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INTERRELATION OF MITOCHONDRIAL DNA AND TELOMERE LENGTH ALTERATIONS IN AGEING HUMAN POPULATION

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Abstract

Ageing has always been a topical subject for both discussion and exploration. The nature and evolution have set a natural limit to our lifespan, which is not very long, but we can do many things to prolong our lives and to improve our health when we are getting older. To do so even better, we must understand how and why we are ageing, and this knowledge may help us to discover and develop novel approaches to change it. Until now, there were no studies about a possible interplay between dynamics of telomere length (TL) and mitochondrial DNA (mtDNA) during the ageing process in Latvian population.

In my work I have looked into two important factors of ageing: TL and mtDNA. It is a well-known fact by now that telomeres and mitochondria is, in some level, connected and possibly drives the ageing process. These pathways are influenced by small differences in our nuclear or mitochondrial genomes that can lead to accelerated ageing or diseases. For example, people belonging to mtDNA haplogroup (hg) J have significantly longer telomeres than non-J carriers but are more prone to Leber's optic atrophy; hg H carriers are more prone to some cancer types; and mitochondrial heteroplasmy and hgs might influence mtDNA copy number (CN) in cells, which in turn can, hypothetically, influence longevity. But many details are not known yet, and in different studies we can see controversial results. During my work I have confirmed the results shown in other studies that both mtDNA and telomeres-related factors are indeed connected with our longevity. But I also have shown that this connection of TL and mtDNA alterations is different and not as clear for the very old or nonagenarians (people between 90 and 100 years old). During my research I did not see that benign and small differences of mtDNA are related to the TL, the total amount of mtDNA or age for individuals in our sample cohort, but some studies have shown such effects and that is why these could be population specific traits. Large studies in different populations are required to confirm these findings.

Abstrakts

Novecošanās vienmēr ir bijusi aktuāla diskusiju un izpētes tēma. Lai arī evolūcija ir parūpējusies par to, lai lielākajai daļai dzīvnieku dzīves ilgums nebūtu pārlieku garš, mēs varam daudz ko darīt, lai mūsu dzīvildzi pagarinātu un uzlabotu veselību novecojot. Lai to izdarītu pēc iespējas efektīvāk, mums ir jāsaprot, kā un kādēļ mēs novecojam. Līdz šim nav veikti pētījumi Latvijas populācijā, kas aprakstītu telomēru garuma (TG) un mitohondriālā DNS (mtDNS) izmaiņas, un to iespējamo saistību novecošanas procesā.

Savā darbā esmu apskatījusi divus svarīgus novecošanās faktorus: TG variācijas un mtDNS sekvenču un daudzuma izmaiņas Latvijas populācijā dažādās vecuma grupās, kā arī šo faktoru iespējamo mijiedarbību novecošanas laikā. Tas ir jau labi zināms fakts, ka telomēri un mitohondriji ir savā starpā saistīti un virza izmaiņas šūnu līmenī gadu gaitā. Šie signāl-ceļi, savukārt, var būt atkarīgi no nelielām izmaiņām kodola vai mitohondriālā genomā, tādejādi novedot pie paātrinātas novecošanās vai slimībām. Piemēram, J mitohondriālas haplogrupas (hg) nēsātājiem ir novēroti garāki telomēri, bet viņiem ir lielāka iespēja saslimt ar Lēbera pārmantoto optisko neiropātiju; H hg nēsātāji ir vairāk pakļauti iespējai saslimt ar kādu no vēža veidiem; kā arī mitohondriālā heteroplazmija un hg var ietekmēt mtDNS kopiju skaitu šūnā, kas var hipotētiski ietekmēt dzīvildzi, lai arī daudzas detaļas vēl ir nezināmas, un daudzos pētījumos ir novērojamas pretrunas. Sava darba ietvaros esmu apstiprinājusi citu autoru darbu, ka šie divi šūnu komponenti ir svarīgi un ir saistīti ar mūsu novecošanos. Turklāt, mani novērojumi liecina, ka ka TG un mtDNS izmaiņas ir savādākas cilvēkiem, kuri nodzīvo pāri pa deviņdesmit gadiem. Es arī parādīju, ka labdabīgas vai nelielas mtDNS sekvenču izmaiņas neietekmē TG, mtDNS daudzumu asins šūnās vai ilgdzīvotību cilvēkiem mūsu paraugu kopā. Ņemot vērā, ka atsevišķi citu autoru pētījumi ir parādījuši šādu ietekmi, tas var liecināt, ka šo faktoru ietekme varētu būt populāciju specifiska. Nākotnē būtu svarīgi veikt plaša mēroga pētījumus dažādās populācijās, lai apstiprinātu šos rezultātus.

Contents

Abbreviations

12S - mitochondrial ribosomal RNA
16S - mitochondrial ribosomal RNA
AD - Alzheimer's disease
ALE - average life expectancy
AMPK - 5' AMP-activated protein kinase
ANOVA - two-tailed t-test and Analysis of variance
ATP - adenosine triphosphate
ATPase - adenylpyrophosphatase
bp – base pair
CAD - coronary artery disease
CJD - Creutzfeldt Jacob disease
CN - copy number
COX - cytochrome c oxidase
DCK1 - dyskerin pseudouridine synthase 1
D-loop - displacement loop
DNA - deoxyribonucleic acid
FECD - Fuchs' endothelial corneal dystrophy
FTD-ALS - Frontotemporal dementia – Amyotrophic Lateral Sclerosis
Gau - Gene Antisense Ubiquitous
HepG2 cells - liver tissue cell line
hg - mitochondrial haplogroup
HSP1, HSP2 - H-strand transcription promoters
H-strand - heavy strand
HVS - hypervariable regions
HVS-I - hypervariable segment I
HVS-II - hypervariable segment II
IGF-1 - insulin-like growth factor 1
INK4a/ARF – p16 cyclin-dependent kinase inhibitor 2A/ADP ribosylation factor
Jak3 - Janus kinase 3
LHON - Leber hereditary optic neuropathy
L-strand - light strand
MEF cells - mouse embryonic fibroblasts
MTATP6, MTATP8 - mitochondrial membrane ATP synthase 6, 8
mtDNA - mitochondrial DNA
mTOR - mechanistic target of rapamycin
NADH - nicotinamide adenine dinucleotide
NCR - non-coding region
NHP2 - NHP2 ribonucleoprotein
NK - natural killer
NOP10 - NOP10 ribonucleoprotein
NRF1 - nuclear respiratory factor 1
O_H - origin of heavy strand synthesis
O_L - origin of light strand synthesis
OXPHOS - oxidative phosphorylation
p53 - tumour protein P53
PD - Parkinson's disease
PGC-1α/β - peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and beta
Poly γ - DNA Polymerase γ
POT1 - protection of telomeres 1
qPCR - real-time quantitative polymerase chain reaction
Rap1 - repressor activator protein 1
RC - respiratory chain
PCR-RFLP – polymerase chain reaction-restriction fragment length polymorphism
ROS – reactive oxygen species
rRNA - Ribosomal ribonucleic acid
SCO2 - cytochrome c oxidase 2
SEM - standard error of the mean
SIRT1 - Sirtuin 1
SNPs - single nucleotide polymorphisms
SSC - saline-sodium citrate buffer
TERC - telomerase RNA component
TERRA - (telomeric repeat-containing RNA)-like transcripts
TERT - telomerase reverse transcriptase
TFAM - mitochondrial transcription factor A
TIN2 - TERF1-interacting nuclear factor 2
TL - telomere length
TPP1 - tripeptidyl peptidase 1
TRF1 - telomeric repeat factor 1
TRF2 - telomeric repeat binding factor 2
TRFs - Southern blots of terminal restriction fragments
tRNA - transfer RNA

