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Birth cohort differences in the prevalence of longevity-associated variants in *APOE* and *FOXO3A* in Danish long-lived individuals

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Abstract

Gene variants found to associate with human longevity in one population rarely replicate in other populations. The lack of consistent findings may partly be explained by genetic heterogeneity among long-lived individuals due to cohort differences in survival probability. In most high-income countries the probability of reaching e.g. 100 years increases by 50–100% per decade, i.e. there is far less selection in more recent cohorts. Here we investigate the cohort specificity of variants in the *APOE* and *FOXO3A* genes by comparing the frequencies of the *APOE* ε4 allele and the minor alleles of two variants in *FOXO3A* at age 95+ and 100+ in 2,712 individuals from the genetically homogeneous Danish birth cohorts 1895–96, 1905, 1910–11, and 1915.

Generally, we find a decrease in the allele frequencies of the investigated *APOE* and *FOXO3A* variants in individuals from more recent birth cohorts. Assuming a recessive model, this negative trend is significant in 95+ year old individuals homozygous for the *APOE* ε4 allele (P = 0.026) or

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Conflict of Interest
The authors declare no conflict of interest.

for the *FOXO3A* rs7762395 minor allele (P = 0.048). For the *APOE* ε4 allele, the significance is further strengthened when restricting to women (P = 0.006). Supportive, but non-significant, trends are found for two of the three tested variants in individuals older than 100 years.

Altogether, this indicates that cohort differences in selection pressure on survival to the highest ages are reflected in the prevalence of longevity gene variants. Although the effect seems to be moderate, our findings could have an impact on genetic studies of human longevity.

Keywords

Human Longevity; Genetics; Cohort Effects; Selection; Apolipoprotein E (*APOE*); Forkhead Box O3A (*FOXO3A*)

1. Introduction[1]

Only two genes have been consistently found to associate with human lifespan: the highly validated apolipoprotein E (*APOE*) gene, which repeatedly has been shown to associate with survival into old age (Schachter *et al.* 1994; Gerdes *et al.* 2000; Bathum *et al.* 2006; Jacobsen *et al.* 2010; Deelen *et al.* 2011; McKay *et al.* 2011; Nebel *et al.* 2011; Soerensen *et al.* 2013), and the forkhead box O3A (*FOXO3A*) gene, which more recently has been found to associate with human longevity in various populations (Willcox *et al.* 2008; Anselmi *et al.* 2009; Flachsbart *et al.* 2009; Li *et al.* 2009; Pawlikowska *et al.* 2009; Soerensen *et al.* 2010).

The limited number of genes known to associate with human longevity could potentially be explained by the complexity of the trait. Another possibility is that human longevity is likely to be affected by many small and low-frequent genetic effects (Christensen *et al.* 2006) as well as structural genetic variations and epigenetic changes (Murabito *et al.* 2012; Tan *et al.* 2013).

However, the lack of reliable and significant findings could also in part be due to heterogeneity among long-lived cases. Genetic studies of human longevity tend to focus on individuals having reached a given age, e.g. 90, 95 or 100 years, without considering the fact that the survival probability has changed dramatically over cohorts. During the past two centuries, record life expectancy in the industrialized countries has improved with a remarkable rate of 3 months per year (Oeppen & Vaupel 2002; Christensen *et al.* 2009), resulting in an increase of 50–100% per decade in the number of individuals surviving to extreme ages, e.g. 100 years, in many countries (Jeune & Kannisto 1997). Therefore, an interaction between birth cohort and the effect of longevity genes may be expected, and the reduced selection pressure on high age survival for more recent birth cohorts could be important to consider in genetic studies of long-lived individuals. A decrease in selection pressure for more recent birth cohorts could, on the one hand, be expected to increase the survival of persons carrying frailty genes, i.e. the frailty gene frequency would also increase

[1]Abbreviations: *APOE*, apolipoprotein E; *FOXO3A*, forkhead box O3A; LSDC, Longitudinal Study of Danish Centenarians; DCRS, Danish Civil Registration System; 5-COOP, 5-Country Oldest Old Project.

