



The FoxO3 gene and cause-specific mortality

Bradley J. Willcox,^{1,2} Gregory J. Tranah,³ Randi Chen,¹
 Brian J. Morris,^{1,2,4} Kamal H. Masaki,^{1,2} Qimei He,¹
 D. Craig Willcox,^{1,2,5} Richard C. Allsopp,⁶ Stefan Moisyadi,⁶
 Leonard W. Poon,⁷ Beatriz Rodriguez,^{1,2} Anne B. Newman,⁸
 Tamara B. Harris,⁹ Steven R. Cummings,³ Yongmei Liu,¹⁰
 Neeta Parimi,³ Daniel S. Evans,³ Phil Davy,⁶
 Mariana Gerschenson¹¹ and Timothy A. Donlon¹

¹Department of Research, Kuakini Medical Center, Honolulu, HI 96817, USA

²Department of Geriatric Medicine, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96817, USA

³California Pacific Medical Center Research Institute, San Francisco, CA 94107, USA

⁴School of Medical Sciences and Bosch Institute, University of Sydney, Sydney, NSW 2006, Australia

⁵Department of Human Welfare, Okinawa International University, Ginowan, Okinawa 901-2701, Japan

⁶Institute for Biogenesis Research, University of Hawaii, Honolulu, HI 96813, USA

⁷Institute of Gerontology, University of Georgia, Athens, GA 30602, USA

⁸Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA 15261, USA

⁹Laboratory of Neurogenetics, Intramural Research Program, National Institute on Aging, Bethesda, MD 20892, USA

¹⁰Division of Public Health Sciences, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

¹¹Department of Cell & Molecular Biology, University of Hawaii, Honolulu, HI 96813, USA

Summary

The G allele of the FOXO3 single nucleotide polymorphism (SNP) rs2802292 exhibits a consistently replicated genetic association with longevity in multiple populations worldwide. The aims of this study were to quantify the mortality risk for the longevity-associated genotype and to discover the particular cause(s) of death associated with this allele in older Americans of diverse ancestry. It involved a 17-year prospective cohort study of 3584 older American men of Japanese ancestry from the Honolulu Heart Program cohort, followed by a 17-year prospective replication study of 1595 white and 1056 black elderly individuals from the Health Aging and Body Composition cohort. The relation between FOXO3 genotype and cause-specific mortality was ascertained for major causes of death including coronary heart disease (CHD), cancer, and stroke. Age-adjusted and multivariable Cox proportional hazards models were used to compute hazard ratios (HRs) for all-cause and cause-specific mortality. We found G allele carriers had a combined (Japanese, white, and black populations) risk reduction of 10% for total (all-cause) mortality (HR = 0.90; 95% CI, 0.84–0.95; P = 0.001). This effect size was consistent across populations and mostly contributed by 26% lower risk for CHD death (HR = 0.74; 95% CI, 0.64–0.86; P = 0.00004). No other causes of death made a significant contribution to the survival advantage for G allele carriers. In conclusion, at older age, there is a large risk reduction in mortality for G allele carriers, mostly due to lower CHD mortality. The findings support further research on FOXO3 and FoxO3 protein as potential targets for therapeutic intervention in aging-related diseases, particularly cardiovascular disease.

Key words: FOXO3; heart disease; longevity; mortality.

Introduction

The aging of the world's population has enormous implications for society, social insurance programs, and health care (National Institute on Aging, 2007; US Department of Health and Human Services, 2012). Thus, understanding how humans can age more healthfully is of vital importance. Genetic factors that affect the rate of aging and/or risk for age-related diseases are important, as they account for approximately one-third of the variability in human lifespan (Brooks-Wilson, 2013; Shadyab & LaCroix, 2015). However, despite almost three decades since the first finding of longevity-associated genotypes in long-lived persons (Takata *et al.*, 1987), the effects on longevity of only the apolipoprotein E gene (APOE) and the forkhead box O-3 (FoxO3) gene (FOXO3) have been consistently replicated across multiple, diverse human populations (Flachsbart *et al.*, 2009; Pawlikowska *et al.*, 2009; Murabito *et al.*, 2012; Brooks-Wilson, 2013; Morris *et al.*, 2015; Shadyab & LaCroix, 2015).

FoxO3 is an evolutionarily conserved transcription factor in the insulin signaling pathway (Kenyon, 2010). It regulates expression of genes controlling a multitude of processes that could enhance health and lifespan (Willcox *et al.*, 2008; Kenyon, 2010; Eijkelenboom & Burgering, 2013). A previous study of American men of Japanese ancestry was the first to find an association of three single nucleotide polymorphisms (SNPs) of FOXO3 with human longevity, the strongest SNP being rs2802292 (Willcox *et al.*, 2008). The association was replicated in 11 other independent studies of populations of diverse ancestry (Bao *et al.*, 2014). A meta-analysis found these and two other FOXO3 SNPs retained statistical significance, the strongest association with longevity involving the original G allele of rs2802292 in men (odds ratio (OR), 1.54; 95% confidence intervals (CIs), 1.33–1.67) (Bao *et al.*, 2014). A meta-analysis of genomewide association studies of longevity yielded an OR of 1.17 ($P = 1.9 \times 10^{-10}$) for this allele (Broer *et al.*, 2015). The effect size has not been well quantified in terms of mortality risk over time. The mechanisms by which the protective allele(s) reduce mortality to promote human longevity are also not known. Identifying the cause of death in longevity-allele carriers vs. noncarriers may provide clues as to why FOXO3 SNPs strongly protect against mortality.

We hypothesized that the longevity-associated FOXO3 genotype would be associated with a sizable risk reduction for mortality and with one or more major age-associated clinical causes of death, such as coronary heart disease (CHD), cancer, and stroke. To test this hypothesis we utilized an extensive, prospectively collected dataset from our long-lived cohort of American men of Japanese ancestry, well characterized for aging phenotypes, drawn from the Honolulu Heart Program (HHP) prospective cohort study (Kagan, 1996). We genotyped this study population to prospectively assess the following: (i) the effect size of the

Correspondence

Bradley J. Willcox, MD, Kuakini Medical Center, 347 North Kuakini Street, HPM-9, Honolulu, Hawaii 96817, USA. Tel.: 808 523 8461; fax: 808 528 1899; e-mail: willcox@hawaii.edu

Accepted for publication 26 December 2015

protective (longevity-associated) *FOXO3* genotype on total (all-cause) mortality in 17 years of follow-up; and (ii) the effect of the protective *FOXO3* genotype on cause-specific mortality. We then attempted a replication of major findings in a suitable cohort of elderly white and black Americans of both sexes in the Health Aging and Body Composition (ABC) cohort study, which had 17 years of follow-up.