Introduction

Population studies have demonstrated that telomere length (TL) displays a great diversity among different populations and distribution of mitochondrial haplogroups (hgs) across the globe is a variable trait. Until now, there were no studies that investigated the variability of TL and mitochondrial DNA (mtDNA) alterations in an ageing Latvian population. All the controversial findings that associate longevity with specific mtDNA hgs, heteroplasmy, TL or other mtDNA alterations during ageing require for more studies in field of population ageing. These observations may be influenced by population diversity, geographic location, health care and/or specific historic background. It is well known that TL shortens with age, but there is not as clear picture for mtDNA amount in our cells. While it is often shown that TL correlates with mtDNA amount in younger people, there have not been studies in nonagenarians or centenarians about the TL and mtDNA correlation in blood cells. Nonagenarians and centenarians may have differences in senescence-related pathways and systems, which may function as a protective mechanism that allows them to live longer. Also, not always it has been proven that heteroplasmy or other changes in mtDNA is connected with shorter lifespan, thus more attention from scientists is required.

Importance of this work. As every population is different by its genetical markers like, for example, mtDNA hgs or environment where it is located, it is important to study as many populations as possible to understand how different factors influence cell functions including ageing. For example, there is very high rate of mortality from cardiovascular diseases in Latvia, which is connected with TL and mitochondrial dysfunction. It is also important to explore at which point in our lifetime we should focus on maintaining the right cellular function: for example, when we are young maybe it is TL but with age it could be more important to convey more energy to mitochondrial maintenance for healthier and longer lives. From another point of view this work is also important because if there are so many inconsistent results among laboratories around the world, then, in order to make the right conclusions, there is a considerable need to do as many research studies as possible.

Aims of the study:

1. The first aim was to determine telomere length and mtDNA copy number in a Latvian population to observe any variations in different age groups.

2. The second aim of this study was to analyse the most prevalent mitochondrial haplogroups in different age groups in Latvian population and to estimate their possible associations with telomere length and mtDNA copy number amount and ageing.

3. The third aim was to examine the connection between mtDNA alterations and telomere length in ageing population.

Tasks to reach the aims:

1. Optimize and validate all necessary methods and experimental approaches.

2. Determine telomere length and mtDNA amount in samples collected from the Latvian population.

3. Determine mitochondrial haplogroups and heteroplasmy in samples from the Latvian population.

4. By using statistical methods, find possible links between these cell components in an ageing Latvian population.

1. LITERATURE REVIEW

1.1 Ageing

Ageing is consequences from the interaction of processes that occur over time and genetics interacting with various disease states and the individual's lifestyle. As the population ages, it is essential to think how to raise the quality and years of healthy life (Peel et al. 2005). Ageing we can see as the unavoidable passage of time; cell senescence as a process in which cells stop dividing and undergo distinctive phenotypic alterations. Evidence shows that the effects of cellular senescence are continuously acquired and that senescent cells exist in tissues even early in life. From energy point of view having senescent cells is energy less expensive for tissue than cycling cells (reviewed in Eisenberg 2011; Dimri et al. 1995). There are several hallmarks of cellular senescence: replicative senescence (telomere attrition); DNA damage-induced senescence (radiation or multiple drugs); oncogene-induced senescence (activation of oncogenes or inactivation of tumour suppressors); oxidative stress-induced senescence (oxidizing products of the cell metabolism or oxidative agents); chemotherapy-induced senescence (anticancer drugs); mitochondrial dysfunction-associated senescence; epigenetically induced senescence (inhibitors of DNA methylases or histone deacetylases); and paracrine senescence (senescence-associated secretory phenotype) (reviewed in Hernandez-Segura et al. 2018).

There are generally accepted nine hallmarks of ageing (Fig. 1): genomic instability (nuclear, mitochondrial DNA and nuclear architecture alterations); telomere attrition; epidemiologic alterations (alterations in DNA methylation patterns, posttranslational modification of histones, and chromatin remodelling and transcriptional alterations); loss of proteostasis (chaperone-mediated protein folding and stability, proteolytic systems); deregulated nutrient sensing (the Insulin- and IGF-1-signaling pathway, mTOR, AMPK, and Sirtuins); mitochondrial dysfunction (reactive oxygen species (ROS), mitochondrial integrity and biogenesis, mitohormesis); cellular senescence (telomere loss, INK4a/ARF Locus and p53); stem cell exhaustion; altered intercellular communication (inflammation and other types of intercellular communication) (reviewed in López-Otín et al. 2013).

Fig.1. Hallmarks of ageing. Picture adapted from López-Otín et al. 2013.

