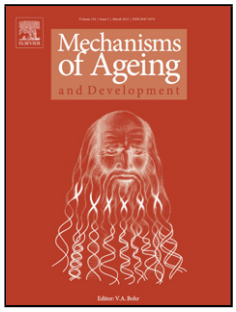

Title: Review and meta-analysis of genetic polymorphisms associated with exceptional human longevity

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Review and meta-analysis of genetic polymorphisms associated with exceptional human longevity

Short title: Review of genetic studies on human exceptional longevity

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Highlights

- This comprehensive review seeks to determine the genetic variants associated with exceptional longevity.
- Meta-analyses of genetic polymorphisms previously associated with exceptional longevity (aged 85+) were undertaken.
- Five polymorphisms, *ACE* rs4340, *APOE* ε2/3/4, *FOXO3A* rs2802292, *KLOTHO* KL-VS and *IL6* rs1800795 were significantly associated with exceptional longevity, with the pooled effect sizes (odds ratios) ranging from 0.42 (*APOE* ε4) to 1.45 (*FOXO3A* males).
- The observed modest effect sizes of the significant variants suggest many genes of small influence play a role in exceptional longevity, which is consistent with many other polygenic traits.

Abstract

Background Many factors contribute to exceptional longevity, with genetics playing a significant role. However, to date, genetic studies examining exceptional longevity have been inconclusive. This comprehensive review seeks to determine the genetic variants associated with exceptional longevity by undertaking meta-analyses.

Methods Meta-analyses of genetic polymorphisms previously associated with exceptional longevity (85+) were undertaken. For each variant, meta-analyses were performed if there were data from at least three independent studies available, including two unpublished additional cohorts.

Results Five polymorphisms, *ACE* rs4340, *APOE* ε2/3/4, *FOXO3A* rs2802292, *KLOTHO* KL-VS and *IL6* rs1800795 were significantly associated with exceptional longevity, with the pooled effect sizes (odds ratios) ranging from 0.42 (*APOE* ε4) to 1.45 (*FOXO3A* males).

Conclusion In general, the observed modest effect sizes of the significant variants suggest many genes of small influence play a role in exceptional longevity, which is consistent with results for other polygenic traits. Our results also suggest that genes related to cardiovascular health may be implicated in exceptional longevity. Future studies should examine the roles of gender and ethnicity and carefully consider study design, including the selection of appropriate controls.

Keywords centenarians, longevity, meta-analysis, *ACE, APOE, FOXO3A,*

1. Abstract

Background Many factors contribute to longevity, with genetics playing a significant role. However, to date, genetic studies examining longevity have been inconclusive. By undertaking meta-analyses this comprehensive review seeks to identify the genetic variants that are associated with longevity.

Methods Meta-analyses on polymorphisms previously associated with longevity in at least three independent studies were undertaken. Eight genes with a total of nine polymorphisms were investigated.

Results Four polymorphisms, *ACE* rs4340, *APOE* ε2/3/4, *CETP* rs5882 and *FOXO3A* rs2802292 were significantly associated with longevity, although the pooled effect sizes were modest. The remaining five polymorphisms (*CETP* rs708272, *IL6* rs1800795, *KLOTHO* rs9536314, *SIRT1* rs3758391, *TNFa* rs1800629) did not reach statistical significance.

Conclusion The effect sizes suggest many genes of small influence play a role, which is consistent with results for other polygenic traits. Our results also suggest that genes related to cardiovascular health may be implicated in exceptional longevity. Few studies have stratified their analyses by gender. Future studies should also examine different ethnicities, given the few non-Caucasian studies undertaken to date. Additionally, methodological issues may contribute to inconsistencies observed in the literature, such as the selection of appropriate controls.

Keywords centenarians, longevity, meta-analyses, *ACE, APOE, FOXO3A*

1. Introduction

Life expectancy in most societies has increased steadily in the last century due to improvements in medical care, nutrition and other factors, with many individuals living to an advanced old age in developed countries (e.g.Oeppen & Vaupel, 2002). However, during ageing there is a loss of homeostasis, which leads to diminished capacity to respond to stressors and increased vulnerability to age-related decline, disease and multimorbidity (Fabbri *et al.*, 2015). Thus, there is concern about an ageing population posing an increasing medical and economic burden on society. However, many exceptionally long-lived individuals have delayed morbidity or have escaped age-related diseases (Andersen *et al.*, 2012). They represent a unique human paradigm for identifying the determinants of longevity and healthy ageing. Studying these rare individuals may reveal novel pathways that lead to

exceptional ageing, which ultimately may suggest strategies to mitigate or prevent age-related decline and disease and to promote healthy ageing.

1.1 The heritability of longevity

Family and twin studies suggest that genetics plays a role in life expectancy with heritability estimated at ~20-30% (Murabito & Lunetta, 2012). Interestingly, the genetic contribution is modest early in life but increases at a greater age (> 60) (Hjelmborg *et al.*, 2006). Two studies of very long-lived individuals, the New England Centenarian Study and the Okinawa Centenarian Study have shown that siblings of centenarians have an increased probability of reaching 100 years of age when compared to individuals without such family histories of longevity (Perls *et al.*, 2002; Willcox *et al.*, 2006). Interestingly, there are gender differences in the roles genes play, with the heritability of becoming a centenarian higher for men (~0.48) than women (~0.33) (Sebastiani & Perls, 2012). Murabito et al., (2012) also found that heritability appears to increase with each 10-year increment in survival age for men but not women, suggesting that genetic effects on aging may be more substantial for men than women.

1.2 Genes associated with exceptional longevity

Genetic studies to date have focussed on linkage analysis, candidate gene approaches or genome-wide association studies (GWAS) to identify exceptional longevity genes. In general, these have produced inconsistent results apart from the apolipoprotein E (*APOE*) (e.g.Beekman *et al.*, 2013) and the forkhead box O3 (*FOXO3A*) genes (e.g.Willcox *et al.*, 2008). The aim of this review is to summarize our present understanding of the genetic factors affecting human exceptional longevity by undertaking a comprehensive meta-analysis reviewing all the major polymorphisms that were investigated in three or more independent human studies of individuals aged 85+ and above, who have exceeded the average life expectancy for individuals born in the early 20th century (Newman & Murabito, 2013).

2. Methods

2.1 Literature Search

A comprehensive search of electronic databases (MEDLINE, NCBI and EMBASE) was conducted to identify all publications on genes associated with exceptional human longevity up to December 30th, 2017. The search strategy was based on combinations of the following keywords "longevity", "centenarian", "ageing", "aging", "gene", "genetic", "polymorphism" and "SNP". The search was extended to include the bibliographies of all eligible studies. Reviews on longevity were also hand-searched to identify additional potentially relevant studies. Where necessary, authors were contacted directly for any additional data required. In addition, unpublished data from our own studies were included in this review (see below, section 2.4).

2.2 Study Selection: Inclusion and exclusion criteria

The following inclusion criteria were used to select articles for the meta-analysis: (i) information was provided on the association between one or more genetic polymorphism(s) and human "longevity"; (ii) used a case-control design whereby centenarians or aged participants (85+ years) were the cases versus younger adult controls; and (iii) provided sufficient genotype data for calculating the odds ratio (OR) and 95% confidence interval (CI). Studies were excluded if (i) the distribution of genotypes in the control group were not in Hardy-Weinberg equilibrium (HWE); (ii) they lacked a control group; (iii) they had overlapping study populations; (iv) they had fewer than 100 cases; (v) the article was unavailable in English; or (vi) results were only described in conference abstracts.

2.3 Data extraction

The recommendations for Meta-analyses of Observational Studies in Epidemiology (MOOSE) were followed. All relevant studies were obtained and independently inspected by two authors (MR and KM) to determine whether they met the inclusion criteria. When available, the appropriate data were also extracted from published GWAS. Careful attention was taken to avoid overlapping studies. The

following information was extracted: author, publication year, ethnicity of the population studied, sample sizes (cases and controls), baseline characteristics of the study population (e.g. gender) and the genotype/allele frequencies. Information on HWE was also extracted or calculated manually if not explicitly reported. Finally, any discrepancies were adjudicated with another author (AT) until a consensus was reached.