Results

Study participants

The baseline characteristics of each cohort of subjects (Japanese, white, and black) are shown in Table 1. HHP study participants tended to be older and had higher prevalence of hypertension, diabetes, and CHD than Health ABC participants. There was a higher percentage of G allele carriers in the Health ABC population, particularly in blacks, in whom the G allele was the common allele.

Meta-analysis of mortality (all-cause)

Cox proportional hazards models were used to generate hazard ratios (HRs) over a 17-year prospective follow-up for black (Health ABC), white (Health ABC), and Japanese (HHP) ancestry. Tests for heterogeneity for all populations combined yielded $P = 0.96$, demonstrating that the populations could be used for a combined analysis, utilizing a dominant model. During follow-up, mortality risk for G allele carriers ranged from 0.87 (blacks) to 0.91 (whites) with a combined (Japanese, white, and black populations) risk reduction of 10% for total mortality (HR = 0.90; 95% CI, 0.84–0.95; $P = 0.001$) (Fig. 1). Tests of model proportionality were met.

Table 1 Characteristics of the three study cohorts of older Americans

Parameter	Japanese (<i>n</i> = 3584)	Whites (<i>n</i> = 1794)	Blacks (<i>n</i> = 1281)
Age (years)	77.7 ± 4.6	73.8 ± 2.9	73.4 ± 2.9
Body mass index (kg m ⁻²)	23.5 ± 3.2	26.5 ± 4.1	28.6 ± 5.4
History of ever smoking (%)	62.4	56.8	55.2
Alcohol (3+ drinks per week) (%)	40.3	23.3	9.6
Exercise regularly (%)	68.3	49.7	31.2
Glucose (g dL ⁻¹)	113 ± 29.5	100.8 ± 27.3	109.3 ± 41.8
Total cholesterol (mg dL ⁻¹)	189.8 ± 33.1	201.3 ± 37.4	204.9 ± 40.1
CHD (%)*	20.6	13.1	13.7
Stroke (%)*	4.5	1.4	3.7
Cancer (%)*	15.6	24.5	11.0
Diabetes (%)*	28.6	10.7	21.0
Hypertension (%)*, †	73.6	48.7	62.9
<i>FOXO3</i> G allele frequency (%)	27.7	36.5	73.2
<i>FOXO3</i> G allele carrier (%) [‡]	47.0	58.4	92.7

Americans of Japanese ancestry tended to be older, had more prevalent CHD and diabetes, and lower frequency of the longevity-associated *FOXO3* G allele at the baseline examination.

*Prevalent disease (not age-adjusted) was defined and detected based on respective study surveillance programs: CHD: any myocardial infarction, bypass surgery, angioplasty, other heart surgery, or ECG evidence; Diabetes: fasting glucose ≥ 126 mg mL⁻¹ or 2-h glucose ≥ 200 mg mL⁻¹, or on diabetes medication.

†Participants were considered to have hypertension if they had a systolic blood pressure of ≥ 140 mmHg and/or diastolic blood pressure of ≥ 90 mmHg or were on antihypertensive medication.

‡Carriers were persons who had at least one G allele of *FOXO3* SNP rs2802292. Shown are homozygotes and heterozygotes combined.

Total (all-cause) mortality risk for carriers of the *FOXO3* G allele

In the HHP cohort, risk of death over a 17-year prospective follow-up period for carriers (vs. noncarriers as baseline risk group) of the longevity-associated G allele of *FOXO3* (rs2802292) in Japanese American men, utilizing a multivariable Cox regression model, demonstrated an 11% risk reduction (HR 0.89; 95% CI: 0.83–0.97; $P = 0.004$) for all-cause mortality (Table 2A). Five important risk factors for mortality in older persons did not substantially alter mortality risk, suggesting that they are not in the causative pathway between *FOXO3* variation and mortality in older Japanese males.

In the Health ABC cohort, a similar analysis of risk of death over 17 years of follow-up demonstrated a similarly large (9%) risk reduction for all-cause mortality in whites (HR 0.91; 95% CI: 0.80–1.02; $P = 0.06$) and 13% in blacks (HR 0.87; 95% CI: 0.66–1.13; $P = 0.29$), respectively (Table 2B). When entering five important mortality risk factors for mortality at older ages into the model, there was a reduction in statistical significance and effect size (HR) in whites and blacks, suggesting that one or more risk factor variables are in the causative pathway between *FOXO3* variation and mortality in these populations.

Specific causes of death and HR for mortality (age-adjusted)

In the HHP cohort, the G allele conferred a highly statistically significant protective effect against CHD mortality in Japanese American men (Table 3A). Hazard ratio in G (longevity allele) carriers (HR: 0.75; 95% CI 0.63–0.90; $P = 0.001$) was 25% lower than noncarriers.

In the Health ABC cohort, there was a similarly large (24%) risk reduction in CHD mortality for whites (HR: 0.76; 95% CI 0.58–0.98; $P = 0.036$) and even larger for blacks (39%; HR 0.61; 95% CI 0.35–1.04; $P = 0.068$ for trend; Table 3B). While certain other diseases (such as cancer in whites and stroke in blacks) showed lower HRs for mortality, none reached statistical significance.

Meta-analysis of CHD mortality

Cox proportional hazards models were used to generate hazard ratios over the 17-year prospective follow-up period for individuals of black (Health ABC), white (Health ABC), and Japanese (HHP) ancestry. Tests for heterogeneity for all populations combined yielded $P = 0.75$, demonstrating that the populations could be used for a combined analysis, utilizing a dominant model. During follow-up, CHD mortality risk for G allele carriers ranged from 0.61 (blacks) to 0.76 (whites) with a combined (Japanese, white, and black populations) risk reduction of 26% for total CHD mortality (HR = 0.74; 95% CI, 0.64–0.86; $P = 0.00004$) (Fig. 2). Tests of model proportionality were met.

FOXO3 genotype and age at death

Carrier status for the protective G allele increased from age in the 70s to age of ≥ 90 years, consistent with protection against mortality for carriers in the three populations (Fig. 3).