There are also many theories that describe ageing. The theory of programmed ageing, under which are several sub-theories, establishes that ageing is a result of a sequential switching on and off of certain genes, with senescence being defined as the time when age associated deficits are manifested. Endocrine theory says that biological clock is regulated through hormones and controls the pace of ageing. Immunological theory postulates that the immune system is programmed to decline, which in turn leads to an increased vulnerability to infectious disease and thus ageing and death. The damage or error theory emphasizes influence of environment; there are also several sub-theories under it. Stochastic or "wear and tear" theory assumes that cells, tissues and organs wear out resulting in ageing. Rate of living theory predicts that lower energy metabolism and lower oxygen basal metabolism slows ageing rate. Cross-linking theory, according to this theory, an accumulation of crosslinked proteins damages cells and tissues, slowing down bodily processes resulting in ageing. Free radicals or ROS (mitochondria) theory proposes that these chemically active molecules cause damage to the macromolecular components of a cell that leads to accumulated damage causing impairment of cells and, eventually, organs, and ageing as an outcome. Somatic DNA damage theory means that DNA damages take place continuously and accumulate with

increasing age, causing cells to deteriorate and malfunction. Apart from these, even more theories of ageing exist: accumulation of undegradable by-product of metabolism, impairment of regulatory pathways during ageing, and energy restriction and lifespan theories.

The focus for this study is on two well-accepted theories: one involves TL shortening and, the second involves mtDNA alterations. As there are contradictory studies about these theories, there is a necessity for more research in the field of ageing.

1.2. Telomeres

Telomeres are the specialized chromosomal DNA-protein structures that cap and protect the terminal regions of eukaryotic chromosomes (Fig. 2). The sequence of telomeric DNA in vertebrates, including humans, is (TTAGGG)n; in humans, its length ranges from 5 to 15 kb and varies between different tissues. Each time a cell divides the telomeres get shorter due to the chromosome end-replication problem. By removal of the RNA primer, which initiates the last Okazaki fragment, a single-stranded G-rich 3′-overhang is formed when a replication fork has reached the end of the chromosome´s lagging strand (Makarov et al. 1997). The telomeric 3′-overhangs are also observed at the end of the leading strand, although of different size (Chai et al. 2006). As the leading-strand replication generates a blunt-ended telomere, 5`-end resection by Apollo exonuclease is required to form the single-stranded 3′-overhang to protect telomeres. Thereby, the telomeric 3′-overhangs are formed by shelterin-controlled multistep process which is slightly different for each strand (Wu et al. 2012; reviewed in Higa et al. 2017). Human telomeres typically terminate in a 35–600 nt single-strand 3′ overhang of the G-rich sequence, which are bound by a specific protein complex termed ‘shelterin’ (telosome) - proteins connected with telomeres. Their main function is to maintain telomere structure and functions. The main proteins can be divided into three levels: 1. proteins that directly are connected with telomeres (TRF1, TRF2, POT1), 2. proteins that are connected with the first level proteins (TPP1, TIN2, Rap1). And there are proteins that are connected with the telomerase (TERT, TERC, NOP10, NHP2, DCK1, NHP2). Plus, there are many other proteins and enzymes that are connected with telomeres and their maintenance (Martínez and Blasco, 2011; Lange et al. 2006). Telomerase is a specific enzyme that extends telomeres, but it is significantly active only in special cells such as stem cells, gametes, some types of blood cells and most cancer cells (Greider and Blackburn 1985; Kim et al. 1994; Wright et al. 1996; Kaszubowska 2008; Wu et al. 2017).

Telomere maintenance and shortening is a complex process which can be affected by different factors, like stress and genetic background, and is different in diverse tissues, furthermore the relationship between TL and ageing is not fully clear (reviewed Blackburn et al. 2015). Telomeres are dynamic structures that become shorter with every cell division until a critical stage is reached when the cell can no longer divide and enters a phase of senescence. Telomere attrition causes genomic instability, which is associated with a higher risk of age-related diseases and cancer, leads to potentially maladaptive cellular changes, and blocks cell division (Harley et al. 1990; Blackburn 2001; de Lange et al. 2006; reviewed in Samassekou et al. 2010; Blackburn et al. 2015). They are essential for stabilizing eukaryotic chromosomes in different ways. One of many functions of telomeres are to prevent the end of the linear chromosomal DNA from being recognized as a broken end; telomeres are also the "first responders" to threats to genomic stability and problems with DNA maintenance. They are located at the nuclear envelope, and their specific association with the spindle pole body is required for normal recombination, protecting cells from nonhomologous recombination and broken DNA ends ensuring normal mitosis and meiosis (Blackburn 2001).

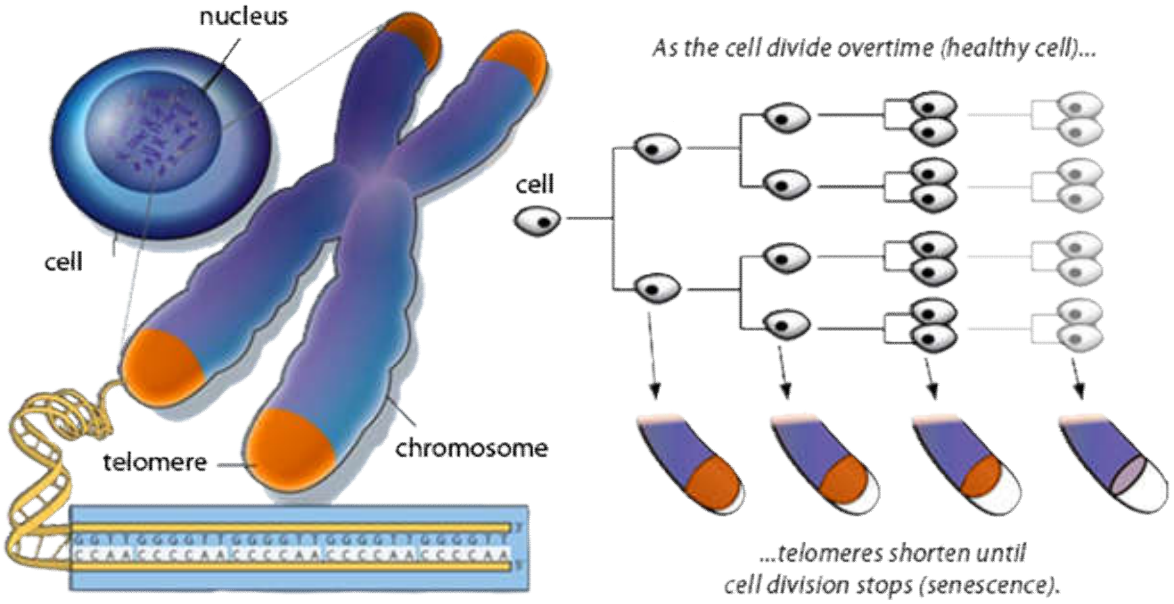

Fig. 2. Telomeres and their shortening. Picture adapted from University of Liège (reflexions.ulg.ac)

