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TATJANA ŠKARIĆ-JURIĆ, ŽELJKA CELINŠĆAK, MAJA ŠETINC, LUKA BOČKOR, ANITA STOJANOVIĆ MARKOVIĆ, MATEA ZAJC PETRANOVIĆ, MARIJANA PERIČIĆ SALIHOVIĆ, JORIS DEELEN, BRANKA JANIĆIJEVIĆ, NINA SMOLEJ NARANČIĆ

SO DIFFERENT BUT EQUAL: 33 LONGEVITY GENES' LOCI IN THE ROMA AND IN THE GENERAL POPULATION OF CROATIA

ABSTRACT: The age pyramid of Roma populations tips strongly towards the younger age groups and is characterized by a low number of elderly individuals. There is a vast range of environmental factors that influence the age structure of Roma populations. To explore whether a genetic risk for premature mortality also exists in this ethnic minority, 33 single nucleotide polymorphisms (SNPs) in 23 putative longevity genes were investigated in 308 adult Roma living in Croatia, and in Croatian population sample, composed of 314 "Old" (85–101 yrs.) and 97 "Young" (20–35 yrs.) subjects. The cumulative effect of the investigated SNPs, which have previously been related to human longevity, was summarized within Genetic Longevity Score (GLS). After Bonferroni correction the "Old" and "Young" Croatian age groups differ only in the allele frequency in MRE11A locus (rs533984), while the Roma had significantly different allele frequencies from the surrounding majority population in most of the investigated longevity genes loci (in 16 out of the 33 SNPs). However, the Roma's GLS is equal to those in the "Young" and "Old" Croatian cohorts implying identical chances of surviving to the age of 85 among Roma as in the majority Croatian population, when only genetics is taken into account.

KEY WORDS: Longevity – Genetic score – Premature mortality – Minority health – Roma – Croatia

INTRODUCTION

Global average life expectancy by 1800 was between 30 and 40 years of age, and over the last 200 years it has almost doubled (Finch 2007). This change is mainly attributed to the increase in food production and the role of the industrial revolution in the regular supply of food, with the improvement of hygienic conditions and advances in medicine. However, there are quite large inequalities within and between countries, and between different

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population groups. There is growing evidence that socioeconomic deprivation is a major cause of differences in mortality (Boruzs *et al.* 2018). Today, the highest life expectancy (over 84 years of age) has been found in some developed countries such as Hong Kong, Japan, Italy, Singapore, and Switzerland, and it is higher in women than in men. With the median age of 43.9 years, Croatia is the 18th country with the oldest population in the world (Central Intelligence Agency 2020). The estimates for 2020 indicate a total fertility rate of 1.42 children born per woman, and that the proportion of people aged 65 years and above in total population amounts to 21.06% (Croatian Bureau of Statistics, 2021).

Roma (Gypsy) are the largest European transnational minority population of common Indian origin consisting of 12 million people. The Roma were migrating throughout most of their history and retained the nomadic way of life as their cultural pattern. Today, most Roma have a permanent residence, but they generally live separated and excluded from the majority communities in several ways: physically – in outskirts of towns and villages, often in overcrowded settlements with poor sanitation and lack of other housing facilities (Anthonj *et al.* 2020), economically – underprivileged, poorly educated and unable to find a regular job (EU-FRA 2014), and socially – discriminated considering the prejudices of majority populations and their hostility (Kende *et al.* 2017). Poverty and social exclusion, along with a number of lifestyle specifics, could have an adverse impact on the health of the Roma population, which indeed shows poorer health status, increased morbidity and mortality compared to other populations, and a shorter average life expectancy.

Most countries do not report detailed ethnic data in their national census data, but one of the rare official documents is the European Public Health Alliance which shows that in all EU member states, the estimated life expectancy of the Roma was lower than that of the surrounding majority non-Roma population (The European Public Health Alliance 2018). The estimated life expectancy gap between Roma and non-Roma varied from 2–10 years lower in the UK Roma than in the UK non-Roma, to as much as 20 years lower life expectancy of the Italian Roma in comparison with Italian non-Roma. The age structure of the Roma in all EU states is much younger than the national average, with a much higher proportion of young people and a relatively smaller size of the elderly population (Frazer, Marlier 2011). In Hungary, both birth rates and mortality are far higher among the Roma population than in Hungarians: there are 2 times more Roma aged <15 years (37% vs. 16.8%) and 5 times less Roma >60 years of age (3.9% vs. 20.2%). A similar trend is observed in Ireland (42% vs. 20% <14-year-olds, and 2.7% vs. 11% >65-year-olds), Italy (45% vs. 15% <16-year-olds, and 0.3% vs. 25% >60-year-olds), and Slovakia (43.6% vs. 25.5% <14-year-olds, and 3.6% vs. 14.5% >60-year-olds), but also in the Czech Republic, Romania and Spain (Frazer, Marlier 2011).

There is a vast spectrum of environmental influences that could cause the observed younger age structure of the Roma population. In Croatia, poor education and traditional attitudes towards female reproductive health all contribute to a high-fertility reproductive pattern present in this population (Škarić-Jurić *et al.* 2007, Klasnić *et al.* 2020). The young-leaning age structure of Roma population is a consequence of high fertility rate, but also of higher mortality at almost all ages (i.e. in periods of childhood, adolescence and early adulthood, as well as in middle ages). The higher risk of mortality in this population has multiple causes: malnutrition, infections, violent deaths, deaths related to multiple parities, and especially higher prevalence of chronic diseases related to several risk factors (Zeljko *et al.* 2008, Zeljko *et al.* 2011). In all ages, a low socioeconomic status and a lack of continuous access to health care (Škarić-Jurić *et al.* 2007) modulate the adverse health outcomes, including mortality rates. Namely, if a person is not employed (as is the case with the vast majority of Roma), the periods in which he/she has health insurance in Croatia are during the maternity period (during pregnancy and until the end of the first year of the child) or while in the education system (for those who do not attend university education health insurance ends at 18 years of age). In addition, citizenship or immigration papers are obligatory to obtain health insurance.

A situation similar to that in Croatia is also present among Roma in other countries: Roma are generally poorly educated and many of them are early school-leavers (more than 70% of 45+ year-old Roma in Greece and Portugal did not complete any level of formal education, FRA 2018), have lower odds of achieving dietary recommendations (Hungary: Llanaj *et al.* 2020) or have inferior diet diversity (Slovakia: Hijova *et al.* 2014; Czeck Republic: Olišarova *et al.* 2018; Romania: Ciaian *et al.* 2018) than non-Roma, and have high prevalence of chronic diseases. Recent research on Hungarian Roma has shown that the prevalence of cardiovascular risk factors and the risk of cardiovascular diseases (estimated by Framingham Risk Score, the Systematic Coronary Risk Evaluation, Pooled Cohort Equations and Revised Pooled Cohort Equations) show

an unfavourable picture in the Roma population in relation to the majority population (Piko *et al.* 2021). Furthermore, recent meta-analyses of seven CVD risk factors showed that Roma, compared to non-Roma from 16 European countries, carry significantly higher burdens of CVD risk factors related to smoking, diabetes, abdominal obesity and metabolic syndrome, with lower burdens for hypertension and BMI ≥ 25 kg/m^2 (Zajc Petranović *et al.* 2021).

In addition to environmental and lifestyle factors, it seems that there may be genetic reasons behind the differences in the frequency of CVD risk factors between Roma and non-Roma populations. The increased prevalence of diabetes in Czech Roma (Hubáček *et al.* 2020), increased mean BMI and waist circumference (Llanaj *et al.* 2020) and the reduced prevalence of hypertension (Soltesz *et al.* 2020) in the Hungarian Roma population, may be related to different frequencies of risk alleles in genes associated with the development of these phenotypes.

The main question of the present study was whether the young age structure and premature mortality characterizing Roma worldwide could be at least partly attributed to the genetic risk load present in this specific population. To investigate the matter, our study explored whether the Croatian Roma have fewer "longevity variants" compared to the surrounding majority (non-Roma) Croatian population, and the principal working hypothesis of the study was that the genetic landscape of the Roma population contributes to their shorter lifespan.

In order to illuminate this issue, a genetic score, composed of summed genotypic values of effect alleles of the selected "longevity variants" was constructed. The genetic score gives an opportunity to summarize and compare the degree of genetic load between different ethnic groups (Werissa *et al.* 2019, Hubáček *et al.* 2020, Soltész *et al.* 2020). The Genetic Longevity Score (GLS) in the present study enabled us to examine the cumulative effect of genetic factors related to human longevity since it sums the genotypic values attributed to each locus, where effect alleles were those found to contribute to longer lifespan in other studies.

Additionally, this study aims to test whether the loci found to be relevant for longevity in other populations had the same effect on the Croatian majority population, by observing the difference between two age extreme cohorts.

Specifically, this study aims to:

(1) present the Croatian Roma longevity variants' allele and genotype frequencies and compare them with those found in the Croatian majority population;
(2) compare longevity variants' allele and genotype frequencies of Croatian "Old" (85+) and "Young" (20–35 yrs.) cohorts;
(3) calculate and compare genetic scores of longevity variants between the Roma minority, Croatian majority "Old" and "Young" cohorts.

MATERIALS AND METHODS

Study populations

The informed consent was obtained from each study participant and the research was approved by the Ethics Committee of the Institute for Anthropological Research, Zagreb.