2.4 Additional unpublished data used in Meta-Analysis

Australian unpublished data were also used in the meta-analyses. Specifically, two studies were utilized that both recruited individuals using the compulsory electoral roll and Medicare lists in order to obtain a representative sample. Cases were obtained from the Sydney Centenarian Study (SCS) (Sachdev *et al.*, 2013) and controls from the Hunter Community Study (HCS) (McEvoy *et al.*, 2010); both of these studies recruited participants from the state of New South Wales, Australia. The SCS is comprised of individuals aged 95 years and over who were recruited into a study of successful ageing in Sydney. More details of the study are found in Sachdev *et al.* (2013). A subsample with available genetic data provided 256 long-lived cases with a European background (age range 95-106, mean age 97.5 years, 31% men). The HCS is a cohort of 3253 individuals (age range 55-85, mean age 66.3 years, 46% male) recruited from Newcastle. For more details of the HCS see McEvoy *et al.* (2010). For the purpose of this study a sub-sample of 1002 individuals aged 55-64 (mean age 59.8 years, 47% male) was used as controls.

Both of these cohorts have genome-wide genotyping data available. HCS samples were genotyped using the Affymetrix Axiom Kaiser array (California, USA) whereas SCS cases were genotyped using the Illumina OmniExpress array (California, USA), according to the manufacturer's instructions. Both studies excluded genotyped SNPs if the call rate was <95%, p-value for HWE was $<10^{-6}$ and minor allele frequency was <0.01%. Relatedness checks were undertaken and only one family member was retained for the analysis if first or second-degree relatives were identified. Ethnic outliers were

detected and omitted via EIGENSTRAT analysis (Price *et al.*, 2006). After QC checks, for SCS there was genotyping data on 640,355 SNPs whilst for HCS there was data on 739,276 SNPs. For both cohorts, imputation was completed using the HapMap2 reference data (release 22, build 36) using the same method as described in Mather *et al.* (2016). *APOE* genotyping in both the SCS and HCS was undertaken using the methods described in Sachdev *et al.* (2010) and Oldmeadow *et al.* (2014) respectively. The results of the analyses using these data are designated as 'Present Study, 2017'.

2.5 Statistical Analysis

Meta-analyses were conducted for polymorphisms investigated in at least three studies. The strengths of the associations between each gene polymorphism and longevity were estimated by allelic odds ratios and 95% CIs. Wherever possible, analyses were also stratified by ethnicity and gender. A fixed-effects model using the inverse variance method was used and the significance of the pooled OR was determined by the Z-test. The I^2 statistic was used to estimate the percentage of variation across the results due to study heterogeneity, rather than sampling error, with the degree of heterogeneity being defined as low (25%), medium (50%) or high (75%). No significant heterogeneity was defined as an I^2 value of less than 50% and/or a p-value <.05. Forest plots were prepared for each study. Evaluation of the winner's curse phenomenon, which refers to the occurrence when the effect size for a newly described genetic association is overestimated by the earliest study compared to later studies, was examined by re-running the meta-analysis omitting the earliest study. Sensitivity analyses were performed after the sequential removal of each included study to assess the influence of each individual study on the pooled OR. Potential publication bias was evaluated by visual inspection of funnel plots and Egger's regression test with $p<0.05$ (two-tailed) considered statistically significant. Pooled effect estimates were also obtained under random effect models using restricted maximum likelihood (REML) for comparison with fixed effects when more than three studies were available for analysis. Meta-regression was performed for stratified analysis. All analyses were conducted using the R metafor package (Viechtbauer, 2010).

3. Results

The initial literature search identified 71 potentially relevant studies (**Fig.1**). After applying exclusion criteria, 65 studies remained that resulted in eight genes with a total of nine variants, which are described in **Table 1**.

3.1 Angiotensin Converting Enzyme *(ACE)*, Deletion/Insertion *Alu* repeats

A total of eight studies were included in the meta-analysis comprising 2043 cases and 8820 controls (Table S1). Heterogeneity between studies was absent. There was no evidence of winner's curse phenomenon when the first study was omitted. There was no evidence of publication bias as observed by the symmetrical funnel plot (Fig. S1). The current meta-analysis shows a modest, albeit statistically significant positive association of the *ACE* D-allele with exceptional longevity (Fig. 2) (OR = 1.11, 95% CI = 1.01-1.22, P = 0.02). A random effects model did not affect the result (Table S11). Details of the gene structure, genomic location and expression across a range of tissues is presented in the Supplementary.

3.2 Apolipoprotein-E (*APOE*), ϵ2/ϵ3/ε4 variants

A total of 12 studies were included in the APOE ϵ2/ϵ3/ε4 meta-analyses comprising 3229 cases and 13685 controls (Table S2).

ϵ4 vs ε3

There was no evidence of winner's curse phenomenon. Heterogeneity between studies was low (I^2 = 37.68%). There was no evidence of publication bias (Fig. S1). A significant negative association of the *APOE* ε4-allele when compared to the ε3-allele with exceptional longevity was observed (OR = 0.42, 95% CI = 0.37-0.48, P < 0.00001) (Fig. 3 upper panel). Heterogeneity was reduced to 32.42% under the random effects model but the overall conclusion remained the same (Table S11). A fixed meta-regression analysis accounting for the ethnicity (Asian n=3 vs non-Asian n=9) showed homogeneity between the two groups (P=0.2669), thus there were no differences between these two ethnic groups.

ε2 vs ε3

There was no evidence of a winner's curse phenomenon. Due to high heterogeneity (65%, Fig S2), the Asian study (Feng et al., 2011) was omitted, resulting in acceptable levels of heterogeneity (35%). There was no evidence of publication bias as the funnel plot suggested no substantial asymmetry (Fig. S1). A positive association of the *APOE* ε2-allele with exceptional longevity was observed (OR = 1.38, 95% CI = 1.21-1.58, P< 0.0001) (Fig. 3 lower panel). Heterogeneity reduced to 27% in the random effects model but the overall effect estimate was similar (Table S11). Meta-regression showed no significant difference between the two ethnic groups (see Table S2), Caucasian (n=9) versus Asian (n=2) (p-value=0.84). Details of the *APOE* gene structure, genomic location and expression across a range of tissues is presented in the Supplementary.

3.3 Cholesteryl ester transfer protein (*CETP*)

***CETP* (I405V polymorphism, G vs A allele, rs5882)**

Initially, a total of 7 studies, with 2110 cases and 2220 controls, were included in the meta-analysis for the *CETP* I405V polymorphism (rs5882) (Table S3, Figure S3 upper panel). There was evidence of winner's curse phenomenon resulting in the Barzilai *et al.* (2003) being omitted from the analysis. However, heterogeneity was high (I^2 = 52.29%); and removal of the one non-Caucasian study by Sun et al. (2013) resulted in heterogeneity dropping to an acceptable level (36.44%). The symmetrical funnel plot suggested no publication bias after the outlier studies were removed (Fig. S1). However, there was no significant association with exceptional longevity for the G allele (OR = 0.93, 95% CI 0.83-1.05, P= 0.27). Comparison with a random effects model did not affect the result (Table S11).

***CETP* (*Taq1B* polymorphism, T vs C alleles, rs708272)**

A total of 4 studies examined the *Taq1B* polymorphism (rs708272) with a total of 1231 cases and 1795 controls (Table S4). There was no evidence of winner's curse phenomenon nor of heterogeneity (I^2 = 0%). The symmetrical funnel plot suggested no publication bias (Fig. S1). The pooled OR for the T allele

(equivalent to B1) compared to the C allele (corresponding to B2) was 0.93 (95% CI 0.83-1.04, P= 0.184) (Fig S3 lower panel). Thus, no statistical significance was observed. A random effects model did not affect the result (Table S11).

