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Complete List of Authors:	Ruberto, Stefano; University of Turin, Department of Life Sciences and Systems Biology Santovito, Alfredo; University of Turin, Department of Life Sciences and Systems Biology
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Title: Association of *TGF β1* codon 10 (T>C) and *IL-10* (G>C) cytokine gene polymorphisms with longevity in a cohort of Italian population

Authors: Stefano RUBERTO[a] and Alfredo SANTOVITO[a]*

[a]University of Turin, Department of Life Sciences and Systems Biology

*Corresponding Author:

Alfredo SANTOVITO

ORCID ID: 0000-0001-5292-5206

University of Turin, Department of Life Sciences and Systems Biology, Via Accademia Albertina n. 13, 10123 Torino, Italy

E-mail: alfredo.santovito@unito.it

Abstract

Longevity is a complex process controlled by both environmental and genetic factors.

We evaluated the association of four cytokine gene polymorphisms with longevity in an Italian cohort. A sample of 1,019 subjects aged 10-100 and belonging to the North-Italian population was genotyped for *IL-6* (G>C, rs1800796), *IL-10* -1082 (G>A, rs1800896), *TNF-α*-308 (G>A, rs1800629), and *TGFβ1* codon 10 (T>C, rs1800471) gene polymorphisms. The association between cytokine gene polymorphisms and longevity was evaluated by dividing the sample into four age groups: 10-24, 25-49, 50-85, and 86-100.

We observed a significant decrease in the frequency of *IL-10 A* allele in the 25-49 ($P = 1.1 \times 10^{-3}$), 50-85 ($P<1 \times 10^{-4}$), and 86-100 ($P = 2 \times 10^{-3}$) age groups compared to that in the youngest age group. Similarly, we found a significant decrease ($P<1 \times 10^{-4}$) in the frequency of *TGFβ1 C* allele in the 50-85 and 86-100 age groups compared to that in the 10-24 and 25-49 age groups.

Previously, high levels of TGFβ1 were detected in elderly subjects, suggesting that this cytokine could counterbalance the harmful effects of inflammation. Similarly, IL-10 has strong anti-inflammatory properties and can inhibit the production of proinflammatory cytokines. In the literature, the lowest levels of functional cytokines were found to be associated with *TGF β1* (T>C) and *IL-10* (G>A) gene polymorphisms, with consequent increase in the duration of inflammation and cancer risk. For these reasons, it is plausible to observe low rates of these mutations in elderly subjects, as found in our work.

Keywords: cytokine; gene polymorphisms; aging

1. Introduction

Longevity is a complex trait determined by both environmental and genetic factors. Environmental factors include dietary habits, general health, and lifestyle, whereas genetic factors are principally referred to gene polymorphisms that play a significant role in homeostasis processes (Budovsky et al., 2013; Santovito et al., 2019). A favorable environment and healthy lifestyle allow genetically predisposed subjects not only to increase their longevity but also to preserve autosufficiency and, in general, a satisfactory health status (Minciullo et al. 2008).

Long-lived individuals seem to be able to avoid age-related diseases. Therefore, the evaluation of genes modulating susceptibility to age-related diseases in global population could be a useful target for human longevity studies. In particular, polymorphisms seem to play an emergent role in genes encoding anti-inflammatory or pro-inflammatory cytokines (Sansoni et al., 2008; Ruan et al., 2014; Minciullo et al., 2016). Indeed, age-associated changes in immune system function contribute to increased mortality in the elderly, and some aspects of aging involve inflammatory processes that could intensify susceptibility to age-related diseases, with consequent decrease in longevity. In this sense, aging and longevity could be associated with processes affecting the balance between anti-inflammatory and pro-inflammatory systems.

Polymorphisms in inflammatory cytokine genes might affect human lifespan, as confirmed by the fact that some variations in cytokine genes have been found to be associated with human longevity (Minciullo et al., 2016; Wei et al., 2016; Khabour and Barnawi, 2010). Aging is characterized by a chronic pro-inflammatory condition, and individuals with systemic inflammation could have an increased risk of age-related diseases. In this context, polymorphisms of inflammation-related cytokine genes could control the balance between anti- and pro-inflammatory processes, i.e., high levels of pro-inflammatory cytokines may increase the susceptibility to age-related diseases, whereas an over-representation of anti-inflammatory cytokines may provide an advantage in the processes related to healthy aging.