(1) The Roma population (age span: 18–75 yrs.). The biological material of 321 adult Roma was collected in multiple field studies (2005–2012), which were part of the ongoing multidisciplinary anthropological, molecular-genetic and epidemiological community-based research of Roma populations in Croatia. The fieldwork was carried out in several regions of Croatia with the highest number of Roma minority inhabitants according to the census data (Croatian Bureau of Statistics 2013). Our sample represents approximately 4.4% of the adult Roma population according to the 2011 Census (when 43.6% of the Roma population was 19 years and older), and if we assume that the same ratio of adult and minor Roma was in 2001 (officially available data from the 2001 Census are not presented separately for minors and adults), 7.8% of the adult Roma population according to the 2001 Census. Therefore, we consider our sample as representative for the adult Roma population living in Croatia. The participants were informed about the goals, methods and expectations of the study with the help of linguistically and culturally competent and trained Roma volunteers.
(2) Croatian population (age span: 20–101 yrs.). The Croatian sample consists of adult unrelated participants of both sexes who belong to two extreme adult age groups: 327 people aged 85 years and older (the "Old" cohort) and 102 young people aged 20–35 years (the "Young" cohort). The "Old" cohort sample was collected in 2007–2009 (for the Croatian project on longevity). In order to counterpart the "Old" cohort representing longevity phenotype, a sample of the "Young" cohort was collected in 2019 (within the course of the CSF project HECUBA), covering a similar age range at the opposite side of the age distribution. Two disparate adult samples were

chosen in order to mimic extreme phenotypes while investigating the possible impact of longevity genes on selective mortality in the Croatian population. The gender asymmetry of the "Old" sample is the result of women being more represented in the population over 85 years of age (Croatian Bureau of Statistics 2001), and the "Young" sample follows this gender distribution.

Genotyping

The genomic DNA was isolated from the peripheral blood using the salting out method (Miller *et al*. 1988). Genotyping was conducted in a commercial company using the Kompetitive Allele Specific PCR (KASP) method. The KASP genotyping assay is a form of competitive allele-specific PCR combined with homogeneous fluorescent SNP genotyping system, which determines the alleles at a specific locus within genomic DNA (Semagn *et al*. 2014).

Selection of markers

The selection of the longevity variants was a result of literature search using publicly available databases (PubMed as well as repositories specialized for human longevity such as https://genomics.senescence.info/longevity/, http://ageing-map.org/). The relevance (strong and/or replicated relation to human longevity) and the involvement into different pathways related to human longevity were the criteria for loci selection. The 33 loci, which have previously been related to the longevity phenotype, were successfully genotyped in both the Croatian and the Roma samples.

Genetic Longevity Score construction

The Genetic Longevity Score (GLS) sums up across all loci the alleles related to human longevity, assuming that each one has the effect of equal size. All genotypes in the file are "oriented" as in the literature, so the effect/longevity allele is the one as declared in source research.

GLS is constructed as the sum of genotypic values for each participant. For each locus, if a longevity allele is homozygous a value of 2 is attributed, if heterozygous a value of 1, and if the longevity allele is not present value 0 is attributed. The summary value for all investigated loci was obtained for each person resulting in an unweighted GLS.

In order to account for the effect size of each locus, the weighted GLS was calculated by multiplying genotypic values with beta values (*Supplementary Table 1*) from the 90th percentile analysis originating from the longevity GWAS summary statistics (Deelen *et al*. 2019). By employing beta values originating from a single study, vast heterogeneity – methodological and populational – present in all here referred to research has been avoided, and more reliable weighted GLS were obtained.

Both scores – unweighted and weighted GLS – are constructed for the Roma minority, as well as for each of the two extreme age groups of the Croatian majority population separately ("Old" and "Young").

In order to remove the noise of multiple linked SNPs in the genetic score values, the linkage disequilibrium (LD) was calculated for all pairs of SNPs located on the same chromosome using Haploview 4.2 (Barrett *et al.* 2005). Only one representative SNP per region of LD (r^2>0.4) was kept which resulted in the exclusion of 8 SNPs, and thus genetic scores calculation was based on the values of 25 longevity genes' loci.

Missing data

The non-successful genotyping has a strong effect on GLS, in terms of sample size. Namely, the missing genotyping data of one locus in one person resulted in the exclusion of all data for this participant. Therefore, we excluded participants who had more than five unsuccessfully genotyped loci. For participants with five or fewer unsuccessfully genotyped loci, the missing data in the construction of GLS score were replaced by median values for each locus calculated separately for each of the three samples separately (Roma, "Old" and "Young" Croatian sample). The final sample sizes used in subsequent analysis were: 308 Roma, 411 Croatian (314 "Old" and 97 "Young"), 719 in total.

Data analysis

Allele and genotype frequencies were calculated by direct counting method. Hardy-Weinberg equilibrium (HWE) was tested using Arlequin 3.5.2.2 (Excoffierr, Lischer 2010). Differences between samples with respect to genotype distribution and allele frequencies were tested in a pairwise fashion by a 2×3 Chi-square test or by Fisher's exact test. The significance of the allele frequency differences was set to p = 0.05; however, after Bonferroni correction for multiple testing (n = 33) a corrected p-value of less than 0.002 was considered significant. Since the normality of distribution assumption for both Genetic Longevity Scores was rejected, study groups were compared by means of nonparametric tests (Kruskal-Wallis and Mann-Whitney U tests). Statistical analyses were performed using the SPSS software package 21.0 (IBM Corp. 2012).

TABLE 1: General information on 33 longevity loci and effect alleles' frequencies in Croatian majority and Roma minority population. The differences between Croatian and Roma effect allele frequencies are evaluated by Chi2-test, and significant p-values are denoted by bold font.

Chr.	Gene	SNP	Effect (longevity) allele	Reference for longevity allele	Croatian effect allele frequency	Roma effect allele frequency	p	Delta (Croatian – Roma difference)	Included in GLS
3	*TERC*	rs12696304	C	(Codd *et al.* 2010, Soerensen *et al.* 2012b)	74.3	70.2	0.284	4.1	yes
3	*TERC*	rs3772190	A	(Soerensen *et al.* 2012b)	22.6	26.7	0.215	-4.1	no
3	*TERC*	rs16847897	G	(Codd *et al.* 2010, Shen *et al.* 2011)	70.5	67.7	0.389	2.8	yes
3	*GHSR*	rs572169	C	(Soerensen *et al.* 2012a)	72.4	80.5	**<0.001**	-8.1	yes
5	*RAD50 (in LD with IL13 region)*	rs2706372	T	(Flachsbart *et al.* 2016)	27.7	23.9	0.090	3.8	yes
5	*LINC02227 (close to EBF1)*	rs2149954	T	(Deelen *et al.* 2014)	38.1	52.9	**<0.001**	-14.8	yes
6	*IRF4*	rs12203592	C	(Law *et al.* 2017)	93.4	89.7	0.005	3.7	yes
6	*TNF-alfa*	rs1800629	G	Yao *et al.* 2020	87.4	95.9	**<0.001**	-8.5	yes
6	*FOXO3A*	rs12206094	T	(Flachsbart *et al.* 2017)	28.8	33.1	0.084	-4.3	yes
6	*FOXO3A*	rs2802292	G	(Bao *et al.* 2014, Revelas *et al.* 2018)	40.9	45.9	0.087	-5.0	no
6	*FOXO3A*	rs2764264	C	(Bao *et al.* 2014)	31.7	40.6	**0.001**	-8.9	no
6	*FOXO3A*	rs10457180	G	(Zettergren *et al.* 2018)	31.6	40.8	**<0.001**	-9.3	no
6	*FOXO3A*	rs13217795	C	(Bao *et al.* 2014)	31.0	39.9	**<0.001**	-8.9	no
6	*FOXO3A*	rs4946935	A	Flachsbart *et al.* 2017, TenNapel *et al.* 2014	29.0	20.4	**<0.001**	8.6	no
6	*IGF2R*	rs9456497	G	(Soerensen *et al.* 2012a)	18.6	19.9	0.384	-1.4	yes
6	*LPA*	rs10455872	A	(König *et al.* 2019)	96.4	98.8	0.003	-2.4	yes
7	*IL6*	rs1800795	G	(Revelas *et al.* 2018, Albani *et al.* 2009a, Fuku *et al.* 2015)	56.8	74.1	**<0.001**	-17.3	yes
7	*IL6*	rs2069837	A	(Zeng *et al.* 2016)	93.2	86.1	**<0.001**	7.1	yes
7	*GHRHR*	rs2267723	A	(Soerensen *et al.* 2012a)	55.6	49.3	0.016	6.3	yes
9	*CDKN2B*	rs4977756	G	(Fortney *et al.* 2015)	39.5	34.8	0.079	4.7	yes
9	*CDKN2B*	rs1333049	G	(Pinós *et al.* 2014)	51.8	52.9	0.672	-1.0	yes
11	*MRE11A*	rs533984	G	(Dato *et al.* 2018)	57.5	58.1	0.788	-0.6	yes
12	*SH2B3/ATXN2*	rs3184504	C	(Kuo *et al.* 2020)	48.5	68.4	**<0.001**	-19.8	yes
13	*KLOTHO*	rs1207362	G	(Soerensen *et al.* 2012a)	69.1	58.2	**<0.001**	10.9	yes
13	*KLOTHO*	rs9536314	T	(Almeida *et al.* 2017, Xu *et al.* 2015)	88.2	83.3	0.008	4.9	no
13	*KLOTHO*	rs9527025	C	(Xu *et al.* 2015, Wolf *et al.* 2019)	11.6	16.8	0.004	-5.2	yes
15	*IGF1R*	rs2229765	A	(Albani *et al.* 2009b)	44.8	29.8	**<0.001**	15.0	yes
17	*TP53*	rs1042522	C	(Van Heemst *et al.* 2005, Reiling *et al.* 2012)	76.2	42.7	**<0.001**	33.6	yes
19	*SIRT6*	rs107251	C	(TenNapel *et al.* 2014)	89.2	95.3	**<0.001**	-6.1	yes
19	*TOMM40*	rs2075650	A	(Flachsbart *et al.* 2016, Shadyab *et al.* 2017)	85.9	82.3	0.080	3.7	yes
19	*APOE*	rs429358	T	(Shadyab *et al.* 2017, Deelen *et al.* 2019)	91.4	80.8	**<0.001**	10.6	yes
19	*APOE*	rs7412	T	(Shadyab *et al.* 2017, Deelen *et al.* 2019)	6.6	6.5	0.915	0.2	yes
19	*APOC1*	rs4420638	A	(Shadyab *et al.* 2017)	87.5	77.9	**<0.001**	9.6	no

