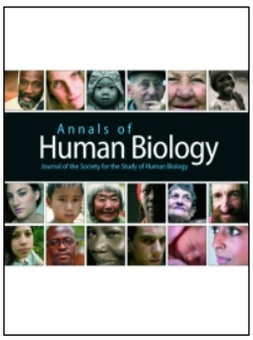
Evaluation of the possible association of body mass index and four metabolic gene polymorphisms with longevity in an Italian cohort: a role for APOE, eNOS and FTO gene polymorphisms



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Title: **Evaluation of the possible association of Body Mass Index and four metabolic gene polymorphisms with longevity in an Italian cohort: a role for *APOE*, *eNOS*, and *FTO* gene polymorphisms.**

Short Title: Gene polymorphisms and longevity

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ABSTRACT

Background: Longevity is considered the result of interactions between environmental and genetic factors.

Aim: To investigate the possible association of body mass index and the frequencies of *APOE*, *ACE*, *eNOS*, and *FTO* gene polymorphisms with longevity.

Subjects and methods: 1,100 healthy volunteers aged 10-100 were recruited. We genotyped subjects for *APOE*, *ACE*, *eNOS*, and *FTO* gene polymorphisms. Data about height and weight were also collected. The sample was split in four age groups: 1-24, 25-49, 50-85 and 86-100.

Results: Significant differences were found in BMI values between age groups. A significant decrease of the *APOE4, eNOS 393* and *FTO A* and allele frequencies was observed in the 86-100 age group compared to the younger groups. For *ACE* gene, no significant differences were found in the allele frequencies between groups. A similar trend was also observed when the sample was subdivided into two main age groups: 1-85 and 86-100.

Conclusion: This study provides evidence for a role of *APOE*, *eNOS*, and *FTO* gene polymorphisms in longevity. It has been estimated that the number of centenarians worldwide will double each decade until 2100, making population data about gene polymorphisms relevant for further studies about longevity.

Keywords: ageing, angiotensin-converting enzyme; apolipoprotein; endothelial nitric oxide; fat mass and obesity-associated gene

1. INTRODUCTION

Longevity is considered to be the result of interactions between environmental and genetic factors. Among the environmental factors, life-style and nutritional status seem to play an important role in the incidence of many age-related diseases. For example, high levels of adiposity, typical of individuals with a body mass index (BMI) more than 30 kg/m^2, are linked to increased incidence of cardiovascular diseases, cancer and neurodegenerative diseases, and therefore a higher mortality rate (Srikanthan and Karlamangla, 2014).

From a genetic point of view, long-lived subjects seem to be characterised by a decreased prevalence of disease-susceptibility alleles and an increased frequency of those variants that confer protection from diseases. Several studies have highlighted that nonagenarians and centenarians do not carry the same risk alleles for common complex diseases as younger subjects (Budovsky et al., 2013).

Among candidate genes, *Apolipoprotein E (APOE)* and *ACE* genes are two of the most consistently associated with human longevity (Fuku et al., 2017). In particular, the *APOE4* allele was found at lower frequency and the *APOE2* allele at higher frequency in the elderly and in centenarians than in younger individuals (Seripa et al. 2006; Santos-Lozano et al., 2016). In this context, *APOE2* is considered as a putative protective factor whereas *APOE*4 is considered as a 'frailty' allele (Gerdes et al., 2000), being associated with higher cholesterol levels and increased risk of metabolic and neurodegenerative diseases (de-Almada et al., 2012; Marrzoq et al., 2011).

For the *ACE* gene, which encodes an enzyme that catalyses the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, the polymorphism linked to longevity is defined by the presence (insertion, I-allele) or absence (deletion, D-allele) of a 287 bp *Alu* fragment in intron 16. This polymorphism is one of the most studied genetic markers for cardiovascular disease risk. Interestingly, the D-allele and the DD-genotype, which theoretically predispose to cardiovascular diseases, were found to be more frequent in some cohorts of centenarians from European countries,

although this putative association has not been replicated in other cohorts of elderly subjects (Garatachea et al., 2013).