(Vaupel *et al.* 1979). On the other hand, the reduction in mortality in later cohorts, e.g. due to improved living conditions and better health care, could imply that the effect of genetic factors as a cause of mortality would increase.

Here we explore the effect of changes in selection pressure over birth cohorts on the frequencies of the *APOE* ε4 allele and the minor alleles of two *FOXO3A* variants, rs7762395 and rs479744, previously reported to associate with longevity in Danish nonagenarians and centenarians (Soerensen *et al.* 2010), using cohorts of Danish long-lived individuals older than 95 years born in 1905 and 1915, and older than 100 years born in 1895–96, 1905 and 1910–11. The selection pressure has changed considerably over these birth cohorts, and together with the genetic homogeneity of the cohorts and the minimal immigration, this is an ideal setup for addressing the aspect of cohort differences in the prevalence of longevity-associated gene variants.

2. Materials and Methods

2.1. Study Population

The study was based on Danish Birth Cohort Study participants born in 1895–96, 1905, 1910, 1911 and 1915.

The Danish 1895–96 Birth Cohort Study, also known as the Longitudinal Study of Danish Centenarians (LSDC), consists of all individuals who had reached an age of 100 years in the period from April 1st 1995 to May 31st 1996 (Andersen-Ranberg *et al.* 2001). A total of 276 eligible centenarians were identified through the Danish Civil Registration System (DCRS) (Pedersen *et al.* 2006) and of these, 207 (75.0%) chose to participate in the intake survey. Blood samples were collected from 154 (74.4%) individuals of whom 132 are included in this study.

The Danish 1905 Birth Cohort Study is an in-depth survey of all Danes born in 1905 and living in Denmark in 1998 (Nybo *et al.* 2001). The study was initiated in 1998 and follow-up studies of the participating survivors were carried out in years 2000, 2003 and 2005. At intake there was a total of 3,600 potential participants, of whom 2,262 (62.8%) agreed to take part in the study, and of these 1,651 (73.0%) provided a biological sample. To match the 1915 Birth Cohort (see below) only individuals who had reached an age of at least 95 years were included. This limited the number of potential participants to 2,205, of whom 1,432 (64.9%) participated in the study and 1,188 (83.0%) of these provided a biological sample. Of those who provided a biological sample, 1,169 are included in the present study. All the 1905 Birth Cohort Study participants included in the group of individuals older than 100 years are included in the group of individuals older than 95 years as well. The Danish 1910 Birth Cohort Study is a survey including all Danes born in 1910 and living in Denmark on September 1st 2010. A total of 400 individuals were identified through the DCRS and invited to participate in the survey, which 273 (68.3%) individuals agreed to (data not published). Blood samples were retrieved from 176 (64.5%) individuals, all of whom are included in the present study.

The Danish 1911–12 Birth Cohort Study is part of the international 5-Country Oldest Old Project (5-COOP), which intends to evaluate the health of the oldest old and make comparisons between the five participating countries, namely Denmark, France, Japan, Sweden and Switzerland (Robine *et al.* 2010). The study includes a random sample of 251 (48.5%) Danish individuals chosen from 518 individuals who had reached an age of 100 years in the period from April 1st 2011 to July 1st 2012 (data not published). Blood samples were collected from 202 (80.5%) individuals, of whom 130 (all born in 1911) are included in this study.

Due to the close proximity in birth year of the study population participants from the 1910 Birth Cohort Study and the 1911–12 Birth Cohort Study, these birth cohorts were grouped together (the 1910–11 cohort) in the present study. Prior to this grouping, the genotype distributions among the birth cohorts were compared (using a Chi^2-test) to ensure that no bias would be introduced by the merging.

The Danish 1915 Birth Cohort Study includes all Danes born in 1915 and living in Denmark on September 1st 2010. A total of 2,509 individuals were identified though the DCRS as eligible participants, with 1,584 (63.1%) individuals participating in the study (Christensen *et al.* 2013). A biological sample was provided by 1,165 (73.5%) individuals, of whom 1,105 are included in this study.

