

Replication studies in longevity: puzzling findings in Danish centenarians at the 3'APOB-VNTR locus

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SUMMARY

In Danes we replicated the 3'APOB-VNTR gene/longevity association study previously carried out in Italians, by which the Small alleles (less than 35 repeats) had been identified as frailty alleles for longevity. In Danes, neither genotype nor allele frequencies differed between centenarians and 20–64-year-old subjects. However, when Danish and Italian data were compared, a significant difference ($p = 0.0004$) was found between the frequencies of Small alleles in youths, which disappeared in centenarians ($p = 0.290$). Furthermore, the demographic-genetic approach revealed in Danes a significant gene–sex interaction relevant to Long alleles (more than 37 repeats). The different findings in Denmark and Italy suggest that gene/longevity associations are population-specific, and heavily affected by the population-specific genetic and environmental history.

INTRODUCTION

Association studies in centenarians are one of the tools currently used to ascertain information on the genetic components of human longevity. Historically, association studies have been developed for investigating complex diseases, and only recently have they been applied to human longevity. A must in association studies is replication: the positive association found between a locus and a complex trait in a certain population must be confirmed in another population. However, both the case/control design

(De Benedictis, 1996) and the genetic-demographic design (Yashin *et al.* 1998, 1999; Toupance *et al.* 1998) currently used in gene/longevity association studies present methodological problems for representing a dynamic process, such as age-related survival selection, within a static framework. Indeed the structure of the centenarian gene pool is the result not only of the genetic history of the population, but also of unstable environmental factors occurring in the last century, which shaped the demographics of the population. Is positive replication expected in such studies?

To investigate this intriguing question we have replicated in Danish centenarians a study carried out in Italian centenarians, where association between longevity and the 3'APOB-VNTR polymorphism (Boerwinkle *et al.* 1989)

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had been observed (De Benedictis *et al.* 1997, 1998*a*). The 3'APOB-VNTR marker is located less than 100 bp downstream of the second transcription termination signal of the Apolipoprotein B (*APOB*) gene (2p24-p23). The *APOB* gene encodes apolipoprotein B, the main protein in Low Density Lipoprotein (LDL), which plays a major role in cholesterol homeostasis (Goldstein & Brown, 1989). It was observed that, in both northern and southern Italians, 3'APOB-VNTR alleles having less than 35 repeats (Small alleles) are significantly rarer in centenarians than in 20-60-year-old subjects, and can therefore be regarded as frailty alleles for longevity. Although northern and southern Italians have different genetic histories (Cavalli *et al.* 1994, pp. 277-280), both belong to the same south-west European cluster, whereas the Danish population belong to the northern Germanic population (Cavalli *et al.* 1994, pp. 75-78 and pp. 268-270). Moreover, it is well known that cultural (e.g. diet) and environmental factors are quite different between southern and northern Europeans. It seemed worthwhile therefore to replicate the Italian study in Danish centenarians.

MATERIALS AND METHODS

Samples

Centenarians

One hundred and seventy five blood samples were analyzed. One hundred and thirty six were randomly retrieved from the 148 samples collected in the Danish Longitudinal Centenarian Study (DLCS) (Bathum *et al.* 1998; Kristensen *et al.* 1998; Andersen-Ranberg *et al.* 1999; Christensen *et al.* 2000; Gerdes *et al.* 2000). Thirty-nine were collected in the feasibility study of Centenarians in the County of Funen (CFCS) (Olsen *et al.* 1996). A detailed description of the criteria of recruitment in the two studies is given in Bladbjerg *et al.* (1999). Briefly, the DLCS included all persons living in Denmark who celebrated their 100th birthday between 1 April 1995 and 31 May 1996 (276 subjects), and who had agreed to donate a blood sample for the

study (148 subjects). The CFCS included all living subjects in the County of Funen, born before 31 December 1894 and alive by 1 May 1994 (58 subjects), who had agreed to donate a blood sample for the study (39 subjects).

Controls

The control group consisted of two hundred 20-64-year-old Danish blood donors (mean age 42 years). Consecutive donors arriving at the blood bank at Odense University Hospital between 0800 and 1200 hours were asked to participate. Blood donors were healthy without chronic diseases and were not on medication.