Other genes that act as regulators of longevity are related to nutritional status and oxidative stress. One of these is the *FTO* gene that is associated with obesity and BMI. The common single-nucleotide polymorphism, rs9939609, located in the first intron of this gene, is one of the most important variants that has been examined by many genome wide association studies. In particular, the minor A allele was found to be strongly associated with various health-damaging effects of adiposity, including cardiovascular diseases and metabolic syndrome (Qi et al., 2014; Reitz et al., 2012), with consequent reduction in longevity.

Finally, it is known that during ageing there is a progressive reduction of nitric oxide (NO) production, where oxidative stress increases as result of the absence of a compensatory enhancement of antioxidant defences. As a result, aged vessels have a compromised vasodilatory function, which induces increased vascular resistance and impaired perfusion, with possible increased risk for hypertension, atherosclerosis, thrombosis, and stroke (Puca et al., 2012). In this scenario, the *nitric oxide synthases* gene (*eNOS*) plays an important role. This gene is polymorphic, and an important polymorphism is represented by a 27-base pair core consensus VNTR in the intron 4: the common wild-type 'b-allele' (*eNOS 4b* allele) has five tandem 27-bp repeats, while the rare 'a-allele' (*eNOS 4a* allele) has only four repeats. Interestingly, the *eNOS 4a* allele and the *eNOS 4a/4a* homozygote genotype were found to be independent risk factors for myocardial infarction and, consequently, were associated with a reduction in longevity (Park et al., 2004; Puca et al., 2012).

The main objectives of this study were to investigate the possible association between BMI, some metabolic (*APOE*, *ACE*, *eNOS*, and *FTO*) gene polymorphisms and longevity in a cohort from northern Italy. The hypothesis was that some anthropometric parameters, such as BMI, and some genetic variants could be differentially represented in long-lived individuals with respect to younger subjects.

2. SUBJECTS AND METHODS

2.1 - Study population

The study was conducted on a cohort of 1,100 healthy Italian subjects (aged 10-100 years; 546 males and 554 females) of European origin from Northern Italy. All the subjects were randomly chosen healthy volunteers, received detailed information about the study, were anonymously identified by a numeric code and gave their informed consent prior to the analyses. Inclusion criteria for the control group were being a man or woman with no history of stroke, cardiovascular disease, diabetes, or neurodegenerative and cancer diseases. The research protocol was approved by the local ethics committee and was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki.

In Italy, in 2017, the average life expectancy at birth was 80.6 years for men and 84.9 years for women (ISTAT, 2017a). Moreover, the fertility rate for women belonging to the 20-49 age group was about 90%, with an average age of mothers and fathers at the birth of their first child of 31.8 and 35.3 years, respectively (ISTAT, 2017a, b). For these reasons, in order to evaluate the possible association of some alleles with longevity, we decided to divide our sample into 4 age groups: 1-24 years, to include subjects in the pre-reproductive phase; 25-49 years, to include subjects in the reproductive phase; 50-85 years, representing the group of subjects in post- reproductive phase; and 86-100 years to represent the group with long-lived individuals.

2.2 - DNA extraction and Genotyping

Peripheral blood samples (5-10 ml obtained by venipuncture) were collected in heparinized vacutainers and stored at -20°C. DNA extraction was conducted using a Chelex solution, according to Santovito et al. (2017). Gene polymorphisms were determined by PCR and RFLP methodologies, using the primers and melting temperatures described in Table 1. PCR reactions were performed in a 25 μL volume containing about 10 ng DNA (template), with a final

concentration of 1X Reaction Buffer, 1.5 mM of MgCl2, 5% of DMSO, 250 μM of dNTPs, 0.5 μM of each primer, and 1 U/sample of Taq DNA polymerase (Thermo Fisher, U.S.). Cycles were set as follows: 35 cycles, 1 min at 95°C, 1 min at 56-65°C depending on the primer sequence (Table 1), 1 min at 72°C, and a final extension step of 10 min at 72 °C. Amplification products were detected by ethidium bromide staining after 4% metaphor agarose gel electrophoresis. The expected PCR and/or RFLP product size for each gene polymorphism analysed are shown in Table 1.

To verify the genotyping results, 10% of the total sample (n = 110) were also analysed by another investigator. The two analyses showed identical results.