Study approvals were received from the Danish National Committee on Biomedical Research Ethics.

2.2. Genotyping

DNA was isolated from whole blood or from blood spot cards using either the QIAamp DNA Mini and Micro Kits (Qiagen, Hilden, Germany), the Extract-N-Amp™ Blood PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) or salting out applying a manual protocol or a semi-automated protocol based on the Autopure System (Qiagen, Hilden, Germany). For 336 of the samples, DNA was amplified using the GenomePlex® Complete Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO, USA) prior to genotyping.

Genotyping of the *APOE* variants rs429358 and rs7412 and the *FOXO3A* variants rs7762395 and rs479744 were primarily carried out using predesigned TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) with genotyping efficiencies of between 96,8% and 99,7%. For 641 of the 1905 Birth Cohort Study participants, genotyping of the *FOXO3A* variants were performed as part of a previous study using the Illumina GoldenGate Technology (Illumina Inc, San Diego, CA, USA) as described by Soerensen et al. 2012 (Soerensen *et al.* 2012).

2.3. Statistical Analysis

Frequencies among individuals older than 95 years from the 1905 and the 1915 birth cohorts were compared using a two-sample z-test, whereas trend analyses of frequencies among individuals older than 100 years born in 1895–1896, 1905 and 1910–1911 were performed applying a Chi^2-test for trend.

An additive model was applied as the baseline model. In addition, dominant and recessive models were applied for the *APOE* gene and a recessive model was applied for the *FOXO3A* gene.

Due to the a priori hypothesis of an association of the investigated genes with longevity, only uncorrected P-values are reported.

Data on changes in survival probabilities for the birth cohorts was retrieved from the Human Mortality Database (www.mortality.org).

3. Results

Characteristics of the study population are shown in Table 1. As expected, a higher number of long-lived women than men are present in the cohorts, with men comprising between 16.5% and 27.3%.

The probability of surviving from birth to age 95 (Figure 1A) and age 100 (Figure 1B) in Denmark is illustrated in Figure 1 (Human Mortality Database, www.mortality.org). Over the last four decades, the probability for both sexes combined of surviving to age 95 and 100 has increased from around 1% to 3.5%, and from around 0.2% to 0.6%, respectively. The increase has been much steeper for women than for men with the probability for reaching 95 years increasing from approximately 1.5% to 6% for women compared to an increase from 1% to 1.5% for men, and with the probability of reaching 100 years increasing from approximately 0.2% to 1% for women compared to an increase from 0.1% to 0.2% for men.

A comparison between the birth cohorts of allele frequencies and frequencies of carriers of the *APOE* ε4 allele and the minor alleles of the *FOXO3A* variants rs7762395 and rs479744 is seen in Figure 2 and 3. In individuals older than 95 years, the prevalence of the *APOE* ε4 allele (Figure 2A) and of *APOE* ε4 allele carriers (Figure 2B and 2C) is generally lower in individuals born in 1915 than in individuals born in 1905, although this tendency is only significant ($P = 0.026$) when assuming a recessive model for men and women combined (Figure 2C). When restricting to women, the negative trend of the *APOE* ε4 allele prevalence or of the prevalence of individuals homozygous for the *APOE* ε4 allele is significant, assuming an additive ($P = 0.034$) or a recessive ($P = 0.006$) model, respectively (separate results for women and men are shown in Supplementary Data, Supplementary Table 1 and 2). For the minor allele frequencies of the *FOXO3A* variants rs7762395 and rs479744 similar negative trends are seen, although these are not statistically significant (Figure 3A). When applying a recessive model (Figure 3B), the prevalence of rs7762395 minor allele homozygotes is significantly lower ($P = 0.048$) among individuals born in 1915 compared to individuals born in 1905, and rs479744 shows a similar borderline significant tendency ($P = 0.073$).