3.4 Forkhead box 03 transcription factor (*FOXO3A,* G vs T alleles, rs2802292)

Fourteen studies were identified giving a total of 7937 cases and 9572 controls examining rs2802292 in both sexes (Table S5). There was no between study heterogeneity (I^2=22.75%). The symmetrical funnel plot suggested no publication bias (Fig. S1). As shown in Figure 4 the G allele compared to the T allele was significantly associated with exceptional longevity (OR = 1.12, 95% CI 1.07-1.18, P <0.0001) (Fig. 4 upper panel). A random effects model produced a similar result (Table S11). Details of the gene structure, genomic location and expression across a range of tissues is presented in the Supplementary.

Analysis separately in males and females

As data was available for sex-specific analyses and there is prior evidence that this SNP may have a gender effect (reviewed in Bao et al, 2014) we undertook analyses stratified by gender. A sub-analysis of six samples comprised of 1739 cases and 2625 controls (Anselmi *et al.*, 2009; Broer *et al.*, 2015; Li *et al.*, 2009; Soerensen *et al.*, 2010; Willcox *et al.*, 2008 and Present Study), examined the association of the rs2802292 polymorphism with exceptional longevity in men (Table S6, Figure S4). The winner's curse phenomenon was observed; thus, the Japanese study was excluded (Willcox *et al.*, 2008). The present study and the MrOS sample from Broer et al. 2015 were also dropped from the meta-analysis due to high heterogeneity (I^2 >50%), which resulted in acceptable heterogeneity (I^2 =0). The final meta-analysis was undertaken including only three studies (Fig. 4 lower panel). The symmetrical funnel plot suggested no publication bias (Fig. S1). A highly significant association with the G allele of this polymorphism and exceptional longevity was observed in males (Fig. 4 lower panel, OR =1.45, 95% CI 1.25-1.68, P <0.0001).

There were only three studies available for a female only meta-analysis (Present Study, Li *et al.*, 2009; Soerensen *et al.*, 2010) but due to high heterogeneity (I^2 = 60.1%, Figure S5), the Chinese study was dropped (Li *et al.*, 2009) resulting in too few studies for a meta-analysis.

3.5 Interleukin 6 (*IL6,* G174C polymorphism, G vs C allele, rs1800795)

A total of 4 studies, with 1377 cases and 2227 controls, were included in this meta-analysis (Table S7). There was no evidence of winner's curse phenomenon. There was no between-study heterogeneity (I^2 = 0%) and the symmetrical funnel plot suggested no publication bias (Fig. S1). The significant pooled OR for the G allele in association with exceptional longevity was 1.13 (95% CI 1.02-1.25, P = 0.015, Figure 5). A random effects model did not affect the result (Table S11). Details of the gene structure, genomic location and expression across a range of tissues is presented in the Supplementary.

3.6 Klotho (*KLOTHO*, KL-VS polymorphism)

The KL-VS polymorphism is a haplotype tagged by a number of SNPs in total linkage disequilibrium (e.g. rs9527026, rs9536314) and hence different SNPs have been used across studies. A total of 3 studies (Invidia *et al.*, 2010; Novelli *al.*, 2008 and Present Study), with 1290 cases and 1797 controls, were included in this meta-analysis (Table S8). There was no evidence of winner's curse phenomenon and heterogeneity was not high (I^2 = 22%) (Fig S1). The KL-VS haplotype was positively associated with exceptional longevity with a modest effect size (OR = 1.18 and 95% CI 1.01-1.37, P = 0.035, Figure 6). Details of the gene structure, genomic location and expression across a range of tissues is presented in the Supplementary.

3.7 Sirtuin protein (*SIRT1*, C vs T allele, rs3758391)

A total of three studies, with 747 cases and 1698 controls (Han *et al.*, 2014; Lin *et al.*, 2016 and Present Study), were included in this meta-analysis of the *SIRT1* polymorphism, rs3758391, with longevity

(Table S9). There was no evidence of heterogeneity (I^2 =0%), publication bias (Fig S1) or Winner's curse. The pooled OR for the C allele in association with exceptional longevity was not significant (OR= 1.08, 95% CI 0.95-1.23 P = 0.25, Fig S6). Thus, no statistical significance was observed.

3.8 Tumour necrosis factor-alpha (*TNFα*, G308A polymorphism, rs1800629)

A total of 4 studies, with 747 cases and 1698 controls, were included (Bruunsgaard *et al.*, 2004; Khabour, 2010; Wang *et al.*, 2001 and Present Study) (Table S10). There was no evidence of winner's curse phenomenon. Heterogeneity was low (I^2 = 11%). The symmetrical funnel plot suggested no publication bias (Fig. S1). The association with exceptional longevity for the G allele compared to the A allele was not significant (OR = 1.18, 95% CI 0.99-1.40; P = 0.07, Fig. S7). A random effects model did not affect the result (Table S11).

4. Discussion

Many genes have been investigated in relation to exceptional longevity, although the results have been inconsistent. Therefore, we assessed the contributions of previously identified genetic variants using meta-analyses. We focused on eight genes with a total of nine polymorphisms, which have been investigated for association with exceptional longevity in at least three published studies. Our results indicate that at least five out of the nine polymorphisms, *ACE* rs4340, *APOE* ε2/3/4, *FOXO3A* rs2802292, the *KLOTHO* KL-VS variant and IL6 rs1800795 were significantly associated with exceptional longevity, although the pooled effect sizes were in general, modest.

The ACE enzyme is a key component of the renin-angiotensin system that regulates blood pressure (Rigat *et al.*, 1990) and plays a key role in sodium homesotasis (Farag *et al.*, 2017). We examined the most frequently studied variant, an insertion/deletion (I/D) polymorphism. Our *ACE* I/D meta-analysis, comprising 8 studies with a total of 10,863 participants, indicated that the *ACE* D-allele shows a modest positive association with exceptional longevity. The deletion allele is missing the 287-bp *Alu*

repetitive element, which leads to higher ACE enzyme activity in blood (Danser *et al.*, 1995) and may increase the risk of cardiovascular disease by up to 10% (You & Shen, 2016; Zhao *et al.*, 2014). However, as demonstrated by our meta-analysis and other studies, the D allele is associated with exceptional longevity (Zajc Petranovic *et al.*, 2012). This suggests that the *ACE* I/D polymorphism may be an example of antagonistic pleiotropy, whereby earlier in life the D allele may increase the risk of disease but later in life it may lead to a survival advantage. It has also been speculated that the D allele may influence longevity by its potential positive effects on tissue repair (Eisenlohr *et al.*, 1992), the immune system (Ehlers & Riordan, 1989), and preservation of muscle strength (Montgomery *et al.*, 1998). Additionally, there is the possibility that this polymorphism is in linkage with another locus that may be driving the observed relationship (Garatachea *et al.*, 2013). Our finding concurs with a meta-analysis undertaken by Garatachea *et al.* (2013), although our meta-analysis included new studies and had a greater number of cases whilst Garatachea et al. had a higher number of controls due to differences in study selection criteria. We used a lower age cut-off for cases (85+ vs Garatachea et al. 100+ years) and did not include small studies (N cases<100). Despite these differences, the observed odds ratios were very similar (OR= 1.11 current study vs 1.16 Garatachea et al).

APOE has been implicated in cardiovascular and neurodegenerative diseases, with the ϵ4 allele variant the major genetic risk factor for Alzheimer's disease. The *APOE* gene encodes the primary cholesterol carrier in the brain as well as contributing to the clearance of beta-amyloid across the blood brain barrier (Forero *et al.*, 2018)and plays an important role in lipid and cholesterol homeostasis (Leduc *et al.*, 2010).

