BIOLOGICAL DETERMINANTS OF LONGEVITY IN A CROATIAN OLDEST-OLD POPULATION

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BIOLOŠKE ODREDNICE DUGOVJEČNOSTI U HRVATSKOJ POPULACIJI OSOBA DUBOKE STAROSTI

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This doctoral dissertation was made at the Institute for Anthropological Research, under the supervision of Tatjana Škarić-Jurić, MD, PhD, scientific advisor with tenure, as a part of the Doctoral programme of Biology at the Department of Biology, Faculty of Science, University of Zagreb.

SUPERVISOR INFORMATION

Tatjana Škarić-Jurić is a scientific advisor with tenure in the field of biomedicine and health and an associate professor of anthropology. She graduated from the School of Medicine, University of Zagreb (UNIZG) in 1989 and finished postgraduate study of Biological Anthropology at the Faculty of Science, UNIZG, in 1993. She obtained her PhD degree in the field of Biomedicine and Public Health in 1999 from the School of Medicine, UNIZG.

Since 1989, dr. sc. Škarić-Jurić has been working at the Institute for Anthropological Research in Zagreb, where she took part in 15 domestic and international scientific projects (having the role of PI in four of these projects). During the three decades of scientific activity, her research interests covered a wide spectrum of topics within fields of biological anthropology and public health. As a young researcher, she focused on exploring the heritable and non-heritable sources of variability in complex human traits by applying quantitative-genetics methodology on family and twin data, and population-genetics methods on population data. Another area of her interest included biological (micro)evolution and its impact on the current structure of human populations, with a special focus on geographical and socio-cultural isolates (Croatian island and Roma populations) which have proved to be very powerful natural experiments. More recently her diversified interests span from genetic analyses on molecular level to the impact of socioeconomic and other environmental factors on complex phenotype formation. Her studies of life-course dynamics of human morphological and physiological characteristics included research on the changes during growth and development period as well as the research centring around ageing process and the phenomenon of human longevity.

She authored 84 scientific and professional papers, co-edited two books, actively participated in 110 international scientific conferences, and supervised three PhD theses, one Master thesis, and three Graduate theses. She was also involved in lecturing activities at the graduate study of Anthropology at the Faculty of Social Sciences and Humanities (courses: «Quantitative Genetics» and «Introduction to Population Genetics»), and postgraduate programs at the Faculty of Science, (course: «Anthropology - Human Biological Variation») and the Faculty of Food Technology and Biotechnology (course: «Nutritional Status»), as well as at the University of Rijeka and J. J. Strossmayer University of Osijek (course: «Medical Anthropology»). She was engaged in number of professional activities: participation in various professional boards and panels, as well as editing and reviewing activities (including for EC framework programs in 2008, 2014, 2016, and 2019).

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University of Zagreb
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Doctoral dissertation

BIOLOGICAL DETERMINANTS OF LONGEVITY IN A CROATIAN OLDEST-OLD POPULATION

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Research of long-lived individuals is key for better understanding of the ageing process and the factors that contribute to successful ageing. In this study, 43 genetic variants with previous association to longevity were explored in the population of Croatian oldest-olds (85+ years), along with health-related parameters and relative telomere length, to determine their impact on longevity and late-life survival. Only one variant, associated with *MRE11* gene, differed between the study group and the young control group. There was a difference in the variants that contributed to reaching longevity (90 years) and extreme longevity (95 years), with the only variant shared between the two models being *TP53* rs1042522. SNP-SNP interactions had a significant effect on survival above the age of 85, with *CDKN2B* being the most important interaction partner. Finally, health-related parameters contributed to survival in advanced age independently of genetic factors, while relative telomere length did not show any association.

(147 pages, 13 figures, 25 tables, 262 references, original in English)
Keywords: oldest-old, longevity, survival, single nucleotide polymorphisms, genetic risk score, genetic interaction, health-related traits

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BIOLOŠKE ODREDNICE DUGOVJEČNOSTI U HRVATSKOJ POPULACIJI OSOBA DUBOKE STAROSTI

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Istraživanja dugovječnih pojedinaca ključna su za bolje razumijevanje procesa starenja, kao i za stjecanje saznanja o čimbenicima koji doprinose uspješnom starenju. U ovom istraživanju 43 genetske varijante prethodno povezane s dugovječnošću, parametri povezani sa zdravljem i relativna duljina telomera istraživani su na hrvatskom uzorku osoba duboke starosti (85+ godina) kako bi se utvrdio njihov doprinos dugovječnosti i preživljenju u dubokoj starosti. Ispitanici su se od kontrolne skupine mladih osoba razlikovali samo u jednoj varijanti, povezanoj s genom *MRE11*. Utvrđena je razlika u varijantama koje su doprinosile doživljenju dugovječnosti (90 godina) i ekstremne dugovječnosti (95 godina), s tim da je jedino rs1042522 u genu *TP53* bila u oba modela. Interakcije između lokusa imale su značajan učinak na preživljenje iznad 85 godina, pri čemu je najvažniji sudionik bio gen *CDKN2B*. Zdravstvene varijable također su doprinosile preživljenju u dubokoj starosti neovisno od genetskih faktora, dok relativna duljina telomera nije pokazala povezanost s preživljenjem.

(147 stranica, 13 slika, 25 tablica, 262 literaturnih navoda, jezik izvornika: engleski)
Ključne riječi: osobe duboke starosti, dugovječnost, preživljenje, polimorfizmi jednog nukleotida, zbroj genetičkih rizika, genetske interakcije, obilježja povezana sa zdravljem

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CONTENTS

1. INTRODUCTION

1.1. Ageing from the evolutionary perspective

Ageing is a highly complex biological process that happens to most living beings. It is characterised by a progressive weakening of all functions of the organism, which ultimately leads to its death. It can also be defined as a time-related deterioration of the physiological functions necessary for fertility and survival (Gilbert, 2000).

Ageing is widespread throughout the animal kingdom, with different species displaying different changes in phenotype as they age. It is, however, not ubiquitous, as some species, like hydra from the Cnidaria phylum, do not exhibit signs of ageing at all (Kirkwood & Tipton, 2017). The life expectancy among species differs greatly as well. The life cycle of the adult stage of the fruit fly, *Drosophila melanogaster*, lasts approximately 3 months (Piper & Partridge, 2018). A laboratory mouse has a maximum lifespan of about 4.5 years (Gilbert, 2000), while their wild counterparts are expected to live only between 1- 1.5 years (Ballenger, 1999). Naked mole rats, the longest-living members of the Rodentia order that are often used as models for longevity, can live up to 30 years (Buffenstein & Jarvis, 2002). Large mammals such as elephants and bowhead whales are also known for their long lifespans, but the title of the longest-living vertebrate goes to the Greenland shark, estimated to reach 500 years of age (Nielsen et al., 2016). In comparison, the maximum lifespan of a human is estimated at 121 years (R. Arking, 1998), with the longest-living person up to date being Jeanne Calment from France, who died aged 122.

Evolutionary theories of ageing have long tried to rationalise ageing by making a connection between ageing process and reproduction. Creating offspring is energy-demanding, so high fertility has often been connected to faster ageing of the organism (Jasienska, 2020; Ryan et al., 2018). This is partially corroborated by the fact that species with shorter life often follow the *r* reproductive strategy – with maximum energy allocated to high numbers of progeny, with short gestation, less parental care, and a short time until sexual maturity (Pianka, 1970). The species with longer lifespans, on the other hand, tend to follow *K* strategy characterised by having few offspring, a long gestational period, intensive parental care, and a long period until sexual maturity (Pianka, 1970). There are some exceptions – for example, long-lived sea turtles reproduce in a way that more

closely resembles *r* strategy. This line of thinking, however, suggesting the trade-off between energy allocated for reproductive fitness and the energy that can be spent for other functions that would contribute to longevity, such as DNA repair, has been proposed by Thomas Kirkwood in 1977 as one of the causes for ageing in a theory known as the disposable soma theory (Kirkwood, 1977). The age-related decline of the organism's vitality has also been rationalised as a consequence of the reproductive exhaustion of the old individuals, who are no longer able to ensure the continuation of their species, and have therefore lost their importance in the light of evolution (Weismann, 1891). It was proposed that such individuals are therefore weeded out by the programmed process of ageing to make room for the reproductively fit individuals of the species. But in the natural world, however, most individuals do not survive to the point of old age and senescence due to extrinsic hazards (Kirkwood, 2005), meaning that there would be no need for a strong natural selection of genes that trigger ageing. Therefore, it is widely accepted that ageing is not programmed, but has rather evolved as a side-effect of declining ability of natural selection to maintain fitness as the organism ages (Flatt & Partridge, 2018).

Regardless of the fact that the ageing process is not programmed by any single gene, the impact of genetics on ageing is substantial. Mutations have been found that modulate lifespan in model organisms (Kirkwood, 2005), as well as certain variants that contribute to longevity in humans (Deelen et al., 2011; Flachsbart et al., 2009; Willcox et al., 2008). Some genes that affect ageing are pleiotropic and affect multiple biological systems within an organism, or are expressed differently at different life stages. Some act in the manner of antagonistic pleiotropy, meaning that they enhance fitness of an organism early in life, but diminish it in later life when the natural selection pressure is weak (Austad & Hoffman, 2018; Kirkwood, 2005). The antagonistic pleiotropy theory of ageing is widely accepted today, with experimental evidence speaking in its favour (Austad & Hoffman, 2018). As more and more ageing-related mechanisms were discovered to conform to the concept of antagonistic pleiotropy (Bartke, 2011; Carter & Nguyen, 2011; Wood et al., 2000), they started to converge and point to the pituitary gland, the main control centre that coordinates the crosstalk between nervous, endocrine and immune systems (Chesnokova & Melmed, 2002), as a potential integrative centre that regulates different processes that accompany ageing.

1.2. Molecular theories of ageing

The study of ageing remains even today one of the most challenging research topics in the fields of biology and medicine. Ageing is fascinating not just because it is ubiquitous and unavoidable (at least for humans), but because it affects numerous phenotypes all at once. It is because of this complexity that, in the history of ageing research, many molecular theories have been proposed to explain why and how ageing happens, and only the most prominent of those theories are described in the continuation of this chapter. Even though each theory could explain some of the changes that happen during the ageing process, none of them could by themselves account for all the age-associated phenotypes. This is why no single theory prevailed, and instead, the molecular background behind ageing is now considered to be a network of interconnected processes.

1.2.1. Somatic mutation theory

Somatic cells are throughout life exposed to different internal and external stressors and mutagens. From the reactive oxygen species that are created as byproducts of oxidative metabolism within the cells, the UV radiation from the sun or the mutagens naturally found in the environment, there are various factors that can damage the DNA molecule and cause mutations (Vijg & Suh, 2013). The DNA can also get damaged spontaneously, for example by heightened body temperature or by hydrolytic cleavage of the glycosidic bonds (a process also known as "depurination"), and mutations can be introduced into the DNA sequence during replication (Vijg, 2000). As these lesions in the DNA can block cell division or transcription of genes, the cells come equipped with a capable system for recognising them, known as the DNA damage response (DDR), as well as a complex system for their repair (Niedernhofer et al., 2018). However, as the organism ages, the efficiency of DNA repair decreases, causing the gradual accumulation of DNA damage. This accumulation of unrepaired DNA lesions manifests as genotoxic stress, which – in order to prevent the replication of damaged DNA – triggers signalling cascades that promote apoptosis or senescence (Yousefzadeh et al., 2021). The inverse situation, on the other hand, in which the damage to the DNA goes unnoticed by DDR, also presents a danger to the organism as it can lead to development of disease, particularly cancer. Thus, it highlights the fine balance between senescence and proliferation, and the key role that DNA repair mechanism play in maintaining this homeostasis. DNA damage is, therefore, considered as one of the main causes of ageing, and this theory has been confirmed by studies that have shown that older individuals, both of model

organisms and humans, exhibit more DNA lesions than their younger counterparts (Hamilton et al., 2001; Jacob et al., 2013). Also, recent studies have shown that some long-lived species could owe that longevity to more efficient DNA repair (Tian et al., 2019). Apart from the accumulation of somatic mutations, ageing is also connected to changes to the epigenetic markers. The strongest evidence exists for the age-related changes in methylation levels across the entire genome (Bell et al., 2012; Christensen et al., 2009; Teschendorff et al., 2010), but ageing has also been connected to the decrease of the number of histones (Larson et al., 2012) and the loss of heterochromatin (Dang et al., 2009; Feser et al., 2010), which could all disturb the epigenetic regulation of gene expression.

1.2.2. The telomere hypothesis of ageing

In 1961, Leonard Hayflick was performing experiments cultivating non-cancerous human and animal cells to see how long they could be maintained in cell culture. Contrary to the theory proposed by Alexis Carrel at the beginning of the 20th century, in which it was stated that normal somatic cells could be maintained in cell culture almost indefinitely (Carrel, 1912), Hayflick found that cells usually underwent 40 to 60 cell divisions before entering senescence (Hayflick, 1965). This limited number of times that a cell can divide before maxing out its proliferative capacity has since been known as the Hayflick limit. The molecular explanation for this phenomenon was given by Alexey Olovnikov in 1971, when he, looking at the trains in the Moscow subway, formulated the end replication problem. He theorised that during replication of the DNA, the very ends of the lagging strand cannot be fully copied by DNA polymerases because the enzyme itself sits on it, like a locomotive engine at the end of the train track (Olovnikov, 1973). He also suggested the ends of chromosomes comprise repeated sequences that serve as buffers that shorten in each cell division, and that the cells enter replicative senescence once this buffer role has been lost (Olovnikov, 1996), thus setting the foundation of the telomere shortening hypothesis of ageing. His theories have been confirmed by further research, and these repetitive DNA sequences at the ends of chromosomes given the name telomeres. Telomeres in humans and other vertebrates consist of hexameric repeats of TTAGGG (Moyzis et al., 1988) that bind proteins of the shelterin complex and serve to protect the ends of the linear DNA molecule from degradation by nucleases (Blackburn et al., 2015). They are shortened in every cycle of DNA replication, and upon reaching a critical length, trigger the activation of the DNA damage response, arrest of the cell cycle and the cells' entry into replicative senescence (Zglinicki & Martin-Ruiz, 2005). Even though the critically

shortened telomeres are recognised as DNA damage, they cannot be mended by regular mechanisms for DNA repair, as this repair requires a special template to rebuild and lengthen the telomeric repeats. Telomerase is a complex enzyme with DNA polymerase activity and an RNA template that enables this lengthening of telomeres, but is not expressed in most human cells (Blackburn et al., 2015). It is, on the other hand, expressed in stem cells (Collins & Mitchell, 2002; Wright et al., 1996) and often in cancer cells (Hahn et al., 1999; Shay et al., 2001), allowing them continuous proliferation far beyond the Hayflick limit. Therefore, telomeres play not only an important role in ageing but also in disease susceptibility and development (Rossiello et al., 2022). Telomeres are the longest at birth, which represents the most important predictor for later life telomere length (Martens et al., 2021). Because of the shortening during the course of life, telomeres could represent a good marker of biological age, as well as an interesting biomarker for ageing research.

1.2.3. Waste accumulation theory

Proteins, the main building blocks of an organism, carry out most of the main structural and functional tasks in a living system, making their proper functioning of utmost importance. In normal cellular metabolism, fresh proteins are synthesised anew, and non-functioning, damaged or redundant proteins are marked for degradation and broken down in proteasomes (Davies, 2001; Shang & Taylor, 2011). The maintenance of this balance, also known as proteostasis, is often dysregulated by ageing (Hipp et al., 2019). Throughout their lifetime, proteins and other macromolecules in the cell are exposed to different conditions and agents that can damage and impair their functions. Elevated temperatures can cause the proteins to lose their 3D conformation, while highly reactive molecules like reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as sugars and aldehydes, can oxidise them and affect their structure. Proteins that are misfolded, unfolded or oxidised by these molecular stressors can then stick together in oligomeric complexes, causing the formation of protein aggregates that impair cellular function and decrease viability (Chondrogianni et al., 2014). To prevent them from further damaging other macromolecules, damaged proteins are either degraded, sequestered in separate compartments within the cell or repaired (Chen et al., 2011). The latter is possible thanks to molecular chaperones, of which most well-known are the ones from the heat-shock protein family (Hsp). They have a vital role in preserving the structural integrity of proteins and help the proteins to achieve their functional conformation by binding to the unfolded regions and oligomerizing with accessory

proteins around this region in order to create an ideal surrounding for the protein to fold (Mayer & Bukau, 2005). They can refold damaged proteins to restore their function, and also help to prevent the accumulation of misfolded proteins (Hut et al., 2005), which is why their expression is required for longevity (Calderwood et al., 2009). The heat shock response, however, has been shown to weaken with age (Calderwood et al., 2009), and, together with the failure of the protein degradation system, leads to collapse of protein quality control, which in turn compromises the cell's functional integrity and can cause the development of protein misfolding diseases, such as Alzheimer's and Huntington's disease (Powers et al., 2009; Taylor & Dillin, 2011).

1.2.4. Mitochondrial theory

Mitochondria are the powerhouse of the cell, cellular organelles where energy in the form of adenosine triphosphate (ATP) is created by oxidative phosphorylation. This process, necessary for the sustenance of eukaryotic life, involves a chain of chemical reactions that include transport of electrons from electron donors to acceptors in a series of redox reactions ending in the release of oxygen (Nunnari & Suomalainen, 2012). This electron transport, happening on protein complexes located on the inner mitochondrial membrane, also known as the respiratory chain, sometimes causes the production of highly reactive byproducts of aerobic metabolism – reactive oxidative species (ROS) (Bratic & Larsson, 2013). Due to their high chemical reactivity, these toxic byproducts can cause oxidative damage to proteins, lipids and nucleic acids. Mitochondria are the only organelle in the animal cells, besides the nucleus, that have their own genome – mtDNA. It is a circular DNA molecule of 16 569 base pairs that encodes 13 proteins with a role in oxidative phosphorylation and 24 RNA components (22 tRNAs and two rRNAs) necessary for mitochondrial protein synthesis (Trifunovic & Larsson, 2008). Due to highly oxidative conditions within the mitochondria inner membrane, the circular, histone-free nature of mtDNA and a more limited set of repair machinery available in the mitochondria (Druzhyna et al., 2008; Lax et al., 2011), mtDNA has a much higher rate of mutation compared to nuclear DNA (Short et al., 2005). This can, as the organism ages, cause the accumulation of mutations in the mtDNA (Trifunovic & Larsson, 2008), and subsequently lead to mitochondrial dysfunction and accelerated ROS generation (Trifunovic et al., 2004; Wallace, 2010). This is also reflected in the fact that, with ageing, the rate of energy production in the mitochondria decreases (Petersen et al., 2003; Short et al., 2005), indicating the weakening of mitochondrial function.

1.2.5. Caloric restriction theory of ageing

One of the first breakthroughs in the field of ageing research was the discovery that rats on a caloric restriction diet could live much longer than their counterparts on normal diets (McCay et al., 1975). It was the first proof of an irrefutable connection between nutrient intake and longevity, as well as the first example of the plasticity of the ageing process. It inspired the scientists to investigate the genes that activate in response to nutrient stimuli, and look for a link between those genes and ageing. This resulted in the discovery of the *age-1* gene in *Caenorhabditis elegans* (Friedman & Johnson, 1988), ushering the genetic era of ageing research. This gene encodes a worm homologue of phosphatidylinositol 3-kinase, a kinase that phosphorylates transcription factors downstream of the insulin/insulin-like growth factor 1 signalling pathway (IIS) that is well-conserved from yeast to mammals (Barbieri et al., 2003). Soon after, DAF-2, a homologue of insulin-like growth factor 1 receptor, and DAF-16, a homologue of the FOXO transcription factor, were discovered as the other key regulatory elements of *C. elegans* lifespan (Kenyon et al., 1993), proving that IIS indeed was the most promising pathway for longevity research. It has since been discovered that IIS is the centre of a much larger regulatory network that spans beyond just one pathway (Figure 1). It is connected to cellular metabolism, stress response, cell cycle control, apoptosis and autophagy via its downstream effectors such as FOXO transcription factors (Martins et al., 2016) and mTOR (Saxton & Sabatini, 2017; Yoon, 2017), as well as cytokine production and inflammatory processes (Spielman et al., 2015; Kim et al., 2008; Manowsky et al., 2016), among others. Because of this involvement in a variety of cellular processes, even though the IIS is evolutionary tied to organismal survival and not ageing per se (Antebi, 2007), the changes in expression of key genes in this signalling network can influence how an organism ages, proving that regulating nutritional signals can indeed influence the ageing process.

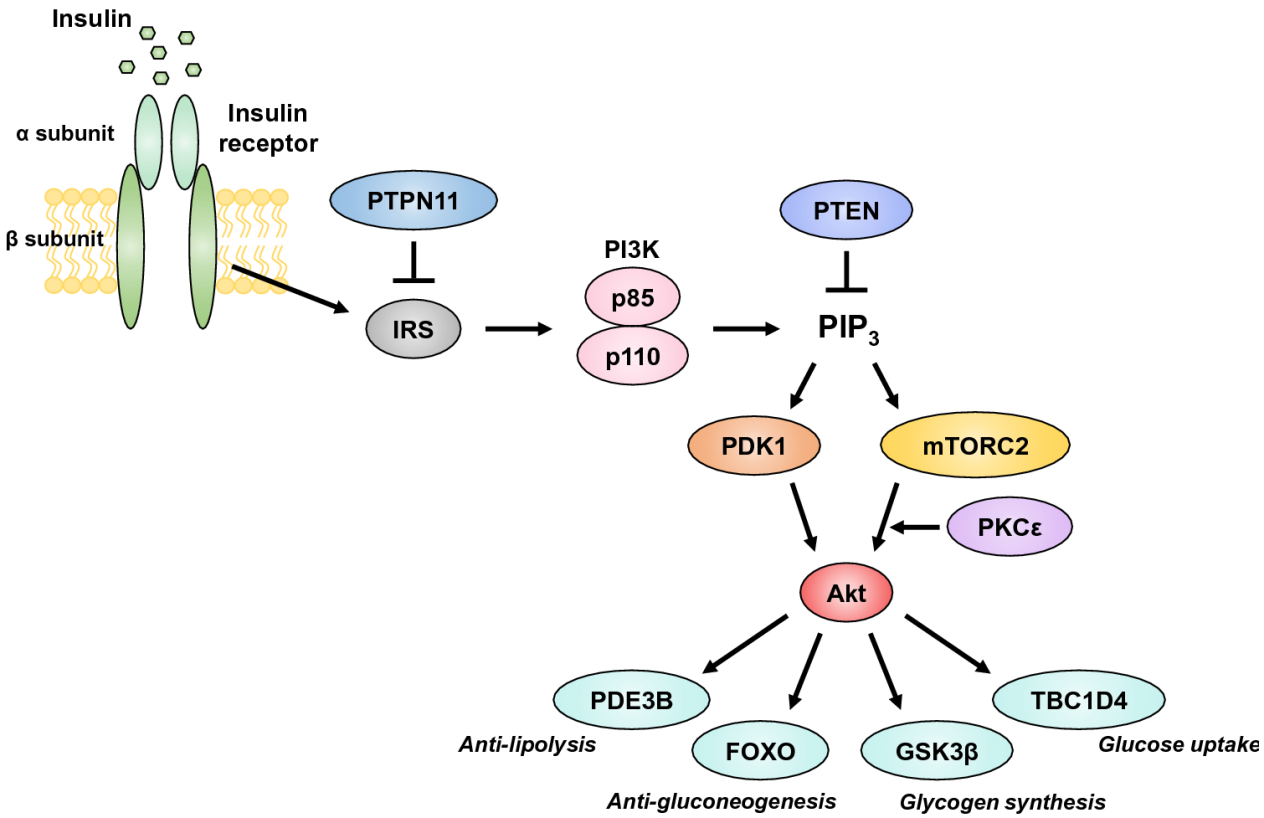

Figure 1. The insulin signalling pathway. Image sourced from Kushi and collaborators (Kushi et al., 2021).

1.3. Hallmarks and biomarkers of ageing

The complexity of the ageing process lies in a wide spectrum of processes and changes that an organism simultaneously undergoes as it ages. These cellular and molecular processes are not independent of each other and often have causal or synergistic effects. They are what determines the aging phenotype, and are considered the hallmarks of ageing (López-Otín et al., 2013, 2023) (Figure 2). DNA instability and telomere attrition are hallmarks of ageing caused by the wear and tear of DNA throughout life, and both represent a limiting factor to the replicative potential of the cells. Epigenetic alterations influence transcription and affect cell division via chromatin organisation (Sen et al., 2016). They are a hallmark of ageing that reflects environmental influence on gene expression. All these changes on the DNA level are the drivers behind cellular senescence and stem cell exhaustion, which are the main hallmarks of ageing on the cellular level. Ageing affects cellular and intercellular signalling pathways, which causes deregulated nutrient-sensing

and altered intercellular communications. It also impacts the cellular systems for maintaining homeostasis, so aged cells often display signs of mitochondrial dysfunction, loss of proteostasis and disabled macroautophagy. On the tissue level, the main hallmark of ageing is chronic inflammation, characterised by an increase in the levels of pro-inflammatory markers in blood and tissues. Chronic inflammation is a strong risk factor for age-related diseases and geriatric conditions that are highly prevalent and causes of disability in elderly individuals, and has therefore even got its own name: inflammaging (Ferrucci & Fabbri, 2018; Franceschi et al., 2000). The ageing-related changes, however, do not stop at the level of the organism itself – the microbiome is also affected by the ageing process through the bidirectional host-bacteria crosstalk. This altered communication results in dysbiosis, a change in the composition of gut microbiota, which makes for a final, holobiont hallmark of ageing (López-Otín et al., 2013, 2023). López-Otín et al. (2023) have grouped these hallmarks of ageing into three categories – primary, antagonistic and integrative (Figure 2). Primary hallmarks are disadvantageous molecular events or changes at the level of individual cells, and are the core drivers of ageing. Antagonistic hallmarks, when they become widespread as a result of the changes incurred by the primary hallmarks, further contribute to the ageing phenotype and exacerbate other ageing-related changes. Finally, integrative hallmarks comprise the changes on multicellular and systemic levels that disrupt normal functioning of the organism as a whole.

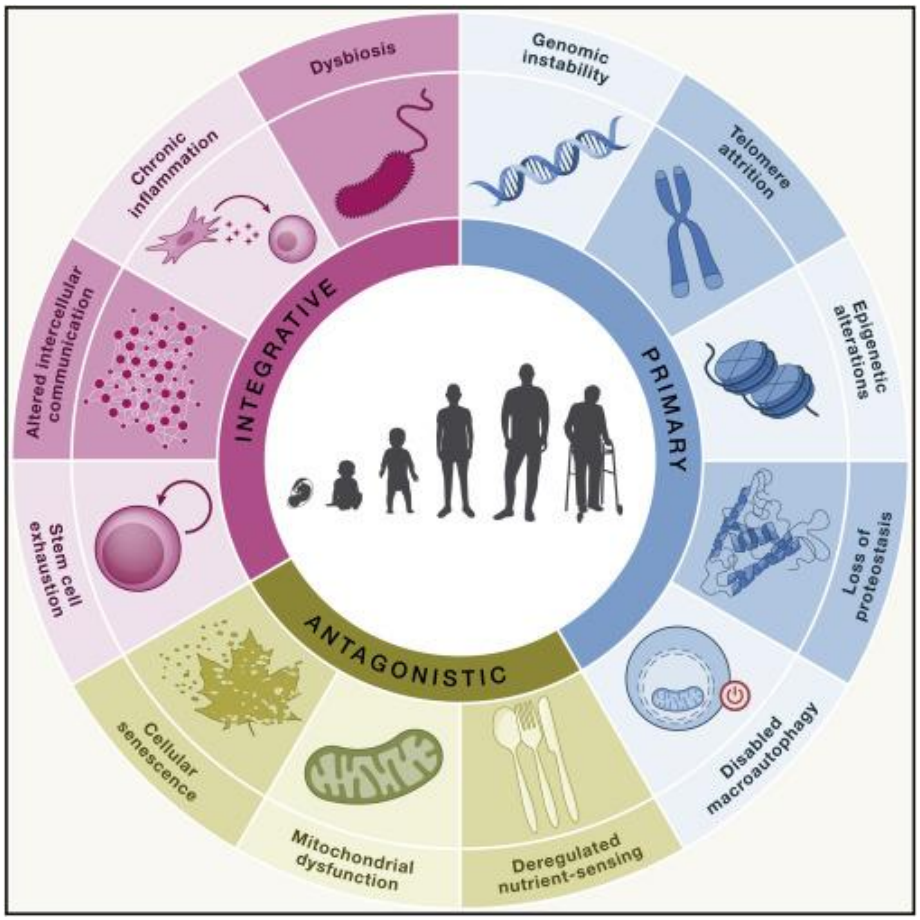

Figure 2. The 12 hallmarks of ageing, as they were defined by López-Otín and collaborators (López-Otín et al., 2023).

The rate of ageing does not only vary greatly between species, but between individuals as well (Belsky et al., 2015). That is why one's biological age, the age that reflects the condition of the organism, does not always match the chronological age (Franceschi et al., 2018). Measuring the rate at which one ages is difficult, and establishing what the biomarkers of ageing are, has caused much debate (Butler et al., 2004; Johnson, 2006; Mather et al., 2011; Sprott, 2010). That is why these main criteria for the determination of ageing biomarkers have been proposed: a) it must predict the rate of ageing; b) it must measure a basic process underlying ageing and not the effects of disease; c) it must be minimally invasive so the measurement can be repeated; d) it has to be a marker of ageing in both humans and model organisms, so it can be verified before being tested on humans (Bürkle et al., 2015). Most importantly, a biomarker of ageing must outperform chronological age in predicting the outcome – remaining lifespan, mortality risk, and age-related

morbidity risk (Lohman et al., 2021). Some of the proposed biomarkers that pass most of these criteria are telomere length (Zglinicki & Martin-Ruiz, 2005), epigenetic clocks measuring the methylation state of CpG islands (Horvath & Raj, 2018), omics-based markers such as those from transcriptomic data measuring the differences in mRNA levels (Harries et al., 2011), proteomic-based estimators that detect age-related changes in protein levels (Tanaka et al., 2018), metabolomic-based estimators (Robinson et al., 2020), as well as biochemical biomarkers from blood (Mamoshina et al., 2018; Putin et al., 2016). Due to the employment of new methods and high-dimensional analyses, this area of ageing research is developing rapidly, so this list of potential biomarkers can be expected to grow even more in the coming years.

1.4. Candidate gene and genome-wide association studies (GWAS)

Living systems rely on tight regulation. All processes happening within an organism are governed by cellular signalling pathways, which are in turn regulated through gene expression, but no organism is completely exempt from the effects of its environment. In the case of a complex process such as ageing, for which time is also an important factor, the effects of environmental factors on the dynamics of the biological process are significant. However, environmental influence is very difficult to study and pinpoint, even if it contributes to the observed phenotype. In the case of human ageing and longevity, such beneficial influence of the environment can be seen in the so called “blue zones”, the areas of the world with markedly higher percentage of long-lived individuals (Buettner & Skemp, 2016). These long-lived individuals, who are living examples of successful ageing, are the most valuable resource for studying the impact of environment and health-related behaviours on ageing phenotypes, but also for studying the genetics of human longevity. Even though the total effect of genetic variation on human lifespan is moderate, with the genetic component estimated at around 20-30% (Herskind et al., 1996), the two approaches for studying the genetic background of human longevity – candidate gene studies and genome-wide association studies (GWAS) – have proven very useful in determining different pathways that are key for healthy ageing and longevity. The goal of these types of studies is to find genetic differences between long-lived individuals, nonagenarians and centenarians, and individuals representing general population (Smulders & Deelen, 2023). While candidate gene studies can identify a limited number of polymorphisms associated with an increased or decreased risk of an outcome by

focusing on pathways with a putative role in said outcome, genome-wide association studies search for association signal through the entire genome in a more unbiased, hypothesis-free way (Smulders & Deelen, 2023; Wilkening et al., 2009). The results of GWAS are considered reliable as these studies usually employ very large samples and their findings have to be replicated, which is why the detected genetic variants can also be used in other studies to predict the target phenotype (Duncan et al., 2019a).

The most well-known gene that influences human longevity is *APOE*, a gene from the family of apolipoproteins that mediates cholesterol transport and metabolism. The ApoE protein has three isoforms, termed ε2, ε3, and ε4, that are defined by two missense single nucleotide polymorphisms (SNPs), rs429358 (Arg112Cys, arginine defines ε4) and rs7412 (Arg158Cys, cysteine defines ε2) (Deelen et al., 2011). Its first connection to longevity was made 30 years ago, when Schachter et al. (1994) reported on a very low frequency of the allele defining the ε4 among French centenarians (Schächter et al., 1994). It was considered as a candidate gene in ageing studies because isoform ε4 (Arg112, Arg158), which is the ancestral variant (Huebbe & Rimbach, 2017), was associated with an increased risk of cardiovascular disease (Bennet et al., 2007; Corder et al., 1993; Eichner et al., 1993; Wilson et al., 1994) and Alzheimer's disease (Farrer et al., 1997; Zuo et al., 2006), which are both age-related conditions. Isoform ε2 (Cys112, Cys158) is protective against these diseases, while ε3 (Cys112, Arg158), which is today the most common of the ApoE isoforms (Mahley & Rall, 2000) is considered neutral. It was determined as the strongest genetic factor influencing longevity (Deelen et al., 2011), and remains the only genetic locus to reach the level of genome-wide significance ($p < 5\times10^{-8}$) in multiple meta-analyses of GWAS results (Deelen et al., 2019). This is why it is today considered a golden standard of longevity research, and is even considered as a potential biomarker of ageing (Bürkle et al., 2015).

The only other longevity-associated locus that has been replicated in several independent studies is *FOXO3* (Smulders & Deelen, 2023). First identified as a genetic factor for longevity by Willcox et al. (Willcox et al., 2008), the variants in *FOXO3* have been associated with longer life in many GWA and candidate gene studies on different populations (Anselmi et al., 2009; Bao et al., 2014; Broer et al., 2015; Flachsbart et al., 2009; Pawlikowska et al., 2009; Soerensen et al., 2010; Zeng et al., 2010). *FOXO3* is the main transcriptional effector of the insulin signalling activated by metabolic stress and nutrient deficiency (Eijkelenboom & Burgering, 2013). It is involved in

regulation a wide variety of cellular processes connected to cellular survival, including metabolism, protein turnover and quality control, as well as cell death (Stefanetti et al., 2018). Because of its involvement in the main pathway implicated in ageing, it is not surprising it has been deemed a candidate gene for longevity.

Variants in other genes that either belong to IIS or are in some way connected to it have been reported to potentially impact the ageing process. For example, variants in *IGF1R* and *IGF2R* genes encoding insulin-like growth factor receptors (Albani et al., 2009a; Bonafè et al., 2003; Li et al., 2016; Soerensen et al., 2012a), an SNP in the gene for hormone receptors that control the secretion of growth hormone (Soerensen et al., 2012a), variants in the genes for metabolic regulators sirtuins (TenNapel et al., 2014), have all been associated with longevity. So have the variants in the *KLOTHO* gene, a silencer of insulin signalling, that has got his name after one of the three Fates from Greek mythology – the one that is tasked with spinning the thread of life (D. E. Arking et al., 2002; Pereira et al., 2020; Soerensen et al., 2012a; Zhu et al., 2019). However, IIS is not the only pathway enriched with longevity genes – strong evidence exists for the connection with ageing and longevity of variants in genes involved in cell cycle control – *TP53* (Groß et al., 2014), as well as *CDKN2B* (Fortney et al., 2015; Pilling et al., 2016; Pinós et al., 2014) located in a region that also happens to be implicated in cardiovascular disease risk (Burton et al., 2007; Helgadottir et al., 2007; McPherson et al., 2007). The association with longevity is also reported for variants in the *IL6* gene, encoding a cytokine with both pro- and anti-inflammatory properties (Albani et al., 2009b; Christiansen et al., 2004; Revelas et al., 2018), as well as for variants in genes involved in DNA repair, for example *ERCC2* and *MRE11* (Dato et al., 2018). Most of these, however, could not yet be replicated in independent studies or in diverse populations.

1.5. Healthy ageing

Since its inception, humans have been fascinated with the idea of eternal life. For centuries, alchemists have tried to create the Philosopher's stone, a magical object that would, apart from transmuting other materials into gold, grant its owner the Elixir of life, and thus, immortality. While their efforts have been unsuccessful, the quest for both genetic and environmental factors that can positively influence the ageing process and contribute to longevity is today more relevant than ever

before. This research also goes beyond merely reaching longevity; it is a quest for a long life spent in good health and with a good quality of life.

The past century has seen the greatest longevity leap in the history of humankind, which resulted in a much larger number of people surviving to older age. But in order to truly reap the benefits of this longer lifespan, it is of utmost importance to remain in good health for as long as possible (Beard et al., 2016). This is why the main term in the research of healthy ageing is healthspan – the years of life lived in good health, free of disease (Garmany et al., 2021), and why a whole branch of ageing research that specialises in this connection between ageing and disease – geroscience – has been established (Kennedy et al., 2014). However, while the human lifespan increased drastically, the healthspan has not followed (Garmany et al., 2021). The number of age-specific risk factors representing physiological status has stayed relatively constant (Crimmins & Beltrán-Sánchez, 2011), meaning that today's oldest-old might not be any healthier than previous generations. This is quite problematic, as advanced age is already the main risk factor for chronic diseases such as cancer, cardiovascular and neurodegenerative diseases and type 2 diabetes (Niccoli & Partridge, 2012). The older age group is also a segment of population where multimorbidity, the coexistence of two or more chronic conditions, is very prevalent (Kirchberger et al., 2012; Salive, 2013). In a study that reviewed data from US studies on mortality, length of life and disease, Crimmins and Beltrán-Sánchez (2011) report an increase in prevalence of disease, longer time lived with a disease, and a decline in mobility functioning (Crimmins & Beltrán-Sánchez, 2011), meaning that the increased life expectancy does not automatically mean a better quality of life. They also show that a decline in some risk factors like high cholesterol and hypertension is due to drug usage (Crimmins & Beltrán-Sánchez, 2011), which also highlights another problem that goes hand in hand with multimorbidity in older populations – polypharmacy, or taking of multiple medications at once (Kurczewska-Michalak et al., 2021) – which can have negative side effects of its own. Exceptionally long-lived individuals, however, delay or completely avoid the onset of most age-related diseases (Smulders & Deelen, 2023), which makes them extremely valuable – not only as examples of successful ageing – but for gaining insight into the conditions and factors necessary for acquiring longevity.