Molecular analyses

3'APOB-VNTR typing was performed on PCR-amplified DNA fragments from blood buffy coats, according to the protocols described by Boerwinkle *et al.* (1989) and already used in the two Italian studies previously carried out (De Benedictis *et al.* 1997, 1998*a*). Primers and PCR conditions are available on request (g.debenedictis@unical.it). Allelic designation referred to the number of repeats in the core sequence motif.

Statistical analyses

Allele frequencies were estimated by gene counting from the observed genotypes. Hardy-Weinberg Equilibrium (HWE) was verified by the G test (Sokal & Rohlf, 1981). The significance level of the G-statistic was determined with reference to its empirical distribution generated by a shuffling algorithm (Chakraborty *et al.* 1991). Permutation tests (10000 simulations) were used to check for difference in frequency distributions between centenarians and younger individuals (Weir, 1996).

To compare Danish and Italian findings, following the studies previously carried out (De Benedictis *et al.* 1997 and 1998*a*), 3'APOB-VNTR alleles having less than 35 repeats were grouped as Small alleles. Frequency differences between Danish and Italian data were then checked by χ^2 test. The demographic-genetic

Table 1. Absolute and relative frequencies ($\times 100$) of 3'APOB-VNTR alleles in 20–64-years-old subjects and in centenarians from Denmark. Standard errors in parentheses. Allele nomenclature refers to the number of VNTR repeats

Alleles	Centenarians				Controls			
	Males		Females		Males		Females	
	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
26	1	1.2 (1.2)	1	0.4 (0.4)	—	—	—	—
29	—	—	—	—	1	0.4 (0.4)	—	—
31	9	10.5 (3.3)	18	6.8 (1.6)	16	6.3 (1.5)	12	8.1 (2.2)
33	5	5.8 (2.5)	15	5.7 (1.4)	7	2.8 (1.0)	7	4.7 (1.7)
35	19	22.1 (4.5)	55	20.8 (2.5)	63	25.0 (2.7)	36	24.3 (3.5)
36	—	—	—	—	—	—	1	0.7 (0.7)
37	36	41.9 (5.3)	109	41.3 (3.0)	103	40.9 (3.1)	59	39.9 (4.0)
39	3	3.5 (2.0)	10	3.8 (1.2)	13	5.2 (1.4)	5	3.4 (1.5)
41	1	1.2 (1.2)	5	1.9 (0.8)	3	1.2 (0.7)	1	0.7 (0.7)
45	—	—	1	0.4 (0.4)	2	0.8 (0.6)	1	0.7 (0.7)
47	5	5.8 (2.5)	22	8.3 (1.7)	15	6.0 (1.5)	13	8.8 (2.3)
49	7	8.1 (2.9)	26	9.8 (1.8)	20	7.9 (1.7)	11	7.4 (2.2)
51	—	—	—	—	4	1.6 (0.8)	1	0.7 (0.7)
53	—	—	2	0.8 (0.5)	4	1.6 (0.8)	1	0.7 (0.7)
55	—	—	—	—	1	0.4 (0.4)	—	—
Total	86	100.0	264	100.0	252	100.0	148	100.0

model described by Yashin *et al.* (2000) was used to estimate allelic relative risk (r.r.) and allele–sex interaction relative risk (r.r.g.s.) of specific 3'APOB–VNTR alleles (a description of the model is given in Appendix I). According to 3'APOB–VNTR frequency pattern (peaks at 31, 37 and 49 repeats) the multiallelic system was recoded as triallelic, with Small, Medium and Large alleles (< 35 repeats; 35–37; > 37 repeats respectively).

RESULTS

The observed genotypic frequencies fitted with those expected under HWE in both centenarians and younger subjects, and in both sexes (data not shown). Table 1 shows the allelic distributions in centenarians and controls according to sex. These distributions did not differ between centenarians and controls either in males ($p = 0.759$ by permutation test) or in females ($p = 0.912$ by permutation test). Likewise, no significant difference was found for the genotypic distributions between centenarians and controls.