2.2 - Statistical Analysis

SPSS software statistical package program version 25.0 for Windows (SPSS, Inc., Chicago, USA) was used for all statistical analyses. Pearson's χ^2 test was conducted for Hardy-Weinberg equilibrium (HWE) and to compare allele or genotype frequencies between different age groups, whereas the ANOVA test was used to evaluate possible differences in BMI values between age groups. Finally, multiple regression analysis was also used to evaluate the influence of age on BMI. All P-values were two tailed and the level of statistical significance was set at $P<0.05$ for all tests.

3. RESULTS

In Table 2 the general characteristics of the studied population are reported. A total of 1,100 subjects, 546 males (mean age ± SD: 54.423±21.128, age range: 10-98) and 554 females (mean age ± SD: 57.314±24.038, age range: 10-100) were recruited.

No significant differences were found between males and females in terms of mean age and BMI values. *Vice versa*, significant differences were found in terms of BMI values between all the age groups, with exception of the group 1-24 years with respect to the 86-100 years group; there was no significant difference in BMI value between these two groups. These results were confirmed by

both the ANOVA analysis (P <0.001) and regression analysis (P = 0.021) that showed a significant correlation between age and BMI.

In Table 3 the allele and genotype frequencies for all studied genes are reported. All genotype frequencies were in accordance with the Hardy-Weinberg law.

Analysing the frequencies of the studied gene polymorphisms (Table 4), we observed a significant decrease in the *APO E4* allele frequency and a significant increase in the *APO E2* allele frequency in the 86-100 age group compared with the 1-24 (P = 0.005) and 25-49 (P = 0.007) age groups. Similarly, the older age group showed a significant (P<0.001) reduction of *eNOs* 393 and *FTO A* alleles compared with the 1-24 and 25-49 age groups. Finally, for *ACE*, no significant differences in the allele frequencies between age groups were found.

A similar trend was also observed when the sample was subdivided into age groups 1-85 and 86-100 (Table 4). In this case, we observed a significant (P<0.001) decrease in *APO E4* and a significant increase in *APO E2* allele frequencies in the 86-100 age group compared with the 1-85 age group (P = 0.012). Moreover, the older age group showed a significant (P<0.001) reduction in *eNOS* 393 and *FTO A* alleles with respect to 1-85 age group, whereas for the other three gene polymorphisms studied no significant differences in the allele frequencies were found between age groups.

4. DISCUSSION

Epidemiological studies have shown that about 25-30% of the overall variation in human lifespan can be attributed to genetic factors, which are mainly apparent after the age of 60 years and become more relevant for extreme longevity. In addition to the genetic background, longevity is also determined by environmental factors associated with social structure, culture and lifestyle (Montesano et al., 2012; Deelen et al., 2013). Among these environmental factors, nutritional status seems to be the principal factor associated with variations in lifespan. In particular, among the anthropometric parameters associated with longevity, BMI seems to be the most important (Frayling et al., 2007). Indeed, high values of this index are linked to increased incidence of cardiovascular and cancer diseases, and, in general, to high mortality rates (Kong et al., 2017). In our sample we observed an increase in BMI with age (Table 2), but the oldest group did not show significant differences with respect to youngest group, indicating a possible positive effect of low values of BMI in longevity.

The starting hypothesis of genetic studies about longevity is that the frequency of unfavourable genotypes should be reduced in a population by the effect of a negative selective pressure, whereas favourable alleles should be more represented, particularly in the gene pool of long-lived subjects (Montesano et al., 2012). However, it should be emphasised that the human lifespan is a complex trait which is assumed to be determined by many genes with small individual effects (Deelen et al., 2013). One of the most frequent candidate genes associated with longevity is the *APOE* gene, with the *APOE4* allele that has been linked to atherosclerosis and coronary heart disease, and the *E4/E4* genotype which is linked with higher risk for cardiovascular and Alzheimer's diseases (Marrzoq et al., 2011). For these reasons, long-lived individuals would be expected to show lower frequencies of *E4* allele with respect to younger subjects, as observed in our study. However, the *E4* variant has been found at higher frequencies than might be expected (6-37%) in different populations distributed worldwide. The "antagonistic pleiotropy" has been considered as a possible explanation for the anomalous high frequency of this deleterious variant:

the *E4* allele seems to have a reproductive and survival advantage at younger ages, because of its association with higher female fertility and cognitive ability in both sexes, but was also found to be associated with elevated risk of ageing-related diseases (Tuminello and Han, 2011; Jasienska et al., 2015).