RESULTS

All investigated longevity loci were polymorphic, and they were in Hardy–Weinberg equilibrium in all three study groups. The effect (longevity) allele frequencies of the 33 investigated polymorphisms for the Croatian Roma and Croatian "Young" and "Old" cohorts are presented in *Supplementary Table 2* and *Figure 1*, while the genotype frequencies are presented in *Supplementary Table 3*. The effect allele always refers to that indicated in the literature as listed in the *Supplementary Table 2*. The Roma allele frequencies significantly differed from both Croatian cohorts in number of longevity loci ($p < 0.002$, after Bonferroni correction): in 10 out of 33 loci from the "Young" and in 13 out of 33 loci from the "Old" cohort. On the other hand, the allele frequencies of the Croatian "Young" and "Old" cohorts differed only in the *MRE11A* locus (rs533984) with longevity allele G more frequent in the "Old" cohort.

The effect allele frequencies in Croatian ("Old" and "Young" cohorts combined) and Roma populations, as well as the significance and the absolute values (delta) of their differences are provided in *Table 1*. The effect/longevity allele frequencies differences (delta) between Croatian majority and the Roma minority populations are shown in decreasing order (*Figure 2*). The Croatian general population has significantly ($p < 0.002$) higher allele frequencies than the Roma minority population for seven SNPs, and the largest differences (>10%) were found for loci: rs1042522 (*TP53*), rs2229765 (*IGF1R*), rs1207362 (*KLOTHO*), and rs429358 (*TOMM40/APOE/APOC1*). On the other hand, the Croatian Roma have significantly higher frequencies for nine SNPs, and the biggest differences (>10%) are found for loci: rs3184504 (*SH2B3/ATXN2*), rs1800795 (*IL6*), and rs2149954 (*LINC02227 (EBF1*)). It should be noted that three *FOXO3A* SNPs (rs10457180, rs13217795, and rs2764264) also have substantially higher frequencies in the Roma (delta ranging from 8.9 to 9.3%).

The gender structure of three samples is presented in *Supplementary Table 4*. We also examined gender differences in longevity allele frequencies (data not shown), and the only SNP whose distribution was associated with gender in both Croatian and Roma populations was rs12696304. Longevity allele frequencies for rs12696304 differed between men and women in the "Young" Croatian population ($p = 0.047$; with C allele frequency of 66.1% in men and 80.0% in women) and in the Roma sample ($p = 0.015$; with C

TABLE 2: Descriptive statistics and normality of distribution tests for the unweighted and weighted genetic longevity scores (GLS) in three groups and in the combined sample. Significant p-values of normality tests are denoted by bold font.

GLS	Statistics	Croatian "Old" (N= 314)	Croatian "Young" (N=97)	Roma (N= 308)	Total sample (N=719)
unweighted	Mean±SD	29.75±3.13	29.56±3.05	29.42±3.30	29.58±3.19
	Median	30	30	29	30
	Min. - Max.	21–39	21–36	21–39	21–39
	Kolmogorov-Smirnov normality test p-value	**<0.001**	0.090	**<0.001**	**<0.001**
	Shapiro-Wilk normality test p-value	**0.003**	0.233	**0.027**	**<0.001**
weighted	Mean±SD	2.65±0.39	2.61±0.38	2.55±0.51	2.60±0.44
	Median	2.75	2.72	2.74	2.74
	Min. - Max.	0.87–3.34	1.56–3.37	0.88–3.31	0.87–3.37
	Kolmogorov-Smirnov normality test p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	Shapiro-Wilk normality test p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**

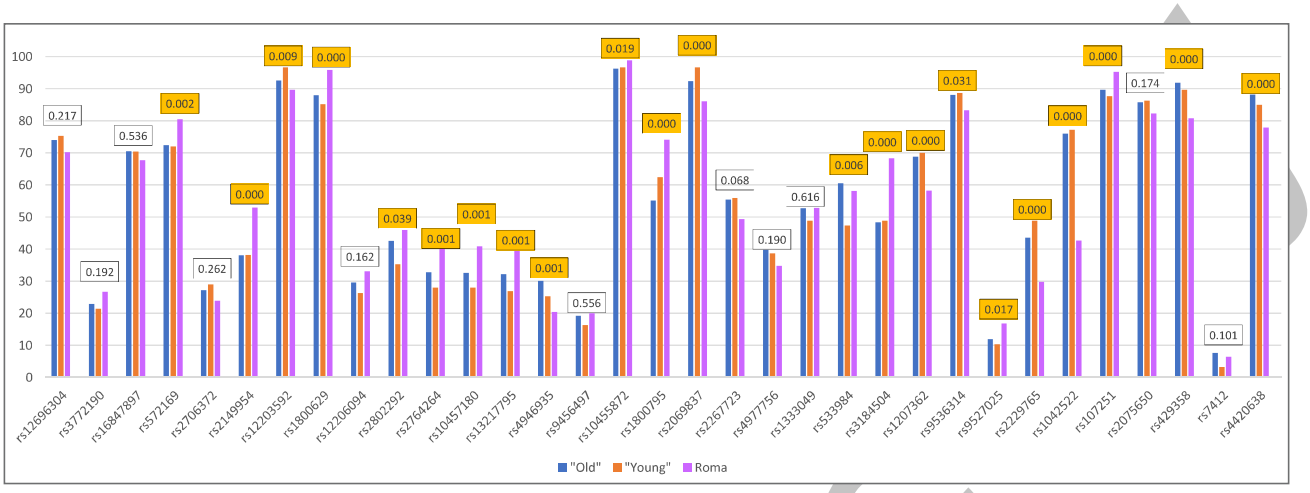

FIGURE 1: Effect alleles' frequencies for 33 longevity loci in Croatian "Old" (85–101 yrs.), "Young" (20–35 yrs.), and Croatian Roma samples. p-values of populations' differences are given for the chi2-test results.

allele frequency of 75.2% in men and 65.9% in women). Additionally, in the Roma population, rs3772190 (p = 0.040) also reached the level of statistical significance when this population was compared by gender, with an A allele frequency of 77.4% in men and 69.8% in women.

Table 2 summarizes unweighted and weighted Genetic Longevity Score (GLS) statistics. Combined unweighted GLS ranged from 21 to 39, on average 29.6), and the mean values in three examined groups were: 29.4 in the Roma, 29.8 in the "Old", and 29.6 in the "Young" Croatian cohort. Normality of distribution tests showed that neither unweighted nor weighted GLS are normally distributed (*Table 2, Supplementary Figure 1*); the exception was unweighted score in the "Young" cohort. Therefore, the nonparametric tests were used for groups' comparisons, and showed that the three compared groups did not differ in GLS, both unweighted and weighted (*Table 3*). Their GLS values also did not show any association with gender (data not shown).

DISCUSSION

The principal aim of the study was to estimate and compare the longevity allele load in the Roma and non-Roma Croatian population. The 33 SNPs were selected from the published genetic data related to human longevity, and the genetic score calculation was based on a subsample of 25 unlinked SNPs. The aim was to evaluate if the short average lifespan of the Roma population demonstrated in different countries by the young age structure, early mortality and small number of older individuals may be related with fewer beneficial longevity genes' alleles present in their gene pool. This study also tests the difference between two extreme age cohorts coming from the majority population of Croatia, with a goal to detect possible selective mortality – related variants.

The presented analysis showed that the Roma minority does not have an increased average genetic risk for premature death in comparison with the majority Croatian population. The results also showed that the

TABLE 3: Genetic longevity scores (GLS) differences among Croatian "Old", Croatian "Young", and Croatian Roma. Significant p-values are denoted by bold font for the nonparametric tests results evaluating differences among groups.