It is widely accepted that TL gradually decreases with age in human cells (except for the terminally differentiated tissues such as brain and myocardium); and for women, telomeres shorten more slowly due to oestrogen effects on telomerase (Kyo et al. 1999; Takubo et al. 2010). Telomere DNA is a target of persistent DNA damage which can induce

cellular ageing and stress-induced senescence (Hewitt et al. 2012). Shortened blood cells` telomeres in population studies were associated with higher rates of mortality from different age-related pathologies, including heart and vascular diseases, diabetes mellitus, Parkinson's disease and Alzheimer's disease, among others (reviewed in Blasco 2007; Jiang et al. 2007; van der Harst et al. 2007). Several studies have reported that telomeres do not shorten as quickly in older individuals and TL does not predict mortality in the very oldest. This observation presumably may be related to a gradual reduction in cell turnover with advancing years. However, the opinion exists that, at a certain age, telomere maintenance is more critical than the exact TL (Mondello et al. 1999; Jiang et al. 1992; Shay et al. 2007; Haussmann and Mauck 2008; Ehrlenbach et al. 2009; reviewed in Simons 2015).

Population studies have demonstrated that TL in blood cells can be quite diverse among different populations. In a study of people over 60 years old, a French population (an average life expectancy (ALE) - 82.4 years; the data of life expectancies for populations was taken from World Health Statistics 2015) contained longer telomeres than an Italian population (ALE – 82.7 years) (Canela et al. 2007). Individuals with an average age of 22.7 years from Southern Europe (ALE – 81.9 years) possessed shorter telomeres than individuals from one part of the Baltic region (ALE - 79.3 years), and individuals from the Middle Europe (ALE - 81.5 years) contained the longest telomeres (Salpea et al. 2008). However, another report that included fourteen European populations with individuals between ages 18-28 showed that TL varied among them drastically, with the shortest telomeres observed in an Italian population (5100 bp (base pairs)) (ALE – 82.7 years) and the longest telomeres observed in a Belgian population (18,640 bp) (ALE - 81.1 years). For an Estonian population (ALE - 77.6 years), telomeres were 7340 bp long (Eisenberg et al. 2011). Pronounced differences in TL were observed for two subpopulations in Finland (ALE – 81.38 years): in an Oulu-based population, the mean TL was 7620 bp, and in a Helsinki-based population, the mean TL was 12,280 bp (Eisenberg et al. 2011). The work of Hansen and colleagues also proved that TL between populations differs. They stated that Europeans (ALE – 71.3-83.4 years) have much shorter telomeres than sub-Saharan Africans (Tanzania) (ALE – 61.8 years) and Afro-Americans (ALE - 75.6 years) in a long age span of 20-80 years (Hansen et al. 2016). In comparison the TL in the USAs population of age range 19-93 years were longer in Afro-Americans (ALE - 75.6 years) than in whites (ALE – 79 years), although Afro-Americans showed a faster rate of TL shortening (Hunt et al. 2008; Chen et al. 2011; data for

ALE in USA taken from Kenneth et al. 2016). In a similar study the same research group again demonstrated that black people have longer telomeres than white people, age range of 19-77 years (Daniali et al. 2013). These differences among populations and ethnicity are poorly understood. It seems that TL in leukocytes of the reviewed populations not always can be explained by life expectancies, while, there is a correlation between age and TL in peripheral blood leukocytes, with correlation coefficient ranged from −0.088 to −0.838 in different studies (reviewed in Müezzinler et al. 2013). In most studies, short telomeres in circulating leukocytes are also associated with high mortality (Cawthon et al. 2003; Rode et al. 2015). Though, telomere shortening may not affect mortality per se but could be controlled by progression of senescence that leads to mortality by other mechanisms (Cawthon et al. 2003). Life expectancy also depends on dynamics of TL or how fast telomeres shorten for each individual as a study on birds has shown (Bize et al. 2009). All these observations of different TL and life expectancies among populations could be explained by differences in genetics, sex, ethnicity, nutrition, economic and social status or stress level and health care systems. It can also be due to lifestyle choices including smoking, alcohol consumption, physical activities, body mass index, diet and supplement intake and environmental pollution that can influence the disparity (reviewed in Epel et al. 2004; Lin et al. 2012; Müezzinler et al. 2013 and Vidacek et al. 2017). For example, Crous-Bou and colleagues showed that women who practised a Mediterranean diet had longer telomeres than those who did not (Crous-Bou et al. 2014). There is evidence that famine can also influence TL. For men who have experienced starvation, TL was shorter than for those who have not. It also seems that men whose parents had experienced recent starvation before conception had shorter telomeres (Kobyliansky et al. 2016).

1.3. Mitochondria

Mitochondria (Fig. 3) are very essential organelles of an eukaryotic cell. It has many functions that ensure differentiation, survival and death of cells. Mitochondria work as factory for ATP (adenosine triphosphate) and metabolites for the cell survival, and release of cytochrome c to initiate cell death. Mitochondria work as signalling organelle releasing proteins, ROS, metabolites or by serving as a scaffold to configure signalling complexes (reviewed in Chandel, 2014). It also regulates bioenergetics through cytosolic calcium regulation (Rizzuto et al. 1993). Mitochondria with its providing of ATP and other

components affect gene expression, chromatin modification related to transcriptional activation, transcription elongation and even alternative splicing (Guantes et al. 2016).