Our meta-analyses examining the *APOE* ϵ2/ ϵ3/ ϵ4 polymorphism (≥11 studies) indicated that when compared to the most common allele, ϵ3, the ϵ4 allele was negatively associated with exceptional longevity, whereas a positive association was observed for *APOE* ϵ2. Similar meta-analysis results were observed when considering the influence of ethnicity, although there were substantially fewer

non-Caucasian studies (Caucasians ≥8studies; Asians 2- 3 studies). Our findings differ from a meta-analysis undertaken by Garatachea *et al.* (2015), where no significant results were observed for either of these analyses. However, we investigated different studies and the age cut-offs for the cases differed as only centenarians were analyzed in Garatachea et al., 2015. Nevertheless, Garatachea et al. did find significant relationships with exceptional longevity when using other genetic models that were consistent with our results (e.g. ϵ4 carriers negatively associated with longevity). In support of these results, a prior genome-wide longevity study found evidence of linkage in nonagenarians (1408 sibling pairs) with a region on chromosome 19 (19q13.11-q13.32), which harbors the *APOE* gene. The same authors using a GWAS in an independent sample also found significant associations with *APOE* SNPs (Beekman *et al.*, 2013). Moreover, exceptional longevity associations with polymorphisms from the *APOE* locus (Broer *et al.*, 2015; Sebastiani *et al.*, 2012; Zeng *et al.*, 2016), *TOMM40* (Deelen *et al.*, 2011) and *APOC1* (Nebel *et al.*, 2011) genes on chromosome 19, which are in strong linkage disequilibrium with the *APOE* gene, have also been observed. It should also be noted that the *APOE* ϵ2/3/4 polymorphism spans a CpG island found in exon 4 and hence epigenetic regulation of *APOE* expression may be involved (Forero *et al.*, 2018). Thus, the contribution of the *APOE* ϵ2/3/4 variant to exceptional longevity deserves a comprehensive assessment including not only genetic variation but epigenetic as well.

FOXO3A is an evolutionary conserved transcription factor and is a strong exceptional longevity candidate. *FOXO3A* has previously been shown to contribute to lifespan extension in a variety of different animal models (Bonafe *et al.*, 2003). *FOXO3A* is a member of the Forkhead family of transcription factors and unlike invertebrates, which have only one *FOXO* gene, mammals have four FOXO genes, (*FOXO1*, *FOXO3*, *FOXO4*, *FOXO6*), all containing the 'forkhead box' DNA binding domain. The *FOXO* family play important regulatory roles in insulin/insulin-like growth factor (IGF1) signaling, which impacts diverse biological processes including cellular homeostasis (Morris *et al.*, 2015), metabolism, proliferation, differentiation, oxidative stress, apoptosis, senescence (Lee & Dong, 2017;

Martins *et al.*, 2016). In our *FOXO3* meta-analysis of over 7900 cases and 9500 controls, using studies of both genders, the G allele of the intronic SNP, rs2802292, was significantly associated with exceptional longevity (OR =1.12). This is consistent with two prior meta-analyses, where Bao *et al.* (2014) and Broer *et al.* (2015) observed similar associations with exceptional longevity (OR = 1.36 & OR =1.17 respectively). The difference between the strength of the observed OR of Bao *et al.* (2014) compared to that reported by Broer *et al.* (2015) and the current study is most likely due to the number of included studies (n=5 for Bao et al; n=20 Broer et al; n=14 current study) and their sample sizes. We had fewer studies than Broer *et al.* (2015), as we included only studies comprising both sexes. The recent Han Chinese centenarian GWAS also found evidence for an association with *FOXO3A* SNPs with exceptional longevity at the p<.05 level (Zeng *et al.*, 2016), providing support that it plays an exceptional longevity role in a Chinese population.

In *FOXO3A* sex-specific analyses, which were also performed by Bao *et al.* (2014), we observed a similar significant positive association for males only (Bao et al. OR =1.54 versus current study OR = 1.45). Due to the winner's curse phenomenon and heterogeneity in our results, our final number of included studies was smaller (n=3 vs Bao et al., n=5). The Willcox *et al.* (2008) examined a Japanese cohort and the frequency of the G allele in the Japanese population is ~24% (compared to 43% in Europeans), which suggests that there may be population-specific effects when considering exceptional longevity. Bao *et al.* (2014) found male-specific associations for two other *FOXO3A* SNPs (rs2764264, rs13217795) that we did not examine due to a lack of studies with the relevant information. Of note, these three SNPs are in moderate to high linkage disequilibrium (r^2 ~0.615-0.889). Data on female studies is lacking with only two studies previously reported for rs2802292, both of which found an association with longevity (Li *et al.*, 2009; Soerensen *et al.*, 2010) and the present study, which did not find a significant association (OR=1.00 [0.78-1.28]). A meta-analysis for females was not undertaken due to high heterogeneity. Further studies are required to investigate the gender effects of *FOXO3A* in exceptional longevity even though recruiting long-lived males may prove difficult as they are less common than their female counterparts.

The investigated *FOXO3A* SNP, rs2802292, is located in intron 2 and is a cis-eQTL for a nearby long non-coding RNA, *LINC0022*, of which little is known. Recently, the presence of the G allele was found to be protective for coronary artery disease mortality in older populations (~76 yrs age), including Japanese and Caucasians (Willcox *et al.*, 2017). The exact mechanism by which such a protective effect occurs is unclear but different lines of evidence suggest *FOXO3* may have beneficial cardiovascular effects (Willcox *et al.*, 2017). Other studies are required to replicate and extend these results.

The majority of prior work examining the influence of the *FOXO3A* gene on exceptional longevity has focused on SNP variants. However, the regulation of *FOXO3A* and its corresponding protein is complex and includes not only genetic variation but also epigenetic (e.g. circular RNA, miRNAs, Yang *et al.*, 2016), and post-translational modifications (e.g. protein acetylation). Recently, a broader chromosomal perspective was undertaken by Donlon *et al.* (2017), who demonstrated long range physical contacts with *FOXO3A* and 46 nearby genes on a large region of chromosome 6 in a Japanese-American cohort. This work suggests that the *FOXO3A* gene may participate in important chromosomal conformational changes that may contribute to regulation of a large number of genes. Indeed, the authors suggest that this set of genes, 'the FOXO3 longevity interactome' may act as an 'ageing hub' (Willcox *et al.*, 2017). The role of rs2802292 as an eQTL of *LINC0022* also deserves attention (see Suppl.). Future comprehensive longevity studies examining the regulation of *FOXO3A* gene expression and its protein product and its influence on human ageing related phenotypes (e.g. age-related disease) are warranted to fully investigate its role in exceptional longevity.

We observed a modest association for rs1800795 (also known as -174G/C) and exceptional longevity (OR=1.13), which is a SNP located in the *IL6* gene promoter (See Suppl.). This gene encodes a member of the interleukin family and has two contrasting actions, as an inflammatory cytokine and also an anti-inflammatory myokine, and plays a critical role in immune defense (Pal *et al.*, 2014). The allele

frequency of rs1800795 varies greatly amongst Caucasian subpopulations but in Asian and African populations this polymorphism is almost monomorphic for the G allele, which is associated with higher levels of IL6 (Albani *et al.*, 2009). Interestingly, Di Bona *et al.* (2009) found a male-specific association with this polymorphism but only in Italian centenarians and not in other European groups, suggesting again that there are longevity gender differences and that environmental factors are also important. Higher levels of IL6 are reported for the G allele. This SNP is also located in an uncharacterized long non-coding RNA, *LOC541472* (alias: *ACO73072*.5), and is an eQTL for this gene and also the *STEAP1B* gene (Fig. S11d). In addition, it should be noted that DNA methylation of the IL6 promoter may also contribute to its transcriptional regulation (Ma & Ordovas, 2017). The role *IL6* plays in exceptional longevity may be due to its role in maintaining homeostasis as it is both pro-inflammatory and anti-inflammatory as well as its function in the maturation of B cells (Minciullo *et al.*, 2016).