Differently to *APOE*, data about the association of *ACE I/D* gene polymorphism and longevity are controversial (for a review see Garatachea et al., 2013). For example, among Europeans, the association of the D allele with longevity was observed in studies about French, British, Italian, and Portuguese centenarians, but not in many other studies (Santos-Lozano et al., 2016), including the present study. These contradictory results could be attributed to different factors, including the ethnic background and sample size of the cohorts.

FTO gene is another longevity-related gene whose common variants were found related to obesity and diabetes (Speakman JR, et al., 2008). In humans, the *A* allele was associated with an increase in adipose tissue and consequently a higher incidence of myocardial infarction (Doney et al., 2009). In this case, we can also hypothesize a pleiotropic effect for *FTO* gene that manifests itself as an increased fitness for the allele *A* carriers at the reproductive age and with a lower fitness for the same carriers in old age. Our data confirm this hypothesis, with a frequency of *A* allele significantly reduced in the older group with respect to the younger.

Finally, we found a significant reduction of the *eNOS 4a* allele in the older group compared with the 1-24 and 25-49 age groups. This result seems to confirm the hypotheses of a possible correlation between this gene polymorphism and natural longevity, as postulated by different authors (Nijiati et al., 2013; Puca et al., 2012; Park et al., 2004). It is known that during ageing, there is a progressive misbalance between NO production, which becomes increasingly reduced, and oxidative stress, which increases without a compensatory enhancement of antioxidant defences. As a consequence, vasodilator function of aged vessels can be compromised, leading to an increase in cardiovasuclar diseases (Forstermann, 2010). In this scenario, *eNOS* may be critically involved in longevity by increasing the deteriorative effects of ageing. In particular, the *eNOS 4a* allele and the

eNOS 4a/4a genotype were found to be independent risk factors for myocardial infarction (Park et al., 2004), and their frequencies are expected to be lower in long-lived subjects, as observed in our sample.

5. CONCLUSIONS

Our results showed an age-related relationship of *APOE*, *FTO* and *eNOS* gene polymorphisms in a cohort of healthy Italian subjects. Despite the limitation of studies like this, due to the difficulty of finding a substantial number of centenarians and, in general, of long-lived people, it is our opinion that the present work could be relevant for further studies about longevity among Italian and European populations. Finally, it has been estimated that the number of centenarians worldwide will double each decade until 2100 (Nijiati et al., 2013), making these studies increasingly important in the future.

CONFLICT OF INTEREST

None declared.

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Table 1 - Primers and annealing temperatures for gene polymorphisms analysed in the present study

Gene	Sequence	T (°C)	Methodology	PCR product (bp)	Reference
ACE	5' - CTGGAGACCACTCCCATCCTTTCT- 3' 5' - GATGTGGCCATCACATTCGTCAGAT – 3'	58	PCR	190 (D-allele) 490 (I-allele)	Álvarez et al., 1998
APOE	5'- TCCAAGGAGCTGCAGGCGGCGCA - 3' 5'- GCCCCGGCCTGGTACACTGCCA - 3'	65	RFLP (*AflIII-HaeII*)	E2 = 168 bp E3 = 145 bp E4 = 195 bp	Zivelin et al., 1997
eNOs	5′ - AGGCCCTATGGTAGTGCCTTT - 3′ 5′ - TCTCTTAGTGCTGTGGTCAC - 3′	56	PCR	420 (five 27-bp repeats) 393 (four 27-bp repeats)	Tsukada et al., 1998
FTO	5'- AACTGGCTCTTGAATGAAATAGGATTCAGA -3' 5'- AGAGTAACAGAGACTATCCAAGTGCAGTAC -3'	63	RFLP (*ScaI*)	TT = 182 AA = 154+28 TA = 182+154+28	Abdelmajed et al., 2017

Table 2 - General characteristics of the studied population.