Serum TNF-α level by *FOXO3* G allele carrier status

In a pilot analysis of HHP Japanese men, carriers of the longevity-associated G allele of *FOXO3* SNP rs2802292 had lower blood levels of the inflammatory cytokine TNF-α (Table S1).

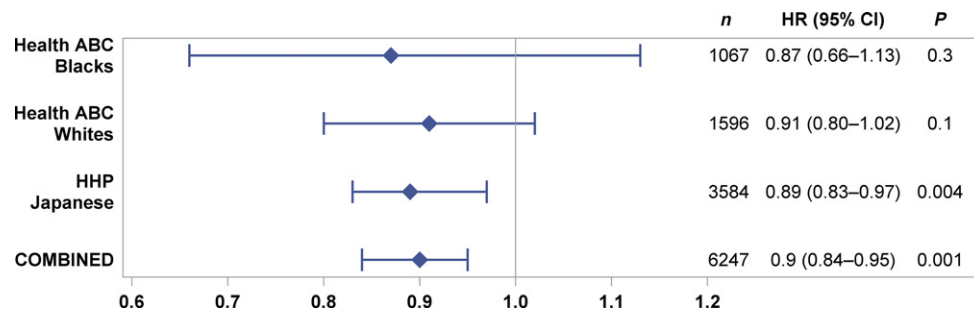


Fig. 1 Meta-analysis of all-cause mortality for *G* allele carriers. Hazard ratios (HRs) for mortality over prospective follow-up periods of 17 years for the cohorts of Americans of black (Health ABC) and white (Health ABC) individuals and 17 years for Americans of Japanese (Honolulu Heart Program (HHP)) ancestry, utilizing a dominant model. Tests for heterogeneity for all populations combined were $P = 0.96$, demonstrating that the populations are appropriate for a combined meta-analysis. During the 17-year prospective follow-up, *G* allele carriers (*FOXO3* SNP *rs2802292*) had HR that ranged from 0.87 (blacks) to 0.91 (whites) and a combined risk reduction of 10% for total mortality (HR = 0.90; 95% CI, 0.84–0.95; $P = 0.001$).

Table 2 All-cause mortality risk for carriers of the *FOXO3* *G* allele in multivariable risk factor model. (A) Honolulu Heart Program cohort. (B) Health ABC cohort

(A) Risk factor	HR (95% CI)		P	
Age (years)	1.11 (1.10–1.12)		< 0.001	
Fasting glucose (per 10 mg dL ⁻¹)	1.06 (1.05–1.08)		< 0.001	
Smoking history* (per 10 pack-years)	1.04 (1.03–1.05)		< 0.001	
Systolic blood pressure (per 10 mm Hg)	1.00 (0.98–1.02)		0.84	
Fasting total cholesterol (per 10 mg dL ⁻¹)	0.98 (0.97–0.99)		0.007	
Body mass index (per kg m ⁻²)	0.95 (0.94–0.97)		< 0.001	
<i>FOXO3</i> <i>G</i> allele carrier (age-adjusted)	0.89 (0.83–0.97)		0.004	
<i>FOXO3</i> <i>G</i> allele carrier [†] (age- and risk factor-adjusted)	0.86 (0.79–0.93)		< 0.001	

(B) Risk factor	Blacks		Whites	
	HR (95% CI)	P	HR (95% CI)	P
Age (years)	1.06 (1.03–1.09)	< 0.0001	1.12 (1.09–1.14)	< 0.0001
Fasting glucose (per 10 mg dL ⁻¹)	1.03 (1.02–1.05)	< 0.0001	1.06 (1.04–1.08)	< 0.0001
Smoking history (ever smoking)	1.26 (1.08–1.46)	0.003	1.37 (1.2–1.55)	< 0.0001
Systolic blood pressure (per 10 mmHg)	1.06 (1.02–1.1)	0.002	1.01 (0.98–1.05)	0.39
Fasting total cholesterol (per 10 mg dL ⁻¹)	0.97 (0.95–0.98)	0.0002	0.97 (0.96–0.99)	0.001
Body mass index (per kg m ⁻²)	0.97 (0.96–0.98)	< 0.0001	0.98 (0.97–1.00)	0.047
<i>FOXO3</i> <i>G</i> allele carrier (age-adjusted)	0.87 (0.66–1.13)	0.29	0.91 (0.91–1.02)	0.063
<i>FOXO3</i> <i>G</i> allele carrier [‡] (age- and risk factor-adjusted)	0.91 (0.69–1.2)	0.51	0.91 (0.81–1.03)	0.15

(A) Risk of death over a 17-year follow-up period for carriers vs. noncarriers of longevity-associated *G* allele of *FOXO3* (*rs2802292*) in HHP population of Japanese Americans.

(B) Risk of death over a 17-year follow-up period for carriers vs. noncarriers of longevity-associated *G* allele of *FOXO3* (*rs2802292*) in Health ABC cohorts.

*Number of packs smoked per day × number of years smoked (pack-years).

[†]Multivariable Cox regression model for subjects with complete risk factor data ($n = 3200$; total deaths = 2345). HR = hazard ratio for all-cause mortality.

[‡]Multivariable Cox regression model for subjects with complete risk factor data. HR = hazard ratio for all-cause mortality.

Discussion

In this, the first large, comprehensive, multiethnic prospective cohort study to quantify the risk reduction associated with the minor allele (*G*) of the *FOXO3* longevity-associated genotype, we demonstrated a large (10%) protective effect against all-cause mortality and 26% for CHD mortality over 17 years of follow-up. The protective effect was, moreover, observed in three genetically different populations. Our study contrasts with the vast majority of prior investigations of *FOXO3* variants and longevity, which have been case:control studies that did not quantify risk over time, but rather simply tested for association with an outcome.

The magnitude of the impact of an *absence* of the *FOXO3* *G* allele was comparable to the increase in risk of death from smoking a pack of cigarettes a day for 25 years in Japanese men. In black males and females, it was equivalent to having a 20 mmHg higher systolic blood pressure, and in white men and women to a 20 mg dL⁻¹ elevation in fasting blood glucose.