Klotho can be found as either a membrane and/or a secreted protein in cerebrospinal fluid, plasma and urine. Interestingly, at least in mice, the membrane klotho protein can be cleaved by proteins of the beta-amyloid pathway, implicated in Alzheimer's disease, namely *ADAM 1*, *ADAM 17* and *BACE* (Bian *et al.*, 2015; Pavlatou *et al.*, 2016). Klotho is involved in diverse pathways including the insulin signaling pathway and in regulation of ion channel activity, calcium and phosphorus homeostasis, inflammation (Hui *et al.*, 2017)and the preservation of stem cells (Bian *et al.*, 2015). It also plays a protective role against oxidative stress, senescence, and cancer (Pavlatou *et al.*, 2016). Animal experiments suggest klotho plays a role in ageing and longevity. For example, increased klotho expression can extend lifespan in *C. elegans* (Chateau *et al.*, 2010; Kuro-o *et al.*, 1997; Kurosu *et al.*, 2005). Two non-synonymous SNPS, rs9536314 (F325V) and rs952705 (C370S), in conjunction with four other SNPs that are in complete linkage disequilibrium define the haplotype "KL-VS" (See Suppl.). This variant alters the structure of the protein and is reported to increase klotho secretion as well as being associated with greater brain cortical volume in humans and slower cognitive decline in older adults (Shardell *et al.*, 2016). Only a few human exceptional longevity studies have examined *KLOTHO*

variation and have published inconsistent results, with some suggesting a KL-VS heterozygote advantage for longevity (e.g. Arking *et al.*, 2002) but others not (e.g. Novelli *et al.*, 2008).

In our meta-analysis a modest association between the *KLOTHO* haplotype, KL-VS, and exceptional longevity (OR =1.18) was noted, however, there was a limited number of studies (n=3) and the result appears to be driven by the present study. There was no evidence of winner's curse or publication bias and between study heterogeneity was acceptable. Despite our positive results for this *KLOTHO* variant, more human *KLOTHO* longevity studies are required to further explore this relationship.

Four polymorphisms (*CETP* rs5882, *CETP* rs708272, *TNFa* rs1800629 and *SIRT1* rs3758392) did not reach statistical significance in our meta-analyses. Possible explanations for this are that there is no effect or insufficient statistical power. In general, our meta-analyses that failed to reach statistical significance had relatively small sample sizes (e.g. *SIRT1*). Thus, more studies are required to conclude that these polymorphisms are in fact not associated with exceptional longevity.

Throughout our meta-analysis we have carefully assessed heterogeneity, winner's curse phenomenon and publication bias. The winner's curse phenomenon was observed for *CETP* (rs5882) resulting in the omission of the first published study (Barzilai *et al.*, 2003), which examined Ashkenazi Jews. Similarly, the first published study for the *FOXO3A* rs2802292 variant (Willcox *et al.*, 2008), examining a Japanese cohort was also excluded due to the winner's curse. Apart from *APOE* (ε2 vs ε3), *CETP* (rs5882) and *FOXO3A* (rs2802292 gender analyses only), study heterogeneity was either absent or defined as low with an I^2 value ranging from 11-22%. For two of the meta-analyses with high heterogeneity, removal of a single study for *APOE* ε2 (I^2 = 65%) and for *CETP* rs5882 (I^2 = 52%) resulted in an acceptable, albeit moderate, level of heterogeneity (35 & 36% respectively). Omission of two studies from the FOXO3A male analysis (the Australian present study & MrOS sample from Broer *et al.* (2015)) resulted in greatly improved heterogeneity but only a small number of studies could then

be assessed (n=3). Omission of the only Chinese study for the *FOXO3A* female analysis resulted in only two acceptable studies. Publication bias was not detected across all studies.

All of the five identified exceptional longevity-related genes in our meta-analysis have been related to cardiovascular health, in particular lipoprotein/cholesterol and blood pressure metabolism. *APOE* has been linked to cardiovascular disease, including heart attack and stroke (Lahoz *et al.*, 2001). *ACE* has been related to hypertension and heart failure (Cambien *et al.*, 1992) and *FOXO3A* has been implicated in coronary heart disease (Donlon *et al.*, 2017). Klotho has been linked to atherosclerosis and premature coronary disease (Pavlatou *et al.*, 2016). The *IL6* rs1800795 SNP has been linked to atherosclerosis (Yin *et al.*, 2013) and coronary artery disease (Hou *et al.*, 2015). These results suggest that cardiovascular-related pathways are important contributors to attaining exceptional longevity. It is of great interest to note that *KLOTHO* interacts with the *FOXO* family. For example, together with foxo, the klotho protein can play a role in the reduction of oxidative stress. Circulating klotho can bind to cell surface receptors, which inhibits phosphorylation of FOXO, resulting in its nuclear translocation. In the nucleus, foxo can then bind to the promoter of the oxidative stress gene, *SOD2*, increasing its expression ultimately resulting in the removal of reactive oxygen species (Pavlatou *et al.*, 2016).

Longevity GWAS meta-analyses have in general had limited success identifying genetic variants. Over the last decade GWAS studies have been performed and have defined exceptional longevity in various ways. When cases were defined as 85 years plus, the *APOE* gene locus was identified (Deelen *et al.*, 2011). When using cases aged 90 years and over GWAS meta-analyses have found SNPs in the *MINPP1* gene (Newman & Murabito, 2013), the *CAMKIV* gene (Malovini *et al.*, 2011), the *APOE/TOMM40/APOC1* gene region (Beekman *et al.*, 2013; Nebel *et al.*, 2011) and in a long-non-coding RNA gene on chromosome 5q33.3 (*RP11-524N5.1*) (Deelen *et al.*, 2011). However, Broer *et al.* (2015) did not find any genome-wide significant results using 90+ year old cases but did replicate candidate gene results for the *APOE* locus and the *FOXO3A* SNP, rs2802292. Focussing on centenarian

cases, genome-wide significant results have also been observed in the APOE/TOMM40 locus (Sebastiani *et al.*, 2012). The most recent study, using a very large sample of Chinese centenarians identified ethnic-specific and other cross-ethnic exceptional longevity SNPs, including *APOE* (Zeng *et al.,* 2016). This study also replicated the findings for *FOXO3* and chromosome 5q33.3 as exceptional longevity loci by Deelen *et al.* (2011). However, it should be noted that the comparison groups (controls) differed widely between these studies and the sample sizes also varied greatly.

There is some uncertainty regarding the concept of 'exceptional longevity' compared to 'longevity'. What is the appropriate age cut-off for exceptional longevity? Presumably, it should be greater than the average life expectancy, which will be specific for each cohort. As previously discussed, there is also evidence that the genetic contribution to longevity increases with age. In our study, we have used a criterion for exceptional longevity of 85 years and over, which would exceed the average life expectancies of most of the participants previously studied. Moreover, the vast majority of exceptionally long-lived individuals included in our meta-analyses were 90 years and older (see Suppl.). If we had used a more extreme age cut-off (e.g. 100+) the number of studies would have been severely restricted for our analyses. Additionally, should the age cut-off take into consideration gender, as prior work suggests that there are sex differences in life expectancy? For example, genetic influences on longevity may be stronger for men (Sebastiani & Perls, 2012). Another major issue of exceptional longevity studies is the optimal study design. A longitudinal birth cohort study would be ideal, enabling selection of early deceased controls and long-lived cases that exceed the average life expectancy from the same cohort. Such a design would control for ethnicity to some extent and birth cohort differences. Alternatively, exceptional longevity can be assessed as a continuous trait (years of survival).

Exceptional longevity is a heterogeneous phenotype; more homogenous exceptional longevity-related phenotypes may be more useful for genetic studies. For example, healthy aging, defined as free of

most common diseases at age (Reed, 2003), has been reported as highly heritable in a male only study. Another exceptional longevity-related phenotype is age-related cognitive performance, which has moderate to high heritability in very old adults (80+) (McClearn *et al.*, 1997). However, most genetic studies have focused simply on exceptional longevity and not longevity-related phenotypes.

Limitations of this meta-analysis include: (i) variations in the definition of cases and controls; (ii) small sample sizes; and (iii) a lack of non-Caucasian studies. In general, racial differences were not able to be assessed except where there were sufficient numbers of studies (*APOE* ε2/3/4). As the majority of studies utilized Caucasian participants with a minority from Asian cohorts, the results from our Caucasian analyses may not be generalizable to non-Caucasian populations. We examined only allelic differences and not different genotypic models or carrier status as not all studies provided the necessary information. Additionally, selection of appropriate controls is problematic. Ideally, cases and controls should be nominated from the same birth cohort avoiding the introduction of survivor bias. Lastly, few studies have examined gender differences and we were only able to examine the association of *FOXO3A* rs2802292 with longevity in males.