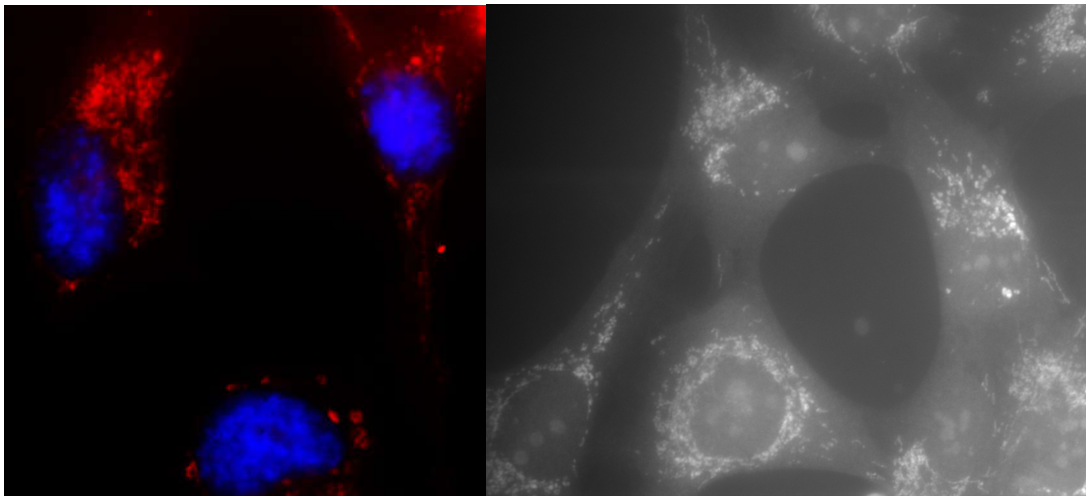

Fig. 3. Mitochondria in cells around nucleus, visualized by MitoTracker Red. Pictures from a private collection of the author.

It has been shown that mitochondria are required for pro-ageing traits of the senescent cell phenotype (Correia-Melo et al. 2016). With age mitochondria become more ineffective and potentially toxic. And they can induce apotheosis or necrosis that can lead to decrease of functions for tissue and the whole body. As oxidative phosphorylation (OXPHOS) takes place in the mitochondria it leads to ROS production which oxidize macromolecules in there and can lead to mtDNA mutations. During ageing mutations in mtDNA accumulates and OXPHOS becomes less effective leading to higher ROS production and that can lead to cell senescence and death (Wallace et al. 2010; Green et al. 2011; Mikhed et al. 2015). Mitochondria are involved in processes of inflammation and autophagy or mitophagy that eliminates dysfunctional mitochondria. Induction of these processes can help to sustain a longer life span because it destroys damaged mitochondria and cells and helps to adapt to stress for the cell. With age mitophagy and autophagy decreases as a result mutated mtDNA and dysfunctional proteins accumulate and cells age (Levine and Kroemer, 2008; Lipinski et al. 2010).

1.4. Mitochondrial DNA

Mitochondrion contains its own circular double-stranded DNA and it is maternally inherited (Giles et al. 1980). It is 16,569 bp long and encodes only 37 genes (Fig. 4A). MtDNA codes the 12S and 16S rRNAs genes, 22 tRNAs, 13 subunits of respiratory chain (RC) - seven subunits of complex I (NADH-ubiquinone oxidoreductase, ND1, 2, 3, 4, 4L, 5 and 6), one subunit of complex III (cytochrome b), three subunits of complex IV (cytochrome c oxidase (COX) I, II and III), and two subunits of complex V (ATPase subunit 6 and 8), 79 subunits are encoded by the nuclear genome (Anderson et al. 1981). 16S also encodes humanin peptide and then is transported into intra- and extra-cellular compartments and function as mitochondrial-nuclear retrograde signalling (Hashimoto et al. 2001; Lee et al. 2013). Also, *cox1* subunit on the antisense strand possibly encodes a short protein named Gau (Gene Antisense Ubiquitous) (Faure et al. 2011). The genome structure of the two strands is distinguished by their nucleotide composition - heavy strand (H-strand) is guanine rich, compared with the cytosine rich light strand (L-strand), 28 genes are on the H-strand and 9 on the L-strand. MtDNA genes lack introns and some genes, like MTATP6 and MTATP8, have overlapping regions. Most genes are very close to each other, separated by one or two non-coding base pairs. MtDNA contains only one major non-coding region (NCR) in which are located the displacement loop (D-loop), 1123 bp long, mostly is in a triple-stranded DNA configuration and includes hypervariable (HVS) regions. The D-loop contains a site of mtDNA replication initiation (O_H - origin of heavy strand synthesis) and is also the site of both H-strand transcription promoters (HSP1 and potential HSP2). O_L is located two thirds of the lengths from O_H. MtDNA replication is not driven by the cell cycle or cell division and is continuously recycled. MtDNA is replicated by DNA Polymerase γ (Poly γ) with a help of Twinkle helicase and mtSSB (single-stranded DNA-binding protein). The mitochondrial genetic three nucleotide code for amino acids is different from nuclear DNA code (Graziewicz et al. 2006; reviewed in Chinnery and Hudson 2013).

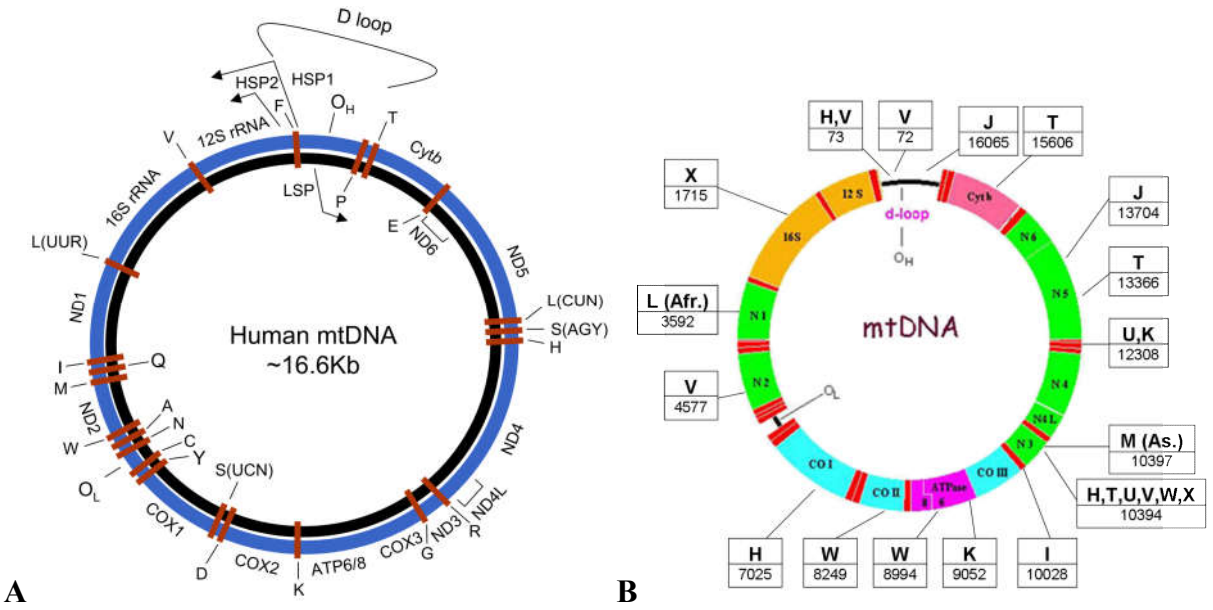

Fig. 4. **A**: Structure of the mtDNA. Picture adapted from Scarpulla, 2008. **B**: Polymorphisms of mitochondrial haplogroups mapped on mtDNA. Picture adapted from Geni (www.geni.com).