Therefore, gerontologists today claim that it is not a lack of disease, but other important factors like the absence of frailty and good functional ability – an individual's ability to perform daily

tasks independently (Clegg et al., 2013; Ramnath et al., 2018) – that are true hallmarks of successful ageing. Parameters like mobility, independence and mental capacity have been shown as good predictors of mortality and some morbidities (Inouye et al., 1998; Reuben et al., 1992), and the same is true for one's perception of their own health (Bardage et al., 2005; Curtin et al., 1999; Kawada, 2003). Finally, all aspects of functional ability are tied to nutritional status, which is especially important for older individuals, as it plays a big role in the preservation of muscle mass and strength during aging (Mithal et al., 2013).

1.6. The global population ageing

In the last 200 years, the average human life expectancy has more than doubled (Figure 3) (Oeppen & Vaupel, 2002). From the world average of around 29 years in 1820, it increased to 73 years in 2020 (World Health Organization, n.d.) in an almost linear fashion. This increase was made possible by civilization advancements like better nutrition and sanitation, education and higher income, as well as development of medicine (Oeppen & Vaupel, 2002). Initially, the biggest contributor to this increase was the decline of juvenile (especially infant) mortality, but in the second half of the 20th century it was the improvements in survival after the age of 65, partly due to improved treatments for ageing-related diseases, that propelled the rise in life expectancy (Wilmoth, 1998). It was this improvement in 65+ survival, together with reduced fertility (Lee et al., 2014), that led to an increase in the share of the elderly in the population. Projections show that by 2050, for the first time in human history, the number of people over the age of 60 will surpass the combined number of adolescents and young adults, and that the world's oldest-old population is likely to triple from 2015 to 2050 (United Nations et al., 2019). It is, therefore, not surprising that centenarians are the fastest growing demographic group in the world's population (Dobriansky et al., 2007).

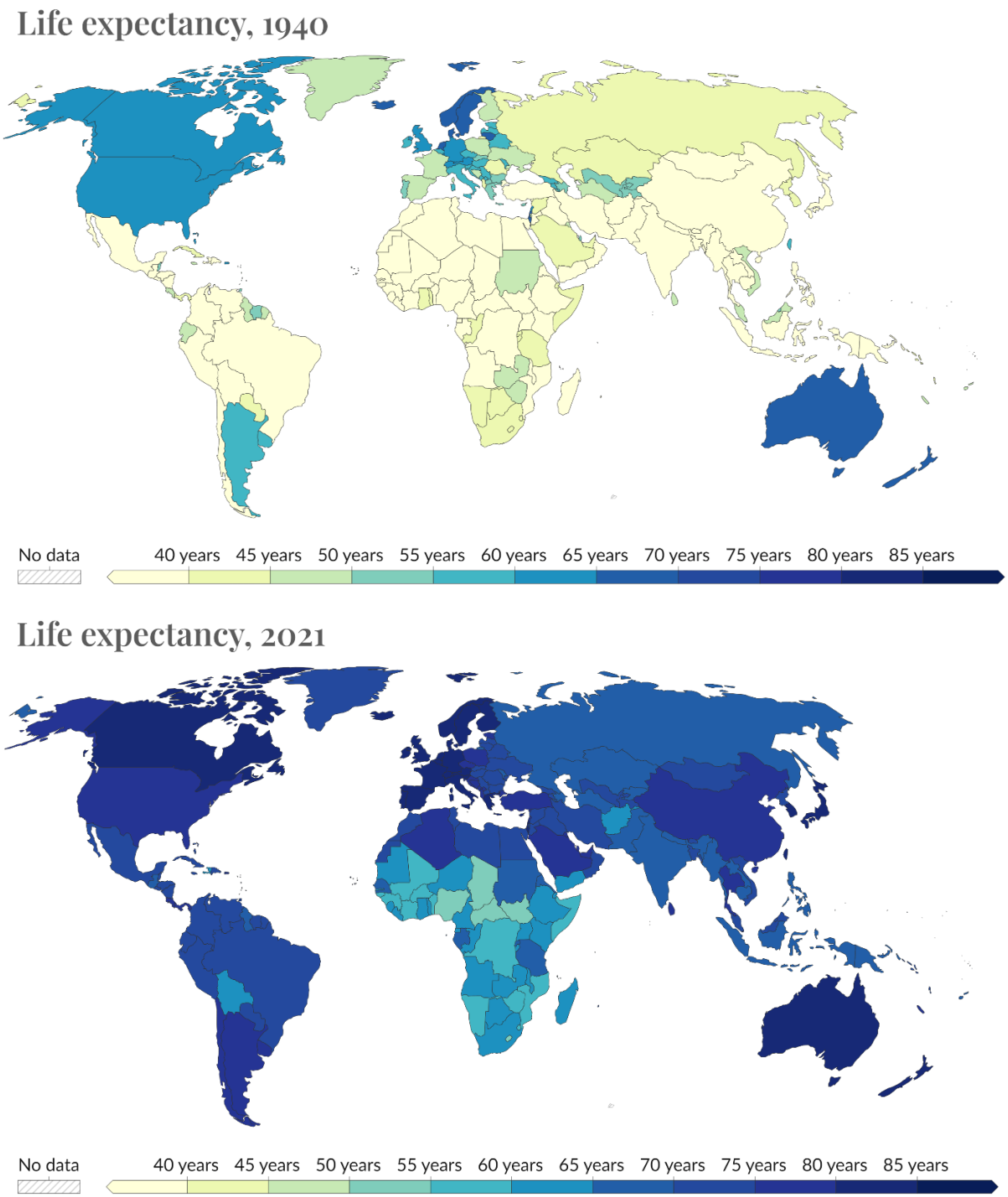

Figure 3. The global life expectancy at birth in 1940, the first year in which data was available for all countries, and 2021. Image was adapted from Dattani and collaborators (Dattani et al., 2023).

As old age is one of the main risk factors for the development of chronic diseases and multimorbidity, demographic ageing of the global population creates a strain on the economic,

social and healthcare systems of many countries. As this global phenomenon of population ageing is expected to continue in the coming decades, this pressure is also expected to increase, so the national governments are being warned of the potential impact these changes will have on the global economy and encouraged to make policies intended to raise fertility (Bhattacharjee et al., 2024). In the face of this future, the need for research into the mechanisms of the ageing process and longevity has never been greater. Successful ageing and the conservation of vitality well into advanced age is going to be of critical importance for the maintenance of economic stability in the coming times, so better understanding of the ageing process and factors which might help to delay the onset of functional disabilities, and thus contribute to successful ageing and longevity, is only going to gain importance.

1.7. Research scope and the aims of the thesis

Croatia is, along with the rest of the more developed countries, facing the ageing of the population. According to the data from the Croatian Bureau of Statistics, there were almost 91,000 people over the age of 85 in 2022 (Croatian Bureau of Statistics, 2023). However, comprehensive genetic research of factors that might contribute to longevity is something that has not yet been done on a sample of the Croatian population.

This study aims to explore whether there is a generational difference in genetic characteristics associated with longevity in the Croatian population, as well as examine whether genetic background influences one's chances of reaching longevity (90+ years) and extreme longevity (95+ years). It will also determine whether the chosen set of genetic variants influences survival in advanced old age. Most importantly, this study will contribute to the general pool of knowledge about the role biological and health factors play in achieving longevity and healthy ageing in the Croatian population.

The hypothesis of this study is that the frequency of single nucleotide polymorphisms associated with longevity differs between oldest-old individuals and a young control group from Croatian population, and that those same polymorphisms have an effect on longevity, while their interactions, relative telomere length and indicators of health status are factors that influence survival in advanced old age.

The main aim of this research is to determine biological contributors to the longevity of the Croatian oldest-old population and it will be achieved through specific goals that include:

1) a comparison of allele and genotype frequencies of variants associated with longevity between a sample of oldest-old individuals and a young control group;

2) testing which of the chosen single nucleotide polymorphisms have an effect on reaching longevity (90+ years) and extreme longevity (95+ years);

3) testing whether the chosen genetic variants or their interactions, relative telomere length, and health-related traits have an effect on survival in advanced old age.

The research that is planned within the framework of this doctoral dissertation is based on a sample of 327 oldest-old people (85 years and older), which was collected between 2007 and 2009 as a part of the project "Complex trait variation and health in children, adults and centenarians". Ten years after the initial survey, within the research project "HEalth, CUltural, and Biological determinants of longevity: Anthropological perspective on survival in very old age (HECUBA)" the age of death of all the respondents was determined from the national mortality register, making it possible to discern a group of long-lived individuals among them (those who lived to be over 90 years of age). Furthermore, within the same project, DNA samples of 100 unrelated young people between the ages of 20 and 35 were collected using the snowball method. This young sample, which should be a representation of individuals with differing chances of reaching longevity, is used as a control group for comparing the frequency of gene variants and as a reference group for calculating the relative telomere length of a group of elderly subjects.

For the genetic analysis, a set of 43 single nucleotide polymorphisms was selected by reviewing the relevant literature in publicly available databases (PubMed, repositories specialising in human genetics such as LongevityMap, https://genomics.senescence.info/longevity/, and Digital Ageing Atlas http://ageing-map.org/ (Budovsky et al., 2013; Craig et al., 2015)). The main criteria for the inclusion in the study was a strong or repeated association with human longevity and involvement in various signalling and metabolic pathways that play a role in the ageing process (e.g., cell cycle regulation, DNA repair mechanisms, the insulin signalling pathway).

The participants from the oldest-old group were users of one of 13 homes for the elderly and infirm from the city of Zagreb and Zagreb County area and participated in the research voluntarily, signing

an informed consent for participation and providing a sample of peripheral venous blood for biochemical, haematological, and molecular genetic analyses. The subjects completed a survey adapted to a sample of elderly people that contained questions related to functional ability, quality of life and health, and contained two internationally standardised questionnaires: Mini Nutritional Assessment (MNA) for assessing nutritional status, and the psychometric test Mini Mental State Examination (MMSE) for assessing the mental state of respondents. They had their blood pressure and bone density of the calcaneus (heel bone) measured, and underwent a short anthropometry. The collected data comprises a database of health variables that cover a wide spectrum of health status data indicating the subject's vitality (i.e., the number of chronic diseases, the number of medications taken daily, the number of prosthetic devices, nutritional status, blood pressure, bone density, etc.), which are in this study used in conjunction with the obtained genetic, biochemical and haematological data.

While some research has previously been conducted on the same long-lived sample – one that has examined the role of four polymorphisms in candidate genes for cardiovascular diseases (CVD) in longevity (Zajc Petranović, 2013) and the other that discussed the influence of five polymorphisms in candidate longevity genes on the subjects' biological age (Krajačić, 2017) – these studies did not cover as wide of a set of longevity-associated polymorphisms, nor could they provide a true insight on the effect these variants have on the lifespan of our oldest-old subjects, which is something this study is able to do.

2. LIST OF PUBLICATIONS

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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**

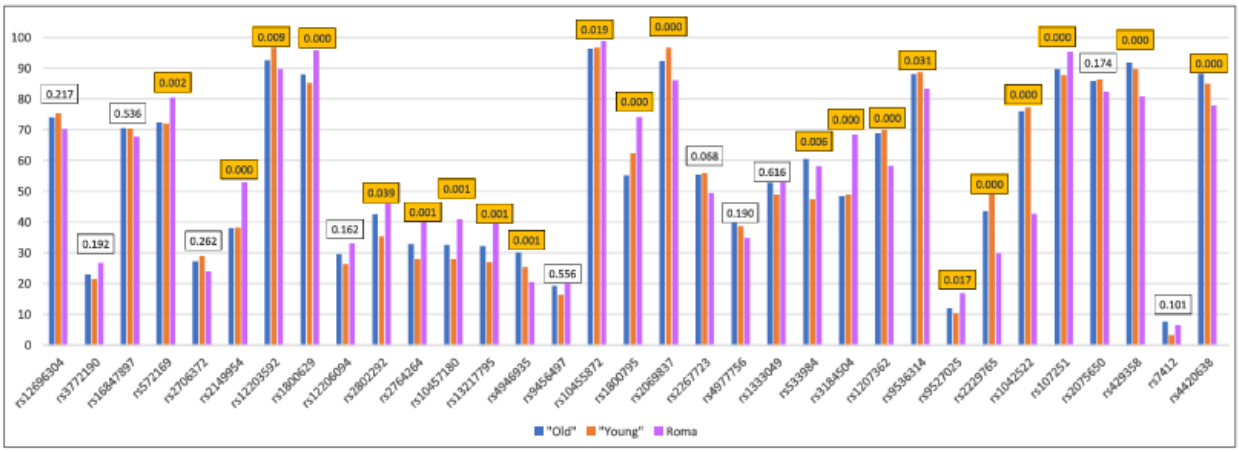

FIGURE 1: Effect alleles' frequencies for 33 longevity loci in Croatian "Old" (85–101 yrs.), "Young" (20–35 yrs.), and Croatian Roma samples. p-values of populations' differences are given for the chi2-test results.

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

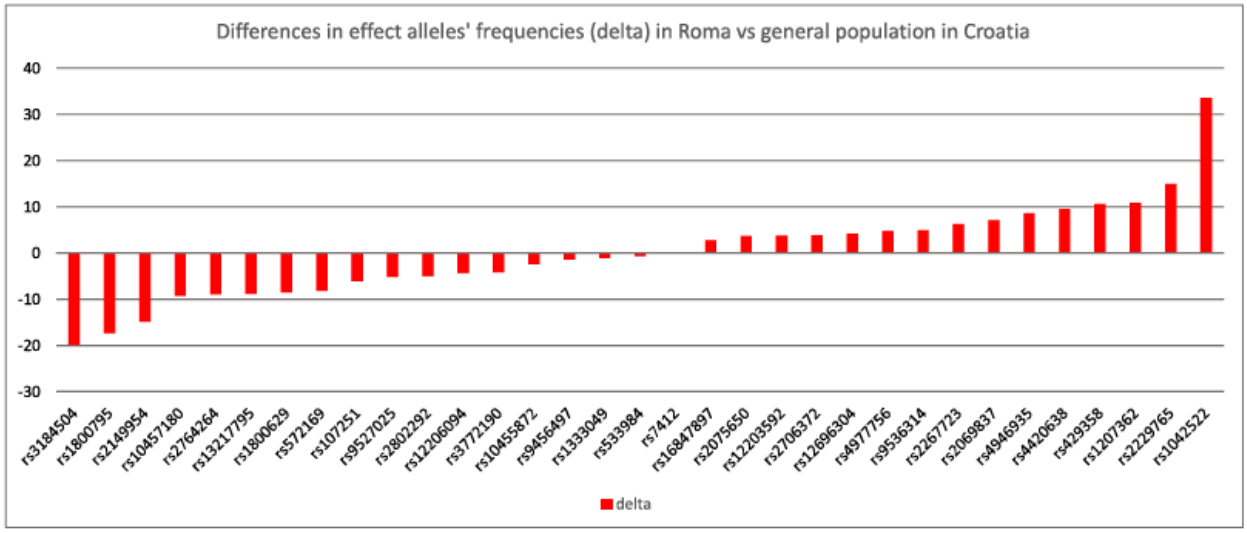

FIGURE 2: Decreasing order of the effect allele frequencies differences (delta) between Croatian (two cohorts combined) and Roma populations for the 33 longevity loci.

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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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				

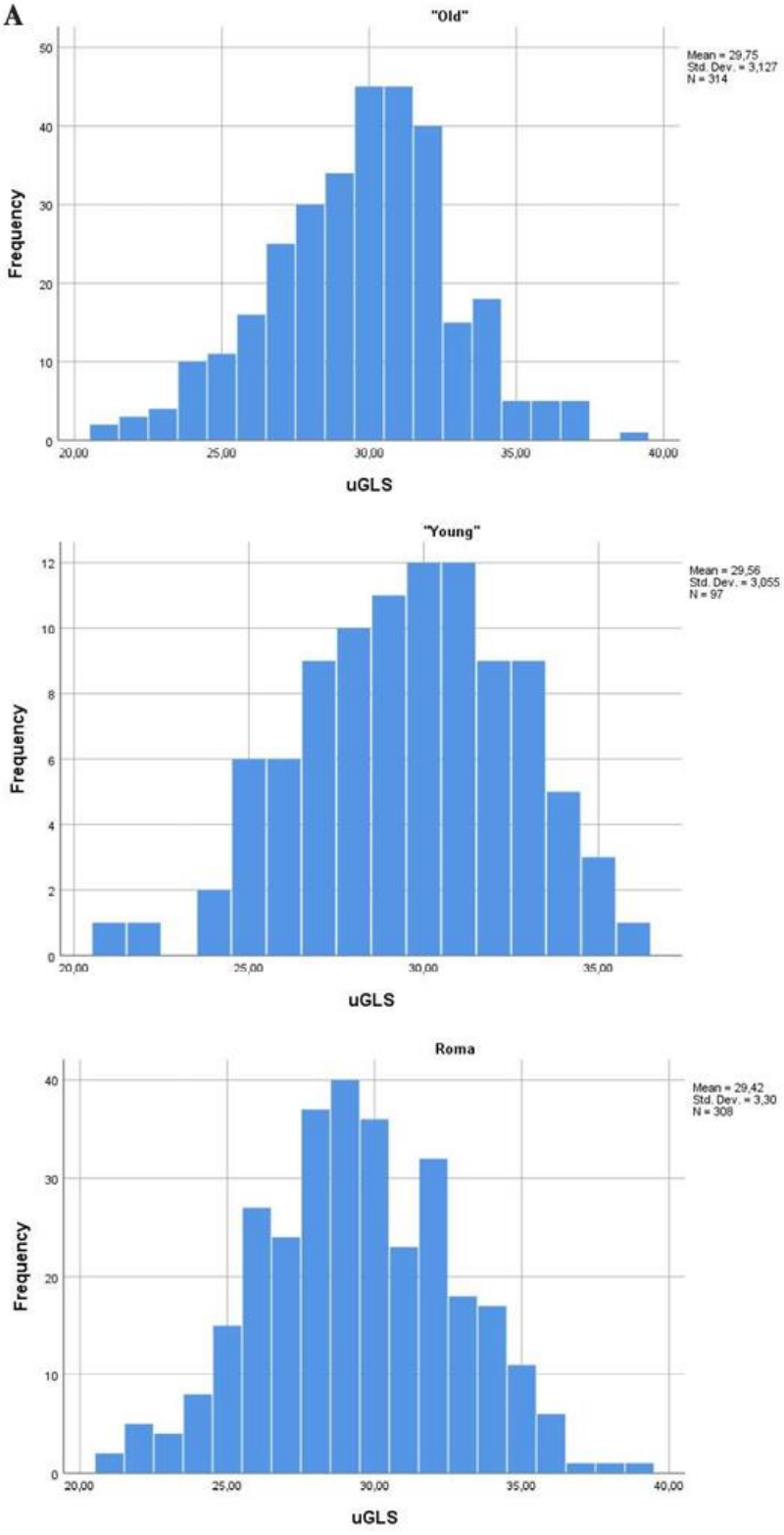
KLOTHO	rs9536314	T:T		253	78,8	77	79,4	214	70,2	0,679	0,160	0,045	
		T:G	no	60	18,7	19	19,6	81	26,6				
		G:G		8	2,5	1	1,0	10	3,3				
KLOTHO	rs9527025	G:G		253	78,8	79	80,6	219	69,7	0,288	0,052	0,029	
		C:G	yes	60	18,7	19	19,4	86	27,4				
		C:C		8	2,5	0	0,0	9	2,9				
IGF1R	rs2229765	G:G		102	32,6	27	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.

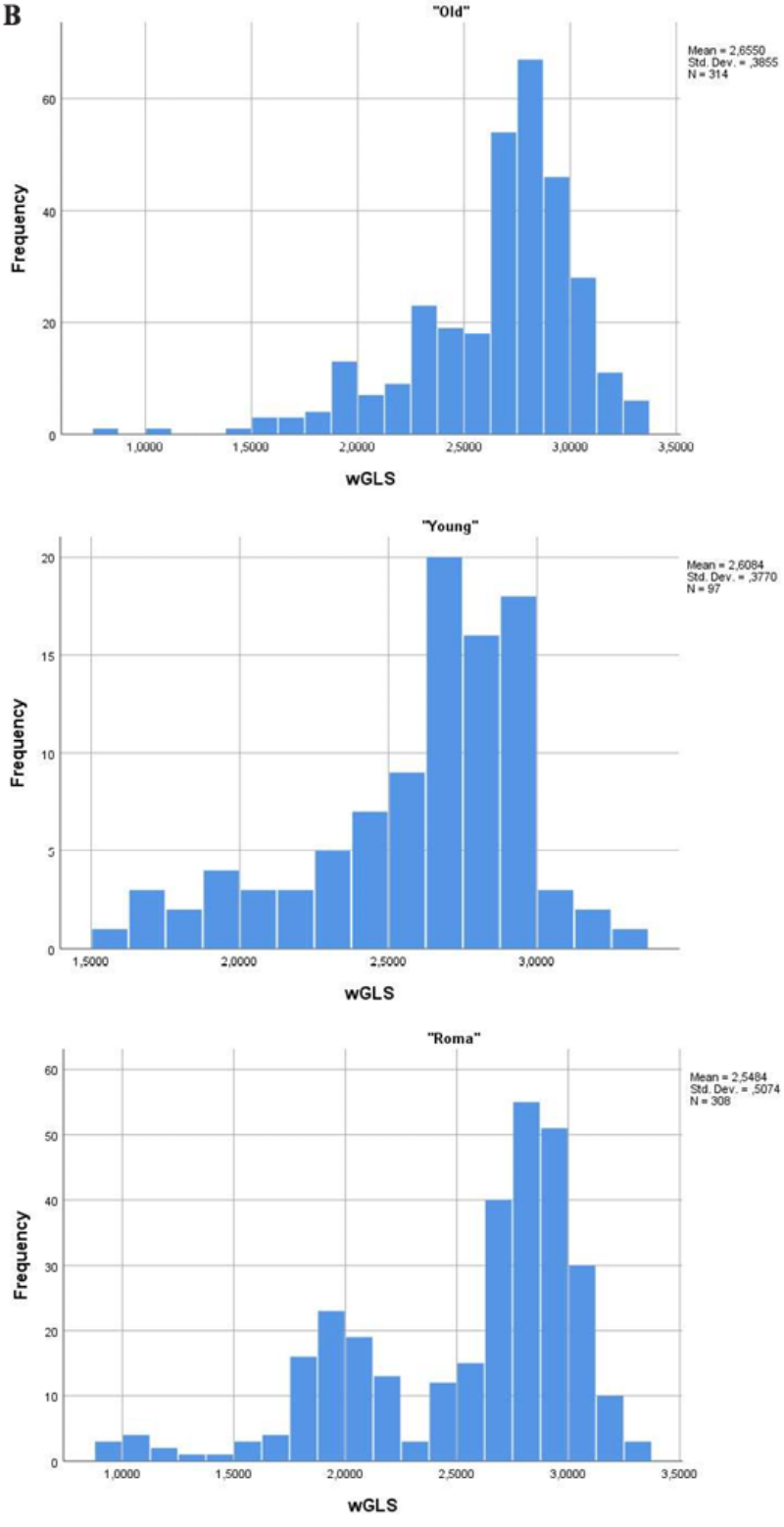
Group	N	Sex		Age				
		Men N (%)	Women N (%)	Mean	Std. Deviation	Minimum	Maximum	Range
Croatian "Old"	314	80 (25.5%)	234 (74.5%)	88.15	3.39	79	101	22
Croatian "Young"	97	32 (33.0%)	65 (67.0%)	24.63	3.76	20	35	15
Croatian – combined	411	112 (27.3%)	299 (72.7%)	73.15	27.23	20	101	81
Roma	308	140 (45.5%)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

28

PLOS ONE

RESEARCH ARTICLE

Genetic scores for predicting longevity in the Croatian oldest-old population

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Abstract

Longevity is a hallmark of successful ageing and a complex trait with a significant genetic component. In this study, 43 single nucleotide polymorphisms (SNPs) were chosen from the literature and genotyped in a Croatian oldest-old sample (85+ years, sample size (N) = 314), in order to determine whether any of these SNPs have a significant effect on reaching the age thresholds for longevity (90+ years, N = 212) and extreme longevity (95+ years, N = 84). The best models were selected for both survival ages using multivariate logistic regression. In the model for reaching age 90, nine SNPs explained 20% of variance for survival to that age, while the 95-year model included five SNPs accounting for 9.3% of variance. The two SNPs that showed the most significant association ($p \leq 0.01$) with longevity were *TERC* rs16847897 and *GHRHR* rs2267723. Unweighted and weighted Genetic Longevity Scores (uGLS and wGLS) were calculated and their predictive power was tested. All four scores showed significant correlation with age at death ($p \leq 0.01$). They also passed the ROC curve test with at least 50% predictive ability, but wGLS90 stood out as the most accurate score, with a 69% chance of accurately predicting survival to the age of 90.

Introduction

Continuous progress in reducing death rates during the early and middle years of life and improvements of the living conditions have resulted in a doubling of global life expectancy over the last two centuries [1], and according to data from the World Health Organization, that trend continues today [2] (accessed on 26th August 2022). This increase in life expectancy has led to a large increase in the percentage of older individuals in the population, and global predictions suggest that by 2050, for the first time in human history, there will be more people over 60 than adolescents and young adults combined. As old age is one of the main risk factors for the development of chronic illnesses such as cancer, cardiovascular and neurodegenerative diseases [3], the ageing of the population represents a significant burden on the social and healthcare systems of many countries [4]. Multimorbidity and frailty are also more prevalent among the elderly population [5, 6], often causing the need for long-term care in the later

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stages of a person's life. This demographic phenomenon has brought to attention the importance of preventing age-related diseases and conditions, identifying phenotypes associated with healthy ageing and genetic variants and biomarkers underlying these traits [7], implementing a sustainable healthcare [8] as well as developing strategies to promote successful ageing. Longevity and healthy ageing, and how to achieve them, are therefore among the principal challenges in human biology and medicine today, and the importance of research on this topic will increase even more in the coming decades.

The first major breakthrough in ageing research was the discovery that caloric restriction could positively affect the lifespan of model organisms [9]. This finding has intrigued scientists for almost a century, and was tested and reproduced in other species as well, with the results from primates being published only recently [10, 11]. In recent years, caloric restriction has also been proposed as an approach to cancer prevention [12, 13] and disease management [14, 15]. Along with increasing lifespan, dietary restriction reduced the occurrence of age-related diseases [16], thus proving that it can also be beneficial for extending the healthspan—a term that refers to the total duration of life spent in good health and the part of the total lifespan free from illness. As human life expectancy continues to increase, the challenges of extending the healthspan become even more important [17, 18] in order to achieve "optimal longevity"–a long and high-quality life [18].

Another major finding that propelled ageing research even further was the discovery of a single gene, aptly named age-1, that affects the lifespan of *Caenorhabditis elegans* [19]. This discovery marked the beginning of a new era of genetics-based longevity research, in which conserved genes and interacting signalling pathways that contribute to longevity have been identified. The ageing process and age-associated phenotypes are linked via gene regulation [20], and the complex network of cellular pathways involved in this regulation has pointed to a much greater plasticity of the ageing process than previously believed [21]. Genetic studies conducted in recent years indicate the same conserved pathways discovered in model organisms may modulate lifespan and healthspan in humans as well [22]. These studies used a candidate gene approach to investigate their association with longevity. Genome-wide association studies (GWAS) are another type of study commonly used to uncover longevity loci in humans [23]. In order to reveal genetic variants that might contribute to reaching an advanced old age, the frequencies of genetic variants are usually compared between an aged group of interest and a younger control group. Studying such longevity loci could prove instrumental for determining the molecular mechanisms underlying healthy ageing, and could also enable accurate prediction of a person's chance of reaching old age.

Polygenic risk score is a sum of an individual's genetic risk for a disease or trait, and it could be a compelling tool for health and lifestyle management [24, 25]. While polygenic risk scores are usually constructed as linear combinations of individual variant effects [26], summing all risk variants reported for a disease on a genome-wide level, a genetic score for predicting the chance of survival to a threshold age of longevity is a sum of significant longevity loci. Genotype data for 43 SNPs previously associated with longevity were obtained for a sample of Croatian elderly individuals (85+ years of age), and this study explores the relation of these longevity variants with the age at death of the studied sample. Its main goals are:

- to find the most influential genetic variants in the Croatian oldest-old sample that are significantly related to longevity (90+) and extreme longevity (95+ years)
- to construct unweighted and weighted genetic scores and test their specificity and sensitivity to predict a chance of survival to the age of 90 and 95 years.

Materials & methods

The study sample comprised 327 unrelated elderly individuals of both sexes aged 85 years and older, residents of the homes for elderly and infirm in Zagreb, the capital of Croatia (detailed description of the sample and study protocol could be found in Perinić Lewis et al. [27]). 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. The field study was conducted between 2007 and 2009, and 10 years after the initial sampling, the age at the time of death of each respondent was determined from the national mortality register.

43 single nucleotide polymorphisms (SNPs) in candidate genes for longevity were selected from publicly available literature databases (PubMed and repositories specialized for human longevity such as https://genomics.senescence.info/longevity/, http://ageing-map.org/). The SNPs were selected based on their strong or repeatedly reported association with human longevity and involvement in various metabolic pathways. S1 Table contains information about the selected SNPs (rs code, chromosome position, nearest gene, allele frequencies, MAF, genotyping success rate, HWE p-values and information on literature mentioning association with longevity).

Each participant provided a peripheral blood sample, and genomic DNA was isolated from leukocytes using the salting-out method [28]. Genotyping was outsourced and done in a commercial laboratory using a Kompetitive Allele Specific PCR (KASP) [29]. It is a genotyping assay that combines competitive allele-specific PCR with a homogeneous fluorescence-based reporting system for the identification and measurement of genetic variation occurring at the nucleotide level to detect SNPs or insertions and deletions (InDels). After genotyping, the final sample comprised 314 participants, as 13 participants had missing data on nine or more SNPs (>20% of unsuccessfully genotyped loci) and were therefore excluded from the analysis. Exclusion criteria were determined according to the principle of parsimony to retain the highest possible number of participants. Because all genetic data for each participant needed to be complete to calculate a genetic score, all missing data for participants with 1–8 unsuccessfully genotyped SNPs were replaced by the median value for that SNP.

Genotype data (available in open access on the online repository Zenodo [30]) were coded for each participant as follows: a value of 2 was assigned to the homozygous genotype of longevity allele, a value of 1 to the heterozygous genotype, and a value of 0 to the homozygous genotype of an allele not associated with longevity in our sample. In cases where less than 10 homozygous genotypes of any type were determined, and in cases of SNPs where dominant or recessive coding proved more significant in further analyses (rs2267723, rs16847897), they were coded with only the values 0 and 1, and the heterozygote was added to the less common homozygote. The coded data were used to perform univariate logistic regression as a means of selecting the SNPs that have a potential influence on longevity, using a cut-off p-value ≤ 0.20 [31]. Two separate analyses were performed with age at death as the dependent variable, for both of which the participants were divided in two groups—a group of those who died before, and a group of those who died after reaching a cut-off age of 90 or 95 years. The number of participants in the two groups according to their age at death was: for the cut-off age 90, there were 103 participants who died before the age of 90, and 211 participants who lived over 90; for the cut-off age 95, there were 230 participants who died before reaching 95 years of age, and 84 participants who lived over 95. All SNPs that had a p-value ≤ 0.20 in univariate analysis were selected for testing in a multivariate logistic regression model. The best models for age thresholds of 90 and 95 years were selected for further calculations.

Genetic Risk Score is called Genetic Longevity Score (GLS) in this study, since "risk" for reaching the age of 90 or 95 is a preferred trait, and thus a more appropriate term was chosen. GLS is a number representing a sum of alleles associated with human longevity across loci

included in the best multivariate logistic model. Unweighted GLS (uGLS) was calculated by summing the coding values assigned to genotypes at all SNPs that accounted for the best logistic regression model for ages at death 90 or 95 (uGLS90 and uGLS95, respectively). Weighted GLS (wGLS90 and wGLS95) was calculated by summing the genotype values for each SNP multiplied by their respective exponentiation of the beta coefficient from the multivariate model. To test the reliability of the scores, additional statistical analyses were performed to evaluate their association with age at death as a continuous or discrete variable (i.e. descriptive analysis, Pearson's correlations, Chi-square test, multiple regression analyses). Receiver operating characteristics (ROC) curve analysis was performed for both unweighted and weighted GLS, with binary age at death set as the dependent variable to calculate the area under the curve (AUC). All statistical calculations were performed using the SPSS software package 21.0.

Results

The general information on investigated 43 SNPs is presented in S1 Table, and univariate logistic regression results for all 43 SNPs with survival ages of 90+ and 95+ years as the dependent variable are shown in S2 Table. In univariate analysis, five SNPs (rs3772190, rs16847897, rs1800629, rs2267723, rs7412) were significantly associated (p-value of ≤ 0.05) with survival to the age of 90, and one SNP (rs429358) to survival to the age of 95. Since only one SNP was entered in the logistic regression in this analysis, this p-value did not have to undergo multiple correction testing. This shows a strong correlation between these SNPs with longevity in the studied population. However, in order to enlarge the qualifying pool of SNPs for further analyses, p-value of ≤ 0.2 was selected as the cut-off value for SNPs to be entered into multivariate logistic regression analysis [31]. With this inclusion criteria for the multivariate analyses, 17 SNPs entered the series of models for survival age 90+, and 10 SNPs entered the models for age 95+. The best multivariate models, which explain the largest proportion of variance in survival age, are presented in Tables 1 and 2, as well as in Fig 1, which shows a forest plot of SNPs that are positively (OR > 1) associated with longevity.

The best model, explaining 20.5% of the variance in survival to 90+ years of the oldest-old Croatian sample, has nine SNPs and is presented in Table 1. The two SNPs that showed the most significant association ($p \leq 0.01$) are: rs16847897, located in the *TERC* gene with the more frequent homozygote (GG) having a 2.128 times higher chance (95% CI 1.249–3.627, p = 0.005), and rs2267723 in the *GHRHR* gene, whose less common homozygote (AA) has a 2.280 times higher chance (95% CI 1.239–4.194, p = 0.008) of reaching 90 years of age. A lower degree of significance ($p \leq 0.05$) has the locus rs7412, in the *APOE* gene, where the carriers of the less frequent allele T (genotypes TT and CT) have a 3.055 times greater chance of living over 90 years (95% CI 1.230–7.587, p = 0.016) than the homozygotic carriers of allele C, and the locus rs1800629, upstream of the *TNF-α* gene, whose more common homozygotes (GG) are 1.898 times (95% CI 1.038–3.468, p = 0.037) more likely to survive up to the age of 90. Finally, rs1042522 located in the *TP53* gene did not reach statistical significance at the level of the entire locus, but heterozygotes for this locus (CG) have a 1.752 times (95% CI 1.010–3.040, p = 0.046) higher chance of reaching 90 years of age. The additional four loci—rs12206094 (in the *FOXO3* gene), rs9536314 (in the *KLOTHO* gene), rs50871 (in the *ERCC2* gene) and rs17202060 (in the *TXNRD1* gene)–are also included in the best model for the survival age of 90 years because they contribute to the quality of the model. Out of five SNPs that were significant at the univariate level, only one wasn't included in the best multivariate model. That SNP was rs3772190, located in the *TERC* gene, which was excluded due to its linkage (calculated in Haploview software [32]) with another *TERC* SNP, rs16847897, which entered the multivariate model since it showed a stronger association with survival to the age of 90.

Table 1. The best multivariate logistic regression model for survival to the age of 90 years in the Croatian oldest-old sample (N = 314).

Closest gene	SNP	Contrasting genotypes	B	p	O.R.	95% C.I. for O.R.	
						Lower	Upper
APOE	rs7412	CC vs TT, CT	1.117	**0.016**	3.055	1.230	7.587
ERCC2	rs50871	CC vs AC vs AA		0.238			
		CC, AA vs AC	-0.407	0.229	0.665	0.343	1.292
		CC, AC vs AA	0.069	0.856	1.072	0.506	2.268
FOXO3	rs12206094	CC vs TC vs TT		0.092			
		CC, TT vs TC	-0.363	0.183	0.696	0.408	1.187
		CC, TC vs TT	0.847	0.159	2.332	0.717	7.583
GHRHR	rs2267723	GG, AG vs AA	0.824	**0.008**	2.280	1.239	4.194
KL (KLOTHO)	rs9536314	TT vs GG, TG	0.454	0.181	1.575	0.809	3.065
TERC	rs16847897	CC, GC vs GG	0.755	**0.005**	2.128	1.249	3.627
TNF-α	rs1800629	AA, GA vs GG	0.641	**0.037**	1.898	1.038	3.468
TP53	rs1042522	CC vs GG vs CG		0.092			
		CC, CG vs GG	0.833	0.248	2.300	0.559	9.456
		CC, GG vs CG	0.561	**0.046**	1.752	1.010	3.040
TXNRD1	rs17202060	TT vs CC vs TC		0.164			
		TT, TC vs CC	0.292	0.480	1.339	0.595	3.012
		TT, CC vs TC	0.705	0.094	2.024	0.887	4.621
Nagelkerke R-squared				0.205			
Hosmer—Lemeshow test				0.536			
% Correct				72.9			

This table shows all the SNPs that together make up the best model for predicting survival to age 90+, the genotypes that were contrasted within the model, beta values, odd ratios and 95% confidence intervals. The p-values of SNPs that passed the significance threshold of $p \leq 0.05$ are highlighted in bold. Nagelkerke R-squared value, indicating the amount of variance explained by the model, is shown at the bottom of the table along with the results of Hosmer—Lemeshow test and the percentage of correctly classified cases.