Of particular interest, in multivariable models of mortality risk factors, five of the top risk factors for mortality at older ages (i.e. smoking, hypertension, fasting glucose, low BMI, low cholesterol) had little influence on the effect on mortality of *FOXO3* genotype in Japanese men. In the Health ABC cohort of white and black men and women, there did appear to be mediator variables, in that the effect was partially

Table 3 Specific causes of death and hazard ratio, age-adjusted, for carriers of the *FOXO3* G allele. (A) Honolulu Heart Program (HHP) cohort. (B) Health ABC cohort

(A)									
Cause of death	No. of deaths			HR* (95% CI)			P-value		
Cancer	546			1.01 (0.85–1.19)			0.93		
CHD (coronary heart disease)	524			0.75 (0.63–0.90)			0.001		
Stroke	315			0.97 (0.77–1.21)			0.76		
Dementia	221			1.01 (0.78–1.32)			0.93		
Other cardiovascular disease (CVD)	213			0.85 (0.65–1.11)			0.23		
Infectious disease	188			0.91 (0.68–1.21)			0.52		
Chronic obstructive pulmonary disease (COPD)	117			0.83 (0.57–1.19)			0.31		
Renal failure	45			0.86 (0.48–1.55)			0.61		
GI (gastrointestinal disease)	39			1.22 (0.65–2.30)			0.54		
Other deaths	480			0.86 (0.72–1.03)			0.09		

(B)									
Cause of death*	Whites			Blacks			Combined		
	n	HR* (95% CI)	P	n	HR* (95% CI)	P	n	HR (95% CI)	P
Cancer	220	0.87 (0.67–1.14)	0.31	179	1.67 (0.82–3.41)	0.16	399	0.97 (0.76–1.24)	0.81
CHD	224	0.76 (0.58–0.98)	0.04	140	0.61 (0.35–1.04)	0.07	364	0.73 (0.57–0.92)	0.0088
Stroke	80	0.87 (0.56–1.35)	0.53	67	0.55 (0.26–1.15)	0.11	147	0.78 (0.53–1.15)	0.21
Other CVD	41	0.96 (0.52–1.8)	0.91	47	1.04 (0.32–3.38)	0.95	88	0.98 (0.57–1.7)	0.96
COPD	42	0.82 (0.44–1.5)	0.51	29	2.07 (0.28–15.3)	0.47	71	0.91 (0.52–1.6)	0.75
Infection	36	1.21 (0.62–2.4)	0.58	24	0.54 (0.16–1.84)	0.33	60	1.03 (0.56–1.91)	0.92
Renal failure	17	0.61 (0.24–1.59)	0.32	25	*N/A	N/A	42	1.01 (0.45–2.26)	0.98
GI	7	0.88 (0.2–3.94)	0.87	9	0.46 (0.05–3.85)	0.47	16	0.72 (0.2–2.59)	0.61
Other	260	1.11 (0.87–1.43)	0.40	167	0.69 (0.4–1.18)	0.18	427	1.02 (0.81–1.29)	0.84

*HR shown is for carriers of the G allele. (A) Number of deaths are all deaths in each disease category from $n = 2688$ total deaths in HHP subjects (mean age 77.7 ± 4.6 years at baseline) followed up for 17 years. Cause of death was coded according to the International Classification of Diseases (ICD) system (version 8) by an expert morbidity and mortality committee after comprehensive review of multiple data sources including death certificates, hospital discharge records and interviews with physicians, among other data. All the study participants were ascertained for vital status at the end of study; in only 17 (1.3%) deaths could a cause not be ascertained. There was a large (25%) risk reduction in CHD mortality. Bonferroni correction of CHD death yielded $P = 0.01$. (B) Number of deaths are all deaths in each disease category from $n = 1614$ total deaths in Health ABC subjects followed up for 17 years. Surveillance for cause of death was similar to HHP methodology. There was a large (24%) risk reduction in CHD mortality for whites (HR: 0.76; 95% CI 0.58–0.98; $P = 0.036$) and a 39% risk reduction for blacks (HR: 0.61; 95% CI 0.35–1.04; $P = 0.068$ for trend). White males and females were aged 73.8 ± 2.9 years at baseline. Black males and females were aged 73.4 ± 2.9 years at baseline. Bonferroni correction of CHD death yielded $P = 0.32$ for blacks and $P = 0.08$ for whites. CVD, cardiovascular disease; GI, gastrointestinal.

attenuated when risk variables were entered into a multivariable model. The data suggest a possible mediator role for hypertension, which we found to be less prevalent in middle-aged women carrying the *FOXO3* G allele (Morris et al., 2016).

Although we found a lower risk for stroke in all three populations, this did not reach statistical significance. The only prior studies by others of *FOXO3* genotype and cardiovascular disease led to conflicting results. One, a 4-year prospective cohort study of Dutch men and women aged ≥ 85 years found that a particular *FOXO3* haplotype conferred an increased risk for incident stroke and also an increased risk of mortality from CHD, stroke, and all-cause (total) mortality (Kuningas et al., 2007). Another investigation, a case:control study of Danish men and women aged ≥ 90 years, failed to find an association of *FOXO3* genotype with CHD or stroke (Soerensen et al., 2015). A case:control study of Han Chinese found no relation with CHD (Zhao et al., 2014), while two other case:control studies found *FOXO3* longevity-allele carriage was associated with lower CHD prevalence (Japanese American males) (Willcox et al., 2008) and with lower CHD and stroke mortality (white males and females) (Pawlikowska et al., 2009).

Although *FOXO3* is a tumor suppressor (Lam et al., 2013; Fruman & Rommel, 2014), we could find only one study that reported an

association of *FOXO3* genotype with all-cancer mortality (Pawlikowska et al., 2009) and another study in which a borderline association with cancer prevalence was found (Willcox et al., 2008). Both were case:control studies of longevity-associated alleles. The current study found no detectable protection against cancer mortality, although larger studies of particular cancer subtypes may be useful.

It should, however, be noted that the prior studies were limited by small sample sizes, short follow-up time, a case:control study design, monoethnic populations, and/or very old age of the study subjects. The current study did not suffer from these challenges. Therefore, while a contributory role for other diseases and disease processes cannot be ruled out, protection against CHD mortality seems paramount.