The trends of decreasing prevalence with more recent birth cohort seen in individuals older than 95 years are generally mirrored in individuals older than 100 years (Figures 2D–F and 3C–D), at least for the *APOE* ε4 allele and the *FOXO3A* rs479744 variant, although none of them reach statistical significance.

No significant differences in the prevalence of the *APOE* ε2 allele or of *APOE* ε2 allele carriers are found across the cohorts at age 95+ or 100+ (data not shown). However, the tendency of a higher prevalence in the more recent cohort seen in the larger sample of nonagenarians is consistent with an overall conclusion of the *APOE* gene becoming more important for mortality in more recent cohorts.

4. Discussion

The aim of this study was to explore whether the pronounced improvement in survival probability and the related reduction in selection pressure seen over recent cohorts of long-lived Danes were reflected in the prevalence of variants in known longevity-associated genes.

Generally, we found that the prevalence of the investigated variants in the *APOE* and *FOXO3A* genes was differing between cohorts born over a 20-year period from 1895 to 1915. A comparison of individuals older than 95 years born in 1905 and 1915 revealed a lower prevalence in the 1915 cohort, both of the *APOE* ε4 allele and of the minor alleles of the *FOXO3A* variants rs7762395 and rs479744. This was particularly evident when applying a recessive model and the trend was generally supported when we compared individuals older than 100 years born with an interval of 5–10 years from 1895 to 1911. None of the comparisons in the 100+ year olds reached statistical significance, probably due to lower power as a consequence of fewer participants compared to the group of 95+ year olds.

The *APOE* ε4 allele has previously been found to associate with increased mortality (Christensen *et al.* 2006), and the lower prevalence seen in the more recent birth cohorts is consistent with ε4 noncarriers having an increasingly larger survival advantage compared to ε4 carriers. It can be speculated that this could cause the mortality of individuals to a greater extent to be dependent on their *APOE* genotype, since competing causes of death have been eliminated due to improved living conditions and better medical treatment in the more recent birth cohorts (Oeppen & Vaupel 2002; Christensen *et al.* 2009). This line of thought is, however, opposite to what would be expected from the heterogeneity hypothesis that a selection pressure in older cohorts would decrease the effect of frailty gene variants such as the *APOE* ε4 allele when compared to younger cohorts (Vaupel *et al.* 1979). This is supported by the findings in a meta-study by Ewbank suggesting that the mortality by *APOE* genotype diminished at the oldest ages (Ewbank 2007). Also, a decline in the risk over age for carriers of the ε4 allele was found when using a statistical model incorporating heterogeneity (Ewbank 2002). The lack of concordance between the effect of the *APOE* gene and the heterogeneity hypothesis seen in the present study has previously been suggested in a longitudinal study of 92+ year olds examining the survival of *APOE* ε4 carriers versus non-carriers (Jacobsen *et al.* 2010). One possible explanation for the discrepancy between Ewbank (Ewbank 2002; Ewbank 2007), and the present study and Jacobsen et al. 2010 may be the different age groups studied, since *APOE* may have different effects on survival at different ages (Jacobsen *et al.* 2010).

For the *FOXO3A* minor alleles, which associate with longevity and not mortality, we found a decrease in minor allele frequency in the more recent birth cohorts. This is what we would

expect from the heterogeneity hypothesis, leaving alive persons with the frailer version of the *FOXO3A* genotype and with other factors compensating for this. Since the role of *FOXO3A* in longevity is still largely unknown, more studies are needed to explain the pattern seen for this gene in the present study.

Since the probability of surviving to extreme ages has increased much faster in women than in men (see Figure 1), sex-stratified analyses were carried out (see Supplementary Data, Supplementary Table 1 and 2). Significant decreases were found for the prevalence of the *APOE* ε4 allele or of individuals carrying two ε4 alleles when we compared 95+ year old women born in 1905 and 1915 and assumed an additive or a recessive model. In contrast, non-significant increases were seen in men. These sex-stratified results are as predicted by the theory presented above, with a bigger decrease in selection pressure resulting in a larger influence of the *APOE* genotype as a cause of death, which is exactly what is seen for women. For the *FOXO3A* variants, no general differences between women and men are seen. There is a vague tendency for bigger differences between the birth cohorts in men, but inference is difficult due to the relatively small number of men.