Most of the mammalian cells contains hundreds to more than a thousand mitochondria each, and each organelle harbours 2-10 copies of mtDNA (Robin and Wong 1988). The mtDNA amount in a cell is heritable, implying genetic regulation of mtDNA levels, and the mtDNA CN in peripheral blood cells is higher for women than men (Ding et al. 2015; Knez et al. 2016). There are debated results from different studies regarding how mtDNA CN in blood cells changes during human ageing. A substantial age-related decline in the abundance of mtDNA has been shown between 17 to 93 years of age (Mengel-From et al. 2014; Ding et al. 2015; Zhang et al. 2017), and low mtDNA content in blood cells was associated with familial longevity in a study from the Netherlands. Based on their results, the authors discussed the necessity of preserving mitochondrial functions rather than enhancing mitochondrial biogenesis via the YY1 transcriptional repressor protein (van Leeuwen et al. 2014). It was reported that an increase in the mtDNA CN is associated with elevated oxidative stress in the human tissues of aged individuals (Barrientos et al. 1997a; Barrientos et al. 1997b). In contrast, He et al. have shown that mtDNA content in blood samples was higher for healthy centenarians than for the younger age groups (He et al. 2014), while Moore and colleagues have reported that association of mtDNA CN with age was not statistically significant among participants of the InCHIANTI study (age range: 29–96) (Moore et al. 2018). High mtDNA CN in leukocytes in many studies has been associated with better health, including higher cognition and lower mortality (Lee et al. 2010; Kim et al. 2011; Mengel-From et al. 2014; Ashar et al. 2015). In a recent study, higher mtDNA was associated with a significantly lower

risk of both solid tumours and other diseases, independent of age and sex (Memon et al. 2017). Centenarians have increased mitochondrial mass and higher ATP level in primary cultures of fibroblasts isolated from the skin (have defective mitochondria but a preserved bioenergetic competence) indicating that longevity is characterized by a preserved bioenergetic function likely attained by a successful mitochondrion remodelling that can compensate for functional defects through an increase in mass, i.e. a sort of mitochondrial "hypertrophy" (Sgarbi et al. 2014). One reason could be due to compensatory mechanisms in centenarians' cells both for higher mtDNA and ATP level, as a normal level of ATP is necessary for a cell to progress through the G1 phase of the cell cycle to proliferate and regenerate, which could help the cells to be more fit during ageing by providing energy reserves (Mandal et al. 2005; Owusu-Ansah et al. 2008; Moiseeva et al. 2009). On the other hand, high mtDNA CN in blood cells was associated with breast, prostate, gastric and colorectal cancer risk (Thyagarajan et al. 2013; Zhou et al. 2014; Zhu et al. 2017; Kumar et al. 2017). Additionally, stress and depression were associated with increased mtDNA amounts in blood cells (Cai et al. 2015) as well as other health-related factors that were shown to have an influence on mtDNA quantity in human cells (reviewed in Malik and Czajka 2013). There are several molecular factors in a cell that can regulate mtDNA CN, such as p53, mitochondrial transcription factor A (TFAM), Twinkle helicase and ATP synthase (Kulawiec et al. 2009; Ekstrand et al. 2004; Tyynismaa et al. 2004; Fukuoh et al. 2014; Moraes 2001). It was also shown that mtDNA CN is regulated by expression of the only mtDNA polymerase γ subunit A (Polγ-A) in a cell-specific manner by nuclear DNA methylation (Kelly et al. 2012). Also, other factors like lifestyle can influence mtDNA amount. Obesity, weight gain and pack years of smoking were associated with reduced mtDNA CN (Meng et al. 2016). Reduced sleep duration and efficiency were associated with lower mtDNA amount (Wrede et al. 2015) but regular exercise for postmenopausal women increases mtDNA CN in leukocytes (Chang et al. 2016).

MtDNA single nucleotide mutations and deletions as well as their accumulation (especially the 4977 bp deletion) were associated with a normal ageing process in several studies and have been proposed either as a useful marker of natural ageing in human subjects or as a factor affecting human longevity (Cortopassi et al. 1992; Meissner et al. 1997; Linnane et al. 1989; Raule et al. 2014; reviewed in DeBalsi et al. 2017). However, mtDNA mutation sites, accumulation rate and impact on cell functionality are affected differently in each tissue

type and there is a great importance of the individuals` ethnic background (Samuels et al. 2013; Raule et al. 2014). Research on the mtDNA mutation signatures suggest a limited role for ROS-induced mutation (Kauppila and Stewart 2015), and accumulation of mtDNA mutations can be related to the down regulation of DNA repair that occurs with cellular senescence. Mice with defective Polγ show aged phenotypes and increased mutational load in the mtDNA (Trifunovic et al. 2004). Somatic mutations can be unevenly distributed and accumulated clonally in cells, causing a mosaic pattern of respiratory chain deficiency in tissues, or accumulation can occur during germline, embryonic or foetal development, in which case, the distribution of mutations would be even between tissues (Trifunovic et al. 2004, Greaves et al. 2014).