GLS	Three groups: Kruskal-Wallis test p-value	Croatian "Old" vs. Croatian "Young": Mann-Whitney U test p-value	Roma vs. Croatian "Young": Mann-Whitney U test p-value	Roma vs. Croatian "Old": Mann-Whitney U test p-value
unweighted	0.307	0.618	0.589	0.123
weighted	0.289	0.210	0.763	0.184

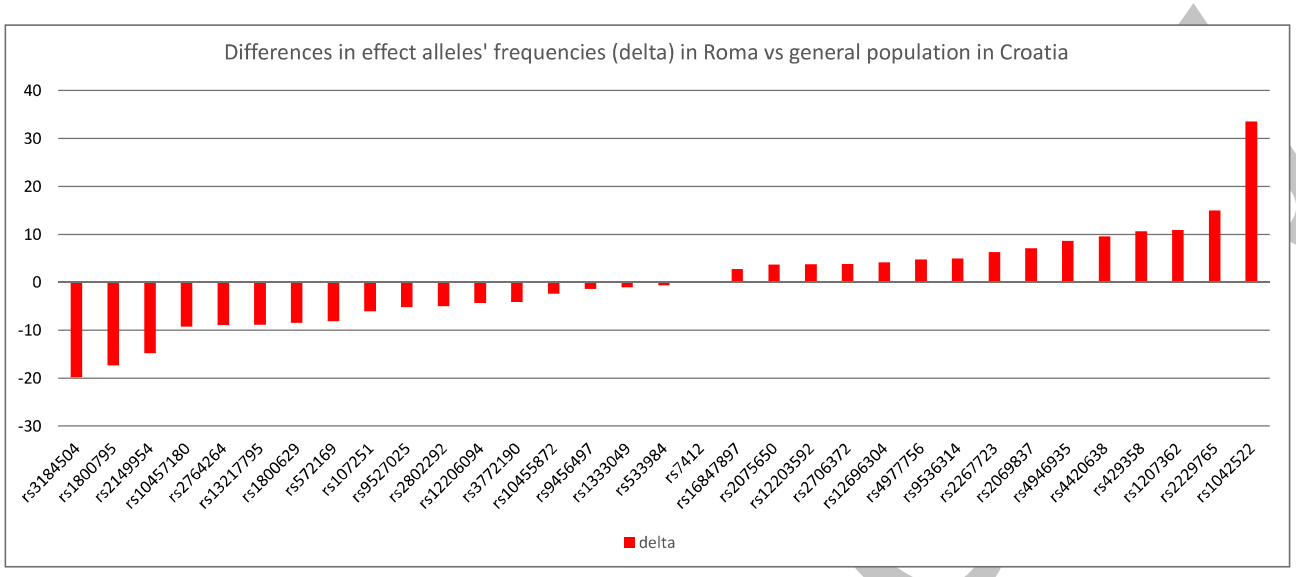

FIGURE 2: Decreasing order of the effect allele frequencies differences (delta) between Croatian (two cohorts combined) and Roma populations for the 33 longevity loci.

Roma population in Croatia has frequencies of alleles in 17 longevity loci similar to those found in the Croatian majority population. However, the allele frequencies of 16 loci significantly differed between the two populations, with seven longevity alleles' frequencies being higher in the Croatian population, while nine longevity alleles are found in higher frequencies in the Roma population. The loci that are the most prominently different (>10%) are specifically addressed here.

Longevity loci markedly more frequent in the general population of Croatia

Among the investigated SNPs, the most notable difference between the majority Croatian population and the Croatian Roma is in rs1042522 in the *TP53* gene. TP53 acts like a tumor suppressor by inducing growth arrest or apoptosis by controlling the set of genes required for regulating cell division. *TP53* rs1042522 is a missense variant, an Arg72Pro substitution (G>C), conferring functional alterations to the protein. The effect of this SNP on longevity was found in many studies, although with some ambiguous results between them (Cho, Suh 2014, Mustafina *et al.* 2011, Groß *et al.* 2014, Reiling *et al.* 2012). In Central Italy the Arg allele was associated with longevity in women aged 91 and over when compared to the group of women aged 73–91 years (Di Pietro *et al.* 2013). On the contrary, Ørsted *et al.* (2007) presented data that Pro allele contributes to longevity through increased survival after cancer diagnosis in the Danish general population, which was later confirmed in Portuguese/Caucasian patients with advanced cervical cancer (Coelho *et al.* 2018). Considering presented evidence, more research is needed to elaborate the true role of rs1042522 in human longevity. Our results show that the C allele resulting in the Arg-Pro substitution is 33.6% more common in the general population of Croatia than in the Croatian Roma.

Most of the loci that have the pronouncedly higher frequencies in the majority Croatian population belong to the growth factor/insulin/IGF-1 signaling (IIS) pathway. In recent years, the IIS pathway has emerged as one of the most notable candidates in longevity research, as several studies have confirmed that multiple SNPs in genes of the IIS pathway are associated with longevity (Soerensen *et al.* 2012a, Sanese *et al.* 2019). rs2229765 is a synonymous substitution with unclear functional significance. Allele A of rs2229765, located in the *IGF1R* gene, has been shown to confer advantage to longevity in males. In an Italian population sample, the frequency of allele A was significantly higher in males over 85 years of age, in comparison to males between the ages of 70 and 85 (Albani *et al.* 2009). It also correlated with a drop in plasma levels of IGF-1 in males, but no correlation was found in females. In the majority Croatian population allele A is 15% more common than in the Croatian Roma.

Another intronic mutation is rs1207362 in the *KLOTHO* gene. Named after the Greek goddess that

spins the thread of life, Clotho, the *KLOTHO* gene was recognized as a candidate for slowing down the ageing process. It encodes the α-Klotho protein, a multifunctional protein that was reported to suppress the signaling downstream of the insulin receptor substrate (IRS) and the IGF-1 receptor (IGF-1R) (Xu *et al*. 2015). In a study by Soerensen *et al*. (2012a), rs1207362, which could possibly cause alternative splicing of the α-Klotho mRNA, was associated with longevity in the Danish population. In our research, longevity G allele in the locus rs1207362 is found with 10.9% higher frequency in the Croatian population than in the Croatian Roma.

ApoE is a polymorphic apolipoprotein essential for plasma lipoprotein metabolism and lipid transport. There are three common allelic variants of the *APOE* gene: ε3 (Cys112, Arg158), ε2 (Cys112, Cys158) and ε4 (Arg112, Arg158). They are defined by combination of SNPs at two independent loci: rs429358 and rs7412. ε2 is defined by T allele on both loci (8.4% worldwide frequency), ε4 by C allele in both loci (13.7% worldwide frequency), while ε3 is defined by C allele at rs7412 and T allele at rs429358 (77.9% worldwide frequency). *APOE* is the first discovered candidate gene for cardiovascular diseases, and ε4 isoform has been associated with Alzheimer's disease and early cognitive decline (Rawle *et al*. 2018). Even larger importance lies in the fact that there is a huge amount of evidence that proves the robust association of the ε4 isoform with longevity. Shadyab *et al*. (2017) found that rs429358 and rs7412 were significantly associated with survival to age 90 in their meta-analysis among American women of African and European ancestry. Deelen *et al*. (2019) reported that rs429358, defining *APOE* ε4, was associated with decreased odds of becoming long-lived and significant association of rs7412, defining *APOE* ε2, with increased odds of becoming long-lived. In the present study T allele of rs429358 is 10.6% more common in the general population of Croatia than in the Roma minority population ($p < 0.001$), while the T allele frequency of rs7412 is similarly distributed in two populations ($p = 0.915$).

Longevity loci markedly more frequent in the Roma minority population

SH2B3 encodes a multi-domain protein involved in blood coagulation and erythropoietin (EPO) signaling pathway (Tong *et al*. 2005). A missense variant (rs3184504) in *SH2B3* has been linked to many common diseases in genome-wide association studies, including several autoimmune and cardiovascular disorders (Laroumanie *et al*. 2018) as well as cancers (Hung *et al*. 2015). Pilling and coworkers in a genome-wide analysis of parental longevity in UK Biobank found that 11 highly correlated genetic variants in the wider *SH2B3/ATXN2/BRAP* locus (including rs3184504) were associated with parent's attained age (Pilling *et al*. 2017). This longevity association has been replicated in other cohorts (Timmers *et al*. 2019). Kuo and coworkers in their study showed that the C allele was associated with lower blood pressure, shorter reaction time (cognitive measure), as well as healthier muscle mass and hematological measures. They also found associations between the C allele and reduced rates of hypothyroidism, hypertension and cardiovascular disease (Kuo *et al*. 2020). The protective rs3184504 C allele is also associated with higher expression of genes involved in toll-like receptor (TLR) signaling (Westra *et al*. 2013). In our study, the rs3184504 longevity related C allele is found in 19.8% higher frequency in Roma than in Croatian population.

Interleukin 6 is a pleiotropic cytokine produced by many cell types. It has been thoroughly researched due to its role in the inflammatory processes (Serrano *et al*. 2008), and recent studies indicate that it might also be a reliable marker for functional decline, and a predictor of morbidity and mortality in old age (Di Bona *et al*. 2009, De Lauretis *et al*. 2013, Fraga *et al*. 2015, Parks *et al*. 2020). rs1800795 is an intronic SNP located in the 5'-flanking region of the interleukin-6 (*IL-6*) gene. Studies of this SNP concerning longevity have contradicting results. Kayaalti *et al*. (2011) found a positive association between the presence of C allele and longevity in the Turkish population, while other studies associated allele G with longevity (Albani *et al*. 2009, Revelas *et al*. 2018). In our research, the G allele was attributed as a "longevity allele" and it is 17.3% more common in Roma than in the Croatian majority population.