Studies previously carried out in Italians

showed that the frequency of Small alleles (alleles having less than 35 repeats) was significantly lower in centenarians than in younger individuals (De Benedictis *et al.* 1997), and differs in the genetic pool according to the age of the cohort (De Benedictis *et al.* 1998a). Data in Table 1 were therefore recoded according to the Italian studies, in order to compare Danish and Italian findings. Results are shown in Table 2. The frequency distribution of Small/non-Small alleles was highly different between Danish and Italian controls ($p = 0.0004$ by χ^2 test with 1 D.F.), whereas it was not different between Danish and Italian centenarians ($p = 0.290$ by χ^2 test with 1 D.F.).

The demographic-genetic analysis was then applied to the data in Table 1, again recoding the multiallelic system according to the previous study (De Benedictis *et al.* 1998a). The results are shown in Table 3; they confirmed that the Small alleles are not risk factors for longevity in Denmark (r.r. = 0.952 with 0.05 s.e.). Moreover, a significant gene–sex interaction was observed for Long alleles (r.r.g.s. = 1.163 with 0.06 s.e.), implying that the risk in male carriers is increased with respect to female carriers.

Table 2. *Absolute and relative frequencies ($\times 100$) of 3' APOB-VNTR alleles having less than 35 repeats (Small, S) in 20–64-year-old subjects and in centenarians from Italy and Denmark. Standard errors in parentheses. p^* refers to the difference between samples of different ages from the same geographic area. p^{**} refers to samples of the same age from different geographic areas*

	Danes				Italians§				p^{**}
	S		Non S		S		Non S		
	Abs	Rel	Abs	Rel	Abs	Rel	Abs	Rel	
20–60 years	43	10.8 (1.5)	357	89.2	64	20.3 (2.3)	252	79.7	0.0004
≥ 100 years	49	14.0 (1.9)	301	86.0	32	11.2 (1.9)	254	88.8	0.290
p^*			0.176				0.002		

§ Data re-elaborated from De Benedictis *et al.*, 1997 and 1998a.

Table 3. *Relative Risk Model applied to Danish 3' APOB–VNTR data. The risk is estimated for carriers of S, M and L alleles (both homozygotes and heterozygotes). Standard error in parentheses*

Alleles	Risk of gene	Risk of gene–sex interaction
Small (< 35 repeats)	0.952 (0.05)	0.959 (0.07)
Medium (35–37 repeats)	1.026 (0.08)	0.966 (0.10)
Large (> 37 repeats)	0.936 (0.05)	1.163 (0.06)

DISCUSSION

On the basis that association studies of complex traits require replication in different populations, we investigated in Danish centenarians the association study between the APOB–VNTR polymorphism and longevity, which had given positive results in Italian centenarians (De Benedictis *et al.* 1997, 1998a).

The data in Table 1 indicated that the 3' APOB–VNTR polymorphism is not associated with longevity in Danes, but an intriguing observation was made when Danish and Italian data were compared (Table 2). The genetic pools of Danish and Italian young individuals were different ($p = 0.0004$), as expected on the consideration of the different genetic and environmental history of these populations, while such a difference disappeared between Italian and Danish centenarians ($p = 0.290$). In individuals who had survived to old age the frequency of Small alleles was almost the same in Denmark and Italy, and

the geographic difference which characterized the gene pools at a young age was lost under survival selection and increasing mortality. Indeed the Italian gene pool seemed to converge with aging towards the values of the Danish gene pool (Table 2). These results may be caused by a different recruitment of Danish and Italian samples, or occur by chance. However, it is worth noting that a similar phenomenon was observed for the Tyrosine Hydroxylase (*TH*) gene, whose HUMTHO–STR marker was found to be positively associated with longevity in Italian centenarians (De Benedictis *et al.* 1998b). Also, in that study, the geographic difference (northern and southern Italy) observed between the gene pools in younger subjects was lost in older individuals: indeed centenarians converged towards the same frequency values, despite the different geographic origin of the population.