The aim of the present study was to evaluate the possible association of polymorphisms of four cytokine genes with human longevity in a sample of Italian population. We postulated that longer survivors could have a higher frequency of anti-inflammatory cytokine genetic variants, such as the T>C (rs1800471) polymorphism in the codon 10 of *TGFβ1* and G>A (rs1800896) polymorphism in the promoter region of *IL-10*, and could have a lower frequency of inflammatory cytokine genetic polymorphisms, such as those in the promoter regions of *IL-6* (-174C>G, rs1800796) and *TNF* (-308 G>A, rs1800629).

TNF is a pro-inflammatory cytokine responsible for various immunoregulatory activities. Upon binding with its receptor, TNF triggers complex signaling pathways, which guide the cell to survival or death (Roy et al., 2017). The -308A allele appears to produce higher transcriptional activity than the -308G allele, and cells with the -308A allele have been demonstrated to release higher levels of TNF (Roy et al., 2017). Similarly, the pro-inflammatory cytokine IL-6 plays a crucial role in acute phase responses and inflammatory processes. The G>C single nucleotide polymorphism at promoter position -174 of this cytokine is associated with different plasma levels of IL-6 in healthy subjects. In particular, the *C* allele of *IL-6* is associated with lower plasma levels of IL-6 and the homozygous CC genotype at codon 10 is strongly associated with decreased production of this cytokine (Terry et al., 2000).

In contrast, IL-10 and TGFβ1 have the capacity to inhibit the synthesis of pro-inflammatory cytokines, such as IL-6 and TNF-α (Sabat et al., 2010). The G to A substitution at the 1082 position of the *IL-10* gene promoter is important for regulation of *IL-10* transcription. Individuals with the AA genotype have lower concentrations of circulating IL-10 (Reid et al., 2002; Galley et al., 2003). The T>C polymorphism in the codon 10 of *TGFβ1* results in a change in the amino acid sequence that alters the plasma levels of TGFβ1. Consequently, the homozygous CC genotype in codon 10 is strongly associated with decreased production of TGFβ1 (Awad et al., 1998).

2. Materials and Methods

2.1. Subjects

In this study, we included 1,019 subjects (aged 10-100 years; 511 males and 508 females) belonging to the population of Northern Italy.

~~At the time of sampling,~~ We recruited subjects who were without diseases (subjects that were not hospitalized at the moment of the sampling), who were natives of Northern Italy localities since at least two generations, and who joined the project. In order to avoid selection bias, subjects were sampled without any discrimination and without *a priori* exclusions, for example in terms of age and sex.

All data from each participant, including ancestry, were collected during an interview in an open-ended manner. We excluded the subjects who claimed to be natives of Southern or Central Italy or who belonged to religious ethnic groups. All subjects received detailed information about the study and gave their informed consent. In case of minors, informed consent was provided by the parents. 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. The possible association of some cytokine genetic polymorphisms with longevity was evaluated by dividing the sample into four age groups: 10-24, 25-49, 50-85, and 86-100, representing individuals in the pre-reproductive, reproductive, and post-reproductive phases, and long-lived individuals, respectively.

2.2. DNA Extraction and Genotyping

Heparinized vacutainers were used to collect 5-10 mL of peripheral blood from each subject. Then, the vacutainers were stored at -20°C prior to analysis.

DNA extraction was conducted using Chelex solution according to the following protocol: 10 µL of peripheral blood was diluted in 990 µL of sterile distilled water for 15 min at room temperature. After centrifugation at 14,000 rpm for 1 min, the pellet was re-suspended in 200 µL of 5% Chelex solution in ultrapure water, heated to 56°C for 15 min, vortexed for 10 s and then heated to 100°C

in boiling water bath for 8 min. For PCR reactions, we used 19 µL of this solution containing extracted DNA, corresponding to about 10 ng DNA (template). PCR reactions were performed in a reaction volume of 25 µL, containing 1X reaction buffer, 1.5 mM MgCl2, 5% DMSO, 250 µM dNTPs, 0.5 µM of each primer, and 1 U/sample of Taq DNA polymerase (Fischer Scientific, Rodano (MI), Italy). The PCR cycles were set as follows: 35 cycles, 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension step of 10 min at 72 °C. The amplification products were electrophoresed using 2.5% agarose gel and detected by ethidium bromide staining. Primer sequences, melting temperatures, PCR methodologies used, and expected PCR product sizes are reported in Table 1.