Based on studies of FoxO3 in model organisms we suspect immune dysregulation may be particularly important. In aging humans, adaptive immunity decreases, leading to immunosenescence, but innate immunity increases, leading to a pro-inflammatory phenotype termed 'inflamm-aging' (Franceschi et al., 2007; Peng, 2007; Salminen et al., 2008). FoxO3 inhibits the production of several inflammatory cytokines linked to human aging. These include interleukin-2 (Oh et al., 2011), interleukin-6 (Dejean et al., 2009), and TNF- α (Lee et al.,

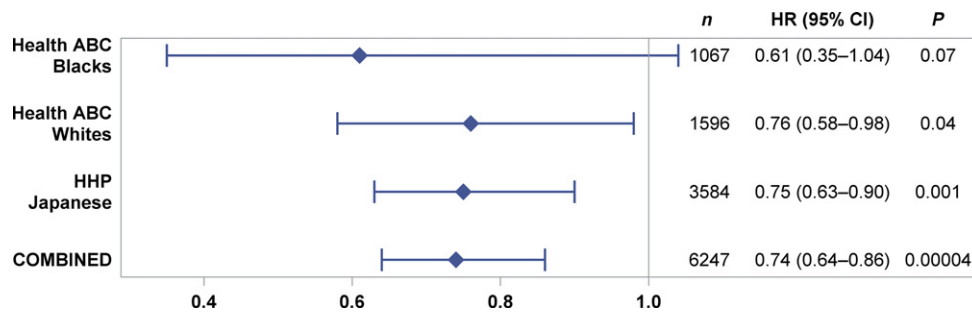


Fig. 2 Meta-analysis of CHD mortality. Hazard ratios (HRs) for CHD mortality over prospective follow-up periods of 17 years for the cohorts of Americans of black (Health ABC; $n = 140$ CHD deaths), white (Health ABC; $n = 224$ CHD deaths) and 17 years for Americans of Japanese (Honolulu Heart Program (HHP; $n = 524$ CHD deaths)) ancestry, utilizing a dominant model. Tests for heterogeneity for all populations combined yielded $P = 0.75$, demonstrating that the populations could be used for a combined analysis, utilizing a dominant model. CHD mortality risk for G allele carriers ranged from 0.61 (blacks) to 0.76 (whites) with a combined (Japanese, white and black populations) risk reduction of 26% for total mortality (HR = 0.74; 95% CI, 0.64–0.86; $P = 0.00004$). Tests of model proportionality were met.

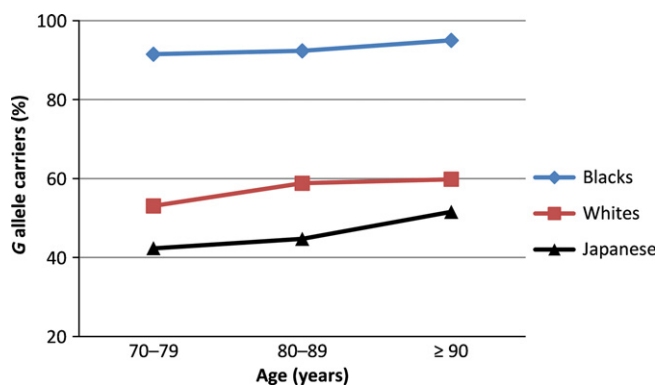


Fig. 3 *FOXO3* G allele carrier status (as % of cohort) by attained age. Percentage of carriers of the longevity-associated *FOXO3* G allele of SNP *rs2802292* increased with age in the pooled population ($P < 0.0001$; logistic regression; adjusted for race).

2013), each of which has been linked to both aging and cardiovascular disease.

In support of this premise, the minor allele of *FOXO3* SNP *rs12212067* (which is in linkage disequilibrium with *rs2802292* and could act as a proxy) was associated with a milder clinical course of seemingly unrelated inflammatory conditions (Crohn's disease and rheumatoid arthritis) (Lee *et al.*, 2013). Minor allele carriage limited inflammatory responses in monocytes through TGF- β 1-induced reduction of pro-inflammatory cytokines, including TNF- α . It also increased production of anti-inflammatory cytokines. Our finding of lower TNF- α in *FOXO3* G allele carriers is consistent with protection against inflammation and thence CHD. More work is needed to confirm a major influence of reduced inflammation conferred by *FOXO3* genotype in protection against CHD, as an explanation for its ability to confer increased lifespan.

Interestingly, the G allele is the ancestral allele as it is the most prevalent allele in African black populations and it persists as the common allele in the American black population (dbSNP, 2016). A question arises as to why the nonprotective (T) allele has become the common allele in whites and Asians. This is likely the result of population bottle necks that have pruned the G allele frequencies in "out of Africa" populations. This process could have fixed the *rs2802292* G allele in a haplotype that contains a functional variant, such as one that results in better old age health. For most of human history infectious disease, of which inflammation is a major component, was likely the major cause of death (Finch, 2010). Perhaps TT

homozygotes may not be able to defend against a high inflammatory load, which can lead to sepsis (among other potentially fatal maladies), and thus, fewer of these survived to pass on T alleles. It is plausible that there might be an unknown antagonistic pleiotropic effect where the allele protects early in life and becomes a risk later in life (Blagosklonny, 2010). There are examples of antagonistic pleiotropy involving the *FoxO3* gene in model organism studies (Shen & Tower, 2010). As yet, we do not know if *FOXO3* genotype protects against mortality at young or middle age, only that it protects against mortality in later life, as no studies have assessed the effects of *FOXO3* genotype over the adult life course in humans. There are also several other plausible explanations of why the G allele has persisted as the common allele in blacks but has decreased in frequency in white and Asian populations. *FoxO* is critical in maintaining stem cell populations (Miyamoto *et al.*, 2007). Perhaps human populations have only recently been able to survive long enough for the beneficial effects of the *FOXO3* G allele on stem cell populations to be realized. Another potential explanation may be the fact that *FoxO3* regulates stress resistance and protects against ultraviolet damage (Yang *et al.*, 2006). Ultraviolet radiation would have been a major biological stressor in African populations. Future work may be able to answer such questions.

Our study has several strengths, including rigorous ascertainment of cause of death, large sample size that was adequate for detection of dominant genetic effects of *FOXO3* on cause-specific mortality, and replication of the main findings across cohorts. Another major strength is the prospective, population-based cohort design, with virtually complete mortality ascertainment in near-extinct cohorts. This eliminated recall bias concerning prevalent diseases and risk factors and also allowed us to quantify cause-specific mortality risk over time. The relative homogeneity of the HHP population was also an advantage, and prior work has found no evidence of a population stratification artifact in this cohort (Willcox *et al.*, 2008). Population substructure was also controlled for in the Health ABC population. The replication we saw in a methodologically similar cohort of elderly white and black Americans demonstrates that the findings are robust and generalizable to other genetic populations.