Mitochondrial heteroplasmy is the presence of multiple mtDNA variants with mutations or SNPs in a single cell or among cells within an individual (Potter et al. 1975). The frequency of heteroplasmy is the same between women and men (de Camargo et al. 2011; Ding et al. 2015). The highest degree of polymorphisms is concentrated within two hypervariable segments of the control region: hypervariable segment I (HVS-I) and hypervariable segment II (HVS-II) (Wilkinson-Herbots et al. 1996). The results of studies looking on the association between mtDNA heteroplasmy and population ageing are not fully consistent. Blood cells` mtDNA heteroplasmy becomes more common with increasing age (Ding et al. 2015; Sondheimer et al. 2011; Zhang et al. 2017), and increased mtDNA heteroplasmy was associated with impaired functioning and increased risk of mortality (Tranah et al. 2017). MtDNA heteroplasmy at specific DNA sites in platelets was associated with reduced neurosensory and mobility functions in older people (Tranah et al. 2015). However, there is also possibility that some mtDNA polymorphisms are selected with age and can compensate for the defects induced by various types of mtDNA mutations, helping an individual to survive longer (Ono et al. 2001; Sondheimer et al. 2011; Rose et al. 2007). Several studies have shown that the total heteroplasmy values and patterns are maintained in centenarian families (Rose et al. 2007; Giuliani et al. 2014). It was reported that heteroplasmy can be inherited from a mother with 30% possibility; however, in 70% of cases, a mother does not pass the mutated site to her children, and heteroplasmic SNP changes can accrue spontaneously during the lifetime (Sondheimer et al. 2011; Ding et al. 2015).

In diabetic patients, a correlation between the mtDNA CN and SNPs in mtDNA has been observed in blood vessels tissue (Chien et al. 2012). Measuring mtDNA CN by shotgun sequencing, Wachsmuth and collaborators found that there was a correlation between mtDNA CN with the total number of heteroplasmies in blood and skeletal muscle cells; in the other tissues, heteroplasmy was correlating with age, indicating that this correlation could be explained mostly by age and not as much by the increase in the number of heteroplasmic sites (Wachsmuth et al. 2016). Another study recorded lower mtDNA CN in individuals having higher numbers of heteroplasmies but posits that decrease of age-related mtDNA CN and increase of heteroplasmy are independent from one another (Zhang et al. 2017).

The mtDNA sequence is diverse among populations and individuals. There are 9 major mitochondrial hgs found in the human population of Europe (H, I, J, M, T, U, V, W, and X) (Torroni et al. 1994; Richards et al. 1998). A human mtDNA hg is defined by differences in mtDNA sequences or SNPs. Previous studies showed that the mitochondrial genotype, or hg, can be associated with longevity and pathologies that can influence healthy ageing and mortality; however, the observed results are controversial. It has been detected that defined mutations in mtDNA which are associated with hgs D, D1, D4 (more abundant in Asian populations) and H1 (in European populations) are more frequently found among centenarians, while the frequencies of other hgs such as M9, N9 and B4a (which are more abundant in Asian populations) decrease in a centenarian group (Tanaka et al. 1998; Tanaka et al. 2000; Cai et al. 2009). Gender may also play a role in the distribution of hgs among centenarians (Fernández-Moreno et al. 2017). In Amish populations, the X hg was associated with successful ageing whilst hg J had opposite effects (Courtenay et al. 2012). Regarding European populations, it has been observed that in Italy hg J is more abundant among centenarians, while hg U frequency decreases (de Benedictis et al. 1999; Rose et al. 2001). A study that has been performed in Finland showed that hgs H and HV were less frequent among centenarians than hgs U, J and U8 (Niemi et al. 2003). Nevertheless, in Spain, Pinós et al. have not identified such observations in relation to hg J and have suggested that longevity is population-specific (Dato et al. 2004; Pinós et al. 2012). In relation to hgs H and U, other researchers have not found significant associations with longevity (de Benedictis et al. 1999; de Benedictis et al. 2000; Pinós et al. 2012). Benn with colleagues also made an assumption that there are no associations of hgs with mortality and longevity (Benn et al. 2008). Beckstead et al. indicated that individuals with hg H may live longer in comparison to

individuals with hg U under calorie restriction (Beckstead et al. 2009). Some researchers say that human adaptation to the chronic cold and irregular caloric availability, due to changes in seasons, could influence the evolution and distribution of mitochondrial hgs and longevity, especially in the North (Wallace 2005; Robine et al. 2012). In various studies, it has been revealed that hgs are definitely associated with healthy ageing and may have a protective effect on the occurrence of some diseases and tumours (e.g., Czarnecka and Bartnik 2011). For example, Hg J is associated with Leber's hereditary optic neuropathy (LHON), while hg H has a protective effect on LHON (Torroni et al. 1997; Hudson et al. 2007). In another study, there was no such observation; although, the lack of observation could be explained by the rarity of hg J in the population (Aitullina et al. 2013). Hg J shows a protective effect against the development of osteoarthritis (Fernández-Moreno et al. 2017). Rosa et al. found that hg H1 has a protective effect on ischaemic stroke while hgs HV and U have been involved in increased risk of it (Rosa et al. 2008). Hg U and its branch U8 could possess protective properties against Alzheimer's disease in patients with the ε4 allele (Carrieri et al. 2001). Furthermore, the results of two studies showed the protective properties of hgs U8 and J against Parkinson's disease in Italians and Poles, respectively (Ghezzi et al. 2005; Gaweda-Walerych et al. 2008). In contrast, a newer study from the UK did not find any linkage between hgs and Parkinson's disease (PD), Alzheimer's disease (AD), frontotemporal dementia-amyotrophic lateral sclerosis (FTD-ALS) or Creutzfeldt Jacob disease (CJD) (Wei et al. 2017). Hg T is associated with coronary artery disease (CAD) and diabetic retinopathy (Kofler et al. 2009). Moreover, among different types of tumours, some hgs may play a dual role of being either protective or not. Vulvar, prostate and renal cancers are associated with hg U, while hg H is more often represented in individuals suffering from head and neck cancers. However, hg H is underrepresented in other cancers such as vulvar, breast and endometrial cancers (Booker et al. 2006; Klemba et al. 2010).