Intron variant of long intergenic non-protein coding RNA 2227 (*LINC02227*) was found to be connected with longevity in a genome-wide association meta-analysis of 7,729 long-lived individuals of European descent (≥ 85 years) and 16,121 younger controls (<65 years) and the results were replicated in an additional set of 13,060 long-lived individuals and 61,156 controls (Deelen *et al*. 2014). In a study performed by Shadyab *et al*. (2017), only seven SNPs in LD with rs2149954 were significantly associated with survival to age 85 after correction for multiple testing. Nygaard *et al*. (2017) found a protective effect of the rs2149954 minor allele T on mortality independent of cardiovascular disease. In the middle-aged individuals they also found

a significant association between the rs2149954 minor allele dose and a lower risk for hypertension, and in the elderly individuals they additionally found indications of an association with a lower risk of cancer and increased physical performance represented by a higher Activities of Daily Living (ADL) score and improved chair stand. In our research the T allele was attributed as "longevity allele" and it is 14.8% more common in Roma than in the Croatian majority population.

Although the three *FOXO3A* loci did not meet the criteria of 10% difference, large enough population differences (8.9%–9.3%) and importance of this gene evoke some remarks. The *FOXO3A* was proven to be a longevity gene by multiple studies performed on different populations (Sanese *et al.* 2019). Product of the *FOXO3* gene is a transcription factor that regulates stress responses and affects lifespan, but the exact mechanisms through which *FOXO3* modulates ageing have not yet been identified (Grossi *et al.* 2018, Flachsbart *et al.* 2017). *FOXO3* is evolutionarily highly conserved, so most variations of the *FOXO3* gene, including the variations that may play a role in longevity, were found in its non-coding elements. *FOXO3* mediates gene expression as a response to hormones, growth factors and nutrients. Impairment of the IIS signaling pathway and PI3K signaling cascade are thought to modulate *FOXO3* expression in a way that is beneficial to longevity in a variety of organisms (Sanese *et al.* 2019). Three of the *FOXO3* SNPs have markedly higher frequencies in the Croatian Roma than in the majority Croatian population. rs10457180 is the most commonly mentioned longevity SNP (Flachsbart *et al.* 2017, Sanese *et al.* 2019), and it is 9.3% more common in the Roma. rs13217795 was also more common in the Roma (for almost 9%,) and this SNP was found to be associated with male longevity and healthy ageing (Willcox *et al.* 2008, Bao *et al.* 2014). rs2764264 was the first *FOXO3* SNP to be associated with longevity (Soerensen *et al.* 2010), and it is 8.9% more common in the Roma than in the Croatian non-Roma population.

MRE11A – the only locus differing in two extreme age cohorts

The only longevity locus that is more frequent in "Old" compared to "Young" cohorts of the Croatian majority population is *MRE11A* (rs533984). MRE11A is a component of the MRN complex. MRE11A provides single-strand (ss) endonuclease activity and double-strand-specific 3'-5' exonuclease activity and is therefore essential in double-strand break repair, recombination and maintenance of telomere integrity and meiosis (De Jager *et al.* 2001, Trujillo *et al.* 1998, Coquel *et al.* 2018, Paull, Gellert 1998, Carney *et al.* 1998). *MRE11A* rs533984 has been implicated in longevity by Dato and coworkers, who performed data analysis on 1,058 tagging SNPs in 140 genes of 1825 subjects (Dato *et al.* 2018). By the multidimensional reduction (MDR) analysis, they showed that the *MRE11A* rs533984 variant with the G allele was significantly associated with extreme survival in females.

Evolutionary considerations

The specific focus of this study was to test if the genetic load of the group of loci previously reported as putative longevity genes are comparable in a European population (such as Croatians) with the amount of beneficial alleles present in the representative sample of the Roma population. The rationale for posing this question is the shorter average life-span of the Roma population in comparison with surrounding majority populations found in all European countries that collect ethnic-specific mortality data. The second reason is the genetic specificity of the Roma population, which has been confirmed in all previously conducted studies (for Croatian Roma, e.g. Salihović *et al.* 2011, Klarić *et al.* 2009, Barešić, Salihović 2014).

The complex social structure and cultural specificities of the Roma made genetic drift an extremely powerful evolutionary force shaping the genetic architecture of this ethnic group worldwide. Namely, their at least a thousand year-long history of migrations was structured in a way that they were spread in numerous small groups (promoting multiple founder effects), sharing strong cultural practice of endogamy (or very selective exogamy; they share brides only with a narrow range of other Roma groups). The Roma also suffered a number of drastic population shrinking events in their history; the prominent one being during the WWII, where they were one of the primary targets of the Holocaust, following the Jewish population. All those reasons confer a possibility that the genetic drift shuffled their genetic structure in a more or in a less advantageous way considering longevity genes.

We do not expect that natural selection for a particular set of genes we have chosen as candidates for the longevity phenotype has occurred in the time span that has elapsed since the formation of the Roma population. In fact, we do not expect that even in one human population, some longevity genes have had the opportunity to be selected due to the fact that the lifespan that humans now enjoy is only a 100 years long phenomenon.

Namely, in the overwhelming majority of the span of human evolution, the circumstances were not permitting the selection process to act on "longevity phenotype" directly, as this is a phenotype that is related to the post-reproductive period of life. However, in the future some evidence might be found for a subsample of those loci indicating that their effect could be considered as a form of antagonistic pleiotropy (AP). A few genes (some are also present in here included group) are already considered as those that might act beneficially in young age, while those genes' action is nocent for the post-reproductive period of life of an individual. To this group of genes belong the genes included in the growth hormone (GH) signaling pathway that also includes insulin-like growth factor 1 (IGF-1). Namely, IGF-1 is a primary mediator of the growth hormone effect, and it plays an important role in growth and development, while in adult age it accelerates the senescence of an individual.

All here considered "longevity genes" have a range of important roles in some of the substantial cellular pathways, and some variants in those genes, in present circumstances, happen to be more common in long-lived persons. And we interpret them – a posteriori – as beneficial, or as those which increase the chance for some individuals to reach a more advanced age.

Strengths and limitations of the study

The most important contribution of this research is the presentation of genetic data on the longevity loci for the Roma which is an ethnic minority population overall underrepresented in genetic studies. Also, to our knowledge, this is the first study that compares the longevity variants loads in different populations, particularly by constructing a genetic longevity score (GLS). The present study investigating the variation of 33 longevity loci in Roma minority and in Croatian majority population revealed the two following main results:

(1) The Croatian Roma and the surrounding majority Croatian population have significantly different allele frequencies in half of the investigated longevity variants' loci (in 16 out of 33 investigated loci: in nine, longevity alleles are more prevalent in the Croatian Roma, while in seven they are more frequently present in the general population of Croatia). In some loci the allele frequency differences between the two populations are actually high (>10%). Therefore, we can point to some longevity alleles at particular loci that are more prevalent in the Roma minority (in genes: *SH2B3/ATXN2*, *IL6*, and *LINC02227* (*EBF1*)) and others that are more frequently present in the Croatian majority population (in genes: *TP53*, *IGF1R*, *KLOTHO*, and *TOMM40|APOE|APOC1*).

(2) Within the context of large allele frequency differences between the Roma minority and surrounding majority Croatian population, the second finding of the study stands prominently and brings even greater importance. Namely, the Roma GLS is the same as in the general population of Croatia, and it is so irrespectively if the "Old" or "Young" cohorts have been considered. This implies the same risk of premature death in two populations, and the identical chance for survival to the age of 85 years, when genetics is exclusively considered. This result indicates that the age structure and mortality pattern found in the Roma population are not the consequence of their increased genetic risk for premature mortality, but rather a combination of extrinsic factors determined by societal circumstances.

Two age cohorts coming from the opposite sides of adult age distribution are deliberately chosen in order to emphasize the possible selective mortality signals present in evaluated longevity genes' loci. However, the principal limitation of this research is the small number of participants in the "Young" cohort, which necessitates that the obtained results including this group should be considered with caution. It should be done so for the lack of the differences in their mean GLS score compared to the "Old" cohort as well as for the single difference in allele frequency separating two age cohorts. Namely, the only longevity locus that is more frequent in "Old" compared to "Young" cohorts of the Croatian majority population is *MRE11A* (rs533984). Having in mind the small "Young" cohort size and the marginal significance of the difference (after Bonferroni correction for multiple testing), this finding, although intriguing, warrants replication in more powered studies.

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Tatjana Škarić-Jurić[1]
Željka Celinšćak[1*]
Maja Šetinc[1]
Luka Bočkor[2]
Anita Stojanović Marković[1]
Matea Zajc Petranović[1]
Marijana Peričić Salihović[1]
Joris Deelen[3, 4]
Branka Janićijević[1]
Nina Smolej Narančić[1]

[1] Institute for Anthropological Research, Ljudevita Gaja 32, 10 000 Zagreb, Croatia
[2] Centre for Applied Bioanthropology, Institute for Anthropological Research, Zagreb, Croatia
[3] Max Planck Institute for Biology of Ageing, Köln, Germany
[4] Cologne Excellence Cluster on Cellular Stress Responses in Ageing-Associated Diseases (CECAD), University of Cologne, Cologne, Germany

*Corresponding author.
E-mail: zeljka.jurcic@gmail.com

SUPPLEMENTARY TABLE 1. Beta values from the analysis of survival to the 90th percentile, obtained from longevity GWA studies performed on several cohorts of European ancestry (Deelen *et al.* 2019). Beta values were used as weights for the calculation of weighted GLS.