Summary and conclusions

The present study is the first to quantify the protection against mortality in a large, well-powered, prospective cohort study of populations of contrasting ethnicities, and to demonstrate that CHD is the main clinical cause of death driving the strong survival advantage seen in carriers of

the major longevity-associated *FOXO3* allele. As CHD is the primary cause of death in most industrialized countries, and death at older ages is increasingly dominated by inflammation-related diseases, the fact that allelic variation in *FOXO3* is now known to significantly modify this risk has important clinical implications, especially in high risk patient populations, such as those with type 2 diabetes (Gregg *et al.*, 2014) and obesity (Olshansky *et al.*, 2005). It has already become apparent that FoxO3 plays an important role in disease progression in age-related diseases, such as cancer, and may be useful for monitoring responses to treatment (Monsalve & Olmos, 2011; Morris *et al.*, 2015). Moreover, therapeutic interventions that modulate biological pathways associated with aging are now becoming a plausible approach to help people age more healthfully (Kennedy & Pennypacker, 2015). Our novel findings prompt consideration of *FOXO3* and its protein (Foxo3) as therapeutic targets in healthy aging with a focus on CHD and possibly other inflammation-related diseases. More study is needed to determine molecular/cellular mechanisms.

Experimental procedures

Study design

We utilized data from the Kuakini Hawaii Lifespan Study, an ancillary study of the genetic and environmental antecedents of healthy aging and longevity, drawn from the Kuakini HHP cohort of aging men (Willcox *et al.*, 2006, 2008; Donlon *et al.*, 2012). The HHP began in 1965 as a population-based prospective study of cardiovascular diseases and included 8006 American men of Japanese ancestry (Kagan, 1996). All study participants were recruited from World War II service records of 9877 men who had valid contact information, were born in 1900–1919, and who were living on the island of Oahu. For the current study, we utilized DNA samples and data collected in 1991–93, the first examination that focused on aging-related measures, which were added at that time to the ongoing HHP study (White *et al.*, 1996). We genotyped 3584 (~80% of available HHP survivors) stored blood samples collected at the 1991–1993 examination. Follow-up monitoring total mortality and cause-specific mortality for 17 years was conducted. All examinations, and the collection of biological samples, were approved by the Institutional Review Board of Kuakini Medical Center. Written informed consent was obtained at each examination from all study participants.

The Health ABC cohort was utilized for a replication study. Health ABC is a prospective observational study of 3075 well-functioning, community-dwelling, male and female, white and black Americans, aged 70–79 years at baseline, recruited from field centers at the University of Pittsburgh, Pennsylvania, and the University of Tennessee, Memphis, and followed up for 17 years.

Genotyping

For HHP subjects, total cellular DNA was isolated from buffy coat of blood using the PureGene system (Gentra Systems, Inc., Big Lake, MN, USA) and quantified using PicoGreen staining (Molecular Probes, Eugene, OR, USA). We selected 32 SNPs that reflected the major genetic variability within intron 2 of *FOXO3*, based on degree of linkage disequilibrium and allele frequencies of $\geq 5\%$. We chose variant *rs2802292* (alleles T/G) as representative of the strongest longevity-associated SNPs and genotyped an extended sample set of 3584 subjects. Genotyping for *rs2802292* involved an allelic discrimination assay.

TaqMan[®] (Applied Biosystems, Inc. [ABI], Carlsbad, CA, USA) reagents (purchased from Life Technologies, Carlsbad, CA, USA) were used for PCR amplification under standard conditions with AmpliTaq Gold[®] DNA polymerase (Perkin-Elmer Corp., Waltham, MA, USA). PCR products were detected by TaqMan[®] assay, using a 6-FAM-labeled FRET probe for one allele and a VIC-labeled probe for the other allele, with minor groove binding (MGB) quenchers to enhance assay signal. PCR products were measured using an ABI Prism 7000 Sequence Detection System. Genotype data were managed through a sample processing and database system with previously proven reliability. All positive controls on each genotyping plate were evaluated for consistency. We have found that these methods are exceptionally robust and have an accuracy of > 99.9% on retesting and a success rate of 99.6% (data not shown). We regenotyped 1% of samples to monitor quality.

For Health ABC samples, genomewide SNP genotyping was performed by the Center for Inherited Disease Research using the Illumina Human1M-Duo BeadChip system. Samples were excluded from the dataset for reasons of sample failure, genotypic sex mismatch, and first-degree relative of an included individual based on genotype data. Health ABC genotyped 1794 self-described white participants at baseline with available DNA and consent to genetics testing. Of these, 1661 passed quality control benchmarks (call rate > 97%, no sex mismatch, and cryptic relatedness). In addition, 1139 self-described African Americans had genotype information available after quality control. Principal component analysis (PCA) was performed using Eigenstrat (Price *et al.*, 2006). PCA was first performed on genotype data from all Health ABC participants and HapMap samples as anchor points to identify population outliers. PCA was then performed on genotype data separately for black and white participants who were not genetic outliers, and the resulting eigenvectors were used as covariates in regression models to adjust for population structure. Imputation was performed for the autosomes using MACH software version 1.0.16. SNPs with minor allele frequency $\geq 1\%$, call rate $\geq 97\%$ and Hardy–Weinberg equilibrium $P \geq 10^{-6}$ were used for imputation. HapMap II phased haplotypes were used as reference panels.

For whites, 914 263 SNPs passed quality control (QC) and were available for imputation using the HapMap CEPH reference panel (phase 2, release 22, build 36). For blacks, 1 007 948 SNPs passed QC and were available for imputation based on a 1:1 mixture of the CEPH:Yoruban (YRI) HapMap phase-2 release-22 build-36 reference panel. A total of 2 543 887 SNPs in whites and 1 958 375 SNPs in blacks were imputed. SNP *rs2802292* was imputed. All fractional values within 0.1 of an integer were rounded to the nearest integer to create the number of *rs2802292* G alleles. The imputation quality measured by MACH r^2 was 0.99 in whites and blacks.

Outcome measures

Total (all-cause) mortality and cause-specific mortality were the principal outcomes for the study. Death occurrence was ascertained by review of obituaries in local newspapers, through the medical examiner's office, by phone calls to family members or primary care physicians, and search of the National Death Index. Cause of death was determined by an expert morbidity and mortality committee, consisting of physicians who adjudicated the cause of death using standardized criteria, through continuous comprehensive surveillance of data collected from multiple sources (Kagan, 1996). These sources included death certificates, medical history from prior examinations, interview of healthcare proxies, hospital discharge records, cancer registry data, and autopsy reports, when available. Similar procedures were utilized in Health ABC.