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Table 2 presents the best model for predicting survival to age 95+, explaining 9.3% of variance. Of the five SNPs contributing to the best model, no locus was significant at the entire locus level (three contrasting genotypes). However, there are several significant associations that elucidate specific genotypes: the rs6067484 locus in the *PTPN1* gene, whose less frequent homozygotes (GG) have a 2.505 times higher chance for reaching 95 years of age (95% CI 1.049–5.981, p = 0.039). Also, for rs4837525 located in the *PAPPA* gene, heterozygotes (AG) have a chance of living over the age of 95, which is 2.703 times (95% CI 1.039–7.033, p = 0.042) higher than those of both homozygotes, and for rs1042522 of the *TP53* gene, the less common homozygote (GG) has a 3.233 times (95% CI 1.013–10.322, p = 0.048) higher chance of surviving to the age of 95 years. Finally, rs429358 in the *APOE* gene, which was also significant on the univariate analysis level, is associated with survival to 95 years at the $p \leq 0.1$ significance level (p = 0.053), with the more frequent genotype (TT) having a 2.345 times (95% CI 0.988–5.563) greater chance of reaching 95 years of age. The association of rs12203592 in the *IRF4* gene, although not statistically significant, contributes to the strength of the model.

It should be noted that the two selected models share only one locus—rs1042522 in the *TP53* gene—which is significantly associated with survival to age 90+ (CG genotype) as well as to age 95+ (GG genotype). However, both models also indicate epsilon diplotypes of the *APOE* gene: the first model at the rs7412 locus and the second at the rs429358 locus. Data on the frequencies of *APOE* gene longevity loci and APOE isoforms in the oldest-old Croatian population are shown in Table 3, with allele distribution frequencies in the European population

Table 2. The best multivariate logistic regression model for survival to the age of 95.0 years in the Croatian oldest-old sample (N = 314).

Closest gene	SNP	Contrasting genotypes	B	p	O.R.	95% C.I. for O.R.	
						Lower	Upper
APOE	rs429358	CC, CT vs TT	0.852	0.053	2.345	0.988	5.563
IRF4	rs12203592	CC vs CT, TT	0.569	0.110	1.766	0.880	3.546
PAPPA	rs4837525	AA vs AG vs GG		0.119			
		AA, AG vs GG	0.766	0.127	2.151	0.804	5.757
		AA, GG vs AG	0.994	**0.042**	2.703	1.039	7.033
PTPN1	rs6067484	AA vs GA vs GG		0.116			
		AA, GG vs GA	0.116	0.685	1.123	0.640	1.970
		AA, GA vs GG	0.918	**0.039**	2.505	1.049	5.981
TP53	rs1042522	CC vs CG vs GG		0.123			
		CC, CG vs GG	1.174	**0.048**	3.233	1.013	10.322
		CC, GG vs CG	0.245	0.375	1.278	0.744	2.197
Nagelkerke R-squared				0.093			
Hosmer—Lemeshow test				0.763			
% Correct				74.2			

This table shows all the SNPs that together make up the best model for predicting survival to age 95+, the genotypes that were contrasted within the model, beta values, odd ratios and 95% confidence intervals. The p-values of SNPs that passed the significance threshold of $p \leq 0.05$ are highlighted in bold. Nagelkerke R-squared value, indicating the amount of variance explained by the model, is shown at the bottom of the table along with the results of Hosmer—Lemeshow test and the percentage of correctly classified cases.

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taken from the gnomAD database [33], and isoform frequencies of 1038 control subjects of European origin under 60 years of age, taken from the paper of McKay et al. [34].

Unweighted and weighted longevity scores were calculated for survival ages 90+ and 95+. Descriptive data of unweighted and weighted GLS90 and GLS95 are shown in Table 4, while the distribution of all four genetic longevity scores by the low/high division of the age-at-death variable is presented in Fig 2. The mean value of the scores within individual age-at-death groups is also presented. Pearson's correlation of all four longevity scores with age at death as a continuous variable is presented in Table 5. With p-value ≤ 0.01, all four GLSs were significantly associated with age at death. There was no significant difference in any calculated GLS between sexes, which is shown in S3 Table.

The distribution of the two unweighted genetic longevity scores (uGLS90 and uGLS95) in the three age-at-death groups ($\leq$ 90.00 years, 90.00–94.99 years, and 95.00+ years) is presented in Fig 3. None of the highest-scoring participants for uGLS90 died before the age of 90, while the entire range of scores were represented in the lowest survival group for uGLS95. The lowest longevity scores were found among participants who died before the age of 90, and were not observed in the other two groups. The distribution is similar between the two scores among those who lived the longest, with no participant having a score below three. In the higher survival groups, distribution curves shift to the right side of the x-axis and higher longevity scores. S1 Fig shows the distribution of median values of genetic longevity scores by three age-at-death groups: <90.00, 90.0–94.99, and 95.00+, presenting the absolute number of individuals in each group. All scores yield analogous results: the group of participants who died before the age of 90 has a higher percentage of below-median longevity scores. Likewise, participants who survived beyond the age of 95 have a higher percentage of above-median scores. The relative age distribution of age-at-death groups (the percentage of each group is equal to 100%) according to the median of weighted genetic longevity scores is presented in S2 Fig, which

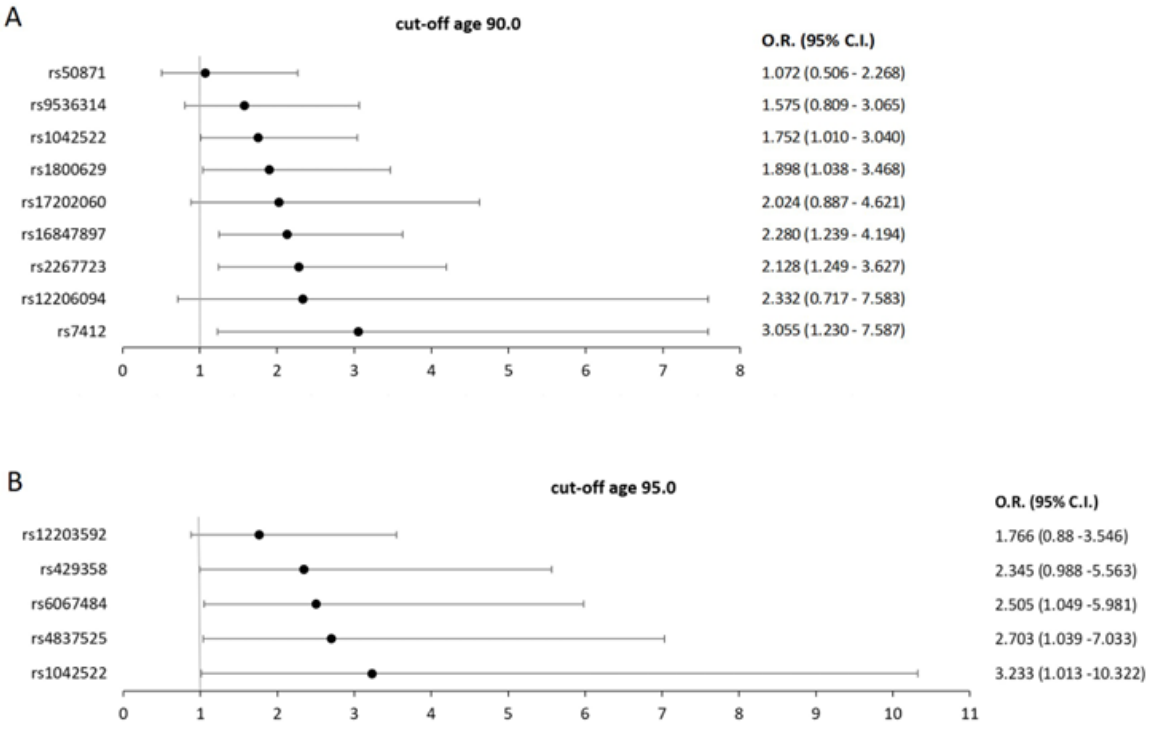

Fig 1. Forest plot of SNPs positively (O.R. > 1) associated with longevity, with multivariate model Odds Ratios (O.R.) and 95% confidence intervals (C.I.) displayed. A) SNPs positively associated with survival to the age of 90, B) SNPs positively associated with survival to the age of 95.

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demonstrates that the weighted longevity score for the threshold of 95 years is gradual and inverse between groups below and above the median. The largest proportion of participants who died before age 90 is in the below-the-median group, and the largest proportion of those who survived over age 95 is in the above-the-median wGLS95 group. On the other hand, the weighted longevity score for the threshold age of 90 years has equal distributions between the 90.00–94.99 group and the 95.00+ group for both below- and above-the-median scores, while the proportion of people who died before 90.00 year of age is substantially higher in the below-the-median wGLS90 group.

Table 3. Frequencies of alleles at loci that determine APOE isoforms in a Croatian sample of oldest-old people, and their frequencies in the general European population.

		Croatian oldest-old sample		European frequencies	
		allele T	allele C	allele T	allele C
APOE loci	rs7412	0.066	0.934	0.077	0.923
	rs429358	0.914	0.086	0.851	0.149
APOE isoform frequencies	ε2 (rs7412-T, rs429358-T)	0.076		0.091	
	ε3 (rs7412-C, rs429358-T)	0.844		0.733	
	ε4 (rs7412-C, rs429358-C)	0.079		0.176	

Allele frequencies in European populations were taken from the gnomAD database [33], and isoform frequencies of 1,038 control subjects of European origin under the age of 60 from a paper by McKay et al. [34].

https://doi.org/10.1371/journal.pone.0279971.t003

Table 4. Descriptive statistics of unweighted (uGLS) and weighted genetic longevity scores (wGLS) for survival to ages 90 and 95.

	TheoreticalMaximum	Minimum	Maximum	Range	Mean	Std. Deviation
uGLS90	18.000	1.000	15.000	14.000	7.869	2.551
wGLS90	36.232	1.330	29.271	27.941	14.336	5.132
uGLS95	10.000	1.000	9.000	8.000	4.379	1.510
wGLS95	25.104	2.703	22.599	19.896	11.112	3.732

https://doi.org/10.1371/journal.pone.0279971.t004

In order to test the predictive power of the obtained genetic longevity scores, we performed ROC curve analysis (Fig 4), which showed that all four scores were satisfactory for predicting the possibility of reaching the longevity milestones (90+ and 95+ years) that were above the theoretical cut-off value for this test of 0.5 [35, 36]. However, with an AUC score of 0.690, wGLS90 is a more predictive longevity score for survival to the age of 90, and uGLS90 with an AUC score of 0.662 is the less predictive. ROC curve analysis showed no differences between

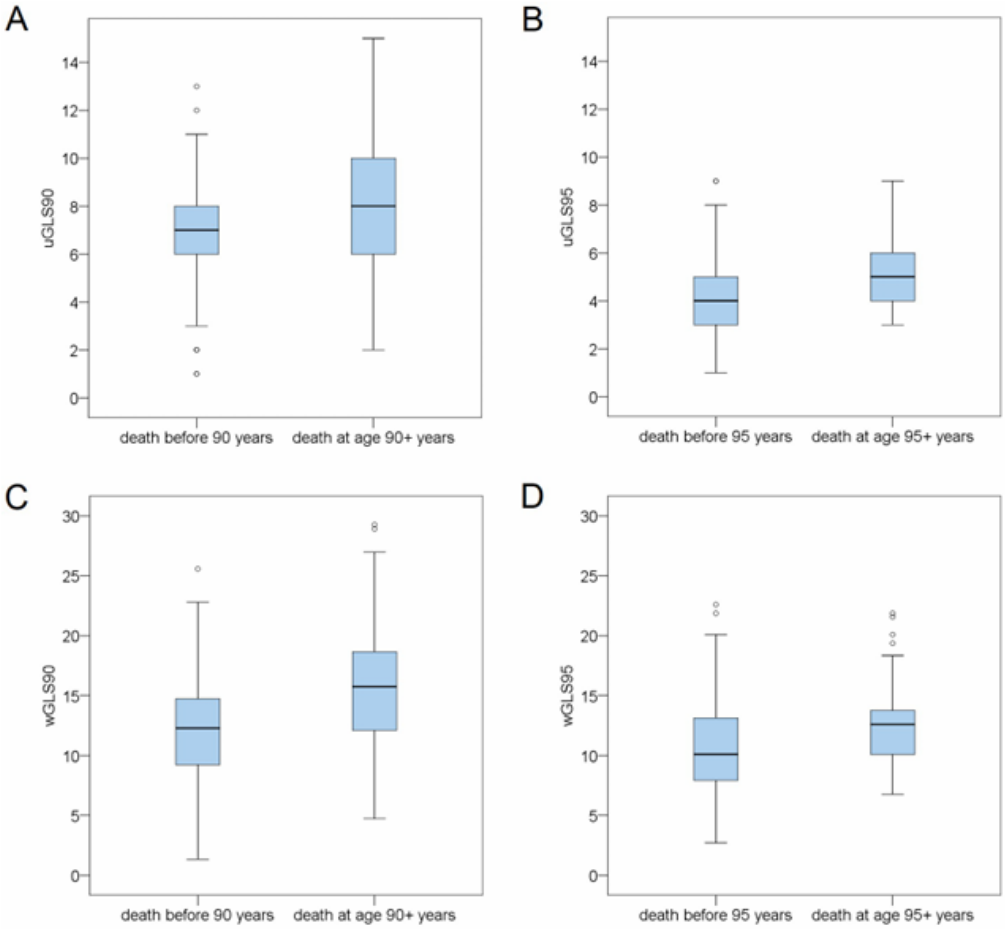

Fig 2. Comparison of the genetic longevity score values between participants who died before and after reaching the cut-off ages of 90 and 95 years. Box-and-whiskers plot showing the median value, quartile and extremes of A) uGLS90, B) uGLS95, C) wGLS90, D) wGLS95.

https://doi.org/10.1371/journal.pone.0279971.g002

Table 5. Correlation between age at death and calculated genetic longevity scores.

	uGLS90	wGLS90	uGLS95	wGLS95
Pearson correlation (r)	0.159	0.178	0.215	0.211
p	0.005	0.002	0.000	0.000

https://doi.org/10.1371/journal.pone.0279971.t005

uGLS95 and wGLS95 as they are equally predictive with AUC scores of 0.649. A multivariate linear regression analysis was also performed, including all genetic scores as independent variables and a continuous age-at-death variable as a dependent phenotype. Beta-values and significance levels obtained from this analysis are presented in Table 6, with wGLS90 and uGLS95 highlighted as more informative scores.

Discussion

With the continuing demographic trend of population ageing, achieving a long and healthy life is becoming more than a personal goal—it is now a research focus of scientists all around the globe. There is a great inter-individual variation in the rate at which one ages, and twin studies have shown that this variation, like longevity, has a genetic component [37, 38]. The principal aims of this study were to investigate which of the alleles associated with longevity in previous studies were important for reaching longevity thresholds in the Croatian oldest-old population (aged 85+), and to calculate and test unweighted and weighted genetic risk scores for predicting survival to the age of 90 and 95 using loci that proved significant in logistic regression analysis. The model for predicting survival to age 90 accounts for two times more variance than the model for predicting survival to age 95, which can be explained by fewer SNPs entering the 95-year model. We also suggest that a decline in the proportion of variance explaining the age at death is due to a shift between survivor and non-survivor groups and a decline in the expected outcome group. However, we cannot rule out the importance of some other stochastic elements that might also increase with advancing age, which reduces the genetic effect.

The two loci most strongly associated with reaching the age of 90 in the studied population are rs16847897 in the *TERC* gene and rs2267723 in the *GHRHR* gene. The *TERC* gene encodes for the RNA component of telomerase, a ribonucleoprotein that elongates telomeric DNA [39,

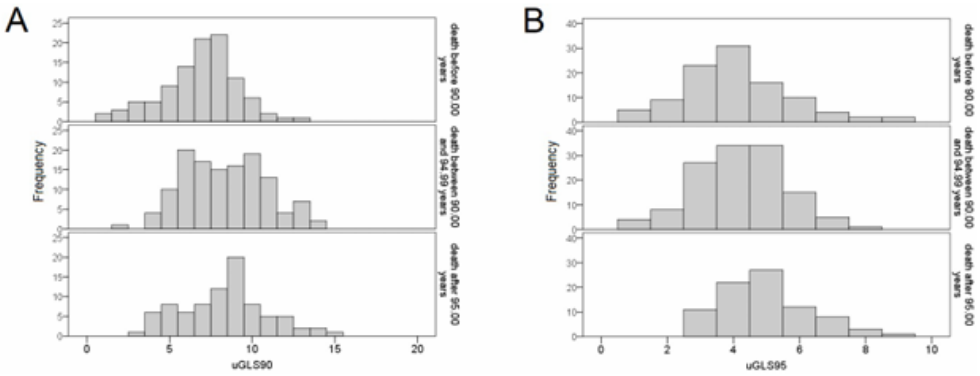

Fig 3. Distribution of two unweighted genetic longevity scores in three age-at-death groups. Histograms show the distribution of A) uGLS90, and B) uGLS95 among the participants belonging to a specific age-at-death group: < 90.00 years, 90.00–94.99 years, and 95.00+ years.

https://doi.org/10.1371/journal.pone.0279971.g003

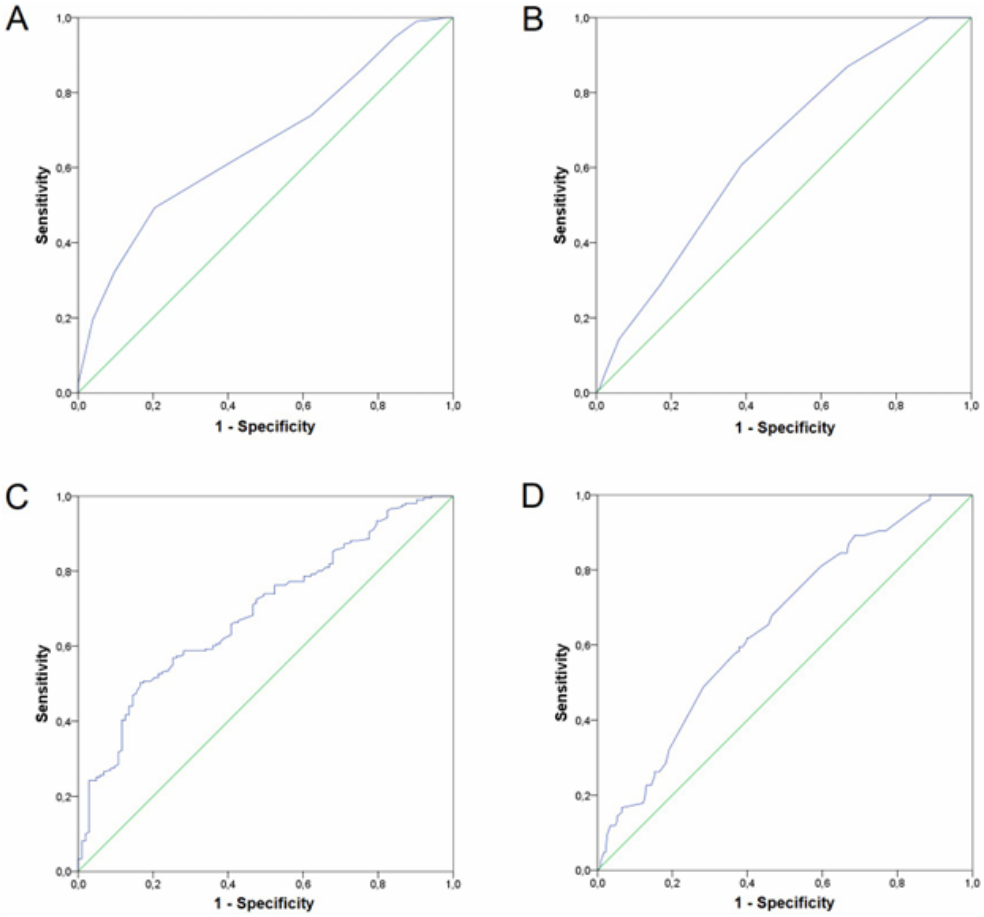

Fig 4. Receiver operating characteristics (ROC) curves for calculated genetic longevity scores. The ROC curve and area-under-curve (AUC) score for: A) uGLS90 (AUC = 0.662), B) uGLS95 (AUC = 0.649), C) wGLS90 (AUC = 0.690), D) wGLS95 (AUC = 0.649).

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40]. Shorter leukocyte telomere length is frequently reported in patients who suffer from age-related diseases such as Alzheimer's disease [41] and vascular dementia [42], and has been proposed as a marker of biological ageing [43]. The intronic SNP rs16847897 located downstream of the *TERC* gene has been associated with leukocyte telomere length in large UK cohorts [44] and in the Chinese Han population [45]. The Chinese Han population study showed that the C allele of rs16847897 was associated with a shorter mean telomere length that equated to approximately 4 years of average age-related telomere attrition. Additionally, a study from

Table 6. The results of a multivariate linear regression analysis including all genetic scores as independent variables and the continuous age-at-death variable as the dependent phenotype.

	uGLS90	wGLS90	uGLS95	wGLS95
Multiple regression (beta)	-0.132	0.143	0.188	-0.106
p	0.513	0.011	0.001	0.734

https://doi.org/10.1371/journal.pone.0279971.t006

Scarabino et al. showed that the C allele increased the risk of earlier onset of Alzheimer's disease in the population from Southern Italy [46]. In the studied Croatian sample, the other allele, G, is beneficial for longevity because it contributes to the chances of reaching the longevity threshold of 90 years. In a follow-up study on a Southern Italian population, however, there was no significant association of rs16847897 with human lifespan [47]. Allele frequencies of this SNP vary considerably in different populations, so that the longevity allele G is a major allele in all mentioned European populations and a minor allele in the Chinese Han population.

The *GHRHR* gene encodes a growth hormone-releasing hormone receptor, a G protein-coupled receptor located on the membrane of somatotropic cells, cells that produce growth hormone in the anterior pituitary gland [48]. It binds growth hormone-releasing hormone, a peptide hormone produced in the hypothalamus. This binding is necessary for the proliferation of somatotrophs and for synthesis and secretion of growth hormone (GH) [49]. It is a part of the growth hormone/ insulin-like growth factor 1/insulin (GH/IGF-1/INS) signalling axis. Research from over 40 years ago showed that the secretion of GH and IGF-1 slowly decreases after an organism matures to adulthood, reaching its absolute lowest level in people over the age of 60 [50]. This biological phenomenon, which has been confirmed both in humans and other mammals [51], was even given a name–'somatopause' [52]. The decrease of GH/IGF-1 signaling has proven to extend longevity in many model organisms, including yeast, worms, fruit flies, and mice [53]. The minor allele A in the intronic rs2267723 of the *GHRHR* gene was significant for longevity in the Danish population [54]. It was also among the top-ranked interactions in a study that explored the combined effect of SNPs from candidate pathways on longevity [55]. This is in line with our study, where the minor allele A is advantageous for reaching 90 years of age.

The impact that changes in the immune system can have on ageing and reaching longevity has been clearly demonstrated in multiple studies [56]. With advancing age, the effectiveness of the immune response decreases, while inflammatory processes increase, which is described by the term 'inflamm-aging' [57]. This lack of equilibrium in the organism's response to stressors contributes to the development of chronic diseases with inflammatory pathogenesis, which are a major characteristic of ageing [58]. A significant association with survival to age 90 was found for rs1800629, located in a regulatory region upstream of the *TNF-α* gene. The *TNF-α* gene encodes a proinflammatory cytokine involved in many biological processes—from regulating proliferation, differentiation and apoptosis, to playing a role in lipid metabolism and coagulation. It has been linked to a number of conditions, including autoimmune disorders, insulin resistance, and cancer [59]. According to functional studies, the uncommon allele A of rs1800629 is a far more effective transcriptional activator than the common allele G [60]. However, a study conducted on an English Longitudinal study sample showed that the A allele is a risk factor for frailty [61], and similar results were obtained in a study of longevity and ageing of the Chinese population, where homozygous carriers of the A allele had worse results in physical function tests (Timed Up and Go Test and 5-meter walking test) [62]. In the Croatian oldest-old sample, the allele beneficial for reaching 90 years of age was the major allele G, which is in concordance with the previously mentioned research by Melki et al. and Yao et al. [61, 62].

rs1042522 was the only SNP significantly associated with survival to both ages 90 and 95. It is a missense variant with a very diverse distribution in world populations [63]. This SNP is located in the *TP53* gene, a gene that encodes the p53 protein that acts as a tumour suppressor by blocking cell cycle progression and promoting apoptosis. The p53 protein plays a central role in cellular regulatory pathways and is an important regulator of the expression and activity of several replication and transcription factors. Its activation is triggered by stress signals that

arise in response to the cell's conditions and environment. Some stressors, for example, are genotoxic damage, oncogene activation, replication stress, loss of normal cell connections and hypoxia [64]. It is crucial for determining cell fate by promoting either repair, survival, or elimination of damaged cells [65]. The *TP53* is the most frequently mutated gene in human cancer, and mutations in this gene can be found in >50% of all human cancers [66–68]. It is also of great importance for the ageing process, since apoptosis and cellular senescence strongly influence the homeostasis of tissues, and too much of both can deplete renewable tissues of progenitor or stem cells and reduce their ability for regeneration [69]. In the presence of intracellular reactive oxidative species (ROS), p53 becomes a target of the histone deacetylase SIRT1 [70, 71], whose expression is strongly down-regulated in senescent cells, and is often considered a potential target for longevity extension [70]. Furthermore, it can downregulate the insulin/IGF-1 pathway, which has been shown to increase lifespan [72]. The polymorphism of rs1042522 is a functional mutation that results in either an arginine (Arg) or a proline (Pro) residue at codon 72, with the proline allele showing a weaker response to induce apoptosis and prevent cell transformation [73, 74]. The European distribution of the Arg72Pro substitution is approximately 60%, 30% and 10% for Arg/Arg, Arg/Pro and Pro/Pro, respectively [75], with major C allele coding for arginine and minor G allele coding for proline. In a study by Ørsted et al. of the general Danish population, overall survival was higher for carriers of the G allele, both homozygotes (6% better survival) and heterozygotes (3% better survival), along with reduced mortality after cancer diagnosis [76]. Similar results were shown by a study conducted on a sample from the Leiden 85-plus study, in which the authors showed that carriers of the Pro/Pro genotype (G allele homozygotes) older than 85 years have increased survival compared to the carriers of Arg [77]. In a smaller cohort of 155 long-lived individuals, Groß et al. found that the proline allele was significantly associated with increased survival time in female participants [78]. In our study, rs1042522 was the only SNP significantly associated with both survival up to the age of 90 (CG genotype, Arg/Pro) and to the age of 95+ years (GG genotype, Pro/Pro). Therefore, regardless of the genotype, the G allele, which codes for proline at the 72nd residue of p53, proved to be beneficial for longevity in Croatian oldest-old persons, which coincides with the results of other studies. Interestingly, in the same paper on the Leiden 85-plus cohort, the Pro/Pro genotype was found as a risk for cancer mortality [77]. Furthermore, in a case-control association study of breast cancer performed on a sample of Croatian women, the percentage of Pro/Pro genotype was higher in cases (11.6%) than in controls (4.6%) [68]. Given the previously mentioned characteristic of the Pro allele for a reduced apoptotic response, that is perhaps not surprising. A reduced affinity for inducing apoptosis may cause a malignant cell being more likely to escape programmed cell death, thus increasing the risk of cancer. However, if Pro/Pro genotype triggers less apoptotic events, this might lead to a greater number of cells in general, which becomes increasingly important with old age. As an organism ages, proliferative capacity of tissues goes down, and this process might even be accelerated by an increased clearing of cells by apoptosis. Therefore, while the largest benefit for survival to the age of 90 in our studied population comes from the heterozygous Arg/Pro genotype (pointing to the importance of balance between cell proliferation and programmed cell death, and a possible heterosis effect), for survival to the threshold of extreme longevity (95 years) the maintenance of proliferative abilities that might come from homozygous Pro/Pro genotype seems to be more beneficial than cancer-protective effects of Arg. This could possibly explain the interplay through which p53 affects the ageing process and longevity. Nonetheless, we find it important to note that while the heterozygous CG genotype shows a statistically significant association in our model with reaching 90 years of age, the GG genotype points to an even higher chances of surviving beyond the age of 90. However, the effect of this might not be visible because only 14 out of 314 participants had the GG genotype, and its benefits might

have been masked by a much higher number of participants with a slightly weaker, but overall beneficial effect of the CG genotype.

The only genetic locus to reach the level of genome-wide significance ($p \leq 5\times10-8$) in multiple GWA studies for longevity is apolipoprotein E (*APOE*) [79–81]. APOE is a protein with an important role in cholesterol transport. The *APOE* gene is polymorphic, resulting in three major isoforms of the APOE: APOE2 (ε2), APOE3 (ε3) and APOE4 (ε4) [82, 83]. The three APOE isoforms differ at the 112th and 158th residues of their primary structures, and are determined by two SNPs that cause amino acid substitutions and result in functional changes in the APOE protein: rs429358 and rs7412, respectively. APOE-ε3 (cys112, arg158) is the most common isoform of the APOE gene [84]. The carriers of this isoform have a C allele on rs7412 and T allele on rs429358. APOE-ε2 (cys112, cys158) is an isoform caused by the transition of the C allele to the T allele of rs7412, while the T allele of rs429358 remains unchanged. This mutation causes a substitution of the basic amino acid Arg158 in APOE-ε3 with the neutral amino acid Cys158 in APOE-ε2 [83], resulting in reduced APOE-ε2 receptor affinity. APOE-ε4 (arg112, arg158) isoform is characterised by the C allele of rs7412 and the C allele of rs429358. The ε4 isoform of APOE is associated with increased total cholesterol and low-density lipoprotein cholesterol [82], heart disease [85–87], Alzheimer's disease and dementia [82, 88] and other illnesses [89, 90]. Its frequency varies significantly between young adult populations. APOE-ε4 is expressed in approximately 25% of Finns, 17–20% of Danes and approximately 10% of French, Italians and Japanese. However, in all mentioned populations, the frequency of APOE-ε4 among centenarians is closer to half of these values [91]. Studies have explained this age-related distribution by showing a negative association between chances of reaching extreme longevity and the presence of ε4 [34, 92, 93]. The APOE isoforms make six possible biallelic genotypes: ε3/ε3, ε3/ε4, ε2/ε3, ε4/ε4, ε2/ε4 and ε2/ε2, which are shown here ranked from most to least common among European populations [94]. The T allele of rs7412, which is a minor allele in the Croatian oldest-old population and in all the populations indexed in 1000 Genomes database [63], has been associated with survival to the age of 90 in the studied population, both in homozygous and heterozygous form. This is in line with the findings of Deelen et al. [23], where the minor allele of rs7412 was found to have a beneficial effect on longevity. The same study found that the minor C allele of rs429358 had a deleterious effect on longevity, which is in concordance with our findings. In the Croatian oldest-old population, the rs429358 allele positively associated with survival to age 95 was the major allele T, but it was slightly below the significance level of $p \leq 0.05$. Since the T allele was associated with survival to longevity threshold age in both *APOE* SNPs, this suggests a beneficial effect of the ε2 isoform of APOE on longevity. Comparison of allele frequencies from the studied population with the European average from the gnomAD database shows that the oldest-old Croats have a lower frequency of longevity-related T allele of rs7412. This is also reflected in the lower prevalence of ε2 in the Croatian population older than 85 years compared to the data of 1038 control subjects of European origin younger than 60 years of age from a study by McKay et al. [34]. However, it is apparent that the studied Croatian population owes its longevity to a higher frequency of longevity-associated T allele of rs429358, which is confirmed by a much higher percentage of the neutral isoform ε3, and a lower percentage of the detrimental isoform ε4 (Table 3). Therefore, we can conclude that while only a small percentage of the Croatian oldest-olds benefit from the protective ε2, the majority had a good chance to reach extreme longevity by being spared from the negative influence of ε4.

The two SNPs that were significant only for survival to the age of 95 are locus rs6067484 in the *PTPN1* gene, and rs4837525 located in the *PAPPA* gene. Information on both of these loci is scarce, with only minor A allele of rs6067484 being previously associated with higher levels of total plasma cholesterol and low-density lipoprotein (LDL) cholesterol in men [95].

However, both variants were reported as potential candidates affecting longevity in a paper by Dato et al. which examined the association between SNP-SNP interactions and longevity [55]. rs6067484 is an intronic variant of the *PTPN1* gene that encodes protein tyrosine phosphatase non-receptor type 1, a suppressor of insulin signalling pathways [95]. The *PTPN1* gene is located in the q13.1-q13.2 area of chromosome 20, a region that is gained or amplified in several cancers [96]. rs6067484 of *PTPN1* was significant in interaction with rs12437963 in the *IGF1R* gene. Due to the importance of the insulin/IGF-1 pathway in ageing processes, it is not surprising that a suppressor of this pathway could be associated with longevity. This also explains why the signal for rs6067484 in the study by Dato et al. was paired with a signal for another variant involved in the same metabolic pathway [55]. In our sample, the allele contributing to survival to 95 years of age was the minor G allele, whose frequency varies from 20–30% in European and Latino populations, to only 1–4% in African populations [33, 63]. Additional connection that the *PTPN1* gene has to healthy ageing is its association with Alzheimer's disease. Studies have shown that overexpression of this gene, mediated by knockdown of miR-124, reduces synaptic failure and memory deficits, highlighting it as a promising new therapeutic target for patients with Alzheimer's disease [97, 98]. Another SNP significant in the Croatian oldest-old population is rs4837525 in the *PAPPA* gene, which also had a significant interaction with the *GHSR* gene of the insulin/IGF-1 signalling pathway in the study by Dato et al. [55]. The *PAPPA* gene encodes a zinc metalloproteinase that cleaves inhibitors of IGF, thus enhancing the activity of insulin/IGF-1 pathway [99]. This enzyme was first discovered in the plasma of pregnant women, and since its function was unknown at the time, it was named pregnancy-associated plasma protein-A [100]. A study by Bøtkjær et al. showed that another single nucleotide variant in the *PAPPA* gene caused an amino acid change (Tyr>Ser) that significantly reduced cleavage rates for one of the IGF-binding proteins [101]. The SNP observed in this study, rs4837525, is intronic and therefore does not affect the protein's catalytic activity, but could affect its expression by acting as an enhancer [63]. It is interesting that the protective effect for reaching the age of 95 years among the oldest-olds in Croatia was found in heterozygotes for this SNP, who carry one ancestral allele A and one new allele G. However, both in the studied population and in other European populations, the G allele is major, regardless of the fact that the A allele is the ancestral one.

The second part of this study focused on the calculation of genetic risk scores for reaching the ages of 90 and 95, but the scores are more aptly named genetic longevity scores (GLSs). Unweighted and weighted longevity scores were calculated for reaching the threshold ages of 90 and 95, resulting in four scores. Because longevity is a complex trait, so heavily influenced by lifestyle and environment, it is not surprising that not many studies have been conducted to quantify the chances an individual might have for longevity based on their genetic makeup. But, perhaps the most similar concept to ours is found in a study by Tesi et al., in which a polygenic risk score for predicting the odds of becoming a healthy centenarian was constructed for a population of Dutch origin using 330 genetic variants that significantly discriminated between centenarians and older adults [102]. The calculated polygenic risk score showed a statistically significant association with cognitive healthy aging and prolonged survival of a sample of 343 centenarians in good cognitive health and 2,905 population-matched controls. In the present study of the Croatian sample of the oldest-olds, observation of empirical and theoretical values of calculated longevity scores showed that no participant had a minimum or maximum theoretical value of any score. Nevertheless, participants with the lowest longevity scores fell into the category of those who died before age 90, and their mean GLS values were lower than the mean GLS values of participants who survived past any longevity age threshold. All four GLSs were significantly associated with age at death with a p value of ≤ 0.01 in Pearson's correlation analysis, and all the results presented here support the predictive capabilities

of the calculated GLSs, which were based on only nine or five SNPs selected from a set of as few as 43 longevity variants. Namely, a multivariate linear regression analysis in which all four genetic longevity scores were compared as independent variables for their effect on age at death as a dependent phenotype, highlighted wGLS90 ($p = 0.011$) and uGLS95 ($p = 0.001$) as the most predictive scores for their threshold age for longevity. This finding was also confirmed by ROC curve analysis, with a 69% and 64.5% chance of correctly predicting survival to 90 and 95 years of age, respectively. The predictive power of the calculated genetic longevity scores is unexpectedly high, especially considering that some of the SNPs with a validated association with longevity from other studies are not even included in the models used for calculation of said scores. This might be partially due to the fact that the studied sample is preselected on the basis of a long life, and aged individuals in such sample have already survived the mortality selection caused by chronic diseases that arise during middle and early old ages. Therefore, the SNPs that might have had a strong negative effect earlier in life are not included when sampling a population that has already survived to an advanced old age. It is quite possible that some other loci would have been included in the models if the comparison would contrast the general adult population and the long-lived age groups.

The study of human longevity is a difficult task, as longevity phenotype is dependent on multiple other factors like individual health, genetics, environment, lifestyle differences and even chance. Because of this complexity, the approaches to studying longevity differ greatly—many studies have focused on finding the differences between long-lived individuals and non-long-lived controls [103, 104], some on studying lifespan as a quantitative variable in general populations by using survival models [105, 106], and others by observing the causal effects of specific risk factors on mortality [107]. However, all of these approaches have some shortcomings—either the choice of the right control group for a longevity GWAS, or the limited statistical power for predicting the effect of genetic variants on mortality. In a paper by Timmers et al. [108], a combination of these approaches is used to create the most comprehensible GWAS analysis of human lifespan to date, and even in that case, along with the discovery of some novel SNPs, only some of the previous findings were replicated. This further highlights the importance of undertaking longevity research with various methods, as all of them could contribute to the ever-growing pool of information on human ageing and longevity.