Functional studies are required to follow-up the role of the significant genes identified in this analysis in promoting exceptional ageing. To date few published studies have examined various polygenic risk scores (e.g. cancer, cardiovascular disease, Alzheimer's disease) and exceptional longevity. In addition, more in-depth study of the genes identified in this meta-analysis, as well as examining the role of epigenetics, lipid and protein modifications will be important to address as the field progresses. The more recent use of whole genome sequencing and pooling resources across independent studies to increase the sample size and racial diversity may further reveal the influence of sex, ethnicity and of rare and common variants as well as copy number variation on exceptional longevity.

Conclusions

The meta-analyses performed revealed several genetic variants with consistent associations to exceptional longevity, with the strongest results observed for the *APOE* ε2/3/4 polymorphism and *FOXO3A* rs2802292 in males. However, in general, the effect sizes were not large, suggesting that many genes of small effect play a role, which is consistent with results for other complex traits.

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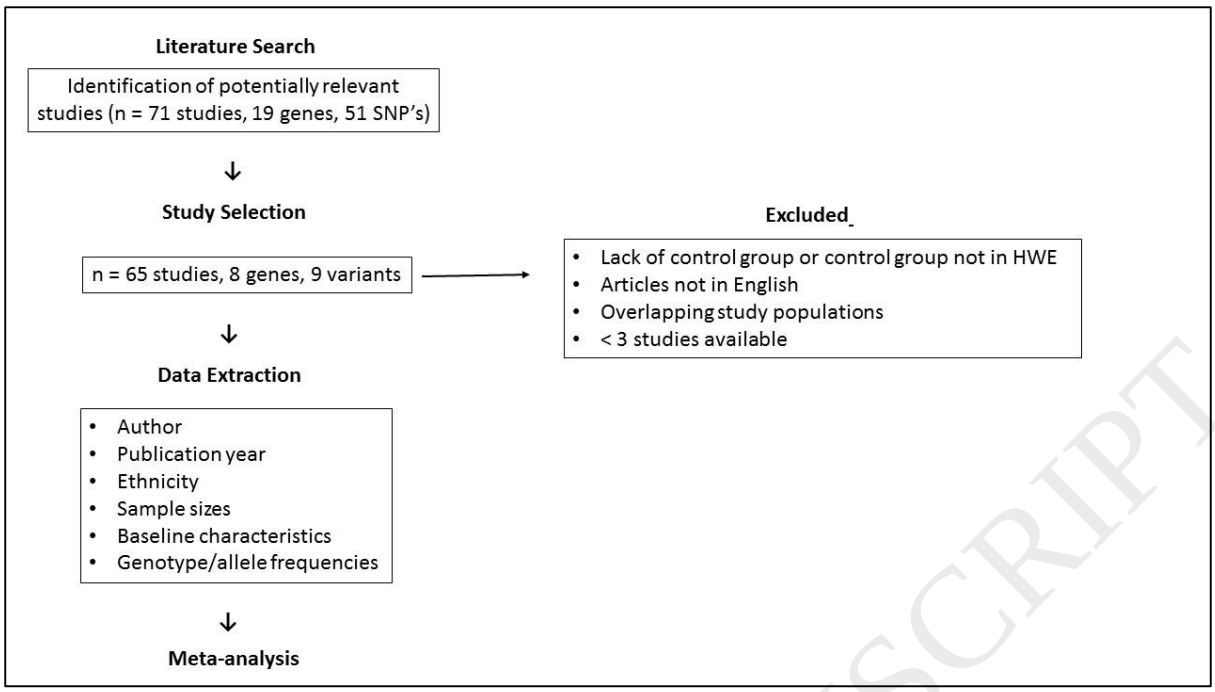


Fig. 1. Literature Search Flow Diagram.

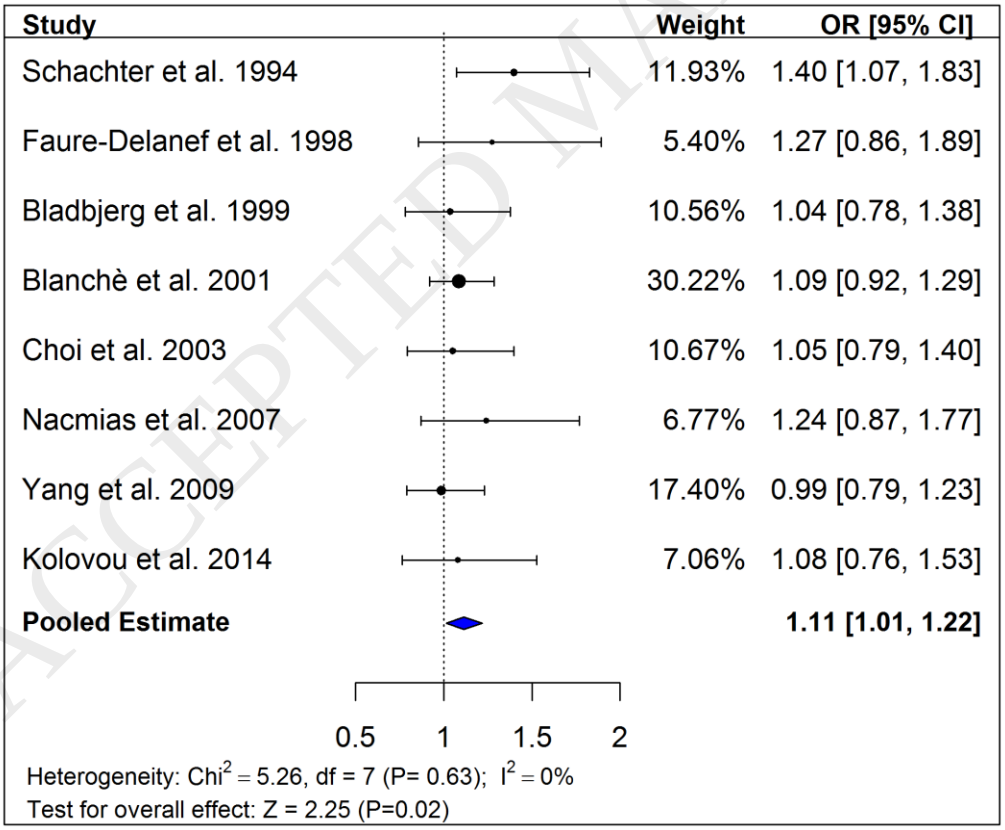
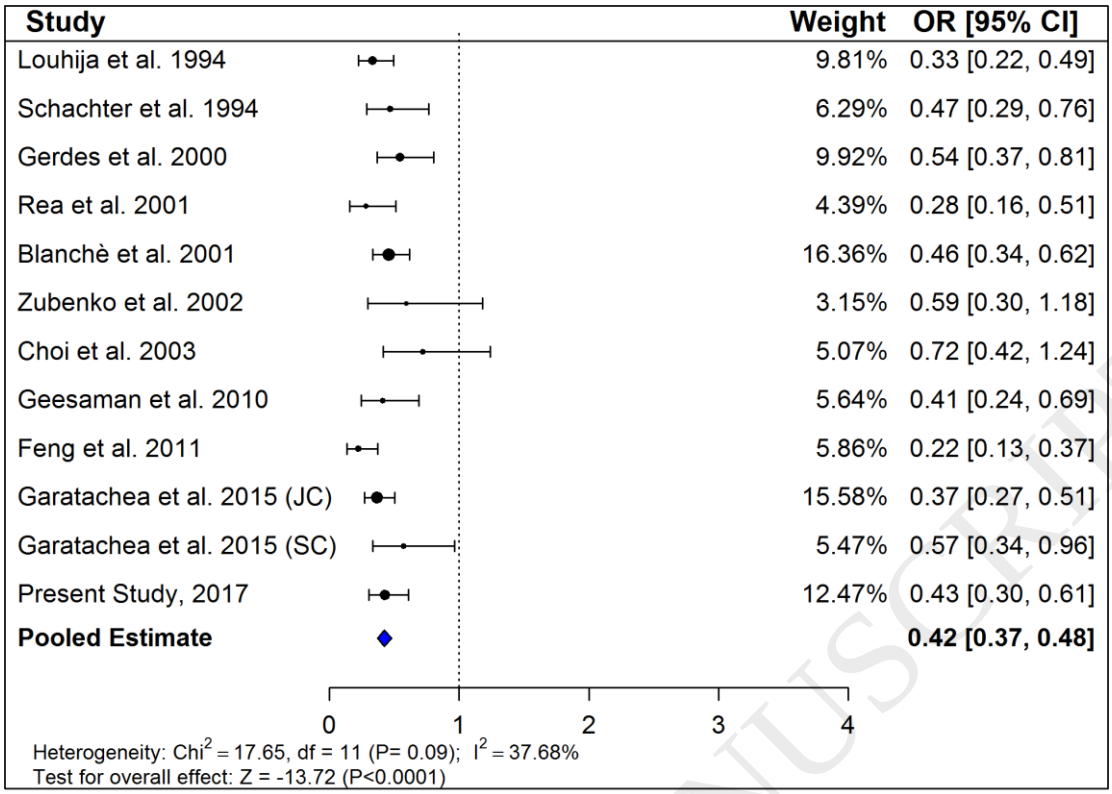