Statistics

Genotypes were evaluated and found to be in Hardy–Weinberg equilibrium. The relation of *FOXO3* genotype with total mortality and cause-specific mortality was determined by Cox regression, adjusting for age, in carriers vs. noncarriers of the *G* allele of *rs2802292* in Japanese, whites, and blacks. For meta-analysis of total mortality, age-adjusted Cox regression models for Japanese, whites, and blacks were utilized, using both dominant and additive models for the effects of carrier status on total mortality. A dominant model was found to be most appropriate. Tests for heterogeneity were performed to assess suitability for a combined analysis. All statistical analyses were performed using the Statistical Analysis System (SAS) version 9.3 (SAS Institute, Cary, NC, USA).

Funding

The work in Honolulu was funded by the Kuakini Medical Center, the US National Institutes of Health (contract N01-AG-4-2149, Grants 5 U01 AG019349-05, 5R01AG027060 [Kuakini Hawaii Lifespan Study], 5R01AG038707 [Kuakini Hawaii Healthspan Study]), contract N01-HC-05102 from the National Heart, Lung, and Blood Institute, and Kuakini Medical Center. We acknowledge the expert assistance of the Hawaii State Department of Health, particularly for supplying death certificate data. Research involving the Health ABC study was supported by National Institutes of Aging contracts N01AG62101, N01AG62103, and N01AG62106 and in part by the Intramural Research Program of the NIH, National Institute on Aging (Health ABC study). The Health ABC genomewide association study was funded by NIA grant 1R01AG032098-01A1 to Wake Forest University Health Sciences, and genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract (number HHSN268200782096C) from the National Institutes of Health to The Johns Hopkins University. We thank all study participants and their families for their cooperation and the Hawaii State Department of Health for death certificate data. We thank Dr. Eunjung Lim for assistance with statistical analysis.

Conflict of interest

None declared.

Author contributions

BJW and TAD contributed to the study concept, data interpretation, study design, and writing the manuscript. KHM supervised recruitment and data collection on the HHP subjects and researchers at the Health ABC study sites recruited and characterized the Health ABC subjects. TAD and YL performed the HHP and Health ABC genotyping, respectively; MG, RA and PD performed cytokine analysis; TAD, YL, RC, EL, QH, NP, GJT, and DE managed data and contributed results; and RC, QH, YL, DES, and NP were responsible for data and statistical analyses. BJW, BJM, and TAD drafted the manuscript. All authors contributed to, read, and approved the final version of the manuscript.

References

Bao JM, Song XL, Hong YQ, Zhu HL, Li C, Zhang T, Chen W, Zhao SC, Chen Q (2014) Association between *FOXO3A* gene polymorphisms and human longevity: a meta-analysis. *Asian J. Androl.* **16**, 446–452.

- Blagosklonny MV (2010) Revisiting the antagonistic pleiotropy theory of aging: TOR-driven program and quasi-program. *Cell Cycle* **9**, 3151–3156.
- Broer L, Buchman AS, Deelen J, Evans DS, Faul JD, Lunetta KL, Sebastiani P, Smith JA, Smith AV, Tanaka T, Yu L, Arnold AM, Aspelund T, Benjamin EJ, De Jager PL, Eiriksdottir G, Evans DA, Garcia ME, Hofman A, Kaplan RC, Kardina SL, Kiel DP, Oostra BA, Orwoll ES, Parimi N, Psaty BM, Rivadeneira F, Rotter JJ, Seshadri S, Singleton A, Tiemeier H, Uitterlinden AG, Zhao W, Bandinelli S, Bennett DA, Ferrucci L, Gudnason V, Harris TB, Karasik D, Launer LJ, Perls TT, Slagboom PE, Tranah GJ, Weir DR, Newman AB, van Duijn CM, Murabito JM (2015) GWAS of longevity in CHARGE consortium confirms *APOE* and *FOXO3* candidacy. *J. Gerontol. A Biol. Sci. Med. Sci.* **70**, 110–118.
- Brooks-Wilson AR (2013) Genetics of healthy aging and longevity. *Hum. Genet.* **132**, 1323–1338.
- Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. dbSNP accession on 2/9/2016: {rs2802292}, (dbSNP Build ID: {100/146}). Available from: <http://www.ncbi.nlm.nih.gov/SNP/>.
- Dejean AS, Beisner DR, Ch'en IL, Kerdales YM, Babour A, Arden KC, Castrillon DH, DePinho RA, Hedrick SM (2009) Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. *Nat. Immunol.* **10**, 504–513.
- Donlon TA, Curb JD, He Q, Grove JS, Masaki KH, Rodriguez B, Elliott A, Willcox DC, Willcox BJ (2012) *FOXO3* gene variants and human aging: coding variants may not be key players. *J. Gerontol. A Biol. Sci. Med. Sci.* **67**, 1132–1139.
- Eijkelenboom A, Burgering BM (2013) FOXOs: signalling integrators for homeostasis maintenance. *Nat. Rev. Mol. Cell Biol.* **14**, 83–97.
- Finch CE (2010) Evolution in health and medicine Sackler colloquium: evolution of the human lifespan and diseases of aging: roles of infection, inflammation, and nutrition. *Proc. Natl. Acad. Sci. USA* **107**(Suppl 1), 1718–1724.
- Flachsbarb F, Caliebe A, Kleindorp R, Blanché H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A (2009) Association of *FOXO3A* variation with human longevity confirmed in German centenarians. *Proc. Natl. Acad. Sci. USA* **106**, 2700–2705.
- Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S (2007) Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mech. Ageing Dev.* **128**, 92–105.
- Fruman DA, Rommel C (2014) PI3K and cancer: lessons, challenges and opportunities. *Nat. Rev. Drug Discov.* **13**, 140–156.
- Gregg EW, Li Y, Wang J, Burrows NR, Ali MK, Rolka D, Williams DE, Geiss L (2014) Changes in diabetes-related complications in the United States, 1990–2010. *N. Engl. J. Med.* **370**, 1514–1523.
- Kagan A, ed. (1996) *The Honolulu Heart Program: An Epidemiological Study of Coronary Heart Disease and Stroke*. Amsterdam, The Netherlands: Harwood Academic Publishers.
- Kennedy BK, Pennypacker JK (2015) Aging interventions get human. *Oncotarget* **6**, 590–591.
- Kenyon CJ (2010) The genetics of ageing. *Nature* **464**, 504–512.
- Kuningas M, Mägi R, Westendorp RG, Slagboom PE, Remm M, van Heemst D (2007) Haplotypes in the human *Foxo1a* and *Foxo3a* genes; impact on disease and mortality at old age. *Eur. J. Hum. Genet.* **15**, 294–301.
- Lam EW, Brosens JJ, Gomes AR, Koo CY (2013) Forkhead box proteins: tuning forks for transcriptional harmony. *Nat. Rev. Cancer* **13**, 482–495.
- Lee JC, Espéllí M, Anderson CA, Linterman MA, Pocock JM, Williams NJ, Roberts R, Viatte S, Fu B, Peshu N, Hien TT, Phu NH, Wesley E, Edwards C, Ahmad T, Mansfield JC, Geary R, Dunstan S, Williams TN, Barton A, Vinuesa CG, Parkes M, Lyons PA, Smith KG (2013) Human SNP links differential outcomes in inflammatory and infectious disease to a *FOXO3*-regulated pathway. *Cell* **155**, 57–69.
- Miyamoto K, Araki KY, Naka K, Arai F, Takubo K, Yamazaki S, Matsuoka S, Miyamoto T, Ito K, Ohmura M, Chen C, Hosokawa K, Nakauchi H, Nakayama K, Nakayama KI, Harada M, Motoyama N, Suda T, Hirao A (2007) *Foxo3a* is essential for maintenance of the hematopoietic stem cell pool. *Cell Stem Cell* **1**, 101–112.
- Monsalve M, Olmos Y (2011) The complex biology of FOXO. *Curr. Drug Targets* **12**, 1322–1350.
- Morris BJ, Willcox DC, Donlon TA, Willcox BJ (2015) *FOXO3*: a major gene for human longevity – A Mini-Review. *Gerontology* **61**, 515–525.
- Morris BJ, Chen R, Donlon TA, Evans DS, Tranah GJ, Parimi N, Ehret GB, Newton-Cheh C, Seto T, Willcox DC, Masaki KH, Kamide K, Ryuno H, Oguro R, Nakama C, Kabayama M, Yamamoto K, Sugimoto K, Ikebe K, Masui Y, Arai Y, Ishizaki T, Gondo Y, Rakugi H (2016) Association analysis of *FOXO3* longevity variants with blood pressure and essential hypertension. *Am. J. Hypertens.* (Epub ahead of print Oct 16, 2015)