To our knowledge, this study is one of the first attempts to calculate genetic risk scores for longevity. The research sample consists of people who, because of their advanced age, already show the characteristics of healthy ageing. In this study, we wanted to further determine the influence of the genetic background on lifespan in this group, which was pre-selected according to age. Furthermore, we aimed to investigate whether there is a difference in genetic factors that contribute to longevity (90+ years) and those that could play a role in reaching extreme longevity (95+ years). The sex distribution of the studied population leans to the female side, with 74.8% percent of female participants, which is in line with the structure of the general population of Croatia for that age group, where 74.5% of people over 85 are women [109]. The main limitation of this study was the small number of SNPs available for analysis, which was compensated by the selection of genetic variants with a strong previously reported association with longevity and a role in various cellular pathways associated with the ageing process. The number of participants was also relatively small, but there was no pooling of data from several studies of independent populations that could weaken estimates of genetic associations due to the different environmental effects or genetic backgrounds [110], which were quite homogenous in this sample.

To summarise, this study indicates which of the previously reported SNPs also correlates with longevity in the Croatian population, a European population whose genetic data are still underrepresented in the available literature. Regardless of the various factors that may

influence age at death, including stochastic events, a selected set of longevity-associated SNPs explains a noteworthy 20% of the variance for survival to age 90 in a Croatian sample of the oldest-old individuals (85+ years). Of the analysed SNPs, rs16847897 in the *TERC* gene and rs2267723 in the *GHRHR* gene were most significantly associated with longevity in the model for survival to the age of 90 with a $p \leq 0.01$, while the set of genes affecting extreme longevity was quite different and with somewhat weaker associations ($0.01. < p < 0.05$). This study also provides unweighted and weighted genetic risk scores for predicting survival to the threshold ages of longevity (90) and extreme longevity (95 years), and while all four calculated scores were significantly correlated with longevity, wGLS90 had the highest predictive accuracy.

Supporting information

S1 Table. Information about the selected SNPs: Rs code, nearest gene, chromosome position, references for literature sources in which association with longevity was reported; along with data that refers to the studied Croatian population: Alleles (major/minor), minor allele frequencies (MAF), genotyping success rate, Hardy-Weinberg equilibrium (HWE).
(XLSX)

S2 Table. Results of univariate binary logistic regression analysis for cut-off ages at death.
(XLSX)

S3 Table. Means, standard deviations and differences in genetic longevity scores between sexes calculated using Student's t-test.
(XLSX)

S1 Fig. Absolute distribution of the four genetic longevity scores median values (equal proportion of participants having genetic longevity scores above and below the median) by three age-at-death groups (<90.00 years, 90.00–94.99 years, and 95.00+ years). A) uGLS90, B) uGLS95, C) wGLS90, D) wGLS95.
(TIF)

S2 Fig. Relative distribution of age-at-death groups by median genetic longevity score (equal proportion of participants with genetic longevity scores above and below the median). A) wGLS90, B) wGLS95.
(TIF)

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Writing – original draft: Maja Šetinc.

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** **S1 Table** from this paper has been updated and included in the next paper included in this thesis.**

S2 Table. Results of univariate binary logistic regression analysis for cut-off ages at death.

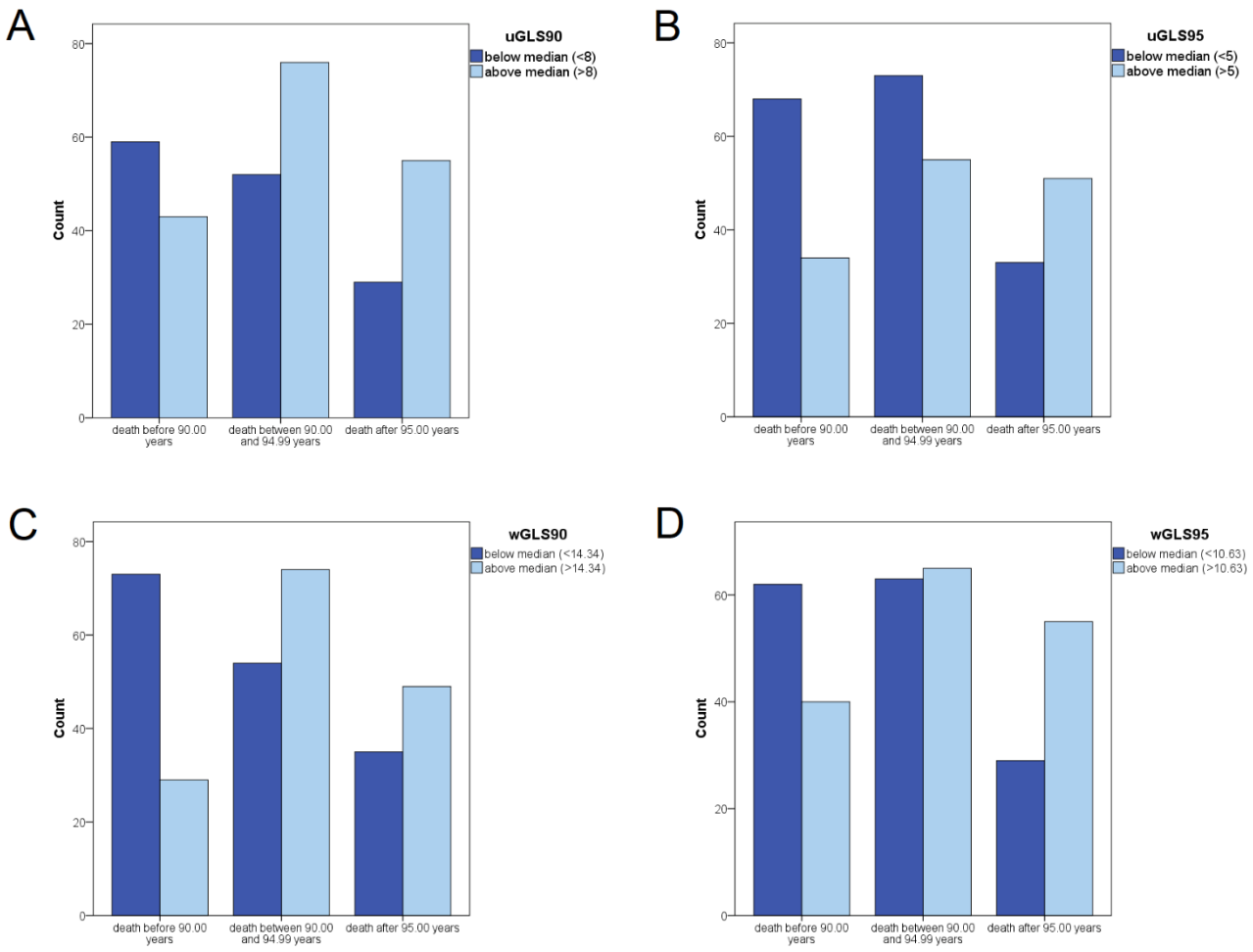
SNP	Closest gene	p-value univariate (90.0+ years)	p-value univariate (95.0+ years)
rs225119	*PARK7*	0.796	0.420
rs2360675	*KLF7*	0.930	0.763
rs12696304	*TERC*	0.199	0.815
rs3772190	*TERC*	0.047	0.980
rs16847897	*TERC*	0.011	0.538
rs572169	*GHSR*	0.587	0.581
rs33954691	*TERT*	0.088	0.773
rs2706372	*RAD50/IL13* region	0.488	0.410
rs2149954	*LINC02227*	0.426	0.714
rs12203592	*IRF4*	0.401	0.058
rs1800629	*TNF-α*	0.036	0.479
rs12206094	*FOXO3A*	0.050	0.668
rs2802292	*FOXO3A*	0.703	0.211
rs2764264	*FOXO3A*	0.427	0.842
rs10457180	*FOXO3A*	0.500	0.650
rs13217795	*FOXO3A*	0.410	0.955
rs4946935	*FOXO3A*	0.054	0.688
rs9456497	*IGF2R*	0.863	0.366
rs10455872	*LPA*	0.318	0.746
rs1800795	*IL6*	0.453	0.840
rs2069837	*IL6*	0.407	0.890
rs2267723	*GHRHR*	0.009	0.759
rs13251813	*WRN*	0.152	0.915
rs4977756	*CDKN2B/ANRIL*	0.946	0.937
rs1333049	*TP53/CDKN2A*	0.575	0.905
rs4837525	*PAPPA*	0.540	0.132
rs533984	*MRE11A*	0.659	0.284
rs17202060	*TXNRD1*	0.052	0.387
rs3184504	*SH2B3/ATXN2*	0.309	0.111
rs1207362	*KLOTHO*	0.783	0.766
rs9536314	*KLOTHO*	0.146	0.339
rs9527025	*KLOTHO*	0.146	0.339

rs2229765	*IGF1R*	0.205	0.531
rs12437963	*IGF1R*	0.552	0.999
rs1042522	*TP53*	0.091	0.126
rs2078486	*TP53*	0.875	0.207
rs107251	*SIRT6*	0.422	0.612
rs2075650	*TOMM40*	0.225	0.176
rs429358	*APOE*	0.246	0.037
rs7412	*APOE*	0.010	0.283
rs4420638	*APOC1*	0.493	0.172
rs50871	*ERCC2*	0.103	0.718
rs6067484	*PTPN1*	0.898	0.107

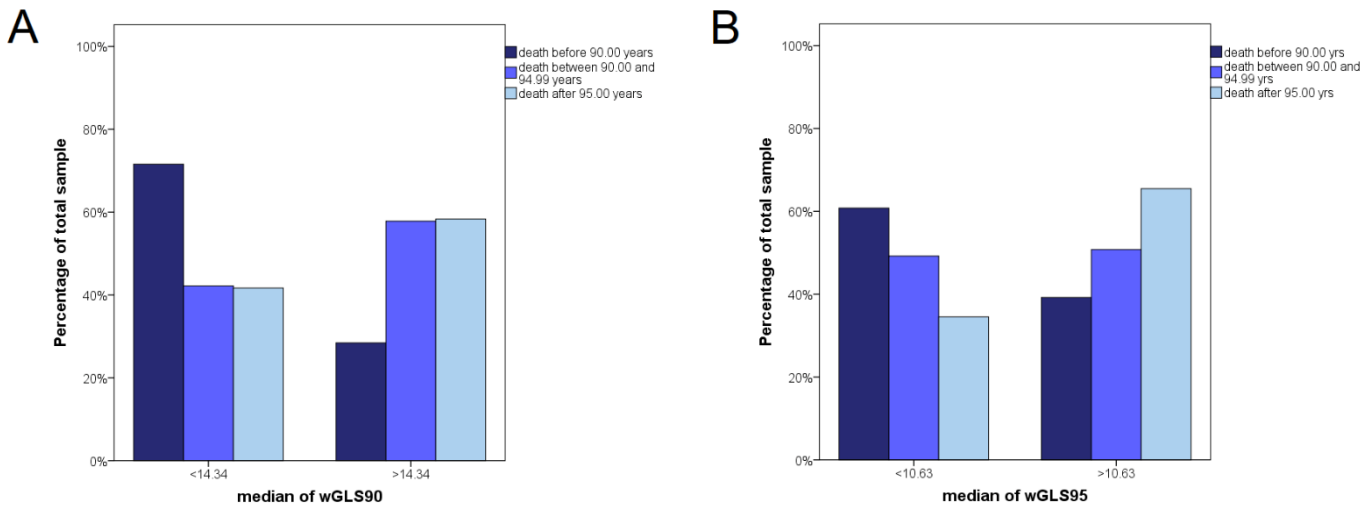
S3 Table. Means, standard deviations and differences in genetic longevity scores between sexes calculated using Student's t-test.

		uGLS90	**uGLS95**	**wGLS90**	**wGLS95**
Total (N=314)	Mean	7.869	4.379	14.337	11.112
	Std. Dev.	2.551	1.510	5.132	3.732
Men (N=80)	Mean	7.588	4.350	13.871	11.011
	Std. Dev.	2.656	1.442	5.241	3.516
Women (N=234)	Mean	7.966	4.389	14.496	11.146
	Std. Dev.	2.513	1.536	5.096	3.809
T-test between sexes	p-value	0.253	0.843	0.348	0.781

S1 Figure. Absolute distribution of the four genetic longevity scores median values (equal proportion of participants having genetic longevity scores above and below the median) by three age-at-death groups (<90.00 years, 90.00–94.99 years, and 95.00+ years). A) uGLS90, B) uGLS95, C) wGLS90, D) wGLS95.



S2 Figure. Relative distribution of age-at-death groups by median genetic longevity score (equal proportion of participants with genetic longevity scores above and below the median). A) wGLS90, B) wGLS95.



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The role of longevity-related genetic variant interactions as predictors of survival after 85 years of age

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ABSTRACT

Genome-wide association studies and candidate gene studies have identified several genetic variants that might play a role in achieving longevity. This study investigates interactions between pairs of those single nucleotide polymorphisms (SNPs) and their effect on survival above the age of 85 in a sample of 327 Croatian individuals. Although none of the SNPs individually showed a significant effect on survival in this sample, 14 of the 359 interactions tested (between SNPs not in LD) reached the level of nominal significance ($p<0.05$), showing a potential effect on late-life survival. Notably, *SH2B3* rs3184504 interacted with different SNPs near *TERC*, *TP53* rs1042522 with different SNPs located near the *CDKN2B* gene, and *CDKN2B* rs1333049 with different SNPs in *FOXO3*, as well as with *LINC02227* rs2149954. The other interaction pairs with a possible effect on survival were *FOXO3* rs2802292 and *ERCC2* rs50871, *IL6* rs1800795 and *GHRHR* rs2267723, *LINC02227* rs2149954 and *PARK7* rs225119, as well as *PARK7* rs225119 and *PTPN1* rs6067484. These interactions remained significant when tested together with a set of health-related variables that also had a significant effect on survival above 85 years. In conclusion, our results confirm the central role of genetic regulation of insulin signalling and cell cycle control in longevity.

1. Introduction

Ageing is a complex process of organismal changes influenced by environmental factors and modulated by a complex system of gene regulation. It is defined by progressive weakening of all the functions of the organism, which ultimately leads to its death (Kirkwood, 2005). Since the world is facing ageing of the global population, with the proportion of elderly expected to almost double by 2050 (World Health Organization, 2023), the importance of research on this topic has never been greater. Most basic ageing mechanisms and candidate genes that affect them were discovered in model organisms (Antebi, 2007), but many retain the same function in humans due to high conservation of those genes among species (Smulders and Deelen, 2023). Research into these mechanisms is essential for a better understanding of what drives the ageing process, as well as for discovering the factors that contribute to successful ageing and longevity. Also, studies of the complex cellular signalling network that regulates the ageing process indicate its plasticity (Campisi et al., 2019) and point to ways in which it can be influenced.

The connection between food intake and lifespan has long been established (Fontana et al., 2010), and with it the involvement of the insulin/insulin-like growth factor signalling pathway (IIS). The key role of the IIS in ageing is exemplified by studies of mice carrying mutations in key genes downstream of insulin receptors (such as IRS1 (Selman et al., 2008)), as well as drugs that modulate insulin sensitivity or boost autophagy (Curtis et al., 2005; Rubinsztein et al., 2011). However, it is likely that many other pathways also play a role. Accumulation of DNA damage and telomere shortening are both time-related processes that

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accompany ageing (Vijg, 2000), and the mechanisms that affect DNA repair and control cell cycle progression are key for maintaining genomic integrity as an organism ages (Lombard et al., 2005). Age is a major risk factor for developing age-related chronic conditions (Niccoli and Partridge, 2012; Dillin et al., 2014; Hou et al., 2019), which is why genes modulating the risk for chronic disease have also been studied as candidate genes for longevity. The most well-known example is the *APOE* gene – associated with the risk for cardiovascular diseases (Eichner et al., 1993; Wilson et al., 1994; Bennet et al., 2007) and Alzheimer's disease (Zuo et al., 2006; Farrer et al., 1997) – which was first identified in candidate gene studies and later confirmed in genome-wide association studies (GWAS) as the most important genetic factor influencing longevity (Smulders and Deelen, 2023).

Longevity is a complex trait, shaped both by the environment and genetic background, as well as by interactions between different genes involved in various signalling pathways (Shadyab and LaCroix, 2015; Brooks-Wilson, 2013). As GWAS focus on identifying the effects of individual SNPs (Lin et al., 2017), the insight gained from these studies is often fragmentary and does not consider the way these genes interact with each other or act in regard to a broader genetic context. When the complexity of the ageing process is considered, it is clear that gene-gene interactions, or epistasis, should also be explored, as complex interactions may be more important than the independent main effects of any one susceptibility gene (Moore, 2003). Analysing statistical interaction between loci can both increase the power to detect effects as well as outline the biological and biochemical pathways that underpin the phenotype (Cordell, 2009). This approach has been successfully used by Dato et al. (2018), who looked at interactions between SNPs belonging to three candidate pathways – IIS, DNA repair and pro/antioxidant pathways – to determine the combined effect of these SNPs on longevity, thus proving the validity of this approach for studying the genetics of ageing (Dato et al., 2018).

In this study, we tested the effect of 43 SNPs, previously reported to have an effect on longevity and associated with genes belonging to different ageing-related pathways, on survival of the oldest-olds, both individually and in SNP-SNP interactions. To this end, we made use of our previously generated dataset on a Croatian sample of individuals aged 85 years and older, which has been used to determine the genetic makeup that contributes to reaching longevity and extreme longevity in the studied sample (Šetinc et al., 2023). Furthermore, the significance of these interactions was tested together with a large set of health status indicators available for the studied population to determine whether the genetic effect was independent of health-related phenotypes.

2. Materials and methods

2.1. Study population

The study sample consisted 327 unrelated oldest-old adults (85 years and older) who were residents of one of the 13 homes for elderly and infirm in Zagreb area (Croatia) in the period between 2007 and 2009 when the field research was carried out. Each subject participated voluntarily, signing an informed consent for participation and an additional consent for providing a peripheral venous blood sample for biochemical, haematological, and genetic analyses. Biochemical and haematological parameters were determined in an accredited laboratory. All subjects were interviewed, a short anthropometry was performed, their blood pressure was measured, and an ultrasound densitometry of the calcaneus (heel bone) was performed using Sahara Bone Densitometer (Hologic, Marlborough, Massachusetts, United States). The comprehensive questionnaire used in the research contained a wide spectrum of questions about functional ability, quality of life, family history of health and longevity, health and health-related behaviours, as well as two internationally standardised questionnaires: Mini Nutritional Assessment (MNA) for assessing nutritional status (Guigoz and Vellas, 1999) and the psychometric test Mini Mental State Examination (MMSE) for assessing the mental state of respondents (Folstein et al., 1975). A detailed description of the sample and study protocol can be found in Perinić Lewis et al. (2022) (Perinić Lewis et al., 2022). Ten years after the initial survey, the date of death for each of the respondents was collected from the national mortality register. Peripheral blood samples of 100 unrelated young people between the ages of 20 and 35 were collected (using the snowball method, with the aim of collecting a sample of individuals with random chances for reaching advanced old age) as a reference group for calculating the relative telomere length of the older adult subjects. The only inclusion criteria for this group were Croatian citizenship (in second generation) and the year of birth, but additional care was taken to make sure that sex distribution and age variance of the control group of young individuals aligned to that of the elderly sample.

The sample collection and the research described here were approved by the Ethics Committee of Institute for Anthropological Research (Zagreb, Croatia) and performed following all institutional guidelines. Ethical approvals obtained on March 4th 2006 (130–981/06) and 22nd November 2018 (20180518).

2.2. DNA isolation and genotyping

DNA was isolated from peripheral blood using the salting-out method (Miller et al., 1988). Forty-three SNPs located in candidate longevity genes were selected by reviewing the relevant literature, with the main criteria for inclusion being a strong or repeated association with human longevity and involvement in various signalling and metabolic pathways that play a role in the ageing process (e.g., cell cycle regulation, DNA repair mechanisms, the IIS). The DNA samples of all subjects were genotyped in a commercial laboratory using Kompetitive Allele Specific Polymerase chain reaction (KASP). Out of the initial 327 subjects from the elderly group, genotyping was unsuccessful for 13 subjects at nine or more loci (over 20% of data was missing) and they were therefore excluded from further analyses, leaving a final sample of 314 participants. All missing data for participants with 1–8 unsuccessfully genotyped SNPs were replaced by the median value for that SNP.

2.3. Measurement of relative telomere length

Relative telomere length (RTL) was measured by quantitative polymerase chain reaction (qPCR) using primers that specifically bind to telomeric repeats (Cawthon, 2002). To calculate the relative telomere length of each subject, two reactions are needed: one in which specific primers multiply telomeric repeats, and another in which a gene that is repeated only once in the human genome is multiplied (in this case, the gene for beta-globin was chosen). We used 200 nM of following primers: *tel1* [5′-CGGTTT(GTTTGG)$_5$GTT-3′] and *tel2* [5′-GGCTTG(CCTTAC)$_5$CCT-3′] for the telomere repeats, and *hbg1* [5′GCTTCTGACACAACTGTGTTCACTAGC-3′] and *hbg2* [5′-CACCAACTTCATCCACGTTCACC-3′] for single-copy gene human beta-globin, as they were listed in the protocol by Lin et al. (2010) (Lin et al., 2010) adapted from Cawthon (2002) (Cawthon, 2002). We used Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix with Low ROX (Agilent Biotechnologies, Santa Clara, California, United States) and added 50 ng of DNA per reaction, which was run on the Agilent AriaMX Real-time PCR System. The thermal cycling profile consisted of: 2 min preheating at 50 °C, 2 min denaturation of the samples at 96 °C, followed by 35 cycles of denaturation at 96 °C lasting 15 s and annealing/extension at 54 °C for 60 s. All samples were run in triplicates. We performed qPCR for both the 85+ sample we wanted to determine the relative telomere length for, and a control group of young people that was used as a reference sample. The relative telomere length was then expressed by fold change which represents the difference between the ratio of multiplied telomeric DNA and reference gene DNA of the target sample compared to the reference sample (Cawthon, 2002), according to the following formula: $2^{-(\Delta Ct(old) - \text{mean } \Delta Ct(young)} = 2^{-\Delta\Delta Ct}$. The fold change

calculated in this manner is proportional to the average length of telomeres in the subjects' leukocytes, and the obtained data was used as a variable in further analyses.

2.4. Statistical analyses

Genotype data (available in open access on the online repository Zenodo) (Setinc et al., 2022) were coded as follows: homozygotes were given a value of 0 or 2, and heterozygotes were assigned a value of 1. The value of 2 was given to the allele that has been associated with increased longevity in previous research (Supplementary Table 1). The participants whose exact date of death was unknown were censored, and the target variable for calculating survival was set as the number of years the participants had lived after the age of 85. First, a Cox regression analysis testing the effect of each SNP on survival above 85 years was performed, with bootstrapping using 1000 samples and correction for gender. In order to avoid false-positive results caused by an extremely small representation of a single genotype, all SNPs with less than 10 cases of homozygous genotypes of either type were excluded from further analyses ($n = 15$). The remaining SNPs were tested for LD using Haploview (Barrett et al., 2005), and all possible SNP-SNP interactions between two SNPs that were not in LD ($r < 0.2$) were tested in survival analysis (359 interactions in total, listed in Supplementary Table 2). The effect of the SNP-SNP interaction on survival was tested using a bootstrapped Cox regression model that included gender, both of the SNPs (to account for their individual effect on the model), and their interaction as the variables. Survival analysis was also performed for RTL, which was tested both univariately and as a part of the health-related dataset. The subset of variables out of this health-related dataset that had a significant effect on survival were tested once again using Cox regression analysis with

Table 1
The results of the Cox regression survival analysis for each of 43 longevity SNPs in the Croatian oldest-old sample.

SNP	Variant type	Associated gene	Gene most likely impacted	Cox regression				included in SNP-SNP analysis
				p-value	Hazard Ratio (HR)	95% CI for HR		
						Lower	Upper	
rs225119	intronic	*PARK7*	*PARK7*	0.626	0.956	0.795	1.142	*
rs2360675	intronic	*KLF7*	*KLF7*	0.740	0.969	0.811	1.151	*
rs12696304	regulatory region variant	*TERC*	*ACTRT3*	0.257	0.897	0.731	1.082	*
rs3772190	intronic	*TERC*	*ACTRT3*	0.095	1.168	0.974	1.408	*
rs16847897	intronic	*TERC*	*ACTRT3*	0.078	0.849	0.705	1.011	*
rs572169	synonymous	*GHSR*	*GHSR*	0.746	1.030	0.845	1.228	*
rs33954691	synonymous	*TERT*	*TERT*	0.928	1.013	0.809	1.297	
rs2706372	intronic	*RAD50/IL13*	*IL13*	0.985	1.002	0.831	1.234	*
rs2149954	intronic	*LINC02227*	*(no data)*	0.464	0.942	0.801	1.114	*
rs12203592	intronic	*IRF4*	*IRF4*	0.059	0.763	0.568	1.016	
rs1800629	regulatory region variant	*TNF*	*HLA-C*	0.622	0.930	0.689	1.212	
rs12206094	intronic	*FOXO3*	*FOXO3*	0.804	1.021	0.870	1.221	*
rs2802292	intronic	*FOXO3*	*FOXO3*	0.487	0.938	0.791	1.122	*
rs2764264	intronic	*FOXO3*	*FOXO3*	0.708	0.969	0.824	1.156	*
rs10457180	intronic	*FOXO3*	*FOXO3*	0.751	0.973	0.815	1.158	*
rs13217795	intronic	*FOXO3*	*FOXO3*	0.603	0.955	0.810	1.133	*
rs4946935	intronic	*FOXO3*	*FOXO3*	0.729	1.031	0.879	1.232	*
rs9456497	intronic	*IGF2R*	*IGF2R*	0.749	0.968	0.773	1.189	*
rs10455872	intronic	*LPA*	*SLC22A3*	0.711	0.919	0.596	1.445	
rs1800795	intronic	*IL6*	*STEAP1B*	0.714	0.971	0.823	1.149	*
rs2069837	non-coding exon variant	*IL6*	*IL6*	0.427	1.128	0.841	1.501	
rs2267723	intronic	*GHRHR*	*GHRHR*	0.681	0.968	0.817	1.133	*
rs13251813	intronic	*WRN*	*WRN*	0.587	1.120	0.741	1.747	
rs4977756	intronic	*CDKN2B*	*CDKN2B*	0.835	1.018	0.851	1.215	*
rs1333049	intronic	*CDKN2B*	*CDKN2B*	0.642	1.037	0.891	1.221	*
rs4837525	intronic	*PAPPA*	*PAPPA*	0.162	0.883	0.751	1.049	*
rs533984	intronic	*MRE11A*	*MRE11A*	0.491	1.064	0.892	1.273	*
rs17202060	intronic	*TXNRD1*	*TXNRD1*	0.407	1.076	0.900	1.309	*
rs3184504	missense	*SH2B3*	*SH2B3*	0.357	0.921	0.780	1.102	*
rs1207362	intronic	*KLOTHO*	*KLOTHO*	0.269	0.911	0.767	1.066	*
rs9536314	missense	*KLOTHO*	*KLOTHO*	0.136	1.200	0.924	1.554	
rs9527025	missense	*KLOTHO*	*KLOTHO*	0.171	0.833	0.649	1.106	
rs2229765	missense	*IGF1R*	*IGF1R*	0.142	1.114	0.955	1.275	*
rs12437963	intronic	*IGF1R*	*IGF1R*	0.791	0.970	0.769	1.213	
rs1042522	missense	*TP53*	*TP53*	0.133	0.870	0.714	1.048	*
rs2078486	intronic	*TP53*	*EFNB3*	0.550	0.899	0.644	1.290	
rs107251	intronic	*SIRT6*	*SIRT6*	0.984	1.004	0.718	1.320	
rs2075650	intronic	*TOMM40*	*TOMM40*	0.709	0.949	0.705	1.225	
rs429358	missense	*APOE*	*APOE*	0.106	0.784	0.556	1.045	
rs7412	missense	*APOE*	*APOE*	0.345	0.879	0.676	1.153	
rs4420638	regulatory region variant	*APOC1*	*APOE*	0.262	0.871	0.668	1.129	
rs50871	intronic	*ERCC2*	*KLC3*	0.654	0.967	0.824	1.127	*
rs6067484	intronic	*PTPN1*	*PTPN1*	0.241	0.894	0.737	1.099	*

In order, the columns show rsID of tested SNPs, variant type, gene (both the gene that has been associated with the SNP in other publications and the gene reported to most likely be affected by the SNP by eQTL or Variant2Gene pipeline in the online database Open Targets Genetics), bootstrap-adjusted p-values, hazard ratios (HR) and adjusted 95% confidence intervals (CI) for HR obtained in a Cox regression analysis of single SNP and gender with survival time after 85 years of age as the time-to-event variable. SNPs that pass the criterion of having over 10 cases of any genotype represented in our sample and have been included in further analyses are marked with an asterisk.

bootstrapping, and the ones that reached statistical significance were added to the regression models with significant SNP-SNP interactions. All statistical analyses were performed using SPSS software package 21.0.

3. Results

3.1. Single SNP and interaction analyses

We first tested each of the 43 genotyped SNPs to determine their individual effect on survival after 85 years of age. However, none of the SNPs showed a significant effect on survival in our sample (Table 1). Relative telomere length, when tested univariately, was also not a significant predictor of survival.

As a next step, we studied the interactions between the SNPs. Out of the 359 tested models (Supplementary Table 2), 14 different SNP combinations showed a bootstrap-adjusted nominally significant interaction effect on survival after 85 years (Table 2). Out of 14 interactions, nine are combinations between three gene pairs – *TERC* and *SH2B3*, *TP53* and *CDKN2B*, and *CDKN2B* and *FOXO3*. Missense variant rs3184504 in *SH2B3* had an effect on late-life survival in interactions with three intronic SNPs located near *TERC*: rs16847897 (p=0.002), rs12696304 (p=0.014) and rs3772190 (p=0.032). A missense mutation in *TP53*, rs1042522, made significant interaction pairs with two intronic SNPs located near *CDKN2B*, rs4977756 (p=0.003) and rs1333049 (p=0.025). The variant rs1333049 near *CDKN2B* also impacted survival above 85 years in separate interactions with intronic *FOXO3* SNPs rs4946935 (p=0.009), rs12206094 (p=0.021), rs13217795 (p=0.043) and rs2764264 (p=0.049). Multiple interactions between a single SNP and variants located in close genomic proximity corroborate the finding that these genes in tandem could affect survival, even though their repeated pairing could also be due to the high LD between the *TERC*, *CDKN2B* and *FOXO3* variants, respectively. Other interaction pairs that affected survival above 85 years were *FOXO3* rs2802292 and *ERCC2* rs50871 (p=0.013), *CDKN2B* rs1333049 and *LINC02227* rs2149954 (p=0.038), *IL6* rs1800795 and *GHRHR* rs2267723 (p=0.038), *LINC02227* rs2149954 and *PARK7* rs225119 (p=0.044), and *PARK7* rs225119 and *PTPN1* rs6067484 (p=0.045). We applied a false discovery rate (FDR) correction to the interaction p-values, but none of the SNP-SNP interactions passed this threshold ($p<1.39 \times 10^{-4}$). Fig. 1 depicts the Kaplan-Meier curve of survival after the age of 85 for the interaction between *TERC* rs16847897 and *SH2B3* rs3184504 (p=0.002, our strongest finding), which shows how different genotype combinations impact the late-life survival. It is visible that the respondents who are carriers of homozygous genotypes associated with longevity for both SNPs in interaction have better survival than carriers of the other genotype combinations.

Table 2
The results of Cox regression analysis of SNP-SNP interactions as predictors of survival above 85 years in a Croatian sample.

First SNP	Second SNP	Interaction p-value	Hazard Ratio (HR)	95% CI for HR	
				Lower	Upper
TERC rs16847897	***SH2B3*** rs3184504	**0.002**	0.665	0.512	0.860
CDKN2B rs4977756	***TP53*** rs1042522	**0.003**	1.512	1.135	2.119
FOXO3 rs4946935	***CDKN2B*** rs1333049	**0.009**	1.306	1.066	1.654
FOXO3 rs2802292	***ERCC2*** rs50871	**0.013**	0.750	0.584	0.940
TERC rs12696304	***SH2B3*** rs3184504	**0.014**	0.708	0.539	0.946
FOXO3 rs12206094	***CDKN2B*** rs1333049	**0.021**	1.292	1.042	1.642
CDKN2B rs1333049	***TP53*** rs1042522	**0.025**	1.336	1.030	1.738
TERC rs3772190	***SH2B3*** rs3184504	**0.032**	1.403	1.007	1.927
LINC02227 rs2149954	***CDKN2B*** rs1333049	**0.038**	0.785	0.619	0.983
IL6 rs1800795	***GHRHR*** rs2267723	**0.038**	1.246	1.000	1.537
FOXO3 rs13217795	***CDKN2B*** rs1333049	**0.043**	1.279	1.021	1.649
PARK7 rs225119	***LINC02227*** rs2149954	**0.044**	0.776	0.605	1.002
PARK7 rs225119	***PTPN1*** rs6067484	**0.045**	1.366	0.986	1.883
FOXO3 rs2764264	***CDKN2B*** rs1333049	**0.049**	1.266	1.005	1.624

Each model included gender, two SNPs and their interaction as predictor variables. Presented in the table are the bootstrap-adjusted p-values from regression models of all significant SNP-SNP interactions, as well as interaction HR and adjusted 95% CI for HR. Significant p-values are marked in bold.

3.2. Health-related measures contributing to survival

Given the large amount of health data collected from the participants, we created a comprehensive set of 33 variables covering a large spectrum of health-related parameters and then used Cox regression to determine which of those factors also contribute to the survival of our oldest-old sample (Table 3). In this model, nine out of the 33 health-related variables tested simultaneously remained significantly associated with survival in advanced old age. Higher odds of surviving past 85 were found for participants who had a family history of longevity, with either a mother or a sibling living beyond 80 years of age. Moreover, participants who were categorised as well-nourished and fell among the first three quartiles of the weight distribution had higher chances of survival as well. In addition, higher chances of survival were found among those who reported taking less than four medicaments daily and

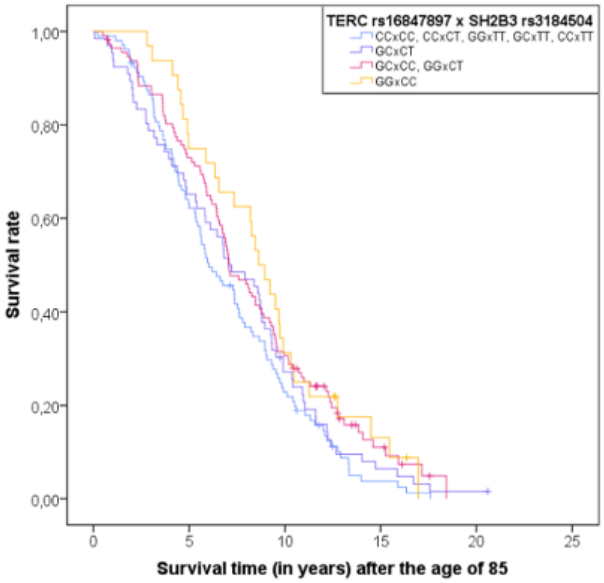

Fig. 1. Kaplan-Meier curve of survival after the age of 85 for the interaction between *TERC* rs16847897 and *SH2B3* rs3184504 (p=0.002). Nine possible genotype combinations are grouped in four categories (4, 2, 1, 0) based on the product value of the genotype scores. The genotype combination with a value of four (marked in orange) has two longevity-associated effect alleles on each locus; the combinations with a value of two (magenta) have two longevity-associated effect alleles on one locus and one on the other; heterozygous genotype combination with the value of one (indigo) have one longevity-associated effect allele on each locus, and the genotypes with a value of zero (blue) have no longevity-associated effect alleles on at least one of the two loci.

Table 3
Cox regression analysis of gender, 33 health-related variables and relative telomere length (RTL) as predictors of survival above 85 years of age, performed with bootstrapping using 1000 samples.