One hundred samples were re-analyzed by another investigator in order to verify the accuracy of the genotyping results. The two analyses showed identical results.

2.3. Statistical Analysis

All statistical analyses were performed using the SPSS software statistical program (version 25.0, SPSS Inc., Chicago, USA). Pearson's $\chi 2$ test contingency table was used to evaluate both the Hardy–Weinberg equilibrium (HWE) and the statistical differences between two age groups at a time. Comparison of genotype frequencies between age groups was carried out using the Fisher's exact test. The relationship between age and a specific genotype was analyzed using both bivariate (age vs each single cytokine) linear regression analysis and multivariate (with all SNPs in the same model predicting age) general linear model with Bonferroni's correction. All *P*-values were two-tailed, and the level of statistical significance was set at $P<0.05$ for all tests.

3. Results

The general characteristics of the studied sample are reported in Table 2. We recruited 1,019 subjects, including 508 males (mean age ± SD: 55.157±19.818, age range: 11-98) and 511 females (mean age ± SD: 58.853±22.553, age range: 10-100).

Allele and genotype frequencies of the analyzed cytokine genes are reported in Table 3. All gene polymorphisms were in the HWE. The frequencies of the cytokine gene polymorphisms among age groups are reported in Table 4, while the frequencies of minor alleles of the analyzed gene polymorphisms are shown in Figure 1.

We observed a general decreasing trend in the frequency of *IL-10 A* allele across all age groups. In particular, we observed a significant decrease in the frequency of *IL-10 A* allele in the 25-49 ($P = 1.1 \times 10^{-3}$), 50-85 ($P<1 \times 10^{-4}$), and 86-100 ($P = 2 \times 10^{-3}$) age groups compared to that in the youngest age group. Similarly, we found a significant decrease in the frequency of *TGFβ1 C* allele in the 50-85($P<1 \times 10^{-4}$) and 86-100 ($P<1 \times 10^{-4}$) age groups compared to that in the 10-24 and 25-49 age groups (Table 4). The bivariate linear regression analysis ($P<1 \times 10^{-3}$ for both gene polymorphisms) and the multivariate general linear model (*IL-10* ($P = 1.4 \times 10^{-2}$) and *TGF β1* ($P<1 \times 10^{-4}$)) confirmed a significant trend with respect to age (see Supplementary Materials).

4. Discussion

Aging and longevity are complex processes that are controlled at different levels, including genetic level (Budovsky et al., 2013). From an immunological point of view, aging could be considered as the result of disequilibrium between pro-and anti-inflammatory processes, which could result in inflammation (Minciullo et al., 2016). In this scenario, cytokine gene polymorphisms could control the levels of pro-inflammatory and anti-inflammatory molecules, playing a significant role in the inflammatory processes (Minciullo et al., 2016).

In this study, we evaluated the role of some cytokine gene polymorphisms in longevity. We found a possible association of *TGFβ1* codon 10 (T>C) and *IL-10* (-1082 G>A) gene polymorphisms with age, with *TGFβ1 C* allele and *IL-10 A* allele significantly underrepresented in the older groups when compared to the younger one.

A possible explanation of our findings is that *TGFβ1* and *IL-10* are potent regulatory cytokines involved in inflammation processes and in immune response homeostasis. High levels of

TGFβ1 have been found in long-lived individuals, suggesting that this cytokine could counterbalance the harmful effects of inflammation (Carrieri et al. 2004; Salvioli et al. 2009; Forsey et al. 2003). T>C polymorphism at codon 10 results in a change in the amino acid sequence that alters the plasma levels of TGFβ1. Consequently, the homozygous CC genotype at codon 10 is associated with decreased production of this cytokine (Awad et al., 1998; Carrieri et al., 2004) and it is significantly underrepresented in our oldest age group.