Traditionally, it has been difficult to replicate primary genetic association findings in aging research, and only a few genes have been found to associate with longevity across populations. The results of this study suggest that birth year and population-dependent differences in selection pressure may be part of the explanation for this general lack of replication, although our data points to only small genetic differences between the investigated birth cohorts. However, the genetic variations related to longevity are currently expected to be rare and/or have small effects, and therefore even modest cohort effects could, when unaccounted for, confound results and leave true associations undiscovered.

A potential problem with this study might be bias due to differences in participation rate, e.g. if fewer sick or disabled individuals are included in a study, it could reduce the *APOE* ε4 frequency. Thus, it cannot be excluded that part of the genetic difference seen between the cohorts is due to cohort differences in the participation rate. However, the significant cohort differences in the prevalence of the investigated alleles are seen in individuals older than 95 years from the 1905 and 1915 birth cohorts, which have only very small differences in participation rates (Christensen *et al.* 2013).

The genetic differences over cohorts found in this study are substantial considering that the birth year intervals are less than two decades. Also, Denmark is among the high-income countries with the smallest increase in survival among the oldest-old and thus larger cohort differences in the allele distribution of longevity-associated genes may be expected in countries with a more pronounced increase in the number of nonagenarians and centenarians.

Altogether, our results point toward the need to consider the birth cohort of long-lived individuals in future genetic studies of human longevity. The results presented in this study are based on a unique collection of samples. Most other longevity samples contain individuals from a wide range of birth cohorts, and therefore the results of this study may be

difficult to replicate directly. Studies of longevity genes may, however, benefit from being stratified into decades of birth year.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The chance of surviving to age 90+ has increased dramatically over the last decades
- We studied 2,712 long-lived individuals from four Danish cohorts born 1895–1915
- Allele frequencies of variants in the longevity genes *APOE* and *FOXO3A* were compared
- Moderate cohort differences in allele frequencies were observed for both genes
- This indicates an interaction between birth cohort and longevity gene effects