Predictor variables (referent values)	According to beta value, longer survival with following characteristics	p-value	Hazard Ratio (HR)	95% CI for HR	
				Lower	Upper
Gender (men)	Women	0.546	0.869	0.489	1.390
Body weight by sex-specific 4th quartile (men = 87.3+ kg; women = 72.6+ kg)	Body weight: men = <87.3 kg; women = <72.6 kg	**0.014**	0.576	0.340	0.949
Waist circumference by median (men = 100.0+ cm; women = 92.0+)	Waist circumference: men = 100.0+ cm; women = 92.0+	0.110	1.352	0.902	2.036
Upper arm circumference by median (men = 27.6+ cm; women = 27.3+ cm)	Upper arm circumference: men = < 27.6 cm; women = <27.3 cm	0.167	0.741	0.441	1.102
Left heel bone mineral density (T-values: > −1.0 OR < −2.4)	Left heel bone mineral density T-values: (−1.0) – (−2.4)	**0.010**	0.631	0.407	0.826
Fasting glucose: 1st, 4th vs 2nd, 3rd quartile (<4.20 mmol/L OR >6.40 mmol/L)	Fasting glucose is within normal range: 4.20 – 6.40 mmol/L	0.471	0.890	0.620	1.228
Total serum cholesterol (<5.0 mmol/L)	Total serum cholesterol: 5.0+ mmol/L	0.354	0.845	0.558	1.240
Bilirubin in serum by sex-specific median (men = <11.0 μmol/L; women = <9.0 μmol/L)	Bilirubin in serum: men = 11.0+ μmol/L; women = 9.0+ μmol/L	0.103	0.758	0.519	1.054
Albumin in serum: 1st, 4th vs 2nd, 3rd quartile (<40 g/L OR >48 g/L)	Albumin in serum: <40 g/L OR >48 g/L	0.464	1.144	0.770	1.603
Iron in serum by sex-specific median (men = <14 μmol/L; women = <12 μmol/L)	Iron in serum: men = 14+ μmol/L; women = 12+ μmol/L	0.225	0.800	0.547	1.160
Unsaturated Iron Binding Capacity: 1st, 4th vs 2nd, 3rd quartile (<26 μmol/L OR >59 μmol/L)	UIBC is within normal range: 26–59 μmol/L	0.626	0.844	0.344	1.726
Folates in serum by median (<=18.1 nmol/L)	Folates in serum: >18.1 nmol/L	**0.017**	0.502	0.245	0.816
Erythrocytes: 1st, 4th vs 2nd, 3rd quartile (< 9.0 *10e12/L OR >15.0 *10e12/L)	Erythrocytes: < 9.0 *10e12/L OR >15.0 *10e12/L	0.549	1.152	0.703	2.028
Basophils by median (0.02+ %)	Basophils: 0.02+ %	0.621	1.102	0.748	1.709
Self-rated health (poor, satisfactory, good)	Self-rated health: very good, excellent	0.556	0.892	0.558	1.349
Self-rated health compared to age-peers (worse or equal)	Self-rated health is better compared to age-peers	0.084	0.727	0.485	1.063
Functional ability (self-rated mobility and independence are both less than excellent)	Self-rated mobility and/or independence are excellent	0.440	0.861	0.556	1.324
Mini Mental State Examination score by median (< 23)	Mini Mental State Examination score: 23+	0.208	0.792	0.498	1.099
Self-rated nutritional status (mildly or severely malnourished)	Self-rated nutritional status: well nourished	**0.028**	0.599	0.334	0.881
Mild or heavy depression (Yes)	Not suffering from depression	0.080	0.745	0.512	1.045
Number of medicaments taken daily (5+)	Number of medicaments taken daily: 0–4	**0.044**	0.685	0.430	0.946
Number of hospital admissions in the past year (2 or more)	One or no hospital admissions in the past year	**0.020**	0.612	0.372	0.900
Experiencing an acute illness in past 3 months (No)	Experiencing an acute illness in past 3 months	0.074	0.701	0.437	1.047
Family history of hypertension (No)	No family history of hypertension	0.948	1.014	0.627	1.662
Family history of diabetes (No)	Have a family history of diabetes	0.277	0.760	0.442	1.311
Smoking status (smoker or ex-smoker)	Smoker or ex-smoker	0.656	1.086	0.724	1.613
For the question: "Do you think that smoking is your health-risk behavior?" (Answer: "Yes")	Does not think that smoking is his/her health risk behavior	0.097	0.468	0.137	1.189
Using denture (No)	Does not use denture	0.348	1.220	0.780	1.976
Number of vitamin supplements daily taken (men: 0–2; women: 0–1)	Number of vitamin supplements daily taken: men: 0–2; women: 0–1	0.570	1.110	0.770	1.639
Regularly taking supplementary vitamin B complex (No)	Regularly taking supplementary vitamin B complex	**0.017**	0.533	0.292	0.966

(continued on next page)

Table 3 (*continued*)

Predictor variables (referent values)	According to beta value, longer survival with following characteristics	p-value	Hazard Ratio (HR)	95% CI for HR	
				Lower	Upper
Regularly taking supplementary magnesium (No)	Regularly taking supplementary magnesium	0.731	0.911	0.471	1.618
Drinking beer (rare or never)	Drinking beer at least once a week	0.426	0.772	0.356	1.380
Age of the oldest living sibling (<80)	Age of the oldest living sibling is 80+ years	**0.035**	0.560	0.289	0.897
Mother's age at death (<80)	Mother's age at death is 80+ years	**0.049**	0.738	0.506	0.983
RTL	RTL	0.491	1.052	0.910	1.250

Except RTL, all predictor variables are binary (0, 1) and the referent value "0" is described in parentheses. Significant p-values ($p<0.05$) are marked in bold.

taking B-complex supplements. Folate levels above 18,1 nmol/L and one or less hospital stays in the year prior to taking the survey were also significantly associated with higher chances of survival. Interestingly, higher chances of survival in advanced old age were found for participants who had osteopenia (lowered bone density), but not osteoporosis. When these nine variables were tested in a separate model (Supplementary Table 3), all of them remained significant. RTL, which was also tested jointly with the health-related variables, did not have a significant effect on survival chances of the studied sample.

3.3. Joint analyses of SNP interactions and health indicators

To determine whether the genetic interactions and the tested health status indicators have an independent effect on survival, significant SNP-SNP interaction models were tested along with the nine significant health-related variables (Supplementary Table 4). Out of 14 interaction models that were significant in the previous step, ten remain significant even in a bootstrapped model containing health variables, and they are presented in Table 4 in a decreasing order of interaction p-values. Five of the interactions that remained significant after the inclusion of health indicators in the analysis included one of the *CDKN2B* SNPs: the interactions of *TP53* rs1042522 with both *CDKN2B* rs4977756 and rs1333049 ($p=0.002$ and $p=0.013$, respectively), the interactions of two *FOXO3* SNPs, rs4946935 and rs12206094, with *CDKN2B* rs1333049 ($p=0.035$ and $p=0.044$, respectively); as well as the interaction between *LINC02227* rs2149954 and *CDKN2B* rs1333049 ($p=0.015$). The interactions between the two SNPs near *TERC*, rs16847897 and rs12696304, and *SH2B3* rs3184504 ($p=0.007$ and $p=0.049$, respectively) also remained significant after the addition of health-related parameters, along with the interactions between *IL6* rs1800795 and *GHRH*R rs2267723 ($p=0.005$), *LINC02227* rs2149954 and *PARK7* rs225119 ($p=0.016$), and *FOXO3A* rs2802292 and *ERCC2* rs50871 ($p=0.047$). While most health-related variables remain significant in models that include genetic interactions, the variable reporting the number of medicaments taken loses significance in both *TERC* – *SH2B3* interaction models, and the maternal age at death is no longer a significant predictor of survival in both interaction models of *CDKN2B* and *FOXO3,* indicating that these genetic interactions likely impact survival through a phenotype that is covered by these health parameters. The number of hospital admissions also loses significance in both *CDKN2B* – *FOXO3* models, the *PARK7* – *LINC02227* model, and one of the *CDKN2B* – *TP53* models, while being only marginally significant in the other.

4. Discussion

In this study, we tested the effect of SNPs associated with longevity in

Table 4
The results of Cox regression analyses of the models combining significant genetic interactions and nine selected health-related variables.

First gene	*CDKN2B* rs4977756	*IL6* rs1800795	*TERC* rs16847897	*CDKN2B* rs1333049	*LINC02227* rs2149954	*PARK7* rs225119	FOXO3 rs4946935	*FOXO3* rs12206094	*FOXO3* rs2802292	*TERC* rs12696304
Second gene	*TP53* rs1042522	*GHRHR* rs2267723	*SH2B3* rs3184504	*TP53* rs1042522	*CDKN2B* rs1333049	*LINC02227* rs2149954	CDKN2B rs1333049	*CDKN2B* rs1333049	*ERCC2* rs50871	*SH2B3* rs3184504
Interaction p-value	0.002	0.005	0.007	0.013	0.015	0.016	0.035	0.044	0.047	0.049
Gender	0.311	0.146	0.078	0.182	0.292	0.082	0.248	0.238	0.183	0.134
Body weight by sex-specific 4th quartile	0.001	0.002	0.001	0.002	0.003	0.001	0.001	0.001	0.001	0.001
Left heel bone mineral density	0.004	0.008	0.007	0.002	0.001	0.005	0.003	0.006	0.002	0.004
Folates in serum by median	0.023	0.001	0.019	0.009	0.011	0.018	0.014	0.023	0.020	0.014
Self-rated nutritional status	0.003	0.008	0.025	0.003	0.007	0.018	0.023	0.019	0.007	0.014
Number of medicaments taken daily	0.030	0.019	0.114	0.039	0.013	0.040	0.017	0.017	0.034	0.074
Number of hospital admissions in the past year	0.069	0.014	0.037	0.050	0.040	0.074	0.095	0.102	0.039	0.037
Regularly taking supplementary vitamin B complex	0.001	0.001	0.001	0.001	0.001	0.003	0.002	0.002	0.006	0.001
Age of the oldest living sibling	0.005	0.003	0.003	0.005	0.005	0.001	0.003	0.001	0.004	0.003
Mother's age at death	0.029	0.017	0.018	0.036	0.049	0.029	0.056	0.063	0.031	0.034

The table shows bootstrap-adjusted p-values of gender, the SNP-SNP interactions, and nine health-related variables that made up the models that were tested by Cox regression analysis. The values of health-related variables are described in Table 3. Significant p-values ($p<0.05$) are marked in bold.

other studies on survival above 85 years of age in a sample of Croatian oldest-old individuals, both individually and in two-SNP interactions. Individually, none of the SNPs had a significant effect on survival in advanced old age. Considering the relatively small sample size, the effect of the individual SNPs was possibly too weak to be detected at this level. However, the predictive power of SNPs can be improved by combining multiple SNPs in a single model (Van Den Broeck et al., 2014), or by testing the interactions between them, as SNP-SNP interactions may be more informative about the target phenotype than a single SNP alone (Gerke et al., 2009). The use of this approach for genetic studies of human longevity was validated in a study by (Dato et al. (2018); Dato et al., 2018), who investigated SNP-SNP interactions impacting longevity in a sample of Danish origin, while focusing on SNPs from three candidate pathways connected to longevity – the IIS, DNA repair, and pro/antioxidant pathways. Their approach was different from the one presented in this paper, as they studied a larger SNP dataset on a much larger sample and used the tagging approach to prioritize SNPs inside the candidate genes. They also applied a multi-dimensional reduction analysis, which we did not do here. We investigated interactions between all the SNPs we had available for our sample. SNP-SNP interactions were not examined for pairs of SNPs in LD, as linkage between loci might also falsely indicate a higher value of interaction (Su et al., 2015). As we also excluded from the interaction analyses all SNPs that had a very low frequency of one of the genotypes (less than 10 carriers of a homozygous genotype) to avoid false-positive results, the final set for interaction analyses comprised 28 SNPs. Of the 359 different models we tested, 10 SNP-SNP interaction pairs were nominally significant predictors of survival beyond the age of 85 years.

4.1. *CDKN2B – the link between pathways with implications for longevity*

Half of the two-SNP interactions that had an impact on survival above 85 years of age had an SNP associated with the *CDKN2B* gene as one of the members of the interacting pair, indicating a key role this gene has in longevity and late-life survival. Intronic variants rs4977756 and rs1333049, previously associated with longevity phenotypes (Pinós et al., 2014; Fortney et al., 2015; Pilling et al., 2016), are located in the chromosomic 9p21.3 region between the genes *CDKN2A* and *CDKN2B,* and are predicted in the online database Open Targets Genetics to most likely impact the expression of these genes, with the strongest evidence existing for *CDKN2B* (Ghoussaini et al., 2021). *CDKN2B* is a tumour suppressor gene that has been strongly associated with risk for coronary heart disease (Helgadottir et al., 2007; Burton et al., 2007; McPherson et al., 2007). It encodes protein $p15^{INK4B}$, an inhibitor of cyclin-dependent kinases 4 and 6 that stops cell cycle progression in response to regulatory signals (Park and Lee, 2003), and has an important role in cell cycle regulation and senescence (McPherson et al., 2007). The expression of $p15^{INK4B}$ is strongly induced by transforming growth factor-β (TGF-β) (Hannon and Beach, 1994), causing G1-phase cell cycle arrest. The genomic region around *CDKN2B* that spans across the two SNPs from this study also encodes a long non-coding RNA, *ANRIL*, that acts in *cis* via epigenetic mechanisms to silence the $p15^{INK4B}$ expression and increase proliferation while slowing down the entry of cells into senescence (Kotake et al., 2011; Yap et al., 2010; Pasmant et al., 2011). Next to *CDKN2B* is the gene *CDKN2A* which encodes protein variants $p16^{INK4A}$ and $p14^{ARF}$ in two different reading frames (Pasmant et al., 2011). While $p16^{INK4A}$ works similarly to $p15^{INK4B}$ as a cell cycle inhibitor, $p14^{ARF}$, on the other hand, acts by activating the p53 tumour suppressor pathway (Gil and Peters, 2006) by inhibiting protein MDM2, the key effector for degradation of p53 (Lohrum et al., 2000).

4.1.1. *Interactions within the cell cycle control network*

In the current study, both SNPs associated with the *CDKN2B* gene interacted significantly with *TP53* rs1042522 to affect survival in the population above 85 years. This variant is a missense mutation causing substitution of arginine (Arg) with proline (Pro) at codon 72 of p53, a key tumour suppressor that blocks cell cycle progression (Lane, 1992; Lavin and Gueven, 2006) and promotes apoptosis in conditions of cellular stress (Shadyab et al., 2017). Under normal conditions, it is present in cells at low levels, but rapidly undergoes stabilising post-translational modifications and activation in response to stimuli (Lavin and Gueven, 2006; Caspari, 2000). The effect of the Arg72Pro substitution is functional, with the proline variant having a reduced apoptotic response compared to the arginine (Marin et al., 2000; Dumont et al., 2003). This variant has also been reported to impact longevity and survival in the oldest-old age group (Van Heemst et al., 2005; Groß et al., 2014). As the potential effect on the expression of *CDKN2A*, and therefore $p14^{ARF}$, has been reported for at least one of the SNPs in the *CDKN2A/B* region, the link between them and the *TP53* rs1042522 that we see in our study could be the via the $p14^{ARF}$/MDM2/p53 axis, and the stabilizing effect $p14^{ARF}$ has on p53. Furthermore, a study by Leeper et al. (2013) found that *CDKN2B* knockdown in human arterial smooth muscle cells resulted in increased expression of p53. They also performed protein microarray analysis of factors related to the p53 signalling and apoptotic pathways, and found that MDM2 protein, ahead of p53 itself, was among the top targets of proteins that are regulated (Leeper et al., 2013). This shows that *CDKN2B* may regulate p53 activity by mediating its degradation via MDM2.

4.1.2. *Interplay between cell cycle control and insulin signalling*

The complex cellular network of insulin signalling and its downstream effects represents probably the best-studied system with implications for longevity. The *FOXO* genes are a group of transcription factors that act downstream of insulin and insulin-like growth factor receptors (Martins et al., 2016). As the most important transcriptional effectors of the IIS, FOXOs are activated by metabolic stress and lack of nutrients (Dong et al., 2008; Eijkelenboom and Burgering, 2013). Insulin or IGF-1 trigger a phosphatidylinositol 3-kinase/protein kinase B (PI3K-AKT) cascade, causing the serine/threonine kinase AKT to phosphorylate FOXO, which is followed by exclusion of FOXO from the nucleus and silencing of the genes targeted by FOXO (Biggs et al., 1999; Brunet et al., 1999; Webb and Brunet, 2014). The genes downstream of FOXO are involved in cellular quality control, proteostasis and autophagy (Mammucari et al., 2007; Kikis et al., 2010). *FOXO3* is a gene whose implication in longevity is well established, and the association of SNPs near *FOXO3* with longevity has been confirmed in diverse populations (Willcox et al., 2008; Anselmi et al., 2009; Flachsbart et al., 2009; Soerensen et al., 2010; Bao et al., 2014; Broer et al., 2015; Zeng et al., 2010). Four variants, whose association with the *FOXO3* gene are also implicated in the Open Targets Genetics database (Ghoussaini et al., 2021), had a significant interaction with *CDKN2B* rs1333049 that contributed to survival above 85 years in our oldest-old sample. This is perhaps not surprising, as *FOXO3* is upstream of the *CDKN2B* gene, acting as a regulator of *CDKN2B* expression (Hornsveld et al., 2018). Additionally, one study showed that FOXOs might be key interacting partners for SMAD transcription factors through which TGF-β pathway activates the *CDKN2B* gene expression (Gomis et al., 2006), which is what might explain the joint effect they have on longevity.

4.1.3. *Genetic risk factors for cardiovascular diseases*

Another significant interaction partner of *CDKN2B* rs1333049 was variant rs2149954, located in the 5q33.3 genomic region, and close to the long intergenic non-coding RNA 2227 (*LINC02227*). This variant was first mentioned in a paper by Deelen et al. (2014) reporting results of GWAS on longevity as a novel locus associated with survival beyond 90 years of age (Deelen et al., 2014). Prior to this, variants in LD with this SNP have been associated with blood pressure and hypertension (Ehret et al., 2011; Wain et al., 2011). Zeng et al. (2016) confirmed the association with longevity in their GWAS on Han Chinese population (Zeng et al., 2016), which was replicated in another study (Liu et al., 2021). There is no data on the functional impact of this variant in the

online databases, but the minor allele of rs2149954 was found to be protective against heart attack and heart failure, and was related with increased physical functioning in the long-lived individuals (Nygaard et al., 2017). Shadyab et al. (2017) found that seven SNPs in LD with rs2149954 impacted the chances of survival to age 85, which was explained by an increased risk of coronary heart disease connected to the one of the alleles (Shadyab et al., 2017). As a connection between genetic variants and CVD risk has been reported for both 5q33.3 region of rs2149954 and 9p21.3 region of *CDKN2B* rs1333049, the significant interaction of these SNPs for survival beyond the age of 85 might have something to do with modulating this risk.

LINC02227 rs2149954 was also significant in interaction with rs225119, an intronic variant associated to the *PARK1* gene. *PARK7* encodes Parkinsonism associated deglycase, also known as DJ-1, an evolutionary conserved enzyme with a cysteine residue that serves as a catalytic nucleophile (Wilson et al., 2003) and a domain that shares a significant homology with a bacterial heat-shock protein (Wei et al., 2007). The cysteine residue is easily oxidised and has been reported to mitigate oxidative stress by serving as a scavenger for reactive oxygen species (ROS) (Clements et al., 2006; Chen et al., 2010; Billia et al., 2013; Shi et al., 2015). DJ-1 has been shown to affect cell survival to some degree by modulating PTEN/PI3K/Akt signalling cascade (Kim et al., 2005) and by altering p53 activity (Shinbo et al., 2005). Dato et al. (2018) have found the interaction of *PARK7* rs225119 with *MRE11A* rs533984 and *GHSR* rs572169 to be associated with longevity (Dato et al., 2018). The connection between *PARK1* and *LINC02227* is not very clear, but perhaps the antioxidative effect of *PARK7* works synergistically with the CVD-protective effect of *LINC02227* rs2149954 to influence survival chances.

4.2. Connection between CVD genetic risk factors and SNPs influencing telomere length

Intronic variants rs16847897, rs12696304 and rs3772190 are located on chromosome 3 near the *TERC* gene. Encoding the RNA component of the ribonucleoprotein telomerase, an enzyme that serves as a template and elongates telomeric DNA (Blackburn and Collins, 2011; Zhang et al., 2012), the *TERC* gene is an important component for telomere maintenance. It is an enzyme that is not expressed in most human cells (Blackburn et al., 2015), but is expressed in stem cells (Wright et al., 1996; Collins and Mitchell, 2002) and often in cancer cells (Hahn et al., 1999). All three of the SNPs have been associated with leukocyte telomere length (Codd et al., 2010; Soerensen et al., 2012; Shen et al., 2011), a phenotype that has been proposed as a marker of biological age (Sanders and Newman, 2013; Lohman et al., 2021) and associated with age-related diseases (Panossian et al., 2003; Aviv, 2012; Rossiello et al., 2022; Jeanclos et al., 1998). Functional analysis, however, links all three of these SNPs to changes in expression levels of another gene, *ACTRT3* (Ghoussaini et al., 2021), whose function has yet to be characterized. The missing link between these SNPs and *TERC* in databases reporting the results of functional analyses could be due to the fact that the product of the *TERC* gene is of RNA nature, and isn't covered in analyses of protein expression. rs16847897, rs12696304 and rs3772190 all interacted with missense rs3184504 in the *SH2B3* gene in a way that significantly affected survival above 85 years of age, with the most significant interaction being between rs16847897 and rs3184504. The *SH2B3* gene encodes SH2B adaptor protein 3 (also known as LNK, lymphocyte adaptor protein), a protein whose main role is negative regulation of inflammatory cytokine signalling and haematopoiesis (Tong et al., 2005; Devallire and Charreau, 2011). rs3184504 is a common missense variant resulting in substitution of tryptophan (Trp) with arginine (Arg) at amino-acid 262, and is predicted to have the strongest impact on the SH2B3 (LNK) itself, disrupting its subcellular localisation and functioning (Dale and Madhur, 2016). This variant has been associated with exceptional human longevity and parental age (Fortney et al., 2015; Pilling et al., 2016). It is also is a top association signal for hypertension in GWAS (Ehret et al., 2011; Levy et al., 2009), and has been linked to cardiovascular and autoimmune disorders (Devallire and Charreau, 2011; Laroumanie et al., 2018). As telomere length and *SH2B3* both impact the chances for developing cardiovascular disease (CVD), the connection between the *TERC* and *SH2B3* genes could lie in disease pathophysiology. Since the incidence of cardiovascular pathologies increases with age (Lye and Donnellan, 2000), with an estimated prevalence of CVD among people over the age of 80 being 82% (Yazdanyar and Newman, 2009), it would make sense for the interactions of these two genes to have an impact on survival in this age group via a joint effect of protective variants in CVD evasion.

4.3. Interactions within broader IIS network

4.3.1. Interplay of SNPs associated with obesity and IIS

The intronic variant rs50871 is located in the *ERCC2* gene, a gene encoding a DNA helicase that is an essential subunit of a complex transcription factor known as the general transcription factor 2 H (TFIIH) in charge of basal transcription, and is also involved in transcription-coupled nucleotide excision repair (NER) (Coin et al., 1998; De Boer and Hoeijmakers, 2000; Keriel et al., 2002; Benhamou and Sarasin, 2002). Functional analyses, however, report that rs50871 impacts the expression of *KLC3* gene (Ghoussaini et al., 2021) encoding kinesin light chain 3, a subunit of the molecular motor protein kinesin. While not much is known about the specific role of *KLC3*, apart from its ability to attach to mitochondria and its involvement in sperm tail formation, this gene has been associated with the development of Alzheimer's disease and obesity metrics (Charisis et al., 2023). While Dato et al. (2018) report that rs50871 had a significant effect on longevity in interaction with *TP53* rs2078486 (Dato et al., 2018), in our study, rs50871 interacted significantly with the *FOXO3* gene rs2802292, which has also been associated with longevity (Flachsbart et al., 2009), especially in men (Willcox et al., 2008; Anselmi et al., 2009; Bao et al., 2014). With FOXO3 being a main connecting link to the IIS, and rs50871 causing changes to the expression of the protein related to obesity, it is possible that the SNP-SNP interaction between these two variants is significant due to the obesity-related changes in insulin signaling (Blackburn and Collins, 2011; Zhang et al., 2012).

4.3.2. Interaction with genes from the growth hormone-IIS axis

Intronic variant rs2267723 is reported to influence the splicing of *GHRHR*, a gene that encodes growth hormone-releasing hormone receptor. A part of growth hormone/insulin-like growth factor 1/insulin signalling axis, this receptor, located in the pituitary gland on the membrane of somatotropic cells, binds growth hormone-releasing hormone which causes synthesis and secretion of growth hormone (GH) (Mayo et al., 2000). rs2267723 interacted significantly with rs1800795, an intronic variant that has previously been associated with the *IL6* gene, but is located closest to the *STEAP1B* gene. While there is evidence it influences the expression of both genes, the effect on *STEAP1B* is much stronger (Ghoussaini et al., 2021). Not much is known of the biological functions of *STEAP1B* genes, apart from their metalloreductase activity and their role in iron and copper homeostasis, (Ohgami et al., 2006; Xu et al., 2022) so it is difficult to assume how the variant associated with this gene works together with *GHRHR* rs2267723 to impact survival of the oldest-olds. Perhaps their interaction is dependent on the effect of rs1800795 on *IL6*, a cytokine with both pro- and anti-inflammatory properties (Minciullo et al., 2016) that has previously been associated with longevity (Christiansen et al., 2004; Albani et al., 2009; Revelas et al., 2018), and can influence insulin signalling and glucose metabolism (Kim et al., 2008).

4.4. Genetic interactions and health status indicators

The key factor for benefitting from the extra years of life attained on account of beneficial genetic background is good health (Beard et al.,

2016). Existence of disease and its onset, functional status and frailty are all indicators of physiological changes that can precede death (Crimmins and Beltrán-Sánchez, 2011), and can be useful as variables for predicting survival. In this study, we tested the dataset of health-related parameters for our oldest-old sample as predictors of survival in advanced old age, both independently and with the significant genetic factors. Of the 33 tested variables, a subset of nine had an effect on survival in a model without genetic factors. These were maternal and fraternal longevity, nourishment status, weight, bone density, folates, number of medicaments taken, taking of B-complex supplements and number of hospital stays in the year prior to taking the survey. For most of these, the category within the variable related to better survival was an expected one, except that higher chances of survival were found for participants who had osteopenia. However, this is not entirely surprising, as osteopenia is a common trait amongst the oldest-old, thus representing normal ageing (Ginsburg et al., 2001; Škarić-Jurić and Rudan, 1997; Raisz and Seeman, 2001). In the joint models of genetic and health-related factors, most of the health-related variables remained significant, proving that the selected health-related traits can indeed robustly and independently of genetic factors predict chances of survival for the oldest-old population. Interestingly, loss of significance of the variable describing the number of medicaments taken in combination with *TERC* and *SH2B3* indicates that this interaction influences a phenotype that is also covered by these health parameters. Perhaps, it might have a role in mediating the number of chronic age-related conditions which are most often the cause of polypharmacy (Kurczewska--Michalak et al., 2021). Furthermore, loss of significance of maternal age at death in models with *CDKN2B – FOXO3* interactions indicates that the phenotype targeted by this genetic interaction has to do with familial longevity and lifespan.

Only four of the genetic interactions stopped being significant with the addition of the health-related variables, probably due to the introduction of variables that impacted the same phenotype as them. The interactions that remained significant, however, highlight the importance of cell cycle control and its interplay with IIS, the two main pathways with implications for longevity, but also indicate the vital role that modulators of cardiovascular risk and proteins with antioxidative effect have in determining survival chances. Furthermore, these findings imply that health status and health-related indicators are not the sole determinants of the dynamics of the ageing process.

4.5. Strengths and limitations of the study

Principal limitations of this study are the relatively small sample size and limited number of genotyped genetic loci, which both lead to the findings that were only nominally significant. Those limitations were partially compensated by generating bootstrap-adjusted results that present a more accurately predicted p-value. This study does, however, focus on SNPs with a strong previous association in studies with more power, and emphasizes the SNP-SNP interactions. By using the two-SNP-interaction method, it was possible to elucidate an effect that might not be detected otherwise. In addition, the analysis of SNP-SNP interactions is a valid method for finding significant genetic contributors in studies with low power, even though the statistical strength of the interaction analysis would also benefit from a larger sample size. Finally, it is, to our knowledge, the first study of genetic makeup contributing to survival of the oldest-olds in the Croatian population, a population otherwise underrepresented in genetic studies. Therefore, our study presents these initial results, but the obtained associations should be replicated in a population with a different genetic background, and a much larger sample size.

5. Conclusion

In conclusion, this study explored the effect of SNP-SNP interactions on survival above 85 years of age in a sample of Croatian oldest-olds. By focusing on genetic interaction between the longevity-associated variants rather than the individual SNPs, it was possible to identify pathways that contribute to survival in advanced old age. We identified a nominally significant interaction between SNPs in *CDKN2B* and *FOXO3*, *TP53* and *LINC02227* SNPs, as well as several other combinations that remain significant even when tested together with health status indicators. This shows that the interplay between genetic variants in different genes may affect survival in a manner that is not explained by biomarkers of health status and should be further explored in studies with larger sample sizes.

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Declarations of interest

None.

The sample collection and the research described here were approved by the Ethics Committee of Institute for Anthropological Research (Zagreb, Croatia) and performed following all institutional guidelines. Ethical approvals obtained on March 4th 2006 (130–981/06) and 22nd November 2018 (20180518).

CRediT authorship contribution statement

Maja Šetinc: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Željka Celinšćak:** Writing – review & editing, Visualization, Validation, Investigation, Data curation. **Luka Bočkor:** Writing – review & editing, Supervision, Resources, Investigation. **Matea Zajc Petranović:** Writing – review & editing, Data curation. **Anita Stojanović Marković:** Writing – review & editing, Validation. **Marijana Peričić Salihović:** Writing – review & editing, Supervision. **Joris Deelen:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Tatjana Škarić-Jurić:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Data Availability

Fully anonymised dataset of genetic data used in this study is publicly available on Zenodo repository (DOI: 10.5281/zenodo.7421684). Data on health-related parameters is available upon request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mad.2024.111926.

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Supplementary Table 1. Information about the selected SNPs**.**

SNP	Associated gene	Chromosome position (GRCh38)	Alleles (major/minor) in Croatian oldest-old population	MAF	Genotyping success rate	HWE p value (Yate's correction*)	Literature source for association with longevity
rs225119	*PARK7*	1:7984301	C/T	0.425	0.979	0.815	[1]
rs2360675	*KLF7*	2:207194916	C/A	0.491	0.972	0.947	[2]
rs12696304	*TERC*	3:169763483	C/G	0.259	0.963	0.663	[3,4]
rs3772190	*TERC*	3:169782699	G/A	0.228	0.966	0.744	[4]
rs16847897	*TERC*	3:169850328	G/C	0.292	0.979	0.996	[3,5]
rs572169	*GHSR*	3:172447937	C/T	0.275	0.963	0.332	[1,6]
rs33954691	*TERT*	5:1255405	G/A	0.103	0.966	0.715 (0.862)	[4]
rs2706372	*RAD50/IL13*	5:132599785	C/T	0.272	0.960	0.997	[7]
rs2149954	*LINC02227*	5:158393594	C/T	0.380	0.982	0.291	[8,9]
rs12203592	*IRF4*	6:396321	C/T	0.074	0.976	0.582 (0.803)	[10]
rs1800629	*TNF*	6:31575254	G/A	0.126	0.972	0.318	[11]
rs12206094	*FOXO3*	6:108584997	C/T	0.295	0.963	0.925	[12,13]
rs2802292	*FOXO3*	6:108587315	T/G	0.424	0.982	0.710	[13–16]
rs2764264	*FOXO3*	6:108613258	T/C	0.331	0.948	0.889	[12,13]
rs10457180	*FOXO3*	6:108643836	A/G	0.329	0.963	0.745	[17,18]
rs13217795	*FOXO3*	6:108652895	T/C	0.324	0.963	0.584	[12,13,19]
rs4946935	*FOXO3*	6:108679539	G/A	0.298	0.979	0.991	[18,20–22]
rs9456497	*IGF2R*	6:160022396	A/G	0.191	0.969	0.854	[6,23]
rs10455872	*LPA*	6:160589086	A/G	0.036	0.969	0.039 (0.209)	[24,25]
rs1800795	*IL6*	7:22727026	G/C	0.448	0.948	0.562	[26–28]
rs2069837	*IL6*	7:22728408	A/G	0.077	0.969	0.781 (0.942)	[9,29]
rs2267723	*GHRHR*	7:30967327	A/G	0.444	0.951	0.999	[1,30]
rs13251813	*WRN*	8:31106695	C/T	0.047	0.976	0.934 (0.970)	[30]
rs4977756	*CDKN2B*	9:22068653	A/G	0.399	0.966	0.959	[24,31]
rs1333049	*CDKN2B*	9:22125504	G/C	0.470	0.972	0.319	[24,31,32]
rs4837525	*PAPPA*	9:116276279	G/A	0.373	0.960	0.686	[1]
rs533984	*MRE11A*	11:94466106	G/A	0.396	0.960	0.589	[1]
rs17202060	*TXNRD1*	12:104337068	C/T	0.336	0.960	0.812	[1]
rs3184504	*SH2B3*	12:111446804	T/C	0.484	0.966	1.000	[24,25,31,33]
rs1207362	*KLOTHO*	13:33038702	G/T	0.312	0.960	0.669	[6]
rs9536314	*KLOTHO*	13:33054001	T/G	0.118	0.982	0.173	[34,35]
rs9527025	*KLOTHO*	13:33054056	G/C	0.118	0.982	0.173	[36,37]
rs2229765	*IGF1R*	15:98934996	G/A	0.436	0.957	0.851	[38,39]
rs12437963	*IGF1R*	15:98953630	A/G	0.144	0.976	0.960	[1]
rs1042522	*TP53*	17:7676154	C/G	0.238	0.976	0.448	[40,41]
rs2078486	*TP53*	17:7679765	G/A	0.077	0.969	0.996 (0.959)	[1]
rs107251	*SIRT6*	19:4176088	C/T	0.101	0.982	0.984 (0.993)	[22]

rs2075650	*TOMM40*	19:44892362	A/G	0.142	0.982	0.776	[7,24,42–44]
rs429358	*APOE*	19:44908684	T/C	0.081	0.948	1.000 (0.941)	[42,45]
rs7412	*APOE*	19:44908822	C/T	0.076	0.963	0.644 (0.850)	[29,31]
rs4420638	*APOC1*	19:44919689	A/G	0.117	0.963	0.939	[8,24,25,31,44,46,47]
rs50871	*ERCC2*	19:45359257	A/C	0.460	0.966	0.902	[1]
rs6067484	*PTPN1*	20:50536246	A/G	0.279	0.963	0.796	[1]

Table shows rs code, gene associated with the SNP; chromosome position (in GRCh38), references for literature sources in which association with longevity was reported; along with data that refers to the studied Croatian population: alleles (major/minor), minor allele frequencies (MAF), genotyping success rate, HWE.
* Yate's correction for continuity was applied for cases where observed number of individuals in one of the cells of the Punnet Square was smaller than 5.

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Supplementary Table 2. The results of Cox regression survival analyses with bootstrapping for all the 359 tested models of single SNPs and their interactions.

