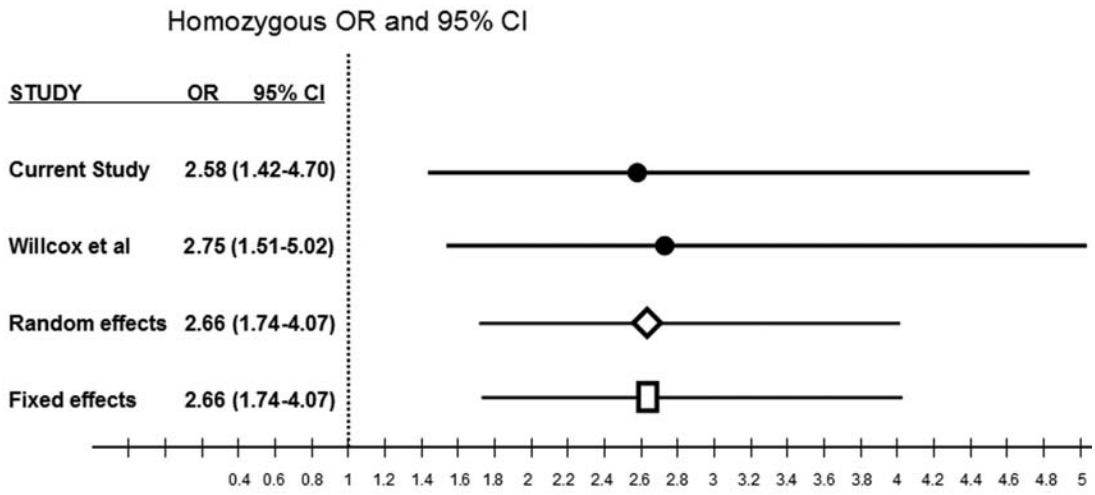

FIG. 3. Meta-analysis of rs2802292 variant in the current investigation and in Willcox et al. study. Each study is represented by the estimate of the homozygous minor versus homozygous major alleles OR and its 95% CI (circle). Summary effects by random effects (diamond) and fixed effects (rectangle) calculations are also shown.

is possible that variants that affect genes of this pathway are gender-specific enriched/depleted as the populations age. Hence, variants that influence longevity in males may not affect female longevity and vice versa.

Recently, Willcox and colleagues demonstrated an association between exceptional longevity and the *FOXO3A* gene in an ethnic Japanese population in Hawaii, focusing on male individuals only.[22] Although there was no population stratification effect observed in this study, it is a possible limitation in most studies. Stratification is the result of imbalance of ethnic background between cases and controls. It is usually modest when the recruitment is consistent among cases and controls, and for populations that did not experience recent immigration. In the present study, we extrapolated the *FOXO3A* locus from our genome-wide association data on a part of the SICS. Particularly, we genotyped (with BeadChip 300k, Illumina) 281 SIC male nonagenarians and 195 SIC male controls. It was encouraging to report the association between rs2802292 at the *FOXO3A* locus and the longevity phenotype was convincingly and independently confirmed in our SICS homozygous minor versus homozygous major alleles ($p = 0.0019$; OR = 2.58; CI 95%, 1.42–4.70). Moreover, to avoid the possible drawback of population structure, we demonstrated a small or null confounding effect of population structure on our association results, thus excluding type I errors (Fig. 1, Supplemental Table 2). Our aim was to replicate rs2802292 in male longevity and to explore the *FOXO3A* locus in males and females to generate eventual new candidate SNPs for longevity and to clarify the gender interaction on the *FOXO3A* locus and longevity. Replication efforts require adequate sample size and our male population was sufficient to achieve >90% power to detect an allele with 40% allele frequency and a factor of 1.5 effect, as previously reported in Willcox paper for rs2802292.[22]

Meta-analysis represents a well-established method for summarizing results and drawing conclusions from different studies for a set of common hypotheses. Therefore, we applied this approach to combine our association results for the SNP rs2802292 with the findings coming from Willcox et al. study and to achieve more statistical power. The null-significance of the Q-statistics[29] ($p = 0.88$) and the inconsistency metric[28] value ($I^2 = 0\%$) confirmed the absence of between-study heterogeneity. In absence of heterogeneity between the two datasets, we obtained identical estimates of the ORs and the 95% CIs for homozygous minor versus homozygous major alleles from both fixed[26] and random effects[27] models (OR = 2.66; 95% CI, 1.74–4.07; $p = 0.0001$) (Fig. 3).

This is the first convincing replication of an association with longevity after the *APOE* association.[5] As stressed by National Cancer Institute–National Human Genome Research Institute (NCI-NHGRI) Working Group on Replication in Association Studies,[39] validation of the postulated gene-investigated phenotype association in a population different from that of the previous study is of great value.

It is also established that mitochondrial Sirtuin 3 interacts with, and under specific cellular conditions regulates, the activity of *FOXO3A*. Additionally, SIRT3 regulates a series of essential intracellular processes that defend the cell against multiple types of cellular damage, including oxidative damage.[40] A rise in the level of reactive oxygen species (ROS) has two important effects: It can damage proteins, lipids, and DNA, leading to cell death or it can trigger the activation of specific physiologic signaling pathway.[41] Moreover, a GH-IGF1 pathway has been implicated in determination of longevity in a variety of species.[18,19,42] Genetic variants that are either positively or negatively selected as population ages impact on survival (demographic selection). In this regard, the *FOXO3A* gene SNP rs2802292 should have a protective role.

These observations imply that the *FOXO3A* gene SNP rs2802292, or SNPs in LD, have a protective role by partially increasing the ability of other proteins to activate *FOXO3A*, or by increasing *FOXO3A* activity on downstream targets. Resequencing of the *FOXO3A* locus will clarify the nature of the genetic variation that is tracked by rs2802292. It is possible that the variation does not need to affect coding genes, because coding genes are less than one-third of the evolutionary conserved genome.[38] Furthermore, future population studies on diseases of aging will clarify if *FOXO3A* associates across diseases, like the longevity gene *APOE* and Alzheimer and cardiovascular diseases. From the data that we obtained in our population,

the association is strictly linked with the male gender. Taking in consideration this new evidence, we would suggest a gender-specific analysis for the longevity phenotype. To conclude, the discovery and replication of a convincing association of *FOXO3A* locus contributes to the hypothesis that the impact of the IGF-I–insulin pathway on longevity is a property that has been evolutionarily conserved throughout the animal kingdom.

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