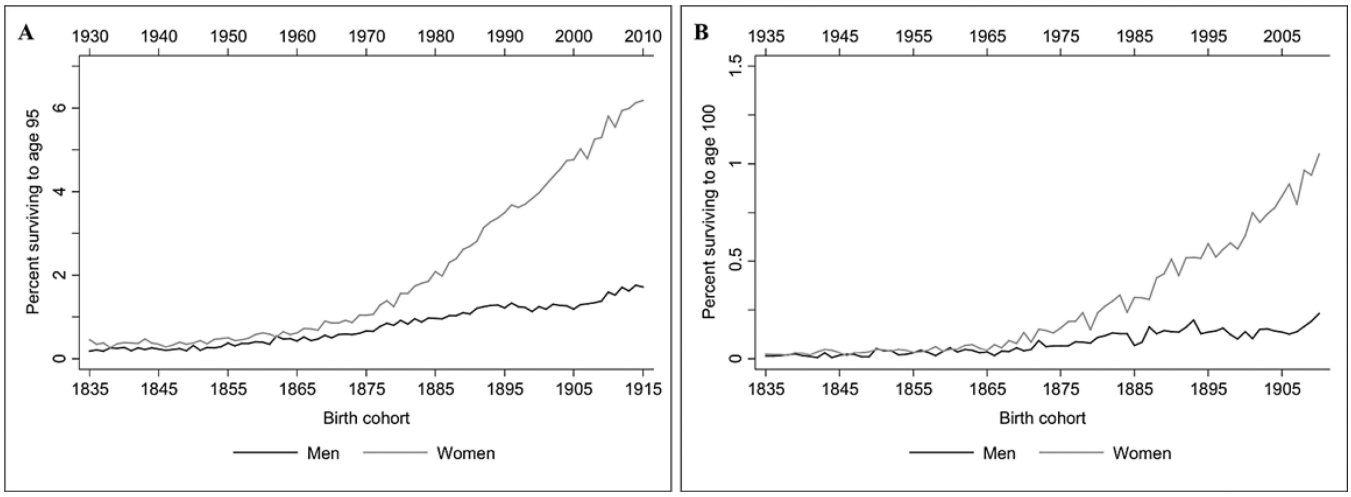

Figure 1.

The probability of surviving in Denmark from birth to age 95 in the period 1835 to 1915 (A) and from birth to age 100 in the period 1835 to 1910 (B).

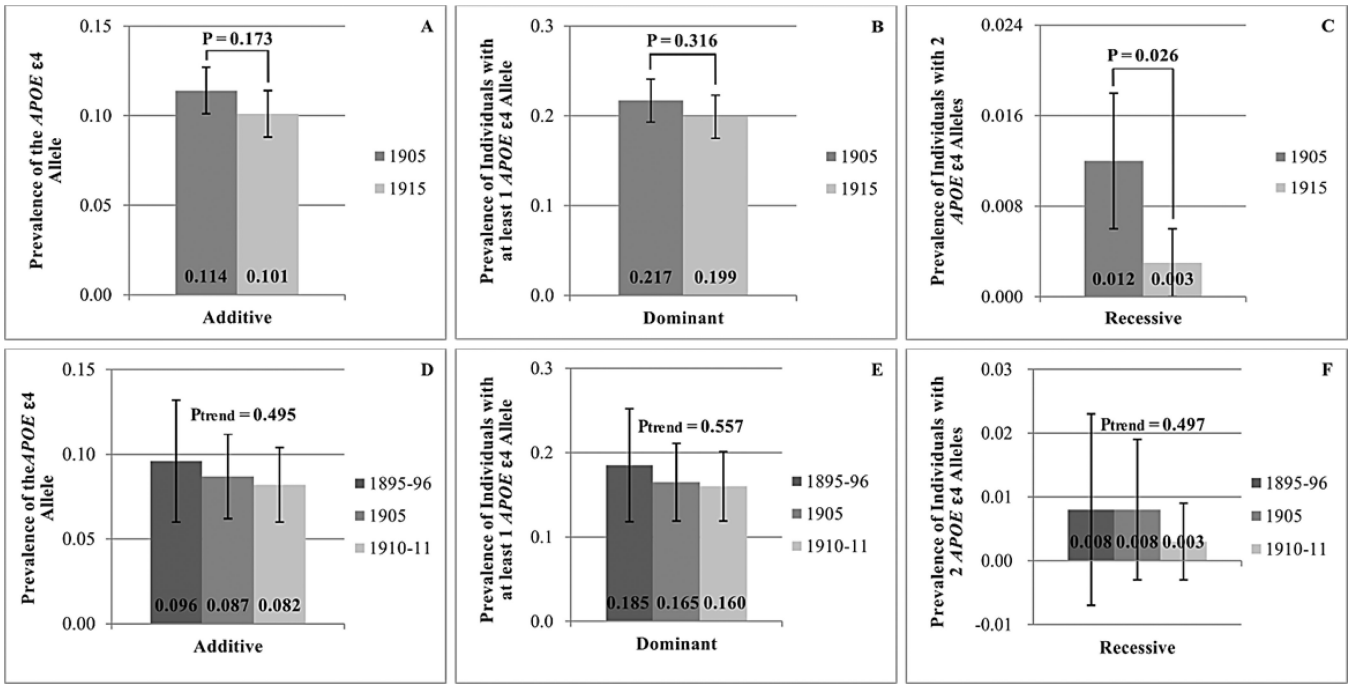

Figure 2.