- Murabito JM, Yuan R, Lunetta KL (2012) The search for longevity and healthy aging genes: insights from epidemiological studies and samples of long-lived individuals. *J. Gerontol. A Biol. Sci. Med. Sci.* **67**, 470–479.
- National Institute on Aging. (2007) Living Long and Well in the 21st Century Strategic Directions for Research on Aging: National Institute on Aging [WWW document]. URL <http://www.nia.nih.gov/about/living-long-well-21st-century-strategic-directions-research-aging> [accessed on 20 December 2014].
- Oh HM, Yu CR, Golestaneh N, Amadi-Obi A, Lee YS, Eseonu A, Mahdi RM, Egwuagu CE (2011) STAT3 protein promotes T-cell survival and inhibits interleukin-2 production through up-regulation of class O forkhead transcription factors. *J. Biol. Chem.* **286**, 30888–30897.
- Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, Hayflick L, Butler RN, Allison DB, Ludwig DS (2005) A potential decline in life expectancy in the United States in the 21st century. *N. Engl. J. Med.* **352**, 1138–1145.
- Pawlikowska L, Hu D, Huntsman S, Sung A, Chu C, Chen J, Joyner AH, Schork NJ, Hsueh WC, Reiner AP, Psaty BM, Atzmon G, Barzilai N, Cummings SR, Browner WS, Kwok PY, Ziv E (2009) Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. *Aging Cell* **8**, 460–472.
- Peng SL (2007) Immune regulation by Foxo transcription factors. *Autoimmunity* **40**, 462–469.
- Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D (2006) Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38**, 904–909.
- Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T (2008) Activation of innate immunity system during aging: NF- κ B signaling is the molecular culprit of inflamm-aging. *Ageing Res. Rev.* **7**, 83–105.
- Shadyab AH, LaCroix AZ (2015) Genetic factors associated with longevity: a review of recent findings. *Ageing Res. Rev.* **19**, 1–7.
- Shen J, Tower J (2010) Drosophila foxo acts in males to cause sexual-dimorphism in tissue-specific p53 life span effects. *Exp. Gerontol.* **45**, 97–105.
- Soerensen M, Nygaard M, Dato S, Stevnsner T, Bohr VA, Christensen K, Christiansen L (2015) Association study of FOXO3A SNPs and aging phenotypes in Danish oldest-old individuals. *Aging Cell* **14**, 60–66.
- Takata H, Suzuki M, Ishii T, Sekiguchi S, Iri H (1987) Influence of major histocompatibility complex region genes on human longevity among Okinawan-Japanese centenarians and nonagenarians. *Lancet* **2**, 824–826.
- US Department of Health and Human Services. (2012) Office of Disease Prevention and Health Promotion. Healthy People 2020 [WWW document]. URL <http://www.healthypeople.gov> [accessed on 20 December 2014].
- White L, Petrovitch H, Ross GW, Masaki KH, Abbott RD, Teng EL, Rodriguez BL, Blanchette PL, Havlik RJ, Wergowske G, Chiu D, Foley DJ, Murdaugh C, Curb JD (1996) Prevalence of dementia in older Japanese-American men in Hawaii: the Honolulu-Asia Aging Study. *JAMA* **276**, 955–960.
- Willcox BJ, He Q, Chen R, Yano K, Masaki KH, Grove JS, Donlon TA, Willcox DC, Curb JD (2006) Midlife risk factors and healthy survival in men. *JAMA* **296**, 2343–2350.
- Willcox BJ, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD (2008) FOXO3A genotype is strongly associated with human longevity. *Proc. Natl. Acad. Sci. USA* **105**, 13987–13992.
- Yang JY, Xia W, Hu MC (2006) Ionizing radiation activates expression of FOXO3a, Fas ligand, and Bim, and induces cell apoptosis. *Int. J. Oncol.* **29**, 643–648.
- Zhao Y, Yu Y, Tian X, Yang X, Li X, Jiang F, Chen Y, Shi M (2014) Association study to evaluate FoxO1 and FoxO3 gene in CHD in Han Chinese. *PLoS One* **9**, e86252.

Supporting Information

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

Table S1. Serum TNF- α level by FOXO3 G allele carrier status.