First SNP	Second SNP	First SNP				Second SNP				Interaction			
		p-value	HR	95% CI Lower	95% CI Upper	p-value	HR	95% CI Lower	95% CI Upper	p-value	HR	95% CI Lower	95% CI Upper
PARK7 rs225119	KLF7 rs2360675	0.879	0.975	0.717	1.320	0.932	0.987	0.752	1.262	0.876	0.979	0.753	1.274
PARK7 rs225119	TERC rs12696304	0.994	1.001	0.608	1.699	0.519	0.920	0.693	1.212	0.856	0.972	0.689	1.320
PARK7 rs225119	TERC rs3772190	0.521	0.925	0.728	1.169	0.591	1.080	0.828	1.470	0.539	1.107	0.802	1.499
PARK7 rs225119	TERC rs16847897	0.413	1.209	0.757	1.912	0.800	0.964	0.730	1.314	0.320	0.858	0.623	1.160
PARK7 rs225119	GHSR rs572169	0.738	0.916	0.526	1.448	0.970	1.005	0.710	1.377	0.870	1.027	0.779	1.452
PARK7 rs225119	RAD50/IL13 rs2706372	0.931	0.908	0.780	1.271	0.739	0.997	0.773	1.429	0.696	1.033	0.720	1.257
PARK7 rs225119	LINC02227 rs2149954	0.212	1.170	0.905	1.498	0.213	1.181	0.908	1.554	**0.044**	0.776	0.605	1.002
PARK7 rs225119	FOXO3 rs12206094	0.904	0.988	0.791	1.237	0.574	1.082	0.817	1.495	0.688	0.940	0.695	1.242
PARK7 rs225119	FOXO3 rs2802292	0.500	0.920	0.714	1.189	0.484	0.900	0.663	1.267	0.720	1.050	0.787	1.369
PARK7 rs225119	FOXO3 rs2764264	0.466	0.926	0.739	1.161	0.389	0.880	0.634	1.186	0.422	1.110	0.832	1.477
PARK7 rs225119	FOXO3 rs10457180	0.356	0.908	0.732	1.116	0.488	0.902	0.676	1.175	0.539	1.087	0.845	1.419
PARK7 rs225119	FOXO3 rs13217795	0.407	0.916	0.736	1.135	0.398	0.889	0.641	1.207	0.569	1.082	0.030	1.438
PARK7 rs225119	FOXO3 rs4946935	0.350	0.910	0.737	1.106	0.748	0.956	0.712	1.290	0.528	1.088	0.842	1.423
PARK7 rs225119	IGF2R rs9456497	0.697	0.953	0.745	1.261	0.754	0.955	0.695	1.309	0.922	1.016	0.748	1.334
PARK7 rs225119	IL6 rs1800795	0.544	1.104	0.790	1.548	0.458	1.093	0.854	1.402	0.266	0.868	0.665	1.129
PARK7 rs225119	GHRHR rs2267723	0.352	0.843	0.576	1.266	0.286	0.881	0.690	1.108	0.351	1.112	0.875	1.439
PARK7 rs225119	CDKN2B rs4977756	0.851	1.028	0.759	1.343	0.495	1.095	0.801	1.468	0.564	0.921	0.706	1.236
PARK7 rs225119	CDKN2B rs1333049	0.615	1.076	0.782	1.409	0.269	1.152	0.890	1.468	0.376	0.891	0.700	1.164
PARK7 rs225119	PAPPA rs4837525	0.935	1.017	0.693	1.510	0.559	0.921	0.688	1.240	0.726	0.957	0.746	1.251
PARK7 rs225119	MRE11A rs533984	0.657	0.908	0.597	1.387	0.657	1.028	0.597	1.370	0.784	1.042	0.710	1.368
PARK7 rs225119	TXNRD1 rs17202060	0.875	1.021	0.767	1.327	0.277	1.187	0.872	1.654	0.487	0.901	0.680	1.194
PARK7 rs225119	SH2B3 rs3184504	0.766	1.049	0.781	1.426	0.903	1.014	0.768	1.301	0.450	0.901	0.692	1.206
PARK7 rs225119	KLOTHO rs1207362	0.726	1.062	0.734	1.507	0.812	0.971	0.720	1.274	0.538	0.929	0.717	1.219
PARK7 rs225119	IGF1R rs2229765	0.594	0.912	0.675	1.283	0.728	1.047	0.808	1.387	0.571	1.072	0.823	1.353
PARK7 rs225119	TP53 rs1042522	0.250	0.874	0.676	1.104	**0.043**	0.722	0.504	0.981	0.180	1.223	0.941	1.732
PARK7 rs225119	ERCC2 rs50871	0.469	0.902	0.671	1.201	0.517	0.929	0.752	1.178	0.631	1.054	0.847	1.302
PARK7 rs225119	PTPN1 rs6067484	0.063	0.804	0.647	1.019	**0.023**	0.698	0.504	0.962	**0.045**	1.366	0.986	1.883
KLF7 rs2360675	TERC rs12696304	0.206	0.761	0.480	1.166	0.129	0.751	0.514	1.088	0.252	1.170	0.890	1.567
KLF7 rs2360675	TERC rs3772190	0.858	1.021	0.805	1.303	0.152	1.324	0.880	1.958	0.438	0.896	0.671	1.181
KLF7 rs2360675	TERC rs16847897	0.431	1.166	0.742	1.744	0.861	0.968	0.649	1.342	0.345	0.874	0.665	1.188
KLF7 rs2360675	GHSR rs572169	0.578	0.865	0.512	1.428	0.781	0.951	0.613	1.353	0.657	1.077	0.802	1.483
KLF7 rs2360675	RAD50/IL13 rs2706372	0.107	0.832	0.660	1.023	0.129	0.754	0.507	1.080	0.063	1.315	0.998	1.804
KLF7 rs2360675	LINC02227 rs2149954	0.626	1.057	0.824	1.320	0.690	1.061	0.796	1.406	0.313	0.884	0.700	1.138
KLF7 rs2360675	FOXO3 rs12206094	0.487	0.927	0.750	1.158	0.767	0.952	0.711	1.294	0.526	1.074	0.845	1.334
KLF7 rs2360675	FOXO3 rs2802292	0.121	0.813	0.620	1.065	0.095	0.765	0.550	1.053	0.098	1.224	0.953	1.581

KLF7 rs2360675	FOXO3 rs2764264	0.679	0.957	0.755	1.215	0.864	0.970	0.715	1.330	0.989	1.001	0.771	1.300
KLF7 rs2360675	FOXO3 rs10457180	0.675	0.956	0.754	1.191	0.746	0.955	0.730	1.300	0.858	1.022	0.800	1.297
KLF7 rs2360675	FOXO3 rs13217795	0.652	0.947	0.749	1.189	0.569	0.922	0.698	1.274	0.724	1.040	0.816	1.293
KLF7 rs2360675	FOXO3 rs4946935	0.917	0.927	0.818	1.209	0.752	0.968	0.868	1.221	0.371	1.069	0.800	1.080
KLF7 rs2360675	IGF2R rs9456497	0.793	1.026	0.855	1.269	0.447	1.162	0.794	1.761	0.259	0.848	0.616	1.135
KLF7 rs2360675	IL6 rs1800795	0.357	0.848	0.592	1.204	0.355	0.866	0.626	1.179	0.395	1.115	0.868	1.459
KLF7 rs2360675	GHRHR rs2267723	0.706	0.938	0.649	1.347	0.683	0.942	0.697	1.276	0.809	1.030	0.808	1.303
KLF7 rs2360675	CDKN2B rs4977756	0.425	1.108	0.875	1.445	0.189	1.215	0.896	1.657	0.175	0.854	0.668	1.077
KLF7 rs2360675	CDKN2B rs1333049	0.813	0.963	0.721	1.281	0.850	1.031	0.765	1.365	0.966	1.006	0.801	1.278
KLF7 rs2360675	PAPPA rs4837525	0.317	0.837	0.607	1.226	0.066	0.775	0.594	1.063	0.243	1.135	0.903	1.411
KLF7 rs2360675	MRE11A rs533984	0.289	0.822	0.500	1.232	0.632	0.926	0.692	1.309	0.318	1.145	0.871	1.483
KLF7 rs2360675	TXNRD1 rs17202060	0.271	0.872	0.685	1.137	0.621	0.922	0.666	1.318	0.215	1.177	0.899	1.520
KLF7 rs2360675	SH2B3 rs3184504	0.709	0.945	0.666	1.302	0.496	0.896	0.621	1.239	0.824	1.029	0.757	1.387
KLF7 rs2360675	KLOTHO rs1207362	0.749	0.937	0.598	1.368	0.462	0.889	0.640	1.231	0.870	1.020	0.785	1.332
KLF7 rs2360675	IGF1R rs2229765	0.361	0.866	0.647	1.177	0.920	0.987	0.742	1.322	0.337	1.124	0.885	1.426
KLF7 rs2360675	TP53 rs1042522	0.426	0.915	0.726	1.137	0.182	0.774	0.541	1.107	0.471	1.116	0.834	1.540
KLF7 rs2360675	ERCC2 rs50871	0.870	1.022	0.780	1.380	0.922	1.015	0.792	1.332	0.591	0.951	0.782	1.154
KLF7 rs2360675	PTPN1 rs6067484	0.741	0.960	0.747	1.210	0.462	0.888	0.662	1.245	0.972	1.005	0.772	1.314
TERC rs12696304	GHSR rs572169	0.442	0.797	0.387	1.232	0.729	0.901	0.444	1.442	0.646	1.083	0.813	1.634
TERC rs12696304	RAD50/IL13 rs2706372	0.131	0.806	0.594	1.053	0.249	0.720	0.392	1.234	0.221	1.244	0.888	1.784
TERC rs12696304	LINC02227 rs2149954	0.257	0.854	0.632	1.113	0.455	0.833	0.509	1.302	0.570	1.088	0.819	1.486
TERC rs12696304	FOXO3 rs12206094	0.487	0.909	0.688	1.195	0.740	1.080	0.676	1.809	0.833	0.969	0.699	1.298
TERC rs12696304	FOXO3 rs2802292	0.953	0.992	0.731	1.368	0.600	1.140	0.708	1.923	0.443	0.886	0.637	1.213
TERC rs12696304	FOXO3 rs2764264	0.850	0.973	0.746	1.269	0.400	1.232	0.748	2.008	0.318	0.859	0.632	1.188
TERC rs12696304	FOXO3 rs10457180	0.947	0.988	0.767	1.314	0.360	1.233	0.766	1.994	0.309	0.862	0.642	1.179
TERC rs12696304	FOXO3 rs13217795	0.858	0.976	0.742	1.283	0.490	1.188	0.719	1.982	0.368	0.870	0.624	1.196
TERC rs12696304	FOXO3 rs4946935	0.540	0.923	0.703	1.191	0.642	1.119	0.720	1.817	0.735	0.950	0.706	1.267
TERC rs12696304	IGF2R rs9456497	0.242	0.854	0.646	1.112	0.487	0.827	0.477	1.452	0.527	1.109	0.780	1.587
TERC rs12696304	IL6 rs1800795	0.791	0.957	0.645	1.416	0.794	1.052	0.676	1.652	0.628	0.940	0.708	1.226
TERC rs12696304	GHRHR rs2267723	0.351	0.841	0.532	1.179	0.575	0.882	0.463	1.326	0.657	1.062	0.821	1.519
TERC rs12696304	CDKN2B rs4977756	0.705	0.946	0.685	1.242	0.568	1.127	0.763	1.791	0.580	0.926	0.682	1.229
TERC rs12696304	CDKN2B rs1333049	0.897	1.022	0.735	1.435	0.270	1.240	0.832	1.895	0.323	0.877	0.652	1.141
TERC rs12696304	PAPPA rs4837525	0.410	0.823	0.527	1.335	0.390	0.809	0.509	1.372	0.718	1.057	0.764	1.392
TERC rs12696304	MRE11A rs533984	0.742	1.068	0.690	1.551	0.144	1.370	0.860	2.106	0.275	0.858	0.649	1.163
TERC rs12696304	TXNRD1 rs17202060	0.321	0.874	0.661	1.122	0.952	1.014	0.630	1.788	0.827	1.039	0.760	1.387
TERC rs12696304	SH2B3 rs3184504	0.192	1.243	0.882	1.732	0.058	1.531	0.970	2.440	**0.014**	0.708	0.539	0.946
TERC rs12696304	KLOTHO rs1207362	0.331	1.196	0.831	1.763	0.318	1.235	0.818	1.910	0.101	0.805	0.599	1.038
TERC rs12696304	IGF1R rs2229765	0.141	0.749	0.477	1.055	0.449	0.823	0.447	1.317	0.238	1.204	0.905	1.735
TERC rs12696304	TP53 rs1042522	0.428	0.897	0.691	1.157	0.605	0.870	0.493	1.442	0.987	1.003	0.723	1.416
TERC rs12696304	ERCC2 rs50871	0.333	0.859	0.587	1.143	0.620	0.915	0.626	1.292	0.717	1.044	0.844	1.343
TERC rs12696304	PTPN1 rs6067484	0.375	0.896	0.676	1.141	0.705	0.911	0.543	1.542	0.941	0.988	0.715	1.411

TERC rs3772190	GHSR rs572169	0.303	1.340	0.781	2.363	0.581	1.061	0.835	1.302	0.581	0.912	0.634	1.294
TERC rs3772190	RAD50/IL13 rs2706372	**0.025**	1.359	1.044	1.809	0.482	1.093	0.853	1.428	0.104	0.762	0.535	1.071
TERC rs3772190	LINC02227 rs2149954	0.122	1.228	0.946	1.618	0.887	0.987	0.798	1.229	0.574	0.925	0.683	1.245
TERC rs3772190	FOXO3 rs12206094	0.255	1.163	0.887	1.523	0.862	1.018	0.821	1.301	0.911	1.017	0.745	1.430
TERC rs3772190	FOXO3 rs2802292	0.715	1.064	0.777	1.436	0.361	0.898	0.705	1.130	0.474	1.119	0.813	1.523
TERC rs3772190	FOXO3 rs2764264	0.417	1.116	0.858	1.455	0.501	0.925	0.725	1.165	0.497	1.112	0.821	1.493
TERC rs3772190	FOXO3 rs10457180	0.486	1.094	0.820	1.405	0.535	0.932	0.738	1.151	0.500	1.108	0.811	1.556
TERC rs3772190	FOXO3 rs13217795	0.427	1.109	0.850	1.446	0.472	0.918	0.728	1.170	0.554	1.093	0.798	1.508
TERC rs3772190	FOXO3 rs4946935	0.216	1.163	0.911	1.492	0.833	1.024	0.826	1.260	0.941	1.010	0.774	1.368
TERC rs3772190	IGF2R rs9456497	0.062	1.278	0.990	1.675	0.635	1.062	0.817	1.401	0.265	0.828	0.600	1.163
TERC rs3772190	IL6 rs1800795	0.759	1.059	0.734	1.560	0.358	0.912	0.748	1.112	0.471	1.092	0.851	1.418
TERC rs3772190	GHRHR rs2267723	0.250	1.247	0.852	1.921	0.919	0.990	0.787	1.247	0.666	0.942	0.701	1.244
TERC rs3772190	CDKN2B rs4977756	0.239	1.188	0.898	1.565	0.746	1.038	0.804	1.322	0.915	0.985	0.769	1.330
TERC rs3772190	CDKN2B rs1333049	0.995	1.001	0.683	1.390	0.859	0.978	0.780	1.220	0.326	1.146	0.895	1.610
TERC rs3772190	PAPPA rs4837525	0.299	1.277	0.788	2.081	0.380	0.908	0.713	1.122	0.688	0.943	0.705	1.305
TERC rs3772190	MRE11A rs533984	0.882	0.971	0.654	1.451	0.830	1.024	0.815	1.292	0.235	1.184	0.876	1.567
TERC rs3772190	TXNRD1 rs17202060	0.090	1.276	0.975	1.745	0.287	1.132	0.888	1.423	0.436	0.870	0.640	1.212
TERC rs3772190	SH2B3 rs3184504	0.387	0.828	0.561	1.237	**0.022**	0.794	0.641	0.974	**0.032**	1.403	1.007	1.927
TERC rs3772190	KLOTHO rs1207362	0.376	0.851	0.578	1.235	0.079	0.811	0.630	1.011	0.072	1.283	0.989	1.742
TERC rs3772190	IGF1R rs2229765	**0.009**	1.556	1.083	2.201	**0.037**	1.201	1.006	1.442	0.052	0.752	0.553	0.990
TERC rs3772190	TP53 rs1042522	0.173	1.194	0.943	1.639	0.292	0.877	0.694	1.099	0.748	0.947	0.666	1.319
TERC rs3772190	ERCC2 rs50871	0.418	1.141	0.840	1.624	0.896	0.988	0.820	1.186	0.828	1.023	0.788	1.250
TERC rs3772190	PTPN1 rs6067484	0.164	1.184	0.950	1.543	0.392	0.892	0.694	1.161	0.915	0.983	0.701	1.351
TERC rs16847897	GHSR rs572169	0.080	0.645	0.366	1.023	0.280	0.761	0.436	1.241	0.215	1.205	0.904	1.670
TERC rs16847897	RAD50/IL13 rs2706372	0.235	0.859	0.660	1.125	0.933	1.021	0.633	1.621	0.924	0.984	0.716	1.354
TERC rs16847897	LINC02227 rs2149954	0.080	0.799	0.625	1.061	0.406	0.832	0.535	1.294	0.538	1.087	0.827	1.423
TERC rs16847897	FOXO3 rs12206094	0.125	0.820	0.636	1.043	0.758	0.935	0.614	1.411	0.661	1.064	0.815	1.411
TERC rs16847897	FOXO3 rs2802292	0.224	0.832	0.586	1.099	0.627	0.905	0.540	1.436	0.863	1.028	0.765	1.449
TERC rs16847897	FOXO3 rs2764264	0.145	0.821	0.611	1.067	0.689	0.918	0.592	1.387	0.815	1.033	0.782	1.395
TERC rs16847897	FOXO3 rs10457180	0.157	0.829	0.631	1.073	0.709	0.922	0.573	1.439	0.824	1.035	0.780	1.398
TERC rs16847897	FOXO3 rs13217795	0.167	0.833	0.624	1.087	0.698	0.916	0.569	1.350	0.871	1.027	0.789	1.402
TERC rs16847897	FOXO3 rs4946935	0.058	0.784	0.595	1.002	0.412	0.844	0.541	1.230	0.340	1.140	0.885	1.508
TERC rs16847897	IGF2R rs9456497	0.111	0.813	0.625	1.029	0.520	0.855	0.495	1.402	0.631	1.082	0.790	1.530
TERC rs16847897	IL6 rs1800795	0.788	1.037	0.719	1.452	0.205	1.251	0.855	1.799	0.111	0.831	0.655	1.052
TERC rs16847897	GHRHR rs2267723	0.082	0.721	0.502	1.052	0.259	0.787	0.525	1.240	0.302	1.146	0.860	1.504
TERC rs16847897	CDKN2B rs4977756	0.212	0.859	0.676	1.117	0.830	1.043	0.760	1.642	0.865	0.981	0.725	1.242
TERC rs16847897	CDKN2B rs1333049	0.283	0.847	0.629	1.194	0.934	1.012	0.739	1.461	0.970	1.004	0.773	1.255
TERC rs16847897	PAPPA rs4837525	0.138	0.736	0.475	1.132	0.206	0.751	0.476	1.257	0.477	1.109	0.815	1.462
TERC rs16847897	MRE11A rs533984	0.847	0.960	0.637	1.456	0.382	1.226	0.797	1.919	0.531	0.905	0.670	1.236
TERC rs16847897	TXNRD1 rs17202060	**0.029**	0.765	0.591	0.973	0.471	0.860	0.569	1.336	0.241	1.176	0.884	1.576
TERC rs16847897	SH2B3 rs3184504	0.143	1.274	0.906	1.835	**0.020**	1.649	1.093	2.537	**0.002**	0.665	0.512	0.860

TERC rs16847897	KLOTHO rs1207362	0.923	1.016	0.654	1.425	0.663	1.084	0.683	1.590	0.293	0.879	0.691	1.157
TERC rs16847897	IGF1R rs2229765	0.164	0.804	0.579	1.106	1.000	1.000	0.684	1.456	0.619	1.061	0.839	1.369
TERC rs16847897	TP53 rs1042522	0.166	0.846	0.660	1.070	0.498	0.866	0.516	1.370	0.994	0.999	0.751	1.357
TERC rs16847897	ERCC2 rs50871	0.261	0.853	0.619	1.114	0.943	0.988	0.690	1.366	0.991	0.998	0.812	1.260
TERC rs16847897	PTPN1 rs6067484	0.248	0.879	0.688	1.099	0.953	0.987	0.660	1.709	0.628	0.931	0.666	1.226
GHSR rs572169	RAD50/IL13 rs2706372	0.211	1.154	0.901	1.430	0.141	1.358	0.869	2.046	0.107	0.806	0.614	1.062
GHSR rs572169	LINC02227 rs2149954	0.565	1.081	0.815	1.397	0.875	1.032	0.649	1.662	0.643	0.939	0.700	1.237
GHSR rs572169	FOXO3 rs12206094	0.965	0.995	0.745	1.313	0.792	0.943	0.618	1.682	0.729	1.056	0.741	1.394
GHSR rs572169	FOXO3 rs2802292	0.916	0.983	0.718	1.388	0.559	0.874	0.568	1.446	0.724	1.050	0.768	1.394
GHSR rs572169	FOXO3 rs2764264	0.670	0.944	0.728	1.241	0.297	0.791	0.543	1.339	0.324	1.151	0.816	1.471
GHSR rs572169	FOXO3 rs10457180	0.559	0.929	0.725	1.185	0.272	0.791	0.512	1.301	0.286	1.157	0.839	1.539
GHSR rs572169	FOXO3 rs13217795	0.730	0.959	0.748	1.242	0.327	0.812	0.541	1.327	0.396	1.120	0.815	1.456
GHSR rs572169	FOXO3 rs4946935	0.826	0.978	0.764	1.235	0.719	0.922	0.633	1.584	0.557	1.081	0.780	1.391
GHSR rs572169	IGF2R rs9456497	0.332	1.120	0.862	1.384	0.300	1.315	0.764	2.117	0.178	0.811	0.592	1.123
GHSR rs572169	IL6 rs1800795	0.261	1.243	0.846	1.835	0.322	1.221	0.830	1.906	0.226	0.856	0.649	1.111
GHSR rs572169	GHRHR rs2267723	0.538	0.898	0.598	1.307	0.293	0.808	0.509	1.217	0.329	1.130	0.875	1.480
GHSR rs572169	CDKN2B rs4977756	0.960	0.993	0.756	1.297	0.831	0.947	0.557	1.624	0.778	1.046	0.769	1.432
GHSR rs572169	CDKN2B rs1333049	0.250	1.184	0.870	1.579	0.173	1.336	0.855	2.052	0.190	0.843	0.643	1.094
GHSR rs572169	PAPPA rs4837525	0.173	0.768	0.498	1.106	**0.036**	0.644	0.400	0.982	0.123	1.245	0.954	1.682
GHSR rs572169	MRE11A rs533984	0.889	0.971	0.616	1.425	0.950	0.983	0.571	1.582	0.704	1.056	0.790	1.439
GHSR rs572169	TXNRD1 rs17202060	0.565	1.086	0.813	1.420	0.377	1.207	0.770	1.855	0.557	0.924	0.700	1.218
GHSR rs572169	SH2B3 rs3184504	0.958	0.992	0.701	1.387	0.558	0.888	0.602	1.335	0.815	1.027	0.794	1.327
GHSR rs572169	KLOTHO rs1207362	0.972	1.005	0.682	1.443	0.542	0.883	0.569	1.373	0.854	1.022	0.771	1.323
GHSR rs572169	IGF1R rs2229765	0.558	1.091	0.793	1.448	0.349	1.198	0.792	1.709	0.697	0.953	0.751	1.270
GHSR rs572169	TP53 rs1042522	0.754	0.962	0.741	1.241	0.249	0.747	0.436	1.192	0.511	1.108	0.845	1.531
GHSR rs572169	ERCC2 rs50871	0.912	1.018	0.745	1.415	0.854	0.950	0.598	1.527	0.934	1.011	0.773	1.296
GHSR rs572169	PTPN1 rs6067484	0.195	1.166	0.924	1.449	0.416	1.213	0.763	1.824	0.128	0.811	0.625	1.084
RAD50/IL13 rs2706372	LINC02227 rs2149954	0.550	0.924	0.696	1.201	0.312	0.891	0.708	1.105	0.478	1.101	0.851	1.474
RAD50/IL13 rs2706372	FOXO3 rs12206094	0.774	0.964	0.728	1.229	0.941	0.993	0.815	1.246	0.671	1.065	0.803	1.476
RAD50/IL13 rs2706372	FOXO3 rs2802292	0.910	0.981	0.715	1.287	0.477	0.930	0.746	1.137	0.876	1.019	0.766	1.452
RAD50/IL13 rs2706372	FOXO3 rs2764264	0.677	0.945	0.689	1.218	0.452	0.918	0.732	1.150	0.465	1.118	0.845	1.559
RAD50/IL13 rs2706372	FOXO3 rs10457180	0.654	0.939	0.708	1.201	0.535	0.933	0.753	1.162	0.538	1.095	0.817	1.539
RAD50/IL13 rs2706372	FOXO3 rs13217795	0.767	0.964	0.741	1.231	0.473	0.927	0.760	1.139	0.644	1.068	0.807	1.500
RAD50/IL13 rs2706372	FOXO3 rs4946935	0.786	0.963	0.731	1.226	0.995	1.001	0.806	1.217	0.672	1.067	0.812	1.474
RAD50/IL13 rs2706372	IGF2R rs9456497	0.491	0.916	0.708	1.161	0.238	0.853	0.640	1.110	0.113	1.283	0.938	1.751
RAD50/IL13 rs2706372	IL6 rs1800795	0.375	1.135	0.812	1.483	0.765	1.033	0.826	1.260	0.371	0.896	0.705	1.161
RAD50/IL13 rs2706372	GHRHR rs2267723	0.202	1.245	0.883	1.791	0.484	1.078	0.860	1.344	0.131	0.822	0.625	1.051
RAD50/IL13 rs2706372	CDKN2B rs4977756	0.576	0.925	0.679	1.244	0.756	0.964	0.773	1.250	0.401	1.119	0.830	1.445
RAD50/IL13 rs2706372	CDKN2B rs1333049	0.867	0.974	0.676	1.344	0.863	1.021	0.833	1.274	0.813	1.028	0.794	1.326
RAD50/IL13 rs2706372	PAPPA rs4837525	0.367	0.830	0.545	1.247	0.083	0.817	0.651	1.028	0.237	1.175	0.902	1.551
RAD50/IL13 rs2706372	MRE11A rs533984	0.749	1.060	0.676	1.528	0.469	1.088	0.855	1.374	0.764	0.956	0.725	1.294

RAD50/IL13 rs2706372	TXNRD1 rs17202060	0.988	0.997	0.747	1.328	0.642	1.063	0.838	1.413	0.890	1.021	0.736	1.336
RAD50/IL13 rs2706372	SH2B3 rs3184504	0.819	0.965	0.670	1.297	0.346	0.904	0.732	1.127	0.774	1.038	0.783	1.357
RAD50/IL13 rs2706372	KLOTHO rs1207362	0.892	0.969	0.625	1.540	0.356	0.897	0.720	1.140	0.869	1.028	0.752	1.379
RAD50/IL13 rs2706372	IGF1R rs2229765	0.375	0.866	0.616	1.175	0.813	1.023	0.838	1.255	0.162	1.186	0.932	1.553
RAD50/IL13 rs2706372	TP53 rs1042522	0.241	0.987	0.624	1.090	0.241	0.842	0.624	1.090	0.736	1.053	0.787	1.523
RAD50/IL13 rs2706372	ERCC2 rs50871	0.276	1.180	0.839	1.575	0.599	1.058	0.862	1.285	0.201	0.854	0.680	1.111
RAD50/IL13 rs2706372	PTPN1 rs6067484	0.535	1.077	0.858	1.395	0.541	0.956	0.626	1.201	0.541	0.902	0.626	1.201
LINC02227 rs2149954	FOXO3 rs12206094	0.378	0.903	0.714	1.178	0.780	0.966	0.743	1.274	0.534	1.078	0.812	1.405
LINC02227 rs2149954	FOXO3 rs2802292	0.503	0.910	0.679	1.210	0.533	0.910	0.664	1.237	0.771	1.042	0.782	1.350
LINC02227 rs2149954	FOXO3 rs2764264	0.946	0.990	0.773	1.302	0.929	1.010	0.778	1.298	0.658	0.945	0.715	1.198
LINC02227 rs2149954	FOXO3 rs10457180	0.904	0.984	0.765	1.275	0.881	1.020	0.766	1.351	0.630	0.938	0.710	1.226
LINC02227 rs2149954	FOXO3 rs13217795	0.970	1.004	0.794	1.331	0.926	1.016	0.790	1.353	0.565	0.921	0.685	1.178
LINC02227 rs2149954	FOXO3 rs4946935	0.482	0.915	0.717	1.185	0.976	0.996	0.778	1.301	0.720	1.050	0.803	1.339
LINC02227 rs2149954	IGF2R rs9456497	0.596	0.946	0.768	1.149	0.866	0.977	0.742	1.320	0.983	0.997	0.782	1.302
LINC02227 rs2149954	IL6 rs1800795	0.686	1.073	0.809	1.540	0.522	1.073	0.866	1.323	0.295	0.881	0.676	1.097
LINC02227 rs2149954	GHRHR rs2267723	0.733	0.949	0.695	1.274	0.760	0.973	0.804	1.208	0.927	0.989	0.787	1.220
LINC02227 rs2149954	CDKN2B rs4977756	0.419	1.105	0.860	1.404	0.104	1.181	0.972	1.462	0.079	0.814	0.642	1,'57
LINC02227 rs2149954	CDKN2B rs1333049	0.164	1.211	0.908	1.642	**0.024**	1.237	1.021	1.490	**0.038**	0.785	0.619	0.983
LINC02227 rs2149954	PAPPA rs4837525	0.187	1.236	0.888	1.709	0.693	1.050	0.829	1.372	0.083	0.813	0.641	1.029
LINC02227 rs2149954	MRE11A rs533984	0.800	0.955	0.670	1.338	0.562	1.078	0.819	1.406	0.928	0.988	0.791	1.293
LINC02227 rs2149954	TXNRD1 rs17202060	0.825	0.976	0.777	1.237	0.295	1.141	0.909	1.519	0.601	0.933	0.702	1.210
LINC02227 rs2149954	SH2B3 rs3184504	0.581	0.931	0.703	1.218	0.439	0.912	0.710	1.148	0.874	1.018	0.826	1.270
LINC02227 rs2149954	KLOTHO rs1207362	0.291	0.811	0.546	1.236	0.129	0.837	0.651	1.076	0.394	1.114	0.863	1.430
LINC02227 rs2149954	IGF1R rs2229765	0.396	0.903	0.694	1.171	0.532	1.068	0.847	1.305	0.631	1.055	0.867	1.315
LINC02227 rs2149954	TP53 rs1042522	0.118	0.838	0.672	1.037	**0.019**	0.723	0.530	0.929	0.069	1.253	0.996	1.674
LINC02227 rs2149954	ERCC2 rs50871	0.589	0.919	0.681	1.274	0.670	0.954	0.785	1.161	0.849	1.022	0.825	1.270
LINC02227 rs2149954	PTPN1 rs6067484	0.918	0.988	0.800	1.240	0.747	0.953	0.723	1.310	0.509	0.905	0.656	1.196
FOXO3 rs12206094	IGF2R rs9456497	0.670	0.955	0.771	1.182	0.348	0.875	0.647	1.156	0.267	1.180	0.871	1.598
FOXO3 rs12206094	IL6 rs1800795	0.578	1.105	0.735	1.543	0.962	1.006	0.783	1.242	0.617	0.942	0.748	1.208
FOXO3 rs12206094	GHRHR rs2267723	0.201	1.231	0.784	1.735	0.568	1.060	0.870	1.326	0.131	0.837	0.649	1.043
FOXO3 rs12206094	CDKN2B rs4977756	0.451	0.910	0.703	1.168	0.491	0.922	0.723	1.161	0.178	1.169	0.922	1.477
FOXO3 rs12206094	CDKN2B rs1333049	0.091	0.788	0.593	1.050	0.235	0.881	0.715	1.082	**0.021**	1.292	1.042	1.642
FOXO3 rs12206094	PAPPA rs4837525	0.721	1.062	0.753	1.637	0.316	0.892	0.709	1.119	0.855	0.980	0.736	1.240
FOXO3 rs12206094	MRE11A rs533984	0.470	1.151	0.788	1.649	0.324	1.128	0.686	1.461	0.486	0.907	0.687	1.207
FOXO3 rs12206094	TXNRD1 rs17202060	0.940	0.989	0.769	1.273	0.703	1.050	0.845	1.335	0.732	1.048	0.793	1.439
FOXO3 rs12206094	SH2B3 rs3184504	0.895	1.020	0.744	1.397	0.507	0.924	0.730	1.165	0.998	0.999	0.780	1.284
FOXO3 rs12206094	KLOTHO rs1207362	0.300	1.214	0.824	1.737	0.871	0.979	0.784	1.218	0.292	0.878	0.678	1.123
FOXO3 rs12206094	IGF1R rs2229765	0.717	0.956	0.750	1.198	0.492	1.067	0.868	1.275	0.423	1.087	0.898	1.332
FOXO3 rs12206094	TP53 rs1042522	0.556	1.071	0.859	1.387	0.421	0.908	0.710	1.183	0.577	0.924	0.677	1.249
FOXO3 rs12206094	ERCC2 rs50871	0.304	1.148	0.899	1.608	0.682	1.034	0.865	1.236	0.268	0.893	0.705	1.102
FOXO3 rs12206094	PTPN1 rs6067484	0.335	1.113	0.903	1.433	0.810	0.974	0.773	1.265	0.279	0.860	0.635	1.138

FOXO3 rs2802292	IGF2R rs9456497	0.242	0.877	0.703	1.106	0.307	0.833	0.570	1.204	0.292	1.172	0.840	1.606
FOXO3 rs2802292	IL6 rs1800795	0.794	1.052	0.716	1.542	0.695	1.054	0.794	1.392	0.458	0.907	0.700	1.174
FOXO3 rs2802292	GHRHR rs2267723	0.707	1.057	0.761	1.442	0.631	1.059	0.839	1.346	0.297	0.892	0.710	1.113
FOXO3 rs2802292	CDKN2B rs4977756	0.391	0.902	0.703	1.145	0.852	0.976	0.748	1.267	0.694	1.049	0.828	1.326
FOXO3 rs2802292	CDKN2B rs1333049	0.086	0.777	0.586	1.050	0.299	0.891	0.708	1.106	0.093	1.203	0.970	1.514
FOXO3 rs2802292	PAPPA rs4837525	0.816	0.956	0.638	1.413	0.411	0.889	0.660	1.178	0.983	0.997	0.751	1.340
FOXO3 rs2802292	MRE11A rs533984	0.542	1.119	0.778	1.645	0.176	1.201	0.910	1.576	0.290	0.867	0.661	1.124
FOXO3 rs2802292	TXNRD1 rs17202060	0.487	0.916	0.698	1.183	0.692	1.054	0.771	1.425	0.856	1.025	0.763	1.355
FOXO3 rs2802292	SH2B3 rs3184504	0.834	0.969	0.728	1.311	0.711	0.949	0.712	1.269	0.776	0.966	0.736	1.231
FOXO3 rs2802292	KLOTHO rs1207362	0.534	1.116	0.737	1.644	0.898	1.016	0.779	1.362	0.332	0.882	0.683	1.179
FOXO3 rs2802292	IGF1R rs2229765	0.371	0.886	0.661	1.213	0.834	1.025	0.821	1.296	0.380	1.105	0.874	1.404
FOXO3 rs2802292	TP53 rs1042522	0.724	0.960	0.757	1.231	0.452	0.899	0.664	1.226	0.803	0.963	0.700	1.290
FOXO3 rs2802292	ERCC2 rs50871	0.091	1.290	0.973	1.758	0.083	1.221	0.975	1.557	**0.013**	0.750	0.584	0.940
FOXO3 rs2802292	PTPN1 rs6067484	0.806	0.970	0.777	1.218	0.757	0.952	0.705	1.339	0.641	0.933	0.687	1.306
FOXO3 rs2764264	IGF2R rs9456497	0.406	0.918	0.754	1.135	0.345	0.867	0.643	1.163	0.398	1.148	0.816	1.594
FOXO3 rs2764264	IL6 rs1800795	0.654	1.085	0.769	1.542	0.667	1.053	0.825	1.335	0.430	0.911	0.706	1.145
FOXO3 rs2764264	GHRHR rs2267723	0.596	0.909	0.623	1.314	0.498	0.926	0.734	1.129	0.708	1.045	0.822	1.351
FOXO3 rs2764264	CDKN2B rs4977756	0.258	0.867	0.676	1.107	0.508	0.925	0.728	1.169	0.217	1.156	0.911	1.467
FOXO3 rs2764264	CDKN2B rs1333049	0.066	0.760	0.567	1.029	0.255	0.882	0.699	1.095	**0.049**	1.266	1.005	1.624
FOXO3 rs2764264	PAPPA rs4837525	0.695	1.076	0.770	1.644	0.473	0.924	0.742	1.179	0.567	0.929	0.705	1.178
FOXO3 rs2764264	MRE11A rs533984	0.856	1.040	0.701	1.548	0.383	1.112	0.867	1.443	0.677	0.945	0.725	1.245
FOXO3 rs2764264	TXNRD1 rs17202060	0.950	0.991	0.756	1.265	0.252	1.163	0.899	1.553	0.729	0.949	0.694	1.274
FOXO3 rs2764264	SH2B3 rs3184504	0.743	0.954	0.712	1.281	0.284	0.886	0.700	1.110	0.894	1.015	0.789	1.297
FOXO3 rs2764264	KLOTHO rs1207362	0.854	1.037	0.674	1.530	0.750	0.964	0.759	1.213	0.666	0.947	0.723	1.242
FOXO3 rs2764264	IGF1R rs2229765	0.498	0.906	0.682	1.170	0.590	1.055	0.865	1.306	0.415	1.096	0.882	1.377
FOXO3 rs2764264	TP53 rs1042522	0.899	0.986	0.784	1.228	0.249	0.854	0.652	1.101	0.953	0.992	0.712	1.357
FOXO3 rs2764264	ERCC2 rs50871	0.594	1.074	0.825	1.464	0.765	1.031	0.861	1.266	0.355	0.904	0.711	1.112
FOXO3 rs2764264	PTPN1 rs6067484	0.472	0.917	0.725	1.164	0.148	0.838	0.651	1.074	0.460	1.101	0.850	1.471
FOXO3 rs10457180	IGF2R rs9456497	0.466	0.924	0.752	1.158	0.402	0.884	0.652	1.179	0.411	1.136	0.820	1.545
FOXO3 rs10457180	IL6 rs1800795	0.556	1.105	0.780	1.496	0.709	1.042	0.829	1.285	0.387	0.899	0.709	1.149
FOXO3 rs10457180	GHRHR rs2267723	0.545	0.902	0.616	1.264	0.540	0.934	0.750	1.149	0.642	1.058	0.852	1.363
FOXO3 rs10457180	CDKN2B rs4977756	0.326	0.882	0.692	1.139	0.534	0.929	0.740	1.174	0.264	1.141	0.906	1.423
FOXO3 rs10457180	CDKN2B rs1333049	0.082	0.778	0.592	1.061	0.296	0.893	0.728	1.111	0.056	1.245	0.980	1.560
FOXO3 rs10457180	PAPPA rs4837525	0.596	1.099	0.771	1.636	0.538	0.939	0.761	1.171	0.484	0.913	0.703	1.160
FOXO3 rs10457180	MRE11A rs533984	0.827	1.043	0.703	1.496	0.395	1.103	0.858	1.395	0.680	0.949	0.726	1.249
FOXO3 rs10457180	TXNRD1 rs17202060	0.890	1.018	0.812	1.288	0.289	1.143	0.878	1.489	0.548	0.916	0.672	1.276
FOXO3 rs10457180	SH2B3 rs3184504	0.630	0.928	0.690	1.246	0.328	0.890	0.706	1.157	0.708	1.049	0.794	1.353
FOXO3 rs10457180	KLOTHO rs1207362	0.547	1.117	0.761	1.744	0.850	0.977	0.777	1.232	0.407	0.900	0.668	1.166
FOXO3 rs10457180	IGF1R rs2229765	0.536	0.919	0.693	1.184	0.557	1.054	0.871	1.284	0.416	1.097	0.882	1.342
FOXO3 rs10457180	TP53 rs1042522	0.751	0.964	0.780	1.226	0.216	0.852	0.657	1.103	0.837	1.035	0.751	1.405
FOXO3 rs10457180	ERCC2 rs50871	0.620	1.073	0.837	1.470	0.802	1.024	0.833	1.234	0.404	0.907	0.715	1.119