Chromosome	Position	Gene	SNP	Literature-defined longevity allele	Effect allele (in Deelen *et al.* 2019)	Non-effect allele (in Deelen *et al.* 2019)	Effect allele frequency	Beta	SE	P-value	Effective N
3	169481271	*TERC*	rs12696304	C	C	G	0,73	0,0252	0,0223	0,2581	11615
3	169500487	*TERC*	rs3772190	A	A	G	0,24	-0,021	0,0223	0,3477	11615
3	169568116	*TERC*	rs16847897	G	C	G	0,28	-0,0193	0,0214	0,3657	11615
3	172165727	*GHSR*	rs572169	C	T	C	0,31	-0,0093	0,0207	0,6549	11615
5	131935477	*RAD50*	rs2706372	T	T	C	0,21	0,0495	0,0245	0,04296	11050
5	157820602	*LINC02227*	rs2149954	T	T	C	0,37	0,0515	0,0205	0,01188	11050
6	396321	*IRF4*	rs12203592	C	T	C	0,14	-0,048	0,0318	0,1305	9923
6	31543031	*TNF-alfa*	rs1800629	G	A	G	0,16	-0,0901	0,0336	0,007318	8400
6	108906200	*FOXO3A*	rs12206094	T	T	C	0,28	0,0776	0,0213	0,0002733	11615
6	108908518	*FOXO3A*	rs2802292	G	T	G	0,62	-0,0787	0,0198	6,81E-05	11615
6	108934461	*FOXO3A*	rs2764264	C	T	C	0,69	-0,0879	0,0209	2,65E-05	11615
6	108965039	*FOXO3A*	rs10457180	G	A	G	0,7	-0,0925	0,0209	9,50E-06	11615
6	108974098	*FOXO3A*	rs13217795	C	T	C	0,7	-0,0941	0,0209	6,85E-06	11615
6	109000742	*FOXO3A*	rs4946935	A	A	G	0,29	0,0934	0,0211	9,59E-06	11615
6	160443428	*IGF2R*	rs9456497	G	A	G	0,82	0,0392	0,0249	0,116	11615
6	161010118	*LPA*	rs10455872	A	A	G	0,94	0,1236	0,0454	0,006513	11050
7	22766645	*IL6*	rs1800795	G	C	G	0,42	0,0279	0,0196	0,1559	11615
7	22768027	*IL6*	rs2069837	A	A	G	0,92	0,0742	0,0357	0,03782	11615
7	31006942	*GHRHR*	rs2267723	A	A	G	0,55	-0,0019	0,0197	0,9238	11615
9	22068652	*CDKN2B*	rs4977756	G	A	G	0,6	-0,0851	0,0196	1,39E-05	11615
9	22125503	*CDKN2B*	rs1333049	G	C	G	0,47	-0,0606	0,0203	0,002851	11050
11	94199272	*MRE11A*	rs533984	G	A	G	0,39	-0,0071	0,0197	0,7181	11615
12	111884608	*SH2B3/ATXN2*	rs3184504	C	T	C	0,49	-0,0438	0,0191	0,022	11615
13	33612839	*KLOTHO*	rs1207362	G	T	G	0,31	-0,0235	0,0208	0,2581	11615
13	33628138	*KLOTHO*	rs9536314	T	T	G	0,84	-0,024	0,0266	0,367	11615
13	33628193	*KLOTHO*	rs9527025	C	C	G	0,16	0,0234	0,0266	0,3796	11615
15	99478225	*IGF1R*	rs2229765	A	A	G	0,46	0,0079	0,0191	0,6779	11615
17	7579472	*TP53*	rs1042522	C	C	G	0,71	-0,0103	0,0231	0,6564	11615
19	4176085	*SIRT6*	rs107251	C	T	C	0,12	-0,028	0,0308	0,3621	11615
19	45395619	*TOMM40*	rs2075650	A	A	G	0,87	0,3799	0,0299	5,16E-37	11075
19	45411941	*APOE*	rs429358	T	T	C	0,87	0,5098	0,0322	1,28E-56	10878
19	45412079	*APOE*	rs7412	T	T	C	0,09	0,2452	0,0367	2,38E-11	11075
19	45422946	*APOC1*	rs4420638	A	A	G	0,83	0,4079	0,0308	4,93E-40	11050

SUPPLEMENTARY TABLE 2. Allele frequencies in general ("Old" and "Young") and Roma population of Croatia. Original α level = 0.05; α level after Bonferroni correction < 0.002.

					Included in	"Old"	"Young"	Roma	"Old" vs "Young"	"Young" vs Roma	"Old" vs Roma
Chromosome	Gene	SNP	Longevity allele	References	GRS	Longevity allele frequency	Longevity allele frequency	Longevity allele frequency	p	p	p
3	*TERC*	rs12696304	C	Codd *et al.* 2010, Soerensen *et al.* 2012	yes	74,0	75,3	70,2	0,773	0,191	0,141
3	*TERC*	rs3772190	A	Soerensen *et al.* 2012	no	22,9	21,4	26,7	0,763	0,173	0,143
3	*TERC*	rs16847897	G	Codd *et al.* 2010, Shen *et al.* 2011	yes	70,5	70,4	67,7	1,000	0,527	0,292
3	*GHSR*	rs572169	C	Soerensen *et al.* 2012	yes	72,5	72,0	80,5	0,926	0,018	0,001
5	*RAD50*	rs2706372	T	Flachsbart *et al.* 2016	yes	27,2	29,0	23,9	0,641	0,174	0,211
5	*LINC02227*	rs2149954	T	Deelen *et al.* 2014	yes	38,1	38,2	52,9	1,000	0,001	<0,001
6	*IRF4*	rs12203592	C	Law *et al.* 2017	yes	92,5	96,6	89,7	0,058	0,004	0,087
6	*TNF-alfa*	rs1800629	G	Yao *et al.* 2020	yes	88,0	85,2	95,9	0,312	<0,001	<0,001
6	*FOXO3A*	rs12206094	T	Flachsbart *et al.* 2017	yes	29,6	26,3	33,1	0,409	0,086	0,193
6	*FOXO3A*	rs2802292	G	Bao *et al.* 2014, Revelas *et al.* 2018	no	42,5	35,3	45,9	0,088	0,013	0,247
6	*FOXO3A*	rs2764264	C	Bao *et al.* 2014	no	32,8	28,0	40,6	0,243	0,002	0,006
6	*FOXO3A*	rs10457180	G	Zettergren *et al.* 2018	no	32,6	28,0	40,8	0,244	0,002	0,004
6	*FOXO3A*	rs13217795	C	Bao *et al.* 2014	no	32,2	26,9	39,9	0,176	0,002	0,007
6	*FOXO3A*	rs4946935	A	Flachsbart *et al.* 2017, TenNapel *et al.* 2014	no	30,1	25,3	20,4	0,228	0,179	<0,001
6	*IGF2R*	rs9456497	G	Soerensen *et al.* 2012	yes	19,2	16,3	19,9	0,444	0,328	0,773
6	*LPA*	rs10455872	A	König *et al.* 2019	yes	96,3	96,7	98,8	1,000	0,090	0,005
7	*IL6*	rs1800795	G	Revelas *et al.* 2018, Albani *et al.* 2009, Fuku *et al.* 2015	yes	55,2	62,5	74,1	0,085	0,003	<0,001
7	*IL6*	rs2069837	A	Zeng *et al.* 2016	yes	92,3	96,8	86,1	0,050	<0,001	0,001
7	*GHRHR*	rs2267723	A	Soerensen *et al.* 2012	yes	55,5	56,0	49,3	0,933	0,128	0,033
9	*CDKN2B*	rs4977756	G	Fortney *et al.* 2015	yes	39,7	38,7	34,8	0,864	0,335	0,075
9	*CDKN2B*	rs1333049	G	Pinós *et al.* 2014	yes	52,7	48,9	52,9	0,403	0,356	1,000
11	*MRE11A*	rs533984	G	Dato *et al.* 2018	yes	60,5	47,3	58,1	0,002	0,011	0,414
12	*SH2B3/ATXN2*	rs3184504	C	Kuo *et al.* 2020	yes	48,4	48,9	68,4	0,934	<0,001	<0,001
13	*KLOTHO*	rs1207362	G	Soerensen *et al.* 2012	yes	68,8	70,0	58,2	0,785	0,005	<0,001
13	*KLOTHO*	rs9536314	T	Almeida *et al.* 2017, Xu *et al.* 2015	no	88,1	88,7	83,3	0,897	0,080	0,021
13	*KLOTHO*	rs9527025	C	Xu *et al.* 2015, Wolf *et al.* 2019	yes	11,9	10,3	16,8	0,602	0,035	0,017
15	*IGF1R*	rs2229765	A	Albani *et al.* 2009	yes	43,6	48,9	29,8	0,232	<0,001	<0,001
17	*TP53*	rs1042522	C	Van Heemst *et al.* 2005, Reiling *et al.* 2012	yes	76,0	77,2	42,7	0,768	<0,001	<0,001
19	*SIRT6*	rs107251	C	TenNapel *et al.* 2014	yes	89,6	87,6	95,3	0,423	0,001	<0,001
19	*TOMM40*	rs2075650	A	Flachsbart *et al.* 2016, Shadyab *et al.* 2017	yes	85,8	86,3	82,3	1,000	0,257	0,100
19	*APOE*	rs429358	T	Shadyab *et al.* 2017, Deelen *et al.* 2019	yes	91,9	89,7	80,8	0,369	0,005	<0,001
19	*APOE*	rs7412	T	Shadyab *et al.* 2017, Deelen *et al.* 2019	yes	7,6	3,2	6,5	0,042	0,105	0,436
19	*APOC1*	rs4420638	A	Shadyab *et al.* 2017	no	88,2	84,9	77,9	0,256	0,037	<0,001

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SUPPLEMENTARY TABLE 3. Genotype frequencies in general ("Old" and "Young") and Roma population of Croatia. Original α level = 0.05; α level after Bonferroni correction < 0.002.