Subjects	**N (%)**	**Age,** Mean ± S.D. (range)	**BMI,** Mean ± S.D.
Total	**1100**	**55.879±22.676**	**26.299±4.262**
Sex			
Males	546 (49.64)	54.423±21.128 (10-98)	26.316±4.114
Females	554 (50.36)	57.314±24.038 (10-100)	26.281±4.406
Age groups			
1-24	117 (10.64)	19.120±4.369	24.730±4.307 [a,b]
25-49	352 (32.00)	37.594±6.985	26.073±3.797 [c,d]
50-85	521 (47.36)	69.317±9.613	27.163±4.359 [e]
86-100	110 (10.00)	89.845±0.279	24.598±4.126

S.D. = Standard Deviation
[a]*P* = 0.015 with respect to 25-49 age group
[b, c] *P* <0.001 with respect to 50-85 age group
[d]*P* =0.006 with respect to 86-100 age group
[e]*P* <0.001 with respect to 86-100 age group

Table 3 – Allele and Genotype Frequencies of the studied metabolic gene polymorphisms (n = 1,100)

Gene polymorphisms	Allele	N	Frequency	Genotype	N	Frequency	HWE *P*-value
ACE	D	1698	0.772	DD	643	0.585	0.110
	I	502	0.228	ID	412	0.374	
				II	45	0.041	
APOE	E3	1855	0.843	E3E3	784	0.713	0.237
	E4	278	0.126	E3E4	227	0.206	
	E2	67	0.031	E2E3	60	0.055	
				E4E4	22	0.020	
				E2E4	7	0.006	
FTO	T	1417	0.644	TT	470	0.427	0.199
	A	783	0.356	TA	477	0.434	
				AA	153	0.139	
eNOs	420	1842	0.837	420/420	721	0.708	0.203
	393	358	0.163	429/393	263	0.258	
				393/393	35	0.034	

HWE = Hardy-Weinberg Equilibrium.

Table 4 - Frequencies of the studied metabolic gene polymorphisms among different age groups

AGE GROUPS						
GENE	**1-24 N = 117**	**25-49 N = 352**	**50-85 N = 521**	**86-100 N = 110**	**1-85 N = 990**	**86-100 N = 110**
ACE						
DD	64	205	310	64	579	64
ID	49	129	192	42	370	42
II	4	18	19	4	41	4
Allele D	177 (0.756)	529 (0.766)	812 (0.778)	170 (0.773)	1528 (0.772)	170 (0.773)
Allele I	57 (0.244)	165 (0.234)	230 (0.222)	38 (0.227)	452 (0.228)	38 (0.227)
APOE						
E3E3	77	244	377	86	698	86
E3E4	32	83	99	13	214	13
E4E4	2	7	12	1	21	1
E2E3	4	16	30	10	50	10
E2E4	2	2	3	0	7	0
Allele E2	6 (0.026)	18 (0.026)	33 (0.032)	10 (0.046)	57 (0.029)	10 (0.046)
Allele E3	190 (0.812)	587 (0.834)	883 (0.847)	195 (0.886)	1660 (0.838)	195 (0.886)
Allele E4	38 (0.162) *	99 (0.141) **	126 (0.121)	15 (0.068)	263 (0.133)	15 (0.068)****
eNOs						
420/420	72	240	371	96	683	96
393/420	40	109	123	12	272	12
393/393	5	3	27	2	35	2
Allele 420	184 (0.786)	589 (0.837)	865 (0.830)	204 (0.927)	1638 (0.827)	204 (0.927)
Allele 393	50 (0.214)	115 (0.163)	177 (0.170)	16 (0.073)	342 (0.173)*	16 (0.073)
	***	***				
FTO						
TT	47	158	199	66	404	66
TA	51	142	245	39	438	39
AA	19	52	77	5	148	5
Allele T	145 (0.620)	458 (0.651)	643 (0.617)	171 (0.777)	1246 (0.629)	171 (0.777)
Allele A	89 (0.380)***	246 (0.349)***	399 (0.383)	49 (0.223)	734 (0.371)***	49 (0.223)

* P = 0.005, with respect to 86-100 age group; ** P = 0.007, with respect to 86-100 age group
*** P < 0.001, with respect to 86-100 age group; **** P = 0.012, with respect to 1-85 age group