FOXO3 rs10457180	PTPN1 rs6067484	0.420	0.913	0.710	1.175	0.135	0.834	0.665	1.100	0.446	1.124	0.839	1.559
FOXO3 rs13217795	IGF2R rs9456497	0.387	0.914	0.731	1.139	0.493	0.900	0.640	1.208	0.496	1.120	0.807	1.608
FOXO3 rs13217795	IL6 rs1800795	0.709	1.073	0.782	1.502	0.744	1.035	0.816	1.296	0.431	0.908	0.704	1.142
FOXO3 rs13217795	GHRHR rs2267723	0.562	0.900	0.619	1.266	0.563	0.943	0.747	1.148	0.716	1.045	0.827	1.349
FOXO3 rs13217795	CDKN2B rs4977756	0.180	0.848	0.651	1.111	0.470	0.917	0.728	1.155	0.175	1.169	0.913	1.484
FOXO3 rs13217795	CDKN2B rs1333049	**0.038**	0.742	0.555	1.012	0.225	0.880	0.705	1.095	**0.043**	1.279	1.021	1.649
FOXO3 rs13217795	PAPPA rs4837525	0.552	1.119	0.781	1.699	0.770	0.966	0.784	1.223	0.347	0.888	0.665	1.138
FOXO3 rs13217795	MRE11A rs533984	0.838	1.036	0.696	1.484	0.367	1.112	0.890	1.418	0.618	0.939	0.731	1.247
FOXO3 rs13217795	TXNRD1 rs17202060	0.979	0.996	0.760	1.289	0.356	1.131	0.882	1.490	0.631	0.932	0.686	1.260
FOXO3 rs13217795	SH2B3 rs3184504	0.485	0.899	0.660	1.194	0.254	0.876	0.698	1.111	0.648	1.063	0.829	1.344
FOXO3 rs13217795	KLOTHO rs1207362	0.737	1.071	0.725	1.614	0.770	0.968	0.757	1.206	0.494	0.916	0.713	1.200
FOXO3 rs13217795	IGF1R rs2229765	0.469	0.905	0.690	1.195	0.538	1.062	0.872	1.281	0.448	1.091	0.875	1.351
FOXO3 rs13217795	TP53 rs1042522	0.631	0.946	0.744	1.184	0.183	0.842	0.654	1.120	0.782	1.039	0.758	1.413
FOXO3 rs13217795	ERCC2 rs50871	0.765	1.044	0.765	1.380	0.880	1.017	0.834	1.219	0.431	0.916	0.736	1.138
FOXO3 rs13217795	PTPN1 rs6067484	0.426	0.905	0.696	1.147	0.176	0.846	0.664	1.105	0.505	1.098	0.821	1.489
FOXO3 rs4946935	IGF2R rs9456497	0.603	0.948	0.776	1.153	0.250	0.842	0.620	1.129	0.153	1.238	0.932	1.672
FOXO3 rs4946935	IL6 rs1800795	0.262	1.191	0.862	1.623	0.702	1.040	0.835	1.276	0.281	0.891	0.725	1.097
FOXO3 rs4946935	GHRHR rs2267723	0.669	1.074	0.774	1.486	0.945	0.992	0.806	1.220	0.688	0.954	0.766	1.191
FOXO3 rs4946935	CDKN2B rs4977756	0.278	0.880	0.689	1.120	0.322	0.896	0.723	1.127	0.063	1.226	0.974	1.534
FOXO3 rs4946935	CDKN2B rs1333049	0.090	0.784	0.581	1.048	0.215	0.880	0.708	1.076	**0.009**	1.306	1.066	1.654
FOXO3 rs4946935	PAPPA rs4837525	0.481	1.152	0.791	1.788	0.449	0.922	0.723	1.155	0.583	0.927	0.696	1.201
FOXO3 rs4946935	MRE11A rs533984	0.999	1.000	0.671	1.411	0.692	1.048	0.822	1.305	0.846	1.027	0.797	1.376
FOXO3 rs4946935	TXNRD1 rs17202060	0.755	1.042	0.815	1.320	0.466	1.087	0.862	1.392	0.904	0.985	0.755	1.310
FOXO3 rs4946935	SH2B3 rs3184504	0.999	1.000	0.749	1.346	0.348	0.903	0.732	1.131	0.781	1.030	0.803	1.283
FOXO3 rs4946935	KLOTHO rs1207362	0.734	1.066	0.717	1.556	0.494	0.929	0.738	1.158	0.827	0.972	0.757	1.279
FOXO3 rs4946935	IGF1R rs2229765	0.732	0.958	0.732	1.228	0.592	1.052	0.874	1.266	0.282	1.121	0.907	1.428
FOXO3 rs4946935	TP53 rs1042522	0.595	1.067	0.852	1.394	0.267	0.886	0.712	1.097	0.759	0.958	0.696	1.319
FOXO3 rs4946935	ERCC2 rs50871	0.498	1.101	0.852	1.492	0.983	1.002	0.843	1.198	0.532	0.938	0.755	1.171
FOXO3 rs4946935	PTPN1 rs6067484	0.739	1.037	0.822	1.347	0.366	0.895	0.706	1.154	0.992	0.998	0.759	1.319
IGF2R rs9456497	IL6 rs1800795	0.345	0.838	0.560	1.155	0.408	0.910	0.718	1.115	0.318	1.154	0.890	1.579
IGF2R rs9456497	GHRHR rs2267723	0.886	1.033	0.647	1.575	0.940	0.992	0.815	1.221	0.727	0.949	0.706	1.290
IGF2R rs9456497	CDKN2B rs4977756	0.997	1.001	0.731	1.372	0.757	1.033	0.852	1.270	0.776	0.963	0.706	1.287
IGF2R rs9456497	CDKN2B rs1333049	0.087	0.699	0.447	1.066	0.531	0.945	0.788	1.137	0.067	1.331	0.973	1.855
IGF2R rs9456497	PAPPA rs4837525	0.619	0.907	0.577	1.280	0.177	0.864	0.691	1.081	0.792	1.037	0.784	1.419
IGF2R rs9456497	MRE11A rs533984	0.534	0.887	0.561	1.296	0.792	1.031	0.811	1.306	0.595	1.081	0.804	1.531
IGF2R rs9456497	TXNRD1 rs17202060	0.787	0.957	0.660	1.324	0.540	1.077	0.860	1.397	0.996	0.999	0.733	1.351
IGF2R rs9456497	SH2B3 rs3184504	0.455	0.879	0.628	1.252	0.245	0.889	0.712	1.089	0.442	1.106	0.838	1.439
IGF2R rs9456497	KLOTHO rs1207362	0.072	0.696	0.443	1.049	0.056	0.818	0.666	1.018	0.075	1.271	0.969	1.723
IGF2R rs9456497	IGF1R rs2229765	0.362	1.146	0.831	1.578	0.054	1.217	0.985	1.496	0.078	0.800	0.614	1.031
IGF2R rs9456497	TP53 rs1042522	0.737	0.957	0.717	1.237	0.183	0.857	0.678	1.082	0.810	1.041	0.737	1.543
IGF2R rs9456497	ERCC2 rs50871	0.731	0.934	0.624	1.358	0.661	0.957	0.785	1.168	0.819	1.032	0.787	1.370

IGF2R rs9456497	PTPN1 rs6067484	0.160	0.809	0.583	1.093	0.053	0.806	0.641	1.006	0.119	1.348	0.947	2.054
IL6 rs1800795	GHRHR rs2267723	0.069	0.767	0.571	1.024	**0.037**	0.752	0.568	0.988	**0.038**	1.246	1.000	1.537
IL6 rs1800795	CDKN2B rs4977756	0.312	0.877	0.664	1.147	0.511	0.904	0.674	1.228	0.298	1.115	0.903	1.385
IL6 rs1800795	CDKN2B rs1333049	0.730	0.951	0.698	1.273	0.887	1.022	0.757	1.377	0.882	1.018	0.816	1.270
IL6 rs1800795	PAPPA rs4837525	0.221	0.800	0.543	1.095	0.072	0.745	0.526	1.037	0.209	1.172	0.922	1.525
IL6 rs1800795	MRE11A rs533984	0.292	0.834	0.571	1.142	0.653	0.935	0.674	1.254	0.289	1.130	0.897	1.455
IL6 rs1800795	TXNRD1 rs17202060	0.581	0.936	0.740	1.198	0.941	1.013	0.742	1.495	0.645	1.057	0.812	1.343
IL6 rs1800795	SH2B3 rs3184504	0.894	1.019	0.761	1.365	0.825	0.971	0.735	1.300	0.707	0.958	0.752	1.191
IL6 rs1800795	KLOTHO rs1207362	0.381	0.877	0.621	1.177	0.210	0.836	0.605	1.119	0.501	1.075	0.862	1.358
IL6 rs1800795	IGF1R rs2229765	0.661	1.058	0.829	1.385	0.134	1.224	0.946	1.637	0.429	0.923	0.747	1.135
IL6 rs1800795	TP53 rs1042522	0.469	0.924	0.730	1.145	0.158	0.791	0.565	1.097	0.477	1.099	0.856	1.480
IL6 rs1800795	ERCC2 rs50871	0.602	0.943	0.748	1.209	0.592	0.933	0.720	1.234	0.754	1.026	0.835	1.235
IL6 rs1800795	PTPN1 rs6067484	0.859	1.020	0.826	1.273	0.988	0.996	0.691	1.474	0.474	0.907	0.686	1.219
GHRHR rs2267723	CDKN2B rs4977756	0.309	0.870	0.654	1.127	0.554	0.894	0.616	1.311	0.351	1.128	0.863	1.446
GHRHR rs2267723	CDKN2B rs1333049	0.937	0.989	0.760	1.311	0.661	1.068	0.799	1.507	0.831	0.977	0.772	1.192
GHRHR rs2267723	PAPPA rs4837525	0.444	1.135	0.811	1.543	0.991	1.002	0.696	1.419	0.337	0.888	0.705	1.134
GHRHR rs2267723	MRE11A rs533984	0.405	0.865	0.584	1.246	0.818	0.959	0.628	1.394	0.432	1.100	0.851	1.459
GHRHR rs2267723	TXNRD1 rs17202060	0.358	1.108	0.881	1.397	0.149	1.360	0.931	2.197	0.162	0.809	0.583	1.060
GHRHR rs2267723	SH2B3 rs3184504	0.570	0.923	0.672	1.241	0.420	0.876	0.608	1.217	0.683	1.048	0.835	1.328
GHRHR rs2267723	KLOTHO rs1207362	0.888	1.021	0.726	1.474	0.803	0.956	0.652	1.430	0.745	0.964	0.747	1.237
GHRHR rs2267723	IGF1R rs2229765	0.415	0.908	0.705	1.145	0.766	1.049	0.760	1.392	0.556	1.055	0.880	1.296
GHRHR rs2267723	TP53 rs1042522	0.808	1.025	0.825	1.290	0.989	0.997	0.654	1.499	0.403	0.885	0.657	1.170
GHRHR rs2267723	ERCC2 rs50871	0.876	1.026	0.764	1.369	0.813	1.031	0.768	1.370	0.585	0.947	0.762	1.169
GHRHR rs2267723	PTPN1 rs6067484	0.908	0.989	0.783	1.240	0.611	0.903	0.594	1.334	0.950	0.990	0.760	1.317
CDKN2B rs4977756	PAPPA rs4837525	0.333	1.186	0.850	1.694	0.901	0.983	0.757	1.284	0.308	0.885	0.692	1.132
CDKN2B rs4977756	MRE11A rs533984	0.332	1.229	0.856	1.980	0.146	1.209	0.940	1.608	0.291	0.854	0.613	1.115
CDKN2B rs4977756	TXNRD1 rs17202060	0.895	0.984	0.760	1.327	0.866	1.025	0.745	1.402	0.628	1.058	0.827	1.358
CDKN2B rs4977756	SH2B3 rs3184504	0.750	0.950	0.690	1.292	0.302	0.868	0.652	1.115	0.619	1.074	0.831	1.448
CDKN2B rs4977756	KLOTHO rs1207362	0.908	1.020	0.739	1.443	0.507	0.918	0.696	1.203	0.951	0.994	0.776	1.266
CDKN2B rs4977756	IGF1R rs2229765	0.716	0.947	0.718	1.279	0.797	1.029	0.826	1.276	0.306	1.128	0.875	1.425
CDKN2B rs4977756	TP53 rs1042522	0.103	0.819	0.643	1.054	**0.003**	0.629	0.465	0.811	**0.003**	1.512	1.135	2.119
CDKN2B rs4977756	ERCC2 rs50871	0.507	0.918	0.698	1.179	0.321	0.900	0.731	1.102	0.299	1.099	0.920	1.332
CDKN2B rs4977756	PTPN1 rs6067484	0.786	1.032	0.824	1.313	0.435	0.901	0.672	1.209	0.910	0.986	0.736	1.328
CDKN2B rs1333049	PAPPA rs4837525	0.957	0.921	0.803	1.276	0.173	0.808	0.744	1.065	0.658	1.093	0.902	1.168
CDKN2B rs1333049	MRE11A rs533984	0.626	1.083	0.768	1.551	0.537	1.109	0.808	1.527	0.787	0.967	0.759	1.231
CDKN2B rs1333049	TXNRD1 rs17202060	0.874	0.982	0.780	1.234	0.897	0.977	0.708	1.429	0.489	1.085	0.851	1.384
CDKN2B rs1333049	SH2B3 rs3184504	0.503	1.105	0.819	1.474	0.890	0.975	0.712	1.298	0.669	0.943	0.728	1.230
CDKN2B rs1333049	KLOTHO rs1207362	0.424	1.140	0.837	1.608	0.916	0.981	0.743	1.314	0.539	0.932	0.739	1.157
CDKN2B rs1333049	IGF1R rs2229765	0.838	1.027	0.808	1.326	0.481	1.088	0.851	1.395	0.812	1.024	0.848	1.230
CDKN2B rs1333049	TP53 rs1042522	0.222	0.881	0.720	1.082	**0.010**	0.654	0.465	0.897	**0.025**	1.336	1.030	1.738
CDKN2B rs1333049	ERCC2 rs50871	0.493	1.086	0.854	1.391	0.870	1.019	0.796	1.279	0.616	0.954	0.799	1.150

CDKN2B rs1333049	PTPN1 rs6067484	0.877	0.985	0.815	1.213	0.150	0.808	0.595	1.094	0.374	1.113	0.861	1.436
PAPPA rs4837525	MRE11A rs533984	0.140	0.738	0.491	1.151	0.644	0.926	0.655	1.376	0.321	1.135	0.862	1.476
PAPPA rs4837525	TXNRD1 rs17202060	0.593	0.928	0.705	1.269	0.440	1.194	0.743	1.984	0.679	0.931	0.663	1.323
PAPPA rs4837525	SH2B3 rs3184504	0.848	1.026	0.776	1.373	0.512	1.114	0.831	1.467	0.165	0.856	0.681	1.060
PAPPA rs4837525	KLOTHO rs1207362	0.636	0.918	0.628	1.354	0.807	0.944	0.745	1.252	0.807	0.972	0.745	1.252
PAPPA rs4837525	IGF1R rs2229765	0.433	0.898	0.672	1.198	0.364	1.152	0.830	1.571	0.827	0.976	0.783	1.231
PAPPA rs4837525	TP53 rs1042522	0.635	0.940	0.743	1.201	0.960	1.010	0.700	1.504	0.287	0.877	0.664	1.120
PAPPA rs4837525	ERCC2 rs50871	0.650	0.930	0.674	1.290	0.854	1.029	0.756	1.430	0.679	0.953	0.749	1.184
PAPPA rs4837525	PTPN1 rs6067484	0.379	0.902	0.727	1.160	0.774	0.946	0.631	1.397	0.780	0.962	0.734	1.278
MRE11A rs533984	TXNRD1 rs17202060	0.349	1.124	0.889	1.492	0.357	1.222	0.793	1.966	0.473	0.899	0.651	1.202
MRE11A rs533984	SH2B3 rs3184504	0.223	0.819	0.573	1.132	**0.038**	0.688	0.463	0.973	0.053	1.283	0.998	1.590
MRE11A rs533984	KLOTHO rs1207362	0.985	1.004	0.658	1.527	0.394	0.866	0.618	1.236	0.774	1.043	0.796	1.391
MRE11A rs533984	IGF1R rs2229765	0.562	1.100	0.809	1.504	0.306	1.177	0.845	1.677	0.690	0.955	0.749	1.212
MRE11A rs533984	TP53 rs1042522	0.306	1.137	0.891	1.523	0.940	1.016	0.658	1.507	0.373	0.879	0.667	1.194
MRE11A rs533984	ERCC2 rs50871	0.193	1.207	0.885	1.642	0.486	1.113	0.807	1.477	0.306	0.890	0.709	1.138
MRE11A rs533984	PTPN1 rs6067484	0.202	1.162	0.922	1.439	0.603	1.109	0.760	1.707	0.243	0.828	0.594	1.146
TXNRD1 rs17202060	SH2B3 rs3184504	0.689	1.066	0.803	1.571	0.464	0.919	0.741	1.155	0.938	1.011	0.736	1.274
TXNRD1 rs17202060	KLOTHO rs1207362	0.973	0.993	0.690	1.458	0.254	0.868	0.670	1.114	0.662	1.059	0.834	1.387
TXNRD1 rs17202060	IGF1R rs2229765	0.101	1.266	0.963	1.723	0.059	1.230	0.982	1.531	0.168	0.857	0.679	1.076
TXNRD1 rs17202060	TP53 rs1042522	0.656	1.052	0.825	1.411	0.221	0.829	0.604	1.088	0.669	1.064	0.790	1.439
TXNRD1 rs17202060	ERCC2 rs50871	0.575	1.114	0.806	1.685	0.974	0.995	0.811	1.260	0.838	0.968	0.717	1.226
TXNRD1 rs17202060	PTPN1 rs6067484	0.774	0.967	0.772	1.271	0.138	0.814	0.631	1.087	0.266	1.206	0.868	1.707
SH2B3 rs3184504	KLOTHO rs1207362	0.655	1.084	0.757	1.560	0.965	1.006	0.773	1.320	0.353	0.890	0.694	1.155
SH2B3 rs3184504	IGF1R rs2229765	0.196	0.851	0.656	1.087	0.682	1.047	0.838	1.327	0.496	1.068	0.882	1.278
SH2B3 rs3184504	TP53 rs1042522	0.131	0.846	0.664	1.058	**0.036**	0.718	0.497	0.972	0.104	1.222	0.954	1.626
SH2B3 rs3184504	ERCC2 rs50871	0.599	1.079	0.825	1.480	0.358	1.126	0.878	1.445	0.168	0.863	0.689	1.050
SH2B3 rs3184504	PTPN1 rs6067484	0.796	0.975	0.800	1.189	0.952	0.991	0.755	1.377	0.390	0.890	0.669	1.140
KLOTHO rs1207362	IGF1R rs2229765	0.070	0.789	0.605	1.043	0.349	0.854	0.595	1.189	0.095	1.210	0.965	1.523
KLOTHO rs1207362	TP53 rs1042522	0.053	0.798	0.626	1.010	**0.021**	0.583	0.363	0.931	0.066	1.319	0.969	1.777
KLOTHO rs1207362	ERCC2 rs50871	0.167	0.828	0.629	1.094	0.310	0.855	0.633	1.156	0.419	1.099	0.880	1.365
KLOTHO rs1207362	PTPN1 rs6067484	0.203	0.871	0.685	1.059	0.231	0.802	0.529	1.172	0.563	1.076	0.828	1.473
IGF1R rs2229765	TP53 rs1042522	0.197	1.134	0.928	1.385	0.343	0.865	0.617	1.145	0.811	0.976	0.791	1.236
IGF1R rs2229765	ERCC2 rs50871	0.751	0.963	0.756	1.239	0.294	0.877	0.689	1.132	0.151	1.140	0.938	1.369
IGF1R rs2229765	PTPN1 rs6067484	0.058	1.138	0.980	1.478	0.841	0.924	0.708	1.476	0.437	0.899	0.665	1.160
TP53 rs1042522	ERCC2 rs50871	0.304	0.853	0.598	1.170	0.706	0.965	0.788	1.179	0.872	1.019	0.815	1.310
TP53 rs1042522	PTPN1 rs6067484	0.227	0.863	0.676	1.093	0.384	0.883	0.674	1.171	0.915	1.018	0.745	1.426
ERCC2 rs50871	PTPN1 rs6067484	0.946	0.995	0.846	1.174	0.651	0.927	0.663	1.338	0.822	0.972	0.763	1.219

Supplementary Table 3. The full model of nine health-related variables tested by Cox regression analysis for their effect on survival above 85 years of age.

	p-value	HR	95% CI for HR	
			Lower	Upper
Gender (men)	0.198	0.816	0.585	1.102
Body weight by sex-specific 4th quartile (men = 87.3+ kg; women = 72.6+ kg)	**0.001**	0.542	0.405	0.750
Left heel bone mineral density (T-values: > -1.0 OR < -2.4)	**0.003**	0.691	0.510	0.884
Folates in serum by median (<=18.1 nmol/L)	**0.011**	0.565	0.342	0.863
Self-rated nutritional status (mildly or severely malnourished)	**0.013**	0.678	0.426	0.893
Number of medicaments taken daily (5+)	**0.030**	0.732	0.528	0.960
Number of hospital admissions in the past year (2 or more)	**0.028**	0.648	0.505	0.973
Regularly taking supplementary vitamin B complex (No)	**0.003**	0.541	0.346	0.815
Age of the oldest living sibling (<80)	**0.002**	0.533	0.290	0.701
Mother's age at death (<80)	**0.033**	0.718	0.582	0.979
Model (overall)	**3.14E-09**			

Supplementary Table 4. The full model of all interactions tested in a Cox regression survival analysis with gender and nine significant health-related variables.

		Interaction	Gender (men)	Body weight by sex-specific 4th quartile (men = 87.3+ kg; women = 72.6+ kg)	Left heel bone mineral density (T-values: > -1.0 OR < -2.4)	Folates in serum by median (<=18.1 nmol/L)	Self-rated nutritional status (mildly or severely malnourished)	Number of medicaments taken daily (5+)	Number of hospital admissions in the past year (2 or more)	Regularly taking supplementary vitamin B complex (No)	Age of the oldest living sibling (<80)	Mother's age at death (<80)	Model (overall)
***CDKN2B* rs4977756 x *TP53* rs1042522**													
	p-value	**0.002**	0.311	**0.001**	**0.004**	**0.023**	**0.003**	**0.030**	0.069	**0.001**	**0.005**	**0.029**	**1.49E-10**
	HR	1.708	0.849	0.537	0.666	0.581	0.580	0.705	0.716	0.493	0.512	0.744	
95% CI for HR	**Lower**	1.230	0.580	0.383	0.495	0.344	0.389	0.500	0.489	0.309	0.308	0.555	
	Upper	2.404	1.159	0.736	0.843	0.903	0.833	0.943	1.019	0.760	0.780	0.981	
***IL6* rs1800795 x *GHRHR* rs2267723**													
	p-value	**0.005**	0.146	**0.002**	**0.008**	**0.001**	**0.008**	**0.019**	**0.014**	**0.001**	**0.003**	**0.017**	**4.09E-10**
	HR	1.444	0.791	0.537	0.700	0.472	0.590	0.695	0.679	0.444	0.482	0.719	
95% CI for HR	**Lower**	1.131	0.560	0.385	0.516	0.282	0.398	0.497	0.489	0.266	0.293	0.536	
	Upper	1.863	1.077	0.732	0.905	0.779	0.826	0.950	0.944	0.697	0.719	0.935	
***TERC* rs16847897 x *SH2B3* rs3184504**													
	p-value	**0.007**	0.078	**0.001**	**0.007**	**0.019**	**0.025**	0.114	**0.037**	**0.001**	**0.003**	**0.018**	**6.77E-10**
	HR	0.677	0.770	0.546	0.675	0.545	0.647	0.772	0.699	0.538	0.521	0.720	
95% CI for HR	**Lower**	0.492	0.561	0.374	0.516	0.315	0.429	0.530	0.494	0.342	0.309	0.534	
	Upper	0.916	1.025	0.733	0.869	0.896	0.918	1.056	0.995	0.798	0.759	0.936	
***CDKN2B* rs1333049 x *TP53* rs1042522**													
	p-value	**0.013**	0.182	**0.002**	**0.002**	**0.009**	**0.003**	**0.039**	**0.050**	**0.001**	**0.005**	**0.036**	**2.12E-09**
	HR	1.428	0.816	0.553	0.658	0.547	0.605	0.720	0.712	0.496	0.515	0.753	
95% CI for HR	**Lower**	1.036	0.584	0.383	0.498	0.324	0.415	0.507	0.496	0.314	0.313	0.574	
	Upper	1.886	1.105	0.762	0.850	0.877	0.854	0.953	1.019	0.782	0.776	0.993	
***LINC02227* rs2149954 x *CDKN2B* rs1333049**													
	p-value	**0.015**	0.292	**0.003**	**0.001**	**0.011**	**0.007**	**0.013**	**0.040**	**0.001**	**0.005**	**0.049**	**1.17E-09**
	HR	0.732	0.851	0.568	0.647	0.535	0.634	0.676	0.713	0.513	0.500	0.775	
95% CI for HR	**Lower**	0.563	0.605	0.391	0.480	0.313	0.439	0.473	0.502	0.326	0.307	0.589	
	Upper	0.953	1.145	0.768	0.834	0.877	0.875	0.897	0.992	0.802	0.732	1.005	
***PARK7* rs225119 x *LINC02227* rs2149954**													
	p-value	**0.016**	0.082	**0.001**	**0.005**	**0.018**	**0.018**	**0.040**	0.074	**0.003**	**0.001**	**0.029**	**2.55E-09**
	HR	0.756	0.779	0.560	0.668	0.557	0.625	0.732	0.731	0.536	0.468	0.742	
	Lower	0.596	0.567	0.391	0.505	0.318	0.417	0.514	0.503	0.335	0.286	0.548	

95% CI for HR	**Upper**	0.972	1.020	0.739	0.855	0.910	0.890	0.975	1.036	0.809	0.674	0.959	
***FOXO3* rs4946935 x *CDKN2B* rs1333049**													
	p-value	**0.035**	0.248	**0.001**	**0.003**	**0.014**	**0.023**	**0.017**	0.095	**0.002**	**0.003**	0.056	**4.92E-09**
	HR	1.277	0.835	0.560	0.670	0.537	0.663	0.696	0.743	0.499	0.483	0.779	
95% CI for HR	**Lower**	1.011	0.581	0.387	0.496	0.306	0.449	0.487	0.512	0.317	0.311	0.598	
	Upper	1.646	1.116	0.765	0.849	0.875	0.918	0.945	1.054	0.805	0.703	1.014	
***FOXO3* rs12206094 x *CDKN2B* rs1333049**													
	p-value	**0.044**	0.238	**0.001**	**0.006**	**0.023**	**0.019**	**0.017**	0.102	**0.002**	**0.001**	0.063	**4.45E-09**
	HR	1.273	0.827	0.562	0.670	0.545	0.648	0.695	0.745	0.500	0.473	0.783	
95% CI for HR	**Lower**	1.015	0.590	0.383	0.507	0.313	0.432	0.485	0.522	0.323	0.294	0.591	
	Upper	1.682	1.116	0.763	0.858	0.901	0.925	0.937	1.079	0.808	0.700	1.015	
***FOXO3A* rs2802292 x *ERCC2* rs50871**													
	p-value	**0.047**	0.183	**0.001**	**0.002**	**0.020**	**0.007**	**0.034**	**0.039**	**0.006**	**0.004**	**0.031**	**1.68E-09**
	HR	0.784	0.800	0.548	0.681	0.588	0.618	0.728	0.696	0.561	0.494	0.738	
95% CI for HR	**Lower**	0.591	0.543	0.380	0.511	0.349	0.420	0.521	0.484	0.358	0.312	0.542	
	Upper	0.999	1.109	0.735	0.879	0.884	0.869	0.967	0.977	0.841	0.708	0.953	
***TERC* rs12696304 x *SH2B3* rs3184504**													
	p-value	**0.049**	0.134	**0.001**	**0.004**	**0.014**	**0.014**	0.074	**0.037**	**0.001**	**0.003**	**0.034**	**1.75E-09**
	HR	0.749	0.790	0.543	0.671	0.586	0.640	0.755	0.707	0.540	0.502	0.744	
95% CI for HR	**Lower**	0.549	0.555	0.384	0.512	0.351	0.429	0.524	0.509	0.356	0.312	0.562	
	Upper	1.036	1.060	0.730	0.858	0.901	0.893	1.010	0.985	0.813	0.760	0.966	
***TERC* rs3772190 x *SH2B3* rs3184504**													
	p-value	0.062	0.101	**0.001**	**0.003**	**0.039**	**0.007**	0.072	0.058	**0.002**	**0.005**	**0.022**	**3.04E-09**
	HR	1.371	0.772	0.541	0.687	0.585	0.618	0.758	0.723	0.520	0.508	0.733	
95% CI for HR	**Lower**	0.956	0.548	0.373	0.519	0.344	0.423	0.520	0.503	0.333	0.308	0.551	
	Upper	1.912	1.036	0.733	0.885	1.016	0.879	0.982	1.014	0.764	0.764	0.956	
***PARK7* rs225119 x *PTPN1* rs6067484**													
	p-value	0.062	0.126	**0.001**	**0.003**	**0.019**	**0.013**	**0.036**	0.108	**0.005**	**0.001**	**0.050**	**2.48E-09**
	HR	1.379	0.774	0.579	0.662	0.562	0.636	0.720	0.743	0.539	0.457	0.758	
95% CI for HR	**Lower**	0.937	0.538	0.405	0.495	0.328	0.435	0.508	0.496	0.363	0.282	0.547	
	Upper	1.895	1.045	0.771	0.849	0.930	0.902	0.933	1.074	0.810	0.668	0.981	
***FOXO3* rs2764264 x *CDKN2B* rs1333049**													
	p-value	0.090	0.305	**0.001**	**0.012**	**0.016**	**0.017**	**0.020**	0.169	**0.002**	**0.003**	**0.015**	**1.62E-09**
	HR	1.238	0.852	0.500	0.729	0.507	0.639	0.689	0.784	0.469	0.516	0.728	
95% CI for HR	**Lower**	0.949	0.588	0.350	0.550	0.283	0.426	0.481	0.542	0.295	0.324	0.544	
	Upper	1.627	1.152	0.666	0.933	0.874	0.924	0.910	1.133	0.716	0.734	0.930	
***FOXO3* rs13217795 X *CDKN2B* rs1333049**													
	p-value	0.128	0.281	**0.002**	**0.002**	**0.016**	**0.022**	**0.022**	0.068	**0.003**	**0.003**	**0.023**	**1.39E-08**
	HR	1.217	0.847	0.561	0.671	0.537	0.656	0.701	0.728	0.512	0.495	0.747	

95% CI for HR	**Lower**	0.940	0.601	0.388	0.497	0.317	0.430	0.498	0.497	0.310	0.317	0.568
	Upper	1.650	1.158	0.753	0.845	0.902	0.921	0.916	1.023	0.816	0.717	0.968

3. DISCUSSION

Ageing is an intricate system of systemic changes that happen to the organism with the progression of time. It is an incredibly complex process with more than one definite cause, resulting in the gradual decline of all the functions of an organism and its death. The reasons behind the ageing process, as well as the mechanisms that drive it forward, have tried to be explained through various theories, with some being widely accepted and validated through experimental evidence. While today ageing is considered in a more holistic approach, as a network of connected processes and events, the need for a deeper understanding of its mechanics has never been greater. As the global population is ageing, more focus is put on research of factors that contribute to living both longer and healthier lives, and importance of such research is only expected to increase in the coming decades as the elderly population, for the first time in history, surpasses the young in numbers.

The end result of a successful ageing process is longevity. As a complex trait shaped by both genetic and environmental factors, longevity is somewhat hard to study. However, long-lived individuals, as examples of successful ageing, are key for gaining a better understanding of what conditions need to be met for achieving a long life. And even though genetic contribution to longevity is only moderate, the study of genetics of longevity allows us to account for at least a part of the variance in the longevity phenotype.

This thesis is, to our knowledge, the first study of the genetic background of human longevity of such magnitude in the Croatian population. By applying different statistical analyses, we aimed to explore different facets of genetic influence on the complex phenotype of longevity. In the era of the global ageing of the population, this kind of research of long-lived individuals is becoming increasingly important, as it can help to shed light on the genetic factors that contribute to successful ageing, as well as give insight in the mechanics of the ageing process by highlighting key genes that are involved in the complex regulatory network behind it.

3.1. The difference in the frequency of longevity-associated variants between the young and the oldest-old

The first aim of this study – to determine whether a difference in the frequency of genetic variants associated with longevity exists between the oldest-old sample (85+ years) and a young adult

control group (aged 20-35 years) – has been covered in the *Anthropologie* paper included in this dissertation (and the Appendices 1 and 2). In this paper, the allele and genotype data for 33 out of the 43 SNPs with association to longevity in other populations were compared between the Croatian oldest-old (N=314) and young (N=97) age groups, as well as with a sample of Roma individuals living in Croatia (N =308). Additionally, a genetic risk score (deemed genetic longevity score) was calculated for all three groups in order to determine whether genetics had a significant role in modulating the chances of achieving longevity in the Roma population. Out of 43 genotyped variants, only 33 consistently reported the same longevity effect allele in published researches, so only they were selected for the construction of our genetic longevity scores. The results for the generational comparison of all 43 individually tested SNPs are presented in the Appendices 1 and 2.

The only difference between the oldest-old and the young Croatian sample was SNP rs533984 associated with *MRE11* gene that passed the Bonferroni correction for multiple testing. The two groups differed significantly in the allele frequency of this SNP, with the G allele being more common in the long-lived sample. This allele of rs533984 has previously been associated with better late-life survival of females in a SNP interaction study by Dato et al. (2018) performed on a sample of Danish nonagenarians (Dato et al., 2018). This variant is an intronic mutation in the *MRE11* gene, and is reported in an online database Open Targets Genetics (Ghoussaini et al., 2021) to most likely impact its expression. *MRE11* encodes a nuclease that is a part of the MRN complex, a "first responder" in case of a double-strand break (Williams et al., 2007). With both exo- and endonuclease activity, MRE11 plays a vital role in the repair of double-strand breaks via homologous recombination repair pathway (HR) and non-homologous end-joining mechanism (NHEJ) (Rass et al., 2009; Shibata et al., 2014). Due to this role in DNA damage repair, the connection between this gene and ageing is not surprising. In fact, a decreased expression of MRE11, along with Ku70 form the NHEJ pathway, has been connected to ageing and cellular senescence (Ju et al., 2006). The rs533984 variant in *MRE11*, however, didn't significantly contribute to the chances of reaching longevity or survival above the age of 85 in our other analyses.

The difference in the allele frequencies between the oldest-old group and the young controls indicates that there could be certain variants that are enriched in the older population, either those that have a beneficial effect as the organism ages, or those that are protective against diseases that

might cause death at an earlier age. In recent years, however, there has been a growing body of evidence to support the former hypothesis, as long-lived individuals have been shown to carry a similar number of risk factors as their younger counterparts (Lin et al., 2021; Revelas et al., 2019). That the oldest-old and the young population in our study differed only in one SNP is surprising, though; the explanation for this might lie in the fact that our sample was very small, especially the control sample of young adults. The main factor speaking in favour of this is the fact that the two groups did not differ in frequencies of either epsilon-defining variants in *APOE* gene, which are considered as a benchmark for studies of longevity (Bürkle et al., 2015).

The main reason for this comparison of the Croatian oldest-old and the young control samples with the Roma ethnic minority group was the fact that the Roma have a much lower life expectancy than the majority populations they live amongst (The European Public Health Alliance, 2018). The Roma, present around the world, are the largest transnational ethnic minority in Europe, numbering between 10 and 12 million people (European Commission, 2020). Originating in India, they migrated to Europe across Central Asia and modern-day Turkey (Fraser, 1992; Matras, 2002). They reached the Balkan peninsula around the 14th century, where the majority of them settled (Hrvatić & Ivančić, 2000), while a smaller number continued further into Central, Western and Northern Europe. Regardless of their long presence on this continent, they continued to live their lives as isolated population groups throughout their history. They tend to follow their characteristic cultural and social practises – such as endogamy, marrying only within a certain tribe or clan – along with social marginalisation and the absence of assimilation with the surrounding populations (Chaix et al., 2004; Fraser, 1992; Gresham et al., 2001). The Roma populations have gone through multiple founder and bottleneck events, which results in specific genetic profiles of the different Roma groups today (Kalaydjieva et al., 2005; Martnez-Cruz et al., 2016). In Croatia, there are two socio-culturally and linguistically different groups of Roma: the Balkan Roma, who are the descendants of the Roma who arrived in the Balkans around the 14th century and speak dialects of the Romani chib language, and the Vlax Roma (also known as Bayash Roma), descendants of the group of Roma who between the 13th and 15th century travelled to the areas of present-day Romania and Moldavia, where they were enslaved for the next 500 years. During this time, they were not allowed to use their language, so they are today characterised by a specific archaic Romanian language – ljimb'd bayash. Of the three separate Roma groups used in this research to represent the Roma population, Roma from the Zagreb area belong to the Balkan Roma, while the Roma of Međimurje

and Baranja belong to the Vlax Roma populations (Stojanović Marković et al., 2022). Apart from the cultural isolation, the Roma populations struggle with worse living conditions than majority populations, often not having appropriate housing and hygienic living environments (Anthonj et al., 2020). They also face challenges regarding social inclusion, which is perpetuated even more by poor education and inability to find a stable job (EU-FRA. European Union Agency for Fundamental Rights, 2014), and further reflected in low socioeconomic status and limited access to healthcare. These reasons, along with a higher prevalence of chronic diseases related to several risk factors (Hubáček et al., 2020; Llanaj et al., 2020; Zajc Petranović et al., 2021; Zeljko et al., 2008; Zeljko et al., 2011), all contribute to negative health outcomes and increase mortality risk (Škarić-Jurić et al., 2007). This increased risk of mortality at all ages is the main reason for lower life expectancy of Roma, and has, along with a high fertility rate (Škarić-Jurić et al., 2007), caused the demographic pyramid of the Roma people to very much skew toward younger ages, with the percentage of elderly being much lower among Roma compared to other European populations.

Because of the genetic specificity of the Croatian Roma population (Barešić & Peričić Salihović, 2014; Martinović Klarić et al., 2009; Peričić Salihović et al., 2011), it is not surprising that, in our study, they differed from the Croatian population in almost a third of examined loci. Frequencies of 10 variants were statistically different between the Roma and the young Croatian control group, and frequencies of 13 were between the Roma and the oldest-old sample. Nonetheless, when a genetic longevity score (both unweighted and weighted) was calculated for all the SNPs not in LD, there was no difference between either of the Croatian groups and the Roma. These results indicate that, even with all the genetic differences between the two groups, the overall number of longevity-associated variants in these populations remains roughly the same, with some more frequent longevity-associated alleles compensating for the lack of others. This might be attributed to the fact that, throughout human evolutionary history, genes associated with ageing were not under selective pressure due to most individuals dying of other causes before reaching old age (Kirkwood, 2005). Thus, a random selection of variants that cumulatively had a balanced effect on the longevity phenotype – not causing premature mortality but also not necessarily contributing to longevity – was passed on to the progeny. Furthermore, it means that the higher mortality and shorter life expectancy of the Roma is rooted in other genetic and non-genetic risk factors, especially those related to cardiovascular diseases, such as diabetes, abdominal obesity and metabolic syndrome (Zajc Petranović et al., 2021).