At present, there are very few general studies of centenarians. Therefore it cannot be assessed whether the genetic pool of centenarians converges towards the same structure in every case. However, if such a convergence is a general phenomenon, how can be it explained? Aging implies a profound remodelling of most physiological parameters (Franceschi & Cossarizza, 1995; Franceschi *et al.* 2000a, 2000b). In this biological remodelling the effect of a certain allele on health and survival may change with aging: alleles which were protective at a young age, and therefore frequent in the young population according to its genetic and environmental history, may change to be disadvantageous in

older people (Williams, 1957; De Benedictis *et al.* 1998a; Yashin *et al.* 1999). These alleles will be lost from the genetic pool as the population ages and survival selection occurs, until the gene pool will arrive at a structure compatible with survival at old age. However, if the structure of the gene pool, as it results from the genetic and environmental history of the population, already has frequency values compatible with survival at old age, no significant difference will be observed between young individuals and centenarians. In the present study, the data in Table 2 are compatible with this hypothesis, where Small alleles show a different frequency between Danes and Italians in young individuals, but the same frequency in centenarians.

The lack of a clear association between Small alleles and longevity in Danes does not imply necessarily that the 3'APOB-VNTR polymorphism is not associated with longevity in this population. Indeed the genetic-demographic approach (see alleles L in Table 3) suggests that the Long alleles at the 3'APOB-VNTR locus differently affect longevity in males and females.

Overall, the present study confirms the complexity of the longevity trait, which is influenced by the genetic and environmental history of the population, the effectiveness of the age-related remodeling pathway, and by chance factors. The complexity of the phenotype should be taken into account when replication studies are carried out.

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APPENDIX I

The demographic-genetic model

We define the relative risk of one observed gene allele or genotype r as the ratio of hazard of death for carriers, $\mu(x, r)$, to that for the non-carriers or the baseline hazard, $\mu_0(x)$. Then, according to the proportional hazard assump-

tion, $\mu(x, r) = r\mu_0(x)$. The corresponding survival function for the carriers is

$$\begin{aligned} s(x, r) &= \exp \left(- \int_0^x \mu(t, r) dt \right) \\ &= \exp \left(- \int_0^x r\mu_0(t) dt \right) \\ &= \exp \left(- r \int_0^x \mu_0(t) dt \right) \\ &= \exp(-rH_0(x)) \\ &= s_0(x)^r. \end{aligned}$$

Here, $s_0(x)$ is the survival distribution corresponding to the baseline hazard function, $H_0(x)$ is the cumulative hazard at age x . Although r can take any value greater than zero, an allele with r larger than 1 (frailty allele) increases the hazard of death, while an allele with r smaller than 1 (robust allele or longevity allele) reduces it.

Since all individuals can be grouped as carriers and non-carriers of an allele, one can introduce the simple two-point distribution for the allele or genotype (Vaupel & Yashin, 1985). Let r be the risk of females carrying the gene, and $r_{g \times s}$, the risk of male carriers to that of female carriers (risk of gene-sex interaction). Then the average survival at age x for the mixed population consisting of both carriers and non-carriers is

$$\begin{aligned} \bar{s}_m(x) &= ps_{0,m}(x)^{rr_{g \times s}} + (1-p)s_{0,m}(x), \\ \bar{s}_f(x) &= ps_{0,f}(x)^r + (1-p)s_{0,f}(x). \end{aligned}$$

Here, p is the proportion of carriers at birth, and $\bar{s}_m(x)$ and $\bar{s}_f(x)$ are survival rates at age x for males and females obtainable from population statistics. The proportion of carriers at age x is $p_m(x) = [ps_{0,m}(x)^{rr_{g \times s}}]/[s_m(x)]$ for males and $p_f(x) = [ps_{0,f}(x)^r]/[\bar{s}_f(x)]$ for females. The likelihood function for the data containing both male and female individuals is given by

$$\begin{aligned} L &\propto \prod_x p_m(x)^{n_m(x)} (1-p_m(x))^{N_m(x)-n_m(x)} \\ &\quad p_f(x)^{n_f(x)} (1-p_f(x))^{N_f(x)-n_f(x)}, \end{aligned}$$

where $n_m(x)$, $n_f(x)$ are numbers of male and female carriers at age x , and $N_m(x)$, $N_f(x)$ are total

number of participants at age x . The maximum likelihood estimate of the parameters p , r , $r_{g \times s}$ is computed by the Gauss software (Aptech Systems 1996).

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