Similarly, IL-10, a potent anti-inflammatory cytokine, is involved in the inhibition processes of pro-inflammatory cytokines production (Saxena et al. 2015). Although high levels of IL-10 diminish resistance to infectious diseases (McElhaney et al. 2012), they were found in centenarians (Salvioli et al. 2009) and were associated with successful aging (Van Den Biggelaar et al. 2004). In particular, *IL-10* (-1082) *A* allele seems to be associated with decreased IL-10 production with respect to *G* allele (Lio et al., 2012) and with decreased longevity in samples of Italian centenarians (Lio et al. 2002) and Jordanian elderly people (Khabour and Barnawi 2010), while the *IL-10* (-1082) *GG* genotype was found to be associated with increased survival in older male subjects (Cederholm et al. 2007). However, in other samples, such as those including Irish and Finnish nonagenarians (Ross et al., 2003; Wang et al., 2001), this association was not observed. Similarly, no association was observed between *TGFβ1* codon 10 genotypes and longevity in a sample of Bulgarian population (Naumova et al., 2011), indicating a possible ethnicity effect on longevity-related genetic processes.

In general, pro-inflammatory cytokines are associated with increased resistance to pathogens, whereas anti-inflammatory cytokines seem to show an advantage, from a longevity point of view, in an environment with low incidence of pathogens (Ruan et al., 2014).

In contrast, gene polymorphisms associated with altered levels of these cytokines, such as *TGFβ1* (T>C) and *IL-10* (G>A) gene polymorphisms, could result in lowest levels of the functional cytokines, and, consequently, could be related to an increase in the duration of the inflammation process and, ultimately, to an increase in cancer risk. For these reasons, it is plausible to observe

low frequencies of these gene polymorphisms in older subjects, as found in our work.

Finally, for the other two studied cytokines, despite their role in inflammatory processes, we found no significant differences in allele frequencies between the age groups. However, in a previously published genome-wide association study, an association between another genetic variant (G>A) located in the intronic region of the *IL-6* gene and longevity was reported in the Han Chinese population. In particular, the minor rs2069837 allele was found to be significantly less frequent among centenarians compared to middle-aged individuals belonging to the Han Chinese population, suggesting a deleterious effect of this locus on longevity (Zeng et al., 2016).

Cytokines involved in inflammatory processes, such as TNF-α and IL-6, show an age-related upregulation (Zanni et al. 2003). High plasma levels of TNF-α were found to be associated with an elevated risk of cardiovascular diseases in elderly subjects (Van Den Biggelaar et al. 2004). Similarly, increased serum levels of IL-6 were found to be negatively associated with aging processes and mortality in old age (Palmeri et al. 2012; Giovannini et al. 2011; Di Bona et al. 2009).

We can hypothesize that both *TNF* and *IL-6* have a pleiotropic effect. If, on the one hand, a pro-inflammatory phenotype provides greater protection against infectious diseases, then on the other hand, the large amount of pro-inflammatory cytokines may contribute to inflammation (Giovannini et al. 2008). In this scenario, high levels of inflammatory cytokines could confer an advantage during the pre-reproductive phase, but may become disadvantageous in the post-reproductive period of life.

5. Conclusions

We found evidence that the polymorphisms of the anti-inflammatory *TGFβ1* (T> C) and *IL-10* (-1082 G> A) could influence the probability of reaching an advanced age in a sample of Italian population. However, a limitation of the present study is the population stratification and the genetic heterogeneity that cannot be excluded. Indeed, it is known that populations could be

heterogeneous in terms of genetic ancestry and, thus, a sampled population may comprise two or more groups with distinct genetic ancestry (Hellwege et al., 2017; Liu et al., 2013). This population stratification is principally due to non-random mating processes following to geographic isolation of subpopulations with low rates of migration and gene flow (Hellwege et al., 2017). As a result, after several generations, allele frequencies change randomly for each population isolate, and the genomes of the individuals in an admixed population become a mosaic composed of chromosomal segments originating from each of the ancestral populations (Hellwege et al., 2017).

Due to this allele frequencies variation among populations of different genetic ancestry, association of alleles with a certain trait - as longevity in the case of the present study - could vary among populations of different genetic ancestry. In a situation like this, we cannot exclude a spurious association of one or more alleles with longevity, resulting from differences in genetic ancestry.

Finally, we would like emphasize that also demographic differences among populations may create stratification, even when populations inhabit the same geographical region. For example, the subdivision of the sample into age groups poses the problem of possible early life differences in mortality in some cohorts. As consequence, elderly subjects could have different infant mortality rates than younger subjects, thus highlighting a false association of some alleles with longevity while, in reality, the association could be simply due to a differential survival in the early stages of life.