3.2. Genetic background for longevity and extreme longevity in Croatian population

Genetic risk scores for longevity were also calculated based on multivariate logistic regression models for predicting the chances of reaching longevity (90 years) and extreme longevity (95 years). Unweighted and weighted genetic longevity scores were calculated for both age threshold ages, and their power of prediction was tested by Receiver Operater Characteristics (ROC) curve, as well as by Pearson's correlation with the participants' age at death. While all four scores could predict the outcome of reaching longevity with relatively good accuracy, the most informative score was weighted score for the survival age of 90, with the area under curve (AUC) of 0.690 (69%). The high power of prediction obtained in our study is quite remarkable – especially considering a relatively small sample size this score was calculated on. Even risk scores calculated on much larger samples achieve only modest predictability (Duncan et al., 2019a), so this speaks in favour of good selection of tested genetic loci. With the advent of GWAS, creating polygenic risk scores for predicting the genetic component to complex traits became an achievable goal, and they are increasingly being used with hopes of predicting health risks and improving time to diagnosis (Duncan et al., 2019b). However, until recently, few polygenic risk scores (PRS) have been done for longevity. Tesi et al. (2021) reported a polygenic risk score for predicting the odds of becoming a healthy centenarian which was based on 330 genetic variants that could discern centenarians from older adults, while most recently Don et al. (2024) created and tested eleven different polygenic longevity scores for predicting parental longevity based on GWAS summary data from four different studies (including the one by Tesi and collaborators) (Don et al., 2024; Tesi et al., 2021). Since these attempts proved that genetic contribution to longevity-related phenotypes can be quantified in this manner, we can expect more PRS for longevity to be calculated in the future.

The multivariate regression analyses that our longevity scores were based on were performed with the goal of determining if any of the chosen variants affect the chances for reaching these thresholds of longevity, and to explore whether the contributing variants differed between the two cut-off ages. In the first round of analyses, we univariately tested of all 43 loci, and the ones that showed moderate association ($p < 0.20$) entered multivariate testing. The resulting best model for predicting the chances of reaching the age of 90 contained nine SNPs and explained 20.5% of variance in

survival to this age, which is quite high considering the moderate overall effect genetics has on this trait (Herskind et al., 1996). Out of these nine loci, four were not significant but contributed to the quality of the model. These were *FOXO3* rs12206094, *KLOTHO* rs9536314, *ERCC2* rs50871 and *TXNRD1* rs17202060. The five loci that had a significant effect on reaching the threshold age of 90 years were intronic SNPs rs16847897 near *TERC* and rs2267723 near *GHRHR*, regulatory region variant rs1800629 near *TNF-α*, and missense variants *APOE* rs7412 and *TP53* rs1042522. The variant in *TP53* is also the only variant that was included in the best model for predicting the chances of reaching the extreme longevity threshold of 95 years, which contained five SNPs and explained 9.3% of the variance in survival to the age of 95. Another four SNPs included in the 95+ model were the intronic rs6067484 locus associated with the *PTPN1* gene and rs4837525 near the *PAPPA* gene, as well as *IRF4* rs12203592 and missense *APOE* rs429358 that were not significant but contributed to the quality of the model. Therefore, our results show that in our studied population there is some difference between the genetic variants that contribute to the chances of reaching the ages of 90 and 95. This could be caused by some of these variants having lesser effect with progressing age. Additionally, some stochastic elements whose effect increases with age cannot be ruled out completely either. The fewer SNPs entering the 95+ model also explains the decrease in the percentage of explained variance between the two models.

3.3. Genetic interactions in survival of the oldest-olds

Thirteen different SNPs associated with twelve different genes were included in the best logistic regression models presented above, and out of these twelve genes that contributed to the longevity of Croatian population, six genes were also part of genetic interactions that affected survival chances above the age of 85. Those six genes are *TERC, TP53, FOXO3, GHRHR, ERCC2* and *PTPN1*.

While all the SNPs were also tested univariately for the effect on survival in advanced old age, only SNP-SNP interaction analyses gave significant results. We theorise that the effect of the individual SNPs was possibly too weak to be detected when examining the impact of a sole SNP on survival, again possibly due to a small sample size. However, analysing the interactions between genetic variants could be more informative than focusing on individual SNPs (Gerke et al., 2009) just as predictive power of the model increases if multiple SNPs are entered (Van Den Broeck et

al., 2014). The analysis of SNP-SNP interactions for studies of longevity was validated by Dato et al. (2018), who investigated the interactions that impacted longevity in a large sample of Danish nonagenarians (Dato et al., 2018).

In our sample, the significant interactions pairs were *TERC* and *SH2B3, FOXO3* and *ERCC2, GHRHR* and *IL6, PTPN1* and *PARK7, PARK7* and *LINC02227,* while the most important interaction partner was *CDKN2B* that interacted significantly with *TP53, FOXO3* and *LINC02227,* and was involved in half of the significant interactions.

Located on chromosome 3, *TERC* gene has a vital role in telomere maintenance. It encodes the RNA component of the ribonucleoprotein telomerase and serves as a template for telomere elongation (Blackburn & Collins, 2011). It is not expressed in most human cells (Blackburn et al., 2015), but is expressed in stem cells (Collins & Mitchell, 2002; Wright et al., 1996) and often in cancer cells (Hahn et al., 1999), as elongation of telomeres is necessary in all cells that continuously divide. All three of the variants near *TERC* that have been chosen for this study – rs16847897, rs12696304 and rs3772190 – have been associated with leukocyte telomere length (Codd et al., 2010; Shen et al., 2011; Soerensen et al., 2012b), which is a phenotype that has been connected to many age-related diseases, including CVD (Aviv, 2012; Jeanclos et al., 1998; Panossian et al., 2003; Rossiello et al., 2022). In our sample, aside from a significant effect on the chances for reaching 90 years of age, the G allele of rs16847897 was associated with better subjective health, functional ability, and scores on the validated Mini Mental State Examination (MMSE) test (Šetinc et al., 2023). All three *TERC* variants had a significant interaction with missense rs3184504 in the *SH2B3* that affected survival above 85 years of age, with the most significant interaction being between rs16847897 and rs3184504. While the connection of all three *TERC* SNPs to *SH2B3* could be due to moderate linkage within the *TERC* gene, it still confirmed a synergistic effect these two genes have on the target phenotype. The *SH2B3* gene encodes SH2B adaptor protein 3 (also known as LNK, lymphocyte adaptor protein) that is a suppressor of inflammatory cytokine signalling and haematopoiesis (Devallire & Charreau, 2011; Tong et al., 2005). This variant, predicted to disrupt the subcellular localisation and functioning of LNK (Dale & Madhur, 2016), has been associated with exceptional human longevity and parental age at death (Fortney et al., 2015; Pilling et al., 2016), as have other variants in the surrounding genomic region (Joshi et al., 2017; Kuo et al., 2020; Pilling et al., 2017; Timmers et al., 2019). However, it has also been reported as a top

association signal for hypertension in GWAS (Ehret et al., 2011; Levy et al., 2009), and is linked to cardiovascular and autoimmune disorders (Devallire & Charreau, 2011; Laroumanie et al., 2018). It is this implication in cardiovascular disorders that could be the connecting link between *TERC* and *SH2B3*. With CVD being the most prevalent chronic conditions in old age – with the incidence increasing to almost 82% among the people over 80 years of age (Lye & Donnellan, 2000; Yazdanyar & Newman, 2009) – the joint effect of protective alleles on these two loci perhaps lessens the risk for development of CVD.

The other locus from our study that has been strongly implicated in CVD risk is *CDKN2B*, located in the 9p21.3 chromosomic region (Burton et al., 2007; Helgadottir et al., 2007; McPherson et al., 2007). This region spans two genes, *CDKN2A* and *CDKN2B,* as well as a long non-coding RNA, *ANRIL*, that can act in *cis* via epigenetic mechanisms to silence the *CDKN2B* expression (Kotake et al., 2010; Pasmant et al., 2011; Yap et al., 2010). Two intronic variants, rs4977756 and rs1333049, that have previously been associated with longevity phenotypes in other populations (Fortney et al., 2015; Pilling et al., 2016; Pinós et al., 2014) and were associated with higher self-rated health and functional ability scores in our sample (Šetinc et al., 2023), are located in the between these genes, and are reported by the online database Open Targets Genomics (Ghoussaini et al., 2021) to most likely impact the expression of these genes, with the strongest evidence existing for *CDKN2B.* The *CDKN2B* gene encodes $p15^{INK4B}$, an inhibitor of cyclin-dependent kinases 4 and 6 that stops cell cycle progression in G1-phase in response to regulatory signals, thus having an important role in regulation of senescence (McPherson et al., 2007). Its neighbouring gene, *CDKN2A*, encodes in two different reading frames $p16^{INK4A}$, which works similarly to $p15^{INK4B}$, and $p14^{ARF}$, which activates the p53 tumour suppressor pathway by inhibiting protein MDM2, the key effector for degradation of p53 (Gil & Peters, 2006; Lohrum et al., 2000; Pasmant et al., 2011). As the variants associated with *CDKN2B* accounted as one member of the interaction pair for half of all the genetic interactions that had a significant effect on survival above the age of 85, it is clear that this gene has an important role in longevity and late-life survival. In our analyses, it interacted significantly with another variant associated with CVD risk factors, rs2149954 (Ehret et al., 2011; Wain et al., 2011), which further strengthens our hypothesis that the link between these variants and longevity lies in the protective effect of longevity-associated alleles against CVD. The central role of *CDKN2B* in our network of longevity-related genetic factors is also backed up by the significance of interaction between *CDKN2B* and *FOXO3*, which is upstream of the *CDKN2B*

and can regulate its expression – either directly (Hornsveld et al., 2018) or via partnering with SMAD transcription factors in response to TGF-β pathway, the main transcription activator of *CDKN2B* (Gomis et al., 2006; Hannon & Beach, 1994).

Another significant interaction partner of *CDKN2B* was rs1042522, a missense variant in *TP53* that causes substitution of arginine (Arg) with proline (Pro) at codon 72 of p53. The *TP53* gene, also known as the 'guardian of the genome', plays a crucial role in determining cell fate by promoting either repair, survival, or elimination of damaged cells (Wu & Prives, 2018), and is the most frequently mutated gene in human cancer (Bišof et al., 2012; Muller & Vousden, 2013; Olivier et al., 2004). The Arg72Pro substitution, which has a very varied distribution throughout the world populations (Auton et al., 2015), does affect the function of the protein, with the proline allele weakening the response that trigger apoptosis (Dumont et al., 2003; Marin et al., 2000). This variant has been reported to impact longevity and survival in the oldest-old age group (Groß et al., 2014; Van Heemst et al., 2005), and was the only variant that was included in both regression models for predicting survival to ages 90 and 95 in our population. The allele that is associated with longevity and better survival is the allele G, the one coding for proline. However, while it was shown that this substitution is beneficial for longevity, it also represented a higher risk for cancer (Van Heemst et al., 2005), which is in line with another study on a Croatian sample that showed a higher percentage of Pro/Pro genotype among patients with breast cancer that among controls (Bišof et al., 2010). This can be explained by the trade-off between a strong apoptosis response, which is important in earlier life stages, and the importance of maintaining proliferative capacity in old age. While weaker apoptotic response can increase the chances of damaged cells escaping the programmed cell death (and thus increase the risk for developing cancer), an overly strong response in an already ageing organism could deplete tissues of proliferative cells, and tip the balance towards prevalence of senescent, non-dividing cells. This is why a weaker apoptotic response might be more beneficial in later stages of life. The implication of *TP53* in survival is further proved by the significant effect the interaction between *TP53* and *CDK2B* had on survival above 85 in our sample, probably due to their synergistic effect as regulators of the cell cycle.

The three remaining SNPs that had a significant effect on both longevity and late-life survival in our sample were associated with genes *ERCC2*, *GHRHR*, and *PTPN1*. What makes them worth mentioning is the fact that the role of these genes in ageing has not yet been thoroughly explored.

Likewise, the variants in these genes have been linked to longevity in only a few studies. Interestingly, both the *ERCC2* variant rs50871 and the *PTPN1* variant rs6067484 have only been reported by Dato and collaborators (2018), while *GHRHR* rs2267723 was first associated with longevity in a study done on Danish population (Soerensen et al., 2012a), and then mentioned again by Dato and collaborators (Dato et al., 2018). Furthermore, their study also focused on variants from specific longevity-related pathways, and explored the genetic interactions between these variants. This has led us to the hypothesis that the effect of these variants is more discreet, and might require a multiple-loci approach to be detected, which is also why they have not yet been reported by any GWAS. Encoding the growth hormone-releasing hormone receptor that is expressed on the membrane of somatotropic cells in the anterior pituitary gland (Martari & Salvatori, 2009), *GHRHR* gene has an important role in the growth hormone/insulin-like growth factor 1/insulin (GH/IGF-1/INS) signalling axis. The connection between growth hormone signalling and ageing has been made almost half a century ago, when it was discovered that the secretion of GH and IGF-1 slowly started to decrease after reaching adulthood, reaching the lowest level after the age of 60 (Zadik et al., 1985). Additionally, the beneficial effect of decreased GH/IGF-1 signalling on longevity has been confirmed in many model organisms (Fontana et al., 2010), so it is not surprising that the variant associated with this gene had an implication for longevity. The connection of *PTPN1* gene and ageing is also quite clear. *PTPN1*, also known as tyrosine phosphatase non-receptor type 1 gene, encodes protein-tyrosine phosphatase 1B (PTP1B), an enzyme that suppresses insulin signalling pathway by dephosphorylating the phosphorylated tyrosine residues of active insulin receptors (Bauer et al., 2010; Bowden, 2009), which is probably how it contributes to lifespan extension. Contrary to these two genes, the *ERCC2* gene is not directly connected to IIS, but contributes to longevity through another important pathway. The product of the *ERCC2* gene is a multifunctional protein with DNA helicase activity, known as XPD. It is both a subunit with a structural role of a nine-piece transcription factor in charge of basal transcription (Benhamou & Sarasin, 2002; Coin et al., 1998; Keriel et al., 2002), and has a crucial role in transcription-coupled nucleotide excision repair (NER) (Coin et al., 1998), one of key mechanisms for protection against genotoxic damage. The mutations in this gene cause Xeroderma pigmentosum, a condition characterised by extreme sensitivity to UV radiation due to dysfunctional NER that cannot repair the occurring DNA lesions (De Boer & Hoeijmakers, 2000).

Based on the vital role this gene plays in preventing DNA damage, it is clear how it could have repercussions on longevity.

3.4. Impact of other factors on survival of the oldest-olds

Apart from testing the impact of genetic variants and their interactions on longevity, we wanted to determine if other parameters, namely telomere length and health-related parameters available for our study population, would also have an impact on survival above the age of 85. The telomere length was determined in the laboratories of the Institute for Anthropological Research (Zagreb) for the purposes of this thesis using a method by Cawthon (2002) that measures relative telomere length - RTL (Cawthon, 2002). Telomere length has been proposed as a potential biomarker for ageing (Butler et al., 2004; Zglinicki & Martin-Ruiz, 2005), but the relation between telomere length and longevity remains unclear. While some studies found a connection between shorter telomeres and increased mortality rate (Arbeev et al., 2020; Cawthon et al., 2003; Kimura et al., 2008), that connection was often not significant for the old or oldest-old age groups (Bischoff et al., 2006; Cawthon et al., 2003; Harris et al., 2006; Martin-Ruiz et al., 2005; Njajou et al., 2009). In our study, relative telomere length was not a significant contributor to the survival of the oldest-old – either by itself or in combination with other health variables – adding to the body of evidence that the effect of longer telomeres is beneficial only earlier in life, possibly because of lowering the risk of CVD (Brouilette et al., 2003; Fitzpatrick et al., 2007). Furthermore, the method for determining relative telomere length is quite sensitive and best done on fresh (or once-thawed) DNA, while our DNA samples were over a decade old. While we checked the validity of the method in our case by comparing variance between the samples and excluded the outliers within each sample, there was a significant amount of DNA fragmentation among the oldest-old sample to begin with, which might have perhaps skewed the analysis. Therefore, it would be beneficial to perform RTL analyses using this method on fresh DNA samples of oldest-old individuals, and use a sample comprised of different age groups originating from the Croatian population as a reference.

The 33 health-related parameters comprised our database for determining the effect of health status on survival in advanced old age. All the variables were tested in a multivariate model without the genetic factors, and a subset of nine showed a significant effect on survival. These were maternal and fraternal longevity, nourishment status, body weight, heel bone mineral density, folates in

serum, number of medicaments taken daily, usage of B-complex supplements and number of hospital stays in the year prior to taking the survey. Since the variables were categorical, we could see which values were connected to better survival, and for all but one, this category was the one we would expect. However, subjects who had osteopenia – intermediate bone mineral density values – were found to have higher chances of survival. This, though, is not such an unexpected finding, since in the aged population, and especially the oldest-olds, osteopenia is considered a normal trait (Ginsburg et al., 2001; Raisz & Seeman, 2001; Škarić-Jurić & Rudan, 1997). The nine significant health-related variables were then added to the models of significant SNP-SNP interactions in order to test if their effect on survival was independent or just a phenotypic manifestation of the tested genetic factors. In these combined models, almost all of the health-related variables remained significant, indicating that effect of health-related variables on survival for the oldest-old population is indeed not just a product of the studied genetic variants, but instead they each influence late-life survival through their own mechanism. With the addition of the health-related parameters, only four of the fourteen genetic interactions stopped being significant, probably due to the introduction of variables that described the same phenotype they affected. The ones that remained significant, however, suggest that the interplay between genetic variants in different genes and longevity-related pathways could affect survival in a way that is not accounted for by health status parameters, and would warrant further investigation.

4. CONCLUSIONS

Our investigation of genetic variants associated with longevity in other populations on a sample of Croatian oldest-olds (85+ years), performed with the aim of giving an overview of the genetic background of longevity in Croatian population, yielded the following results:

- The studied population of Croatian oldest-olds differed significantly from the control sample of young adult individuals only in the variant rs533984 associated with *MRE11*, with allele G being more common among the oldest-old. As this variant is associated to a gene with a vital role in DNA repair, this difference between the study and the control sample is not surprising. However, the lack of other differences between the two groups is; but this can be attributed to a relatively small sample size of the young control group.
- Allele and genotype frequencies of many longevity-associated variants differed significantly between the Croatian Roma and both the Croatian oldest-old and the young control group, proving that the Roma are a genetically distant population.
- Regardless of the numerous differences in allele distribution between the Croatian majority and the Roma minority populations, genetic longevity scores did not significantly differ between them. This has showed that the lower life expectancy of the Roma had more to do with other risk factors for chronic diseases, either genetic, or environmental and lifestyle ones.
- There was a difference in the variants that contribute significantly to the chances of reaching two thresholds of longevity – 90 and 95 years – in our population. Using multivariate logistic regression analyses, we created a best model for each of the age thresholds. Nine SNPs were entered in the model for predicting the chances of reaching 90 years that explained 20.5% of variance, while only five were entered in the best model for survival above 95, which explained 9.3% of variance. The only SNP shared between the two is a missense mutation in *TP53* gene, but both models also had one of the variants that determine the epsilon diplotypes of *APOE*. While we have shown that there is a difference in the genetic background behind longevity and extreme longevity for our studied population, we recognise that this difference might be caused by some of these variants having lesser effect with progressing age as well as by some other stochastic factors contributing to late-life mortality.
- The unweighted and weighted genetic longevity scores for predicting the chances of reaching 90 and 95 years of age, which were based on the models obtained by regression analyses, were

shown as highly predictive. It showed that, although few in number, the genetic loci have been selected well.

- Fourteen SNP-SNP interactions significantly impacted survival above the age of 85. *CDKN2B*, an important regulator of the cell cycle, stood out as the most significant interaction partner, being represented in half of all the significant interactions. It affected late-life survival in interaction with *TP53*, another controller of the cell cycle; *FOXO3*, the main transcription factor downstream of insulin signalling; and a variant in a long non-coding RNA *LINC02227*, associated with cardiovascular diseases. Another interaction pair with a strong effect on survival above 85 were *TERC* gene and *SH2B3*, which could affect survival by modulating CVD risk. These results confirm the central role cell cycle control and insulin signalling have among the ageing-associated pathways, and highlight the importance of alleles protective of CVD for survival in advanced age.
- Parameters describing one's health status could reliably predict late-life survival, while relative telomere length could not. A set of health-related parameters that included maternal and fraternal longevity, nourishment status, body weight, heel bone mineral density, folates in serum, number of medicaments taken daily, usage of B-complex supplements and number of hospital stays in the year prior to taking the survey was significantly associated with survival above the age of 85.
- When these health-related parameters were tested along significant genetic interaction, in most cases, both the genetic and health variables remained significant, which means they affect survival independently and through separate mechanisms. These results indicate that not enough is known about the way these longevity-associated variants impact survival in advanced old age, which is why they warrant further research. Most importantly, they also show that both genetic and health indicators should be used as predictors of survival in future studies of healthy ageing.

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6. CURRICULUM VITAE

Maja Šetinc was born on April 29 1995 in Zagreb, where she completed elementary school and attended the language-focused programme of grammar school Gimnazija Lucijana Vranjanina. In 2019, she graduated from the Department of Biology, Faculty of Science, University of Zagreb, obtaining her Master's degree in Molecular Biology after defending her thesis "Cloning of the ligand JAGGED1 and its effect on the proliferation of lymphocytes". During her studies, she completed a six-month laboratory internship at the Division of Microbiology of Department of Biology and actively participated in the organisation of the yearly manifestation "Night of Biology". She was also a part of the organising committee of the first rendition of "Meet the Biologists".

In 2020, Maja started working as an assistant at the Institute for Anthropological Research in Zagreb under the supervision of Tatjana Škarić-Jurić, MD, PhD, scientific advisor with tenure, within her projects "Health, Cultural, and Biological Determinants of Longevity: Anthropological Perspective on Survival in Very Old Age (HECUBA)" and "Young Researchers' Career Development Project – Training New Doctoral Students" funded by Croatian Science Foundation. That same year, Maja also enrolled in the doctoral programme of Biology at the Department of Biology, Faculty of Science, University of Zagreb. In 2022, Maja spent three months in scientific training as a guest scientist at the Max Planck Institute for Biology of Ageing in Cologne, Germany, under the supervision of Joris Deelen, PhD, for which she was awarded the Scientific Exchange Grant from the European Molecular Biology Organization (EMBO). She has also been chosen as one of the scholarship recipients of the National programme L'Oréal-UNESCO "For Women in Science" for 2024.

Maja Šetinc has so far published nine original scientific papers (three of them as the first author) and one professional review paper. She participated in nine conferences and multiple workshops, and is currently a member of the European Anthropological Association and Croatian Society for Human Genetics.

7. APPENDICES

Appendix 1. Allele frequencies of the 43 longevity-associated SNPs in Croatian oldest-old and the young control sample. The frequencies were compared between the two groups using Chi-square or Fisher's exact test, and the p-values of this comparison are shown in the table.

Associated gene	rsID	**Allele**	Oldest-old sample		Young control		p-value
			N	frequency (%)	N	frequency (%)	
IL6	rs1800795	G	342	55.2	116	63.0	0.062
		C	278	44.8	68	37.0	
KLOTHO	rs9536314	T	566	88.2	173	89.2	0.798
		G	76	11.8	21	10.8	
KLOTHO	rs9527025	G	566	88.2	177	90.3	0.443
		C	76	11.8	19	9.7	
APOC1	rs4420638	A	556	88.3	170	85.0	0.223
		G	74	11.7	30	15.0	
FOXO3	rs2802292	T	370	57.6	125	62.5	0.249
		G	272	42.4	75	37.5	
TERC	rs12696304	C	467	74.1	142	73.2	0.780
		G	163	25.9	52	26.8	
IL6	rs2069837	A	585	92.3	155	96.9	0.036
		G	49	7.7	5	3.1	
CDKN2B	rs4977756	A	380	60.1	123	62.1	0.677
		G	252	39.9	75	37.9	
APOE	rs7412	C	582	92.4	189	96.4	0.049
		T	48	7.6	7	3.6	
APOE	rs429358	T	570	91.9	179	89.5	0.311
		C	50	8.1	21	10.5	
TOMM40	rs2075650	A	551	85.8	168	86.6	0.906
		G	91	14.2	26	13.4	
IRF4	rs12203592	C	591	92.6	180	96.8	0.042
		T	47	7.4	6	3.2	
CDKN2B	rs1333049	G	337	53.0	98	49.5	0.416
		C	299	47.0	100	50.5	
SH2B3	rs3184504	T	326	51.6	101	51.0	0.935
		C	306	48.4	97	49.0	
LPA	rs10455872	A	611	96.4	194	97.0	0.826
		G	23	3.6	6	3.0	
TNF	rs1800629	G	556	87.4	162	84.4	0.277
		A	80	12.6	30	15.6	
TP53	rs1042522	C	486	76.2	150	77.3	0.773

		G	152	23.8	44	22.7	
TP53	rs2078486	G	585	92.3	189	95.5	0.150
		A	49	7.7	9	4.5	
GHSR	rs572169	C	457	72.5	146	73.0	0.928
		T	173	27.5	54	27.0	
TERC	rs16847897	G	453	70.8	141	69.8	0.791
		C	187	29.2	61	30.2	
IGF1R	rs2229765	G	353	56.4	98	51.6	0.245
		A	273	43.6	92	48.4	
ERCC2	rs50871	A	341	54.0	103	52.6	0.744
		C	291	46.0	93	47.4	
FOXO3	rs10457180	A	423	67.1	144	72.0	0.222
		G	207	32.9	56	28.0	
FOXO3	rs12206094	C	444	70.5	147	74.2	0.323
		T	186	29.5	51	25.8	
FOXO3	rs13217795	T	426	67.6	144	73.5	0.133
		C	204	32.4	52	26.5	
FOXO3	rs4946935	G	449	70.2	143	75.3	0.201
		A	191	29.8	47	24.7	
GHRHR	rs2267723	A	346	55.6	111	57.2	0.741
		G	276	44.4	83	42.8	
IGF1R	rs12437963	A	546	85.6	156	79.6	0.057
		G	92	14.4	40	20.4	
IGF2R	rs9456497	A	513	80.9	158	84.0	0.391
		G	121	19.1	30	16.0	
KLOTHO	rs1207362	G	432	68.8	136	70.1	0.790
		T	196	31.2	58	29.9	
KLF7	rs2360675	C	324	50.9	90	48.9	0.676
		A	312	49.1	94	51.1	
LINC02227	rs2149954	C	398	62.0	122	61.6	0.933
		T	244	38.0	76	38.4	
MRE11A	rs533984	G	379	60.4	95	48.0	0.002
		A	249	39.6	103	52.0	
PAPPA	rs4837525	G	394	62.7	119	61.3	0.735
		A	234	37.3	75	38.7	
PARK7	rs225119	C	368	57.5	111	56.1	0.743
		T	272	42.5	87	43.9	
PTPN1	rs6067484	A	454	72.1	147	74.2	0.584
		G	176	27.9	51	25.8	
RAD50/IL13	rs2706372	C	457	72.8	139	70.9	0.648
		T	171	27.2	57	29.1	
SIRT6	rs107251	C	577	89.9	174	87.9	0.429
		T	65	10.1	24	12.1	

TERC	rs3772190	G	488	77.2	146	76.0	0.769
		A	144	22.8	46	24.0	
TERT	rs33954691	G	567	89.7	179	89.5	0.895
		A	65	10.3	21	10.5	
TXNRD1	rs17202060	C	417	66.4	136	71.6	0.186
		T	211	33.6	54	28.4	
WRN	rs13251813	C	608	95.3	196	96.1	0.846
		T	30	4.7	8	3.9	
FOXO3	rs2764264	T	415	66.9	142	71.7	0.221
		C	205	33.1	56	28.3	

Appendix 2. Genotype frequencies of the 43 longevity-associated SNPs in Croatian oldest-old and the young control sample. The frequencies were compared between the two groups using Chi-square or Fisher's exact test, and the p-values of this comparison are shown in the table.

Associated gene	rsID	**Genotype**	Oldest-old sample		Young control		p-value
			N	frequency (%)	N	frequency (%)	
IL6	rs1800795	G:G	99	31.9	36	39.1	0.152
		C:G	144	46.5	44	47.8	
		C:C	67	21.6	12	13.0	
KLOTHO	rs9536314	T:T	253	78.8	77	79.4	0.679
		T:G	60	18.7	19	19.6	
		G:G	8	2.5	1	1.0	
KLOTHO	rs9527025	G:G	253	78.8	79	80.6	0.288
		C:G	60	18.7	19	19.4	
		C:C	8	2.5	0	0.0	
APOC1	rs4420638	A:A	246	78.1	71	71.0	0.259
		G:A	64	20.3	28	28.0	
		G:G	5	1.6	1	1.0	
FOXO3	rs2802292	T:T	103	32.1	38	38.0	0.458
		T:G	164	51.1	49	49.0	
		G:G	54	16.8	13	13.0	
TERC	rs12696304	C:C	170	54.0	52	53.6	0.860
		G:C	127	40.3	38	39.2	
		G:G	18	5.7	7	7.2	
IL6	rs2069837	A:A	269	84.9	75	93.8	0.110
		G:A	47	14.8	5	6.2	
		G:G	1	0.3	0	0.0	
CDKN2B	rs4977756	A:A	113	35.8	40	40.4	0.635
		G:A	154	48.7	43	43.4	

		G:G	49	15.5	16	16.2	
APOE	rs7412	C:C	270	85.7	91	92.9	0.151
		C:T	42	13.3	7	7.1	
		T:T	3	1.0	0	0.0	
APOE	rs429358	T:T	262	84.5	79	79.0	0.262
		C:T	46	14.8	21	21.0	
		C:C	2	0.6	0	0.0	
TOMM40	rs2075650	A:A	238	74.1	72	74.2	0.671
		G:A	75	23.4	24	24.7	
		G:G	8	2.5	1	1.0	
IRF4	rs12203592	C:C	275	86.2	87	93.5	0.142
		T:C	41	12.9	6	6.5	
		T:T	3	0.9	0	0.0	
CDKN2B	rs1333049	G:G	96	30.2	24	24.2	0.509
		G:C	145	45.6	50	50.5	
		C:C	77	24.2	25	25.3	
SH2B3	rs3184504	T:T	84	26.6	27	27.3	0.897
		T:C	158	50.0	47	47.5	
		C:C	74	23.4	25	25.3	
LPA	rs10455872	A:A	296	93.4	94	94.0	0.728
		G:A	19	6.0	6	6.0	
		G:G	2	0.6	0	0.0	
TNF	rs1800629	G:G	246	77.4	67	69.8	0.135
		G:A	64	20.1	28	29.2	
		A:A	8	2.5	1	1.0	
TP53	rs1042522	C:C	181	56.7	58	59.8	0.778
		C:G	124	38.9	34	35.1	
		G:G	14	4.4	5	5.2	
TP53	rs2078486	G:G	270	85.2	90	90.9	0.297
		G:A	45	14.2	9	9.1	
		A:A	2	0.6	0	0.0	
GHSR	rs572169	C:C	171	54.3	55	55.0	0.992
		C:T	115	36.5	36	36.0	
		T:T	29	9.2	9	9.0	
TERC	rs16847897	G:G	160	50.0	49	48.5	0.964
		G:C	133	41.6	43	42.6	
		C:C	27	8.4	9	8.9	
IGF1R	rs2229765	G:G	102	32.6	27	28.4	0.483
		A:G	149	47.6	44	46.3	
		A:A	62	19.8	24	25.3	
ERCC2	rs50871	A:A	94	29.7	29	29.6	0.847
		A:C	153	48.4	45	45.9	
		C:C	69	21.8	24	24.5	

FOXO3	rs10457180	A:A	139	44.1	52	52.0	0.384
		G:A	145	46.0	40	40.0	
		G:G	31	9.8	8	8.0	
FOXO3	rs12206094	C:C	155	49.2	53	53.5	0.513
		T:C	134	42.5	41	41.4	
		T:T	26	8.3	5	5.1	
FOXO3	rs13217795	T:T	140	44.4	53	54.1	0.245
		T:C	146	46.3	38	38.8	
		C:C	29	9.2	7	7.1	
FOXO3	rs4946935	G:G	157	49.1	55	57.9	0.318
		G:A	135	42.2	33	34.7	
		A:A	28	8.8	7	7.4	
GHRHR	rs2267723	A:A	96	30.9	30	30.9	0.771
		G:A	154	49.5	51	52.6	
		G:G	61	19.6	16	16.5	
IGF1R	rs12437963	A:A	233	73.0	61	62.2	0.119
		G:A	80	25.1	34	34.7	
		G:G	6	1.9	3	3.1	
IGF2R	rs9456497	A:A	206	65.0	67	71.3	0.500
		G:A	101	31.9	24	25.5	
		G:G	10	3.2	3	3.2	
KLOTHO	rs1207362	G:G	152	48.4	47	48.5	0.742
		T:G	128	40.8	42	43.3	
		T:T	34	10.8	8	8.2	
KLF7	rs2360675	C:C	84	26.4	18	19.6	0.238
		C:A	156	49.1	54	58.7	
		A:A	78	24.5	20	21.7	
LINC02227	rs2149954	C:C	130	40.5	39	39.4	0.968
		T:C	138	43.0	44	44.4	
		T:T	53	16.5	16	16.2	
MRE11A	rs533984	G:G	110	35.0	22	22.2	0.006
		G:A	159	50.6	51	51.5	
		A:A	45	14.3	26	26.3	
PAPPA	rs4837525	G:G	120	38.2	41	42.3	0.099
		G:A	154	49.0	37	38.1	
		A:A	40	12.7	19	19.6	
PARK7	rs225119	C:C	103	32.2	28	28.3	0.680
		T:C	162	50.6	55	55.6	
		T:T	55	17.2	16	16.2	
PTPN1	rs6067484	A:A	166	52.7	53	53.5	0.508
		G:A	122	38.7	41	41.4	
		G:G	27	8.6	5	5.1	
RAD50/IL13	rs2706372	C:C	166	52.9	53	54.1	0.237

		T:C	125	39.8	33	33.7	
		T:T	23	7.3	12	12.2	
SIRT6	rs107251	C:C	259	80.7	77	77.8	0.618
		T:C	59	18.4	20	20.2	
		T:T	3	0.9	2	2.0	
TERC	rs3772190	G:G	186	58.9	56	58.3	0.764
		G:A	116	36.7	34	35.4	
		A:A	14	4.4	6	6.2	
TERT	rs33954691	G:G	253	80.1	80	80.0	0.930
		G:A	61	19.3	19	19.0	
		A:A	2	0.6	1	1.0	
TXNRD1	rs17202060	C:C	141	44.9	47	49.5	0.269
		T:C	135	43.0	42	44.2	
		T:T	38	12.1	6	6.3	
WRN	rs13251813	C:C	290	90.9	94	92.2	0.814
		T:C	28	8.8	8	7.8	
		T:T	1	0.3	0	0.0	
FOXO3	rs2764264	T:T	137	44.2	51	51.5	0.428
		T:C	141	45.5	40	40.4	
		C:C	32	10.3	8	8.1	

8. PROŠIRENI SAŽETAK

Starenje je biološki proces progresivnog slabljenja svih funkcija organizma kroz vrijeme koji na kraju završava smrću organizma. Ono nema samo jedan uzrok, već više njih, međusobno povezanih i umreženih tako da djeluju na čitav organizam. Stoga ne čudi što je kroz povijest istraživanja starenja razvijeno mnoštvo teorija kako bi se objasnile promjene koje se događaju dok organizam stari, no niti jedna nije uspjela objediniti sve aspekte ovog složenog procesa.

Dugovječnost je složeno obilježje uvjetovano i genetskim i okolišnim čimbenicima, a može se smatrati rezultatom uspješnog procesa starenja. U proteklih 200 godina, očekivani životni vijek čovjeka se više nego udvostručio, što je dovelo do velikog povećanja udjela starije populacije. Kako je životna dob glavni rizični faktor za razvoj kroničnih nezaraznih bolesti, proces starenja svjetskog stanovništva predstavlja teret za zdravstvene i socijalne sustave mnogih zemalja, što naglašava značaj istraživanja zdravog starenja – postizanja dugovječnosti uz održavanje dobrog zdravlja. U istraživanju čimbenika koji doprinose dugovječnosti vrlo značajnu ulogu imaju osobe duboke starosti, upravo kao primjeri uspješnog starenja. Takva su istraživanja dugovječnih pojedinaca ključna za bolje razumijevanje procesa starenja te za stjecanje saznanja o čimbenicima koji doprinose dugom i zdravom životu.

U sklopu ovog istraživanja 43 genetske varijante povezane s dugovječnošću u drugim populacijama istraživane su na hrvatskom uzorku osoba duboke starosti (85+ godina) kako bi se dobio uvid u genetsku pozadinu dugovječnosti u toj populaciji. Cilj je bio utvrditi postoji li razlika u učestalostima genotipova ili alela između osoba duboke starosti i mladog kontrolnog uzorka te utvrditi doprinose li iste varijante postizanju dugovječnosti (90 godina) i ekstremne dugovječnosti (95 godina). Također, genetske varijante te pokazatelji zdravstvenog statusa i relativna duljina telomera testirani su kako bi se utvrdilo utječu li na preživljenje u dubokoj starosti (iznad 85 godina).

Ispitanici duboke starosti od kontrolne su se skupine mladih osoba razlikovali samo u jednoj varijanti, povezanoj s genom *MRE11* koji ima važnu ulogu u popravku DNA. Izostanak drugih razlika vjerojatno je posljedica malog broja ispitanika, pogotovo kontrolne skupine. Devet varijanti doprinosilo je šansama za postizanje dugovječnosti, dok je pet varijanti doprinosilo postizanju ekstremne dugovječnosti. Tim dvama modelima zajednička je bila jedino varijanta rs1042522 u

genu *TP53*, poznatom i kao „čuvar genoma“. Također, 14 je interakcija između dvaju polimorfizma jednog nukleotida imalo značajan utjecaj na preživljenje u dubokoj starosti, među kojima se kao najznačajniji interakcijski partner pokazao *CDKN2B,* gen koji kodira inhibitor kinaza ovisnih o ciklinu te tako sudjeluje u regulaciji staničnog ciklusa. Pokazatelji zdravstvenog stanja također su imali utjecaj na preživljenje u dobi iznad 85 godina, a testiranjem sa značajnim genetskim interakcijama utvrđeno je da su njihovi utjecaji na krajnji fenotip – preživljenje – međusobno neovisni. Time je pokazano da su oba tipa varijabli uključena u dosizanje dugovječnosti. Relativna duljina telomera nije imala utjecaj na preživljenje osoba starijih od 85 godina.