Gene	SNP	Genotype	Included in GRS	"Old"		"Young"		Roma		"Old" vs "Young"	"Young" vs Roma	"Old" vs Roma
				N	frequency (%)	N	frequency (%)	N	frequency (%)	p	p	p
TERC	rs12696304	C:C		170	54,0	52	53,6	160	51,1	0,860	0,866	0,350
		G:C	yes	127	40,3	38	39,2	126	40,3			
		G:G		18	5,7	7	7,2	27	8,6			
TERC	rs3772190	G:G		186	58,9	56	58,3	173	56,0	0,764	0,855	0,213
		G:A	no	116	36,7	34	35,4	112	36,2			
		A:A		14	4,4	6	6,2	24	7,8			
TERC	rs16847897	G:G		160	50,0	49	48,5	140	44,9	0,964	0,727	0,366
		G:C	yes	133	41,6	43	42,6	147	47,1			
		C:C		27	8,4	9	8,9	25	8,0			
GHSR	rs572169	C:C		171	54,3	55	55,0	205	65,9	0,992	0,076	0,004
		C:T	yes	115	36,5	36	36,0	92	29,6			
		T:T		29	9,2	9	9,0	14	4,5			

RAD50/IL13 region	rs2706372	C:C		166	52,9	53	54,1	183	58,8	0,237	0,133	0,318
		T:C	yes	125	39,8	33	33,7	109	35,0			
		T:T		23	7,3	12	12,2	19	6,1			
LINC02227 (EBF1)	rs2149954	C:C		130	40,5	39	39,4	70	22,7	0,968	0,002	<0,001
		T:C	yes	138	43,0	44	44,4	153	49,7			
		T:T		53	16,5	16	16,2	85	27,6			
IRF4	rs12203592	C:C		275	86,2	87	93,5	257	80,8	0,142	0,012	0,161
		T:C	yes	41	12,9	6	6,5	55	17,3			
		T:T		3	0,9	0	0,0	6	1,9			
TNF-alfa	rs1800629	G:G		246	77,4	67	69,8	278	91,1	0,135	<0,001	<0,001
		G:A	yes	64	20,1	28	29,2	27	8,9			
		A:A		8	2,5	1	1,0	0	0,0			
FOXO3A	rs12206094	C:C		155	49,2	53	53,5	150	48,1	0,513	0,062	0,076
		T:C	yes	134	42,5	41	41,4	119	38,1			
		T:T		26	8,3	5	5,1	43	13,8			
FOXO3A	rs2802292	T:T		103	32,1	38	38,0	99	32,0	0,458	0,074	0,077
		T:G	no	164	51,1	49	49,0	137	44,3			
		G:G		54	16,8	13	13,0	73	23,6			
FOXO3A	rs2764264	T:T		137	44,2	51	51,5	116	37,3	0,428	0,009	0,007
		T:C	no	141	45,5	40	40,4	136	43,7			
		C:C		32	10,3	8	8,1	59	19,0			
FOXO3A	rs10457180	A:A		139	44,1	52	52,0	115	36,6	0,384	0,006	0,004
		G:A	no	145	46,0	40	40,0	140	44,6			
		G:G		31	9,8	8	8,0	59	18,8			
FOXO3A	rs13217795	T:T		140	44,4	53	54,1	113	37,2	0,245	0,004	0,007
		T:C	no	146	46,3	38	38,8	138	45,4			
		C:C		29	9,2	7	7,1	53	17,4			
FOXO3A	rs4946935	G:G		157	49,1	55	57,9	196	63,0	0,318	0,388	0,001
		G:A	no	135	42,2	33	34,7	102	32,8			
		A:A		28	8,8	7	7,4	13	4,2			

SUPPLEMENTARY TABLE 3. Continued.

IGF2R	rs9456497	A:A		206	65,0	67	71,3	198	62,5	0,500	0,250	0,743
		G:A	yes	101	31,9	24	25,5	110	34,7			
		G:G		10	3,2	3	3,2	9	2,8			
LPA	rs10455872	A:A		296	93,4	94	94,0	309	97,8	0,728	0,091	0,020
		G:A	yes	19	6,0	6	6,0	7	2,2			
		G:G		2	0,6	0	0,0	0	0,0			
IL6	rs1800795	G:G		99	31,9	36	39,1	174	55,2	0,152	0,018	<0,001
		C:G	yes	144	46,5	44	47,8	117	37,1			
		C:C		67	21,6	12	13,0	24	7,6			
IL6	rs2069837	A:A		269	84,9	75	93,8	239	76,1	0,110	0,002	0,005
		G:A	yes	47	14,8	5	6,2	67	21,3			
		G:G		1	0,3	0	0,0	8	2,5			
GHRHR	rs2267723	A:A		96	30,9	30	30,9	77	24,9	0,771	0,140	0,098
		G:A	yes	154	49,5	51	52,6	152	49,2			
		G:G		61	19,6	16	16,5	80	25,9			
CDKN2B/ANRIL	rs4977756	A:A		113	35,8	40	40,4	139	45,3	0,635	0,697	0,042
		G:A	yes	154	48,7	43	43,4	122	39,7			
		G:G		49	15,5	16	16,2	46	15,0			
TP53/CDKN2A	rs1333049	G:G		96	30,2	24	24,2	101	32,3	0,509	0,249	0,686
		G:C	yes	145	45,6	50	50,5	132	42,2			
		C:C		77	24,2	25	25,3	80	25,6			
MRE11A	rs533984	G:G		110	35,0	22	22,2	114	36,5	0,006	0,029	0,081
		G:A	yes	159	50,6	51	51,5	135	43,3			
		A:A		45	14,3	26	26,3	63	20,2			
SH2B3/ATXN2	rs3184504	T:T		84	26,6	27	27,3	33	10,6	0,897	<0,001	<0,001
		T:C	yes	158	50,0	47	47,5	125	40,1			
		C:C		74	23,4	25	25,3	154	49,4			
KL (KLOTHO)	rs1207362	G:G		152	48,4	47	48,5	104	35,0	0,742	0,013	0,001
		T:G	yes	128	40,8	42	43,3	137	46,1			
		T:T		34	10,8	8	8,2	56	18,9			