~~A limitation of the present study is that the results of this study cannot be generalized due to the different genetic background of the populations distributed all over the world, their substructures, early life differences in mortality rate, as well as the different environments in which they live. However, we believe that data on the frequencies of polymorphisms of cytokine genes in different age groups may be useful to better clarify the role of these genes in longevity-related processes.~~

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure 1 – Distribution of minor alleles among age groups

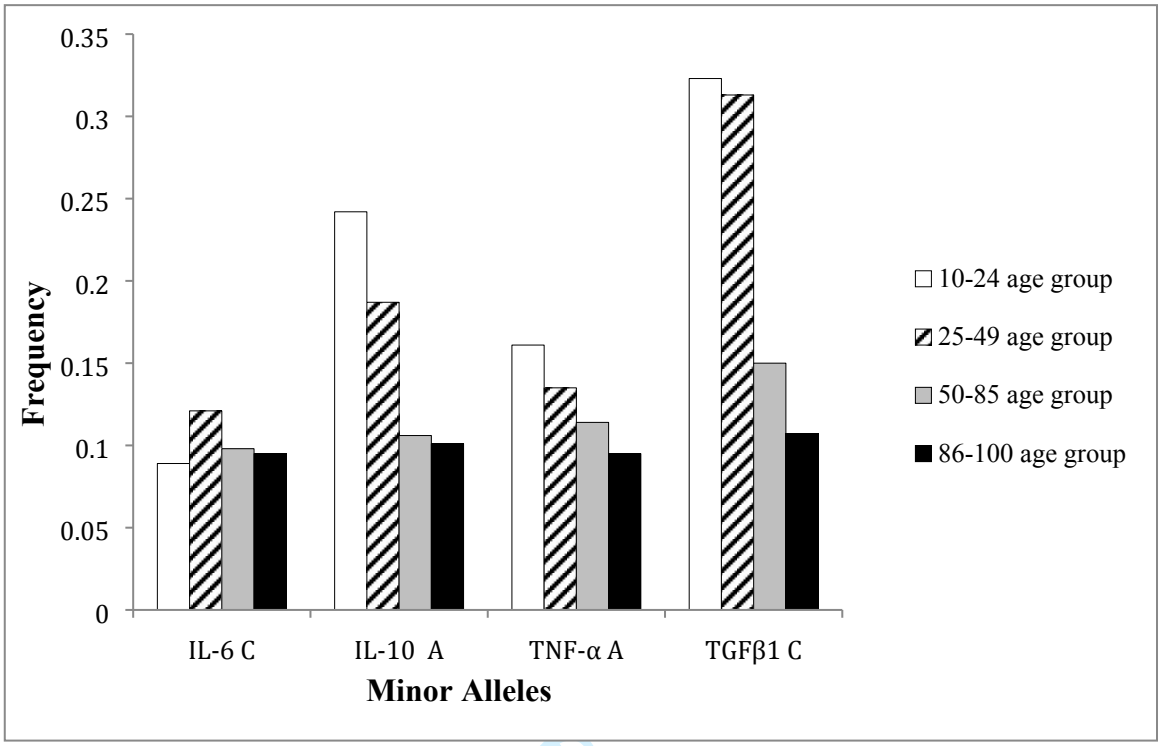

Table 1 - Primers and PCR products for gene polymorphisms analysed in the present study