Fig. 2. Meta-analysis of associations between the *ACE* deletion (D) versus insertion (I) alleles and exceptional longevity.



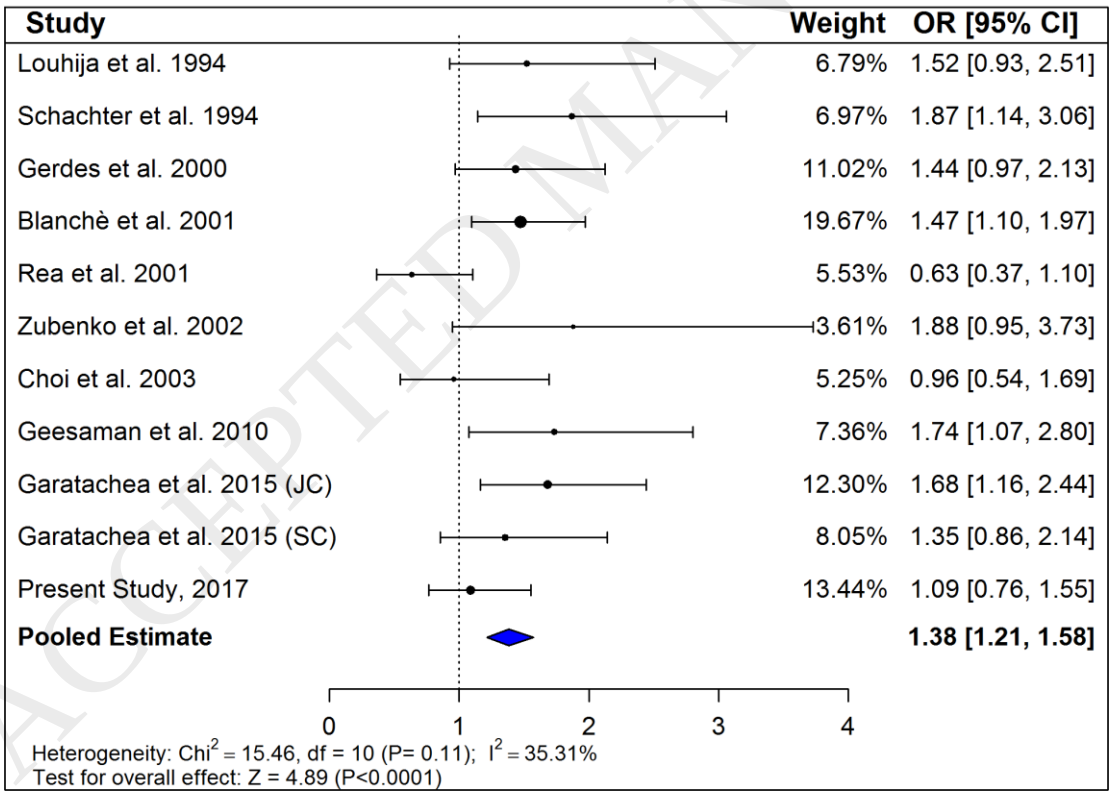


Fig. 3. Meta-analysis of associations between the *APOE* alleles and exceptional longevity: ε4 vs ε3 (upper panel) and ε2 vs ε3 (lower panel).

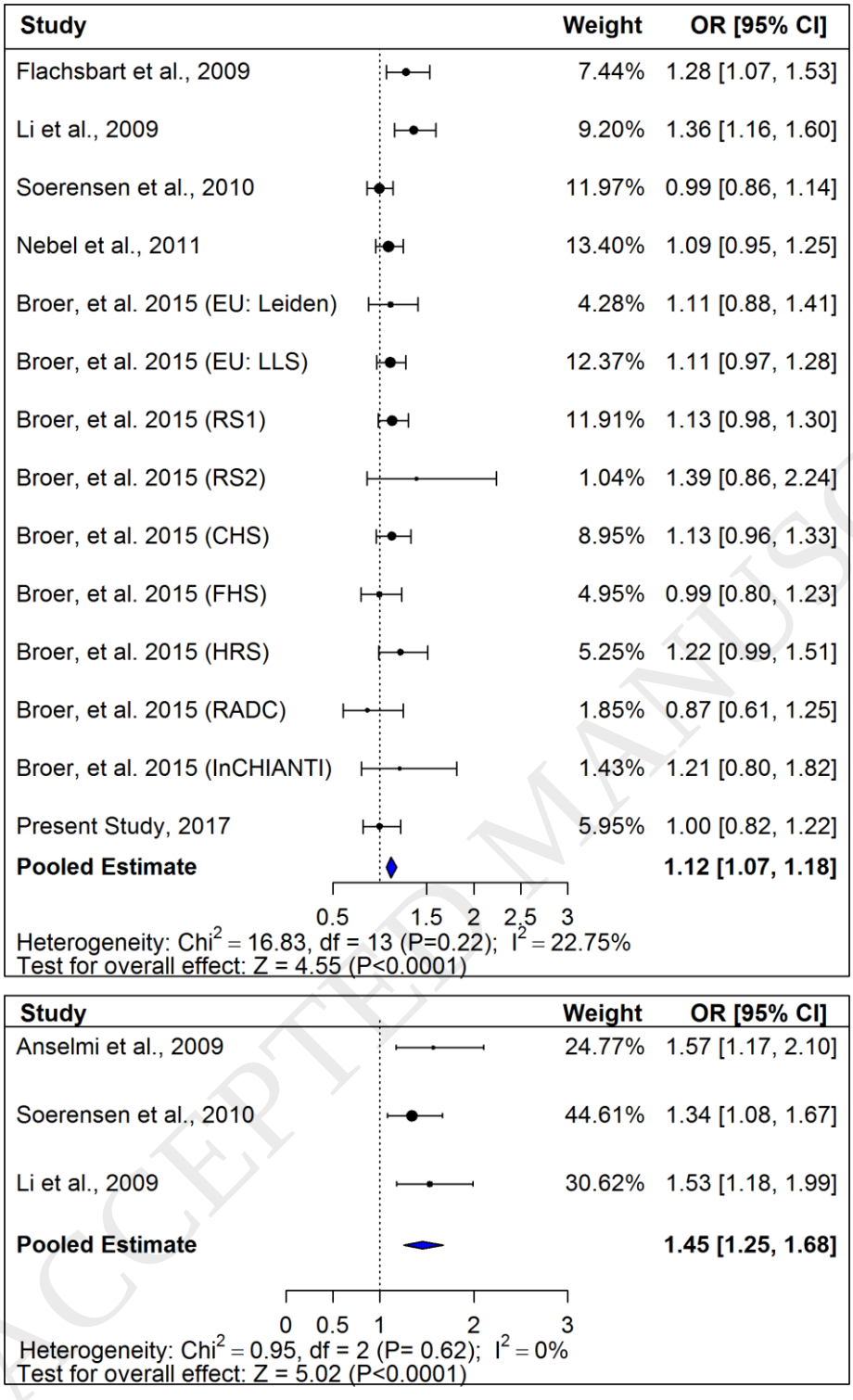


Fig. 4. Meta-analysis of associations between *FOXO3* rs2802292 alleles G vs T and exceptional longevity (top-panel) and within male samples only (lower-panel).