The prevalence of the *APOE* ε4 allele and of *APOE* ε4 allele carriers in individuals older than 95 years born in 1905 and 1915 (A–C) and in individuals older than 100 years born in 1895–96, 1905 and 1910–11 (D–F) assuming an additive (A, D), a dominant (B, E) and a recessive (C, F) model. The 95% confidence interval is shown as well as the P-value for the significance of the difference between the birth cohorts (A–C) or of the significance of the trend (D–F).

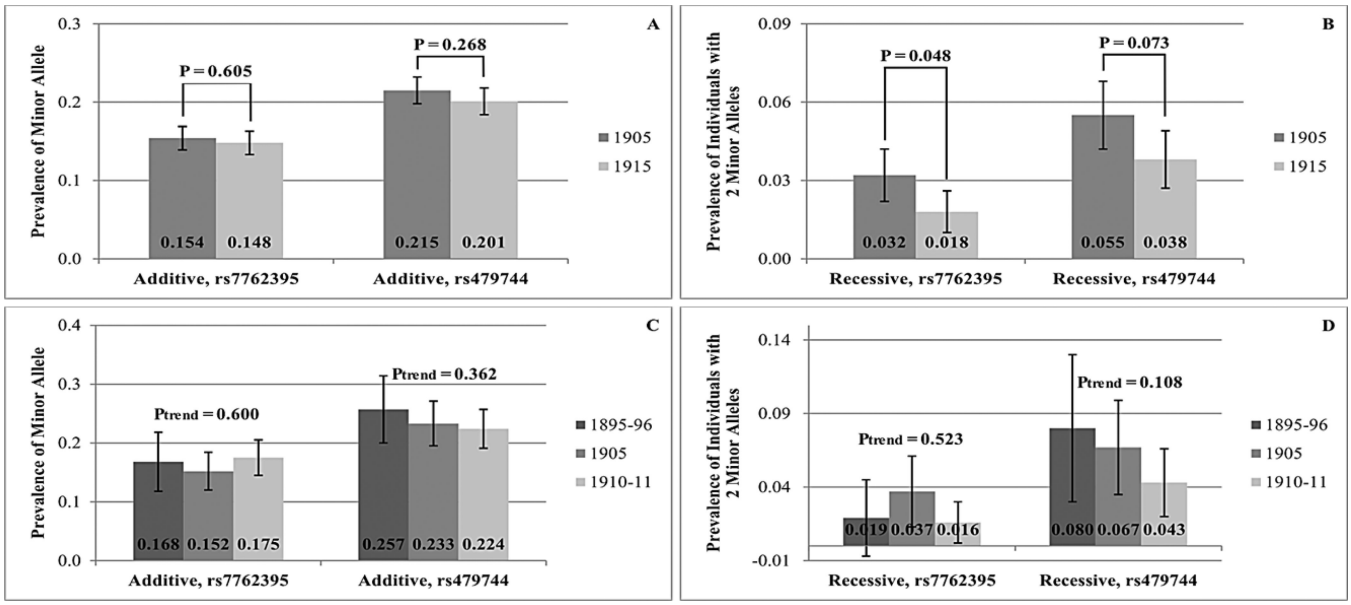

Figure 3.

The prevalence of the *FOXO3A* rs7762395 and rs479744 minor alleles or of minor allele carriers in individuals older than 95 years born in 1905 and 1915 (A, B) and in individuals older than 100 years born in 1895–96, 1905 and 1910–11 (C, D) assuming an additive (A, C) and a recessive (B, D) model. The 95% confidence interval is shown as well as the P-value for the significance of the difference between the two birth cohorts (A, B) or of the significance of the trend (C, D).

Table 1

Characteristics of the study population.

Birth Cohort	Mean Age	Age Range	N	% Men
1895–96	100.1	99.0–100.4	132	22.7
1905	95	-	1169	24.5
1905	100	-	255	16.5
1910	100.2	99.7–100.9	176	24.4
1911	100.2	99.8–101.1	130	23.1
1915	95.3	94.7–95.9	1105	27.3