Gene	Main Function Protein	Reference SNP	Sequence	PCR product (bp)	Reference
IL-6 (-174, G>C) - Antisense primer - G-sense primer - C-sense primer	Pro-inflammatory	rs1800796	5'-TCGTGCATGACTTCAGCTTTA-3' 5'-AATGTGACGTCCTTTAGCATG-3' 5'-AATGTGACGTCCTTTAGCATC-3'	190	Zakharyan et al., 2012
IL 10 -1082 (G>A) - Antisense primer - G-sense primer - A-sense primer	Anti-inflammatory	rs1800896	5'-AGTGCCAACTGAGAATTTGG-3' 5'-CTACTAAGGCTTCTTTGGGAG-3' 5'-ACTACTAAGGCTTCTTTGGGAA-3'	258	Perrey et al., 1999
TNF-α (-308, G>A) - Antisense primer - G-sense primer - A-sense primer	Pro-inflammatory	rs1800629	5'-TCTCGGTTTCTTCTCCATCG-3' 5'-ATAGGTTTTGAGGGGCATGG-3' 5-AATAGGTTTTGAGGGGCATGA-3'	184	Perrey et al., 1999
TGF-β_1 (Codon 10, T>C) - Antisense primer - C-sense primer - T-sense primer	Tissue repair, anti-inflammatory	rs1800471	5'-TCCGTGGGATACTGAGACAC-3' 5'-GCAGCGGTAGCAGCAGCG-3' 5'-AGCAGCGGTAGCAGCAGCA-3'	241	Perrey et al., 1999

For all genes, the annealing temperature was 60°C and the used methodology was ARMS PCR

Table 2 - Demographic characteristics of the studied population.

Subjects	**N (%) – (Females/Males)**	**Age,** Mean ± S.D. (range)
Total	**1,019**	**56.932±21.483**
Sex		
Males	508 (49.85)	55.157±19.818 (11-98)
Females	511 (50.15)	58.853±22.553 (10-100)
Age groups		
10-24	62 (6.08) – (35/27)	19.120±4.369
25-49	352 (34.54) – (156/196)	37.594±6.985
50-85	521 (51.13) – (262/259)	69.317±9.613
86-100	84 (8.24) – (58/26)	89.845±0.279

S.D. = Standard Deviation

Table 3 – Allele and Genotype Frequencies of 4 cytokine gene polymorphisms in an Italian sample (n = 1,019)

Gene polymorphisms	Allele	N	Frequency	Genotype	N	Frequency	HWE *P*-value Pearson's $\chi 2$ test
IL-6	G	1824	0.895	GG	818	0.803	0.841
	C	214	0.105	GC	188	0.184	
				CC	13	0.013	
IL-10 (-1082)	G	1794	0.880	GG	795	0.780	0.277
	A	244	0.120	GA	204	0.200	
				AA	20	0.020	
TNFα-308	G	1788	0.877	GG	777	0.763	0.102
	A	250	0.123	GA	234	0.230	
				AA	8	0.008	
TGFβ1 cod 10	T	1604	0.787	TT	641	0.629	0.188
	C	434	0.213	TC	322	0.316	
				CC	56	0.055	

HWE = Hardy-Weinberg Equilibrium.

Table 4 - Frequency of four cytokine gene polymorphisms among age groups.
Fisher exact test was used in order to perform statistical pairwise comparisons. Only significant results were reported. All statistical results, including non-significant ones, were reported in Supplementary Material.

GENE	10-24 N = 62	25-49 N = 352	50-85 N = 521	86-100 N = 84
IL-6 (-174)				
GG	51	269	430	68
GC	11	81	80	16
CC	0	2	11	0
Allele C	11 (0.089)	85 (0.121)	102 (0.098)	16 (0.095)
IL-10 (-1082)				
GG	40	268	419	68
GA	14	81	94	15
AA	8	3	8	1
Allele A	30 (0.242)	87 (0.187)*	110 (0.106)**	17 (0.101) ***
TNF-α (-308)				
GG	44	258	406	69
GA	16	93	111	14
AA	2	1	4	1
Allele A	20 (0.161)	95 (0.135)	119 (0.114)	16 (0.095)
TGFβ1 cod. 10				
TT	29	165	380	67
TC	26	154	126	16
CC	7	33	15	1
Allele C	40 (0.323)	220 (0.313)	155 (0.150)[a, b]	18 (0.107) [c, d]

*P =1.1 x 10^{-3} significantly decreased with respect to 10-24 age group
** P<1 x 10^{-4} significantly decreased with respect to 10-24 age group)
** P = 2 x 10^{-3} significantly decreased with respect to 10-24 age group

[a] $P < 1 \times 10^{-4}$ significantly decreased with respect to 10-24 age group
[b] $P < 1 \times 10^{-4}$ significantly decreased with respect to 25-49 age group
[c] $P < 1 \times 10^{-4}$ significantly decreased with respect to 10-24 age group
[d] $P < 1 \times 10^{-4}$ significantly decreased with respect to 25-49 age group