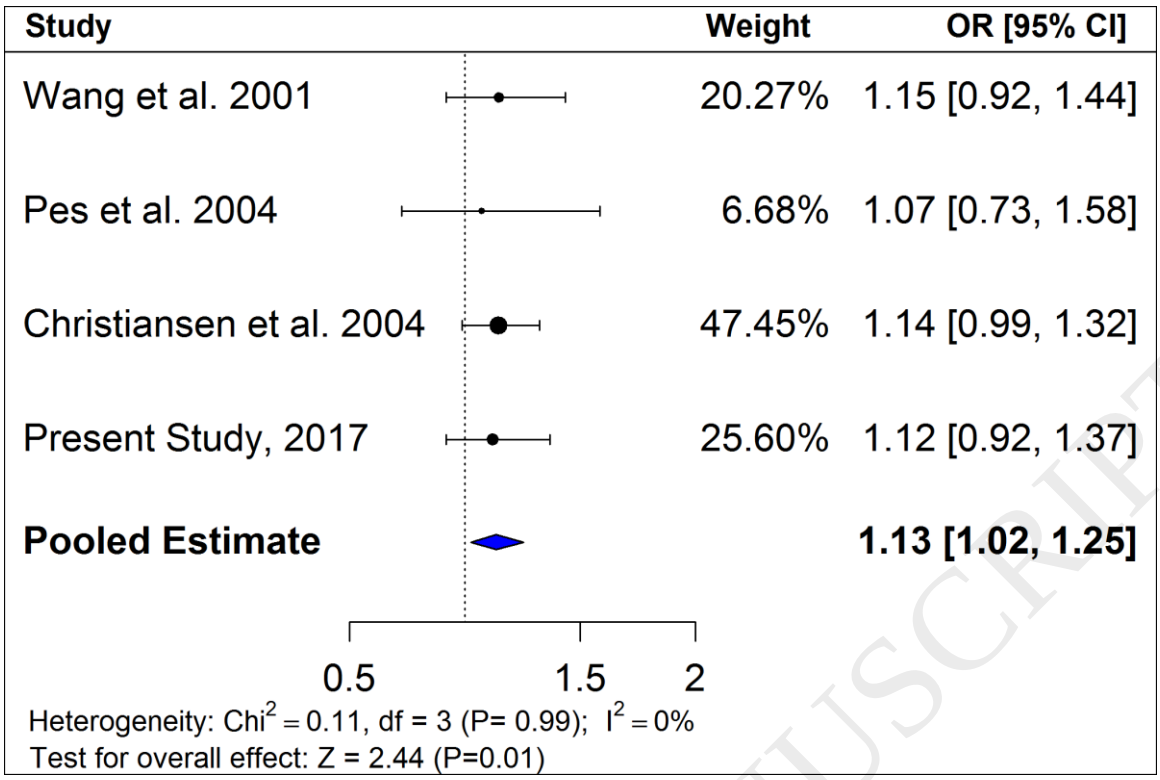


Fig. 5. Meta-analysis of associations between *IL6* rs1800795 alleles G vs C and

et exceptional longevity.

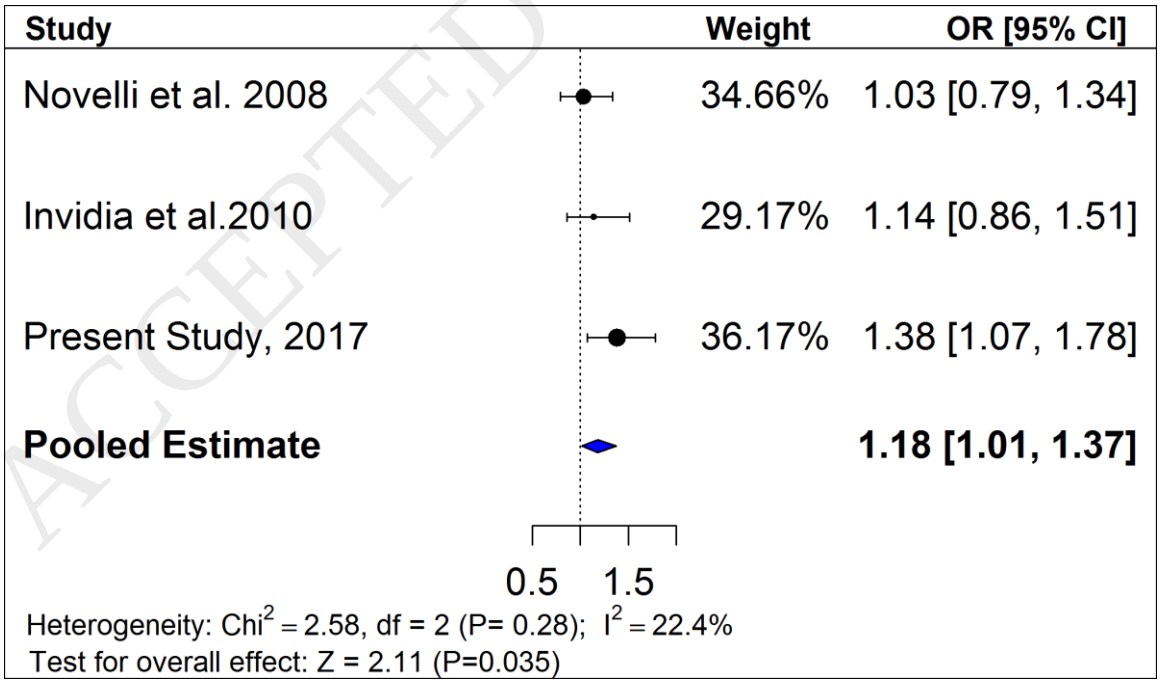


Fig. 6. Meta-analysis of associations between *KLOTHO* KL-VS versus wild-type haplotype and exceptional longevity.

Table 1: Characteristics of investigated exceptional longevity genes

Gene	Chromosome Number	Protein	Variant (rs identification)	Common Variant Name	Function/ pathway
ACE	17	Angiotensin-converting enzyme	**Deletion/Insertion** (Tagging SNP's: rs4340, rs1799752, rs13447447)	Insertion/Deletion (*Alu* repeats)	Regulates blood pressure (Renin-angiotensin system); Balances fluids & salts
APOE	19	Apolipoprotein-E	rs7412 & rs429358 haplotype	ϵ2/ϵ3/ϵ4	Maintaining normal levels of cholesterol; Clearance of amyloid from the brain; Lipoprotein metabolism
CETP	16	Cholesteryl ester transfer protein	rs5882	I405V	Involved in the transfer of neutral lipids (including cholesteryl ester & triglyceride) among lipoprotein particles
CETP	16	Cholesteryl ester transfer protein	rs708272	Taq1B (B1 vs B2)	As above
FOXO3A	6	Forkhead box 03 transcription factor	rs2802292	N/A	Transcription factor involved in diverse cellular pathways e.g. apoptosis
IL6	7	Interleukin 6	rs1800795	G174C	Immune defence: Cytokine pro-inflammatory & anti-inflammatory myokine
KLOTHO	13	Klotho	Haplotype defined by six SNPs in total linkage disequilibrium (e.g. rs9536314, rs9527026)	F352V (KL-VS vs *wt*)	Inflammation, oxidative stress, insulin signalling, calcium & phosphate homeostasis affecting growth & maintenance of bone strength
SIRT1	10	Class 1 sirtuin protein	rs3758391	N/A	(NAD)-dependent deacetylase affecting a variety of substrates
TNFa	6	Tumour necrosis factor-alpha	rs1800629	G308A	Cytokine, Regulation of immune cells & Inflammation