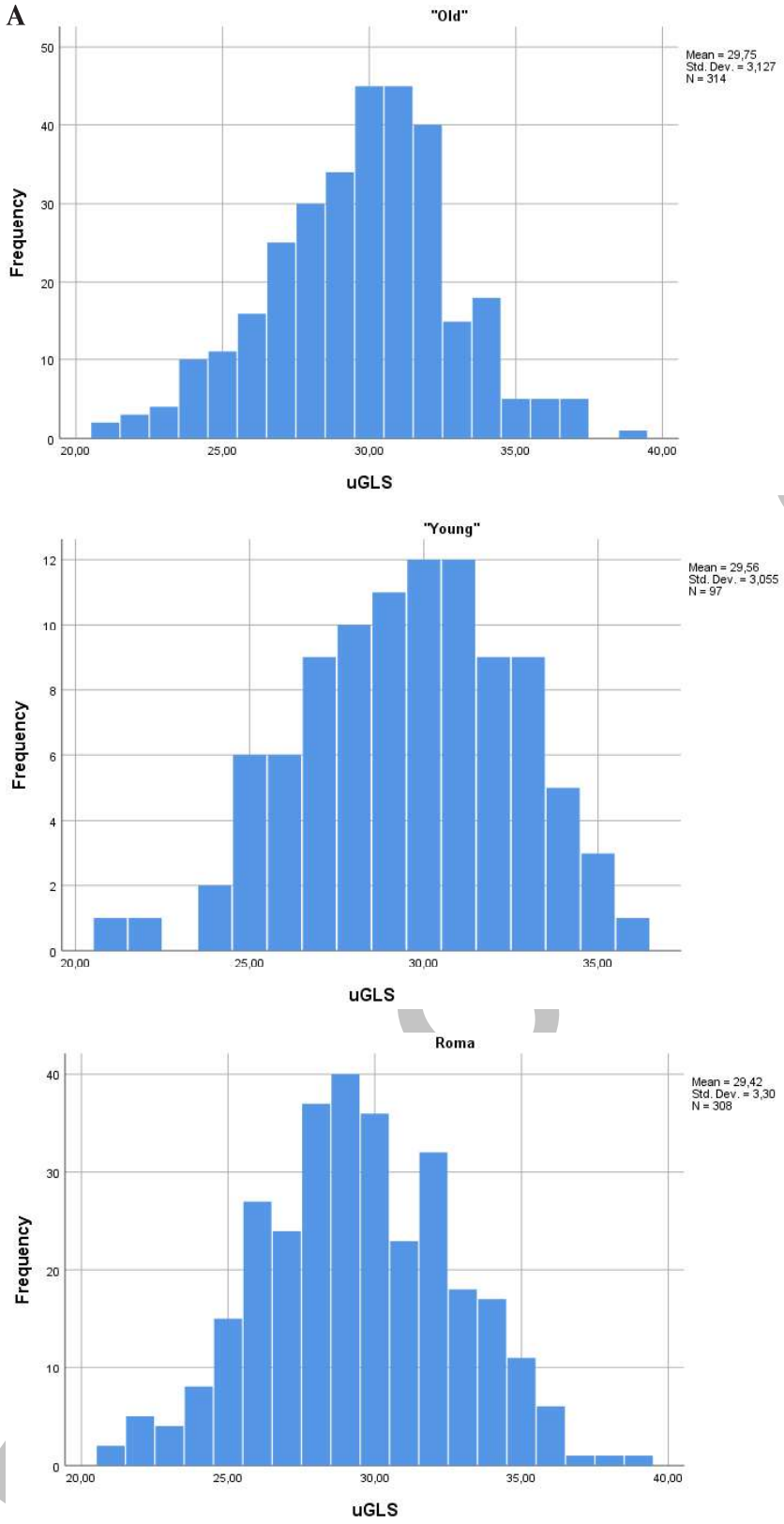
KLOTHO	rs9536314	T:T		253	78,8	77	79,4	214	70,2	0,679	0,160	0,045
		T:G	no	60	18,7	19	19,6	81	26,6			
		G:G		8	2,5	1	1,0	10	3,3			
KLOTHO	rs9527025	G:G		253	78,8	79	80,6	219	69,7	0,288	0,052	0,029
		C:G	yes	60	18,7	19	19,4	86	27,4			
		C:C		8	2,5	0	0,0	9	2,9			
IGF1R	rs2229765	G:G		102	32,6	27	28,4	161	51,8	0,483	<0,001	<0,001
		A:G	yes	149	47,6	44	46,3	116	37,3			
		A:A		62	19,8	24	25,3	34	10,9			
TP53	rs1042522	C:C		181	56,7	58	59,8	50	16,1	0,778	<0,001	<0,001
		C:G	yes	124	38,9	34	35,1	162	52,3			
		G:G		14	4,4	5	5,2	98	31,6			
SIRT6	rs107251	C:C		259	80,7	77	77,8	289	91,2	0,618	<0,001	<0,001
		T:C	yes	59	18,4	20	20,2	28	8,8			
		T:T		3	0,9	2	2,0	0	0,0			
TOMM40/APOE/APOC1	rs2075650	A:A		238	74,1	72	74,2	218	69,0	0,671	0,322	0,303
		G:A	yes	75	23,4	24	24,7	86	27,2			
		G:G		8	2,5	1	1,0	12	3,8			
TOMM40/APOE/APOC1	rs429358	T:T		262	84,5	79	79,0	196	66,0	0,262	0,016	<0,001
		C:T	yes	46	14,8	21	21,0	88	29,6			
		C:C		2	0,6	0	0,0	13	4,4			
TOMM40/APOE/APOC1	rs7412	C:C		270	85,7	91	92,9	272	87,2	0,151	0,147	0,219
		C:T	yes	42	13,3	7	7,1	40	12,8			
		T:T		3	1,0	0	0,0	0	0,0			
TOMM40/APOE/APOC1	rs4420638	A:A		246	78,1	71	71,0	193	62,1	0,259	0,060	<0,001
		G:A	no	64	20,3	28	28,0	98	31,5			
		G:G		5	1,6	1	1,0	20	6,4			

SUPPLEMENTARY TABLE 4. Sex and age distribution of three studied groups.

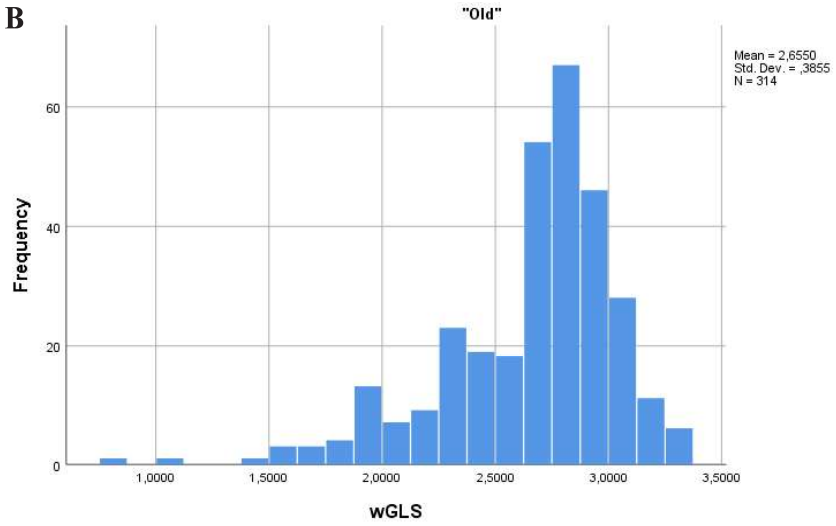
Group	N	Sex		Age				
		Men N (%)	Women N (%)	Mean	Std. Deviation	Minimum	Maximum	Range
Croatian "Old"	314	80 (25.5%)	234 (74.5%)	88.15	3.39	79	101	22
Croatian "Young"	97	32 (33.0%)	65 (67.0%)	24.63	3.76	20	35	15
Croatian – combined	411	112 (27.3%)	299 (72.7%)	73.15	27.23	20	101	81
Roma	308	140 (45.5%)	168 (54.5%)	40.49	13.94	18	75	57

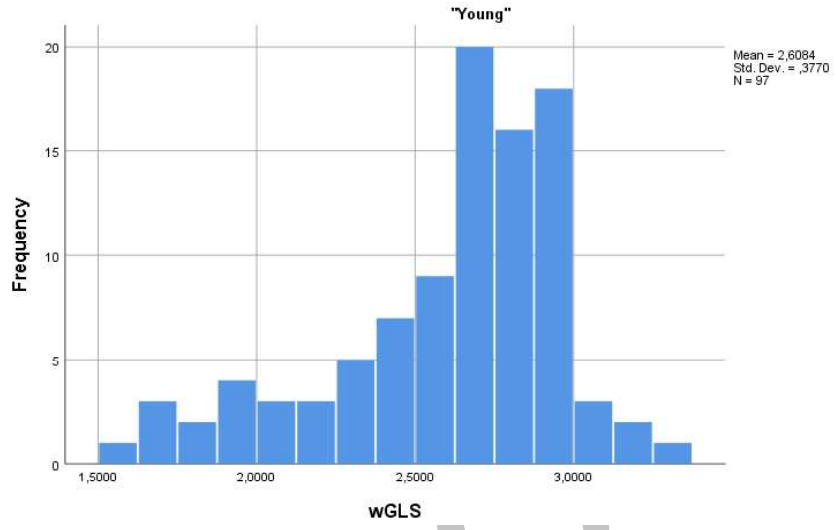
SUPPLEMENTARY FIGURE 1: Unweighted (A) and weighted (B) genetic longevity scores (GLS) distribution in three groups: Croatian "Old", Croatian "Young", and Croatian Roma.

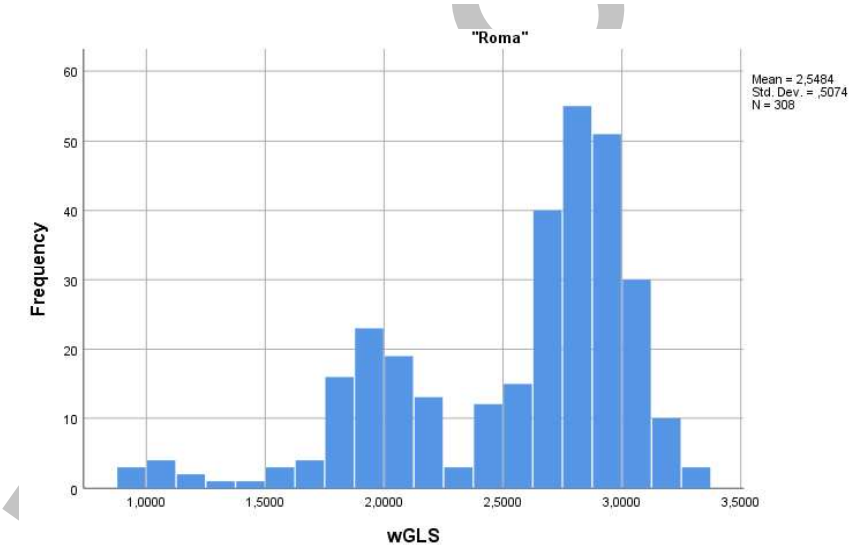
A


"Old"
Mean = 29,75
Std. Dev. = 3,127
N = 314
Frequency
uGLS
"Young"
Mean = 29,56
Std. Dev. = 3,055
N = 97
Frequency
uGLS
Roma
Mean = 29,42
Std. Dev. = 3,30
N = 308
Frequency
uGLS

B


"Old"
Mean = 2,6550
Std. Dev. = ,3855
N = 314
Frequency
wGLS


"Young"
Mean = 2,6084
Std. Dev. = ,3770
N = 97
Frequency
wGLS


"Roma"
Mean = 2,5484
Std. Dev. = ,5074
N = 308
Frequency
wGLS