The opinion exists that hgs might play a protective role for a cell against ROS because of greater heat generation (higher electron transport rates, looser coupling or partially uncoupled OXPHOS). Haplogroup-defining mutations may affect ATP synthesis because certain mitochondrial variants are biochemically different (Tanaka et al. 1998; Tanaka et al. 2000; Cai et al. 2009; Fernández-Moreno et al. 2017). Mitochondrial hgs have different coupling efficiencies (the percentage of oxygen consumption used for ATP synthesis rather than heat generation) or mitochondrial production of ROS. Reduced mitochondrial coupling

in aging tissues has been demonstrated. In addition to the negative consequences of mitochondrial uncoupling, mild uncoupling has been shown to reduce the generation of ROS and provide protection against age-related disease. For the cold-adapted hgs, uncoupling mutations would produce less ATP per calorie consumption, which allows greater heat generation and more oxidization, and fewer ROS would be formed; for example, hg H has higher ATP production in comparison to hg J (Mishmar et al. 2003; Baudouin et al. 2005; Wallace 2005; Brand 2000). Hgs J and U are uncoupled hgs, while hg H is tightly coupled and therefore produces more ROS and less heat. It has been proven that cybrids of hgs H and U had different amounts of mitochondria, where hg U had fewer mitochondria than hg H. Hg U had lower levels of cytochrome *b* mRNA, rRNA, protein synthesis and mitochondrial inner membrane potential (MIMP) than hg H (Martínez-Redondo et al. 2010; Gómez-Durán et al. 2010). In another case due to increased binding of TFAM cybrid cells harboring hg J had two-fold increase of mtDNA amount in comparison with cybrid cells containing hg H (Suissa et al. 2009). The H hg had higher peroxide and superoxide anion production and apoptosis but lower oxidative stress than the J hg (Fernández-Moreno et al. 2017). Martínez-Redondo et al. have also shown that hg H had higher mitochondrial oxidative damage than hg J. In their study, hg H showed higher oxygen uptake than other hgs and therefore more ROS production was observed; the next highest ROS producer was hg V followed by hgs T and U; but the lowest was hg J (Martínez-Redondo et al. 2010). U3a and J2b subhaplogroups with a C150T SNP displayed lower ROS production than hgs without the C150T SNP, and this SNP was associated with longevity (Chen et al. 2012; Zhang et al. 2003). Other research groups found that hg H had more increased mitochondrial function than hg U in human skeletal muscle fibres (Larsen et al. 2014). Studying sperm mobility, scientists found that individuals with hg H had the highest activity of spermatozoids, compared to that of hg V and the lowest activity of spermatozoids has been detected for hg T, which is directly connected with the functionality of the OXPHOS system (Ruiz-Pesini et al. 2000).

By using shotgun sequencing, in recent studies no difference has been found in mtDNA CN among H, U, T, K and J hgs (Wachsmuth et al. 2016; Zhang et al. 2017). Although in another research, it was showed that hgs U5A1 and T2 were significantly associated with higher mtDNA CN by changing the *COXIII* and *COXI* amino acid sequences, respectively (Ridge et al. 2014). Additionally, the frequency of heteroplasmy among hgs did not differ in several studies (de Camargo et al. 2011; Ramos et al. 2013), but one study

claimed that the HV hg had more frequent heteroplasmy while hgs J, T and U8 had less heteroplasmy (Ding et al. 2015). Previously, Fernández-Moreno et al. showed that individuals with hg J had significantly longer telomeres than non-J carriers (Fernández-Moreno et al. 2011).

1.5. Interrelation between telomeres and mitochondria

Heap of evidence suggests a crucial role for signalling from the nucleus to mitochondria in ageing (reviewed in Fang et al. 2016). Telomere theory declares telomere shortening as the main trigger and the best marker of cellular senescence and ageing, an adjacent theory is oxidative stress or mitochondrial theory (reviewed in Bernadotte et al. 2016). And for some time, scientists are trying to find a trade that links telomere attrition to metabolic compromise, which is central in cellular functional decline during ageing. Mitochondrial dysfunction leads to perturbations on the electron transport chain resulting in increased ROS generation and reductions in ATP level (Balaban et al. 2005; Moiseeva et al. 2009). Additionally, increased mitochondrial density and biogenesis are associated with increased ROS production due to an increased number of sites where ROS generation can occur (Passos et al. 2007; Yoon et al. 2010). This, in turn, can further induce DNA damage, and it has been shown that oxidative stress is associated with increased telomere attrition (von Zglinicki 2002; Kawanishi and Oikawa 2004). One of the first studies that reported a connection between mtDNA mutations causing respiratory chain disorders and telomere shortening was about mutations in the mtDNA of LHON, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELS) syndrome patients; the researchers observed shorter telomeres in patients than in a control group (Oexle and Zwirner 1997). Liu et al., at the beginning of this century, demonstrated that mitochondrial dysfunction generates ROS and leads to chromosomal instability through telomere attrition (Liu et al. 2002). The study of Passos and colleagues also showed connections among TL with mitochondrial genetics and ROS in later years (Passos et al. 2007). On opposite side, an evidence exists that shortening of telomeres is a causal factor for mitochondrial dysfunction. Short telomeres, which are sensed as double-strand breaks and genomic DNA instability, suppress peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and beta (PGC-1α/β, which regulate a vast number of mitochondrial functions, including mitochondrial replication/transcription, OXPHOS, oxidative stress and gluconeogenesis (e.g., Wu et al. 1999)) action via the p53

transcription factor (Chin et al. 1999; Sahin and Depinho 2010; Sahin et al. 2011). Short telomeres cause increased p53 activity and high levels of apoptosis (Flores and Blasco 2009), as well as up-regulation of the mTOR and Akt survival pathways and down-regulation of cell cycle and DNA repair pathways (Schoeftner et al. 2009). Further work by Sahin and colleagues confirmed that telomere dysfunction is associated with reduced mtDNA content, while p53 deficiency partially rescues the transcriptional regulation of PGC-1α/β and mtDNA CN (Sahin et al. 2011). P53 also contributes to the stress-induced activation of subtelomeric region and TERRA (telomeric repeat-containing RNA)-like transcripts from multiple chromosomes (Tutton et al. 2016). Subtelomeres are segments of DNA between telomeric caps and chromatin (Mefford and Trask 2002). TERRA has important roles in the regulation of telomerase and in arranging chromatin remodelling throughout development and cellular differentiation. The accumulation of TERRA at telomeres can also interfere with telomere replication, leading to a sudden loss of telomere tracts (reviewed in Luke et al. 2009). P53-binding sites in human subtelomeres provide enhancer-like functions that have direct impact on the local chromatin structure and DNA damage response at subtelomeric DNA (Tutton et al. 2016).

There are suggestions that p53 influences telomere DNA structure and promotes telomere DNA stability, and its binding can be induced by numerous stress conditions (Tutton et al. 2016). In contrast, in mitochondria, p53 plays a direct role in the mito-checkpoint response and positively regulates mtDNA CN; the loss of p53 leads to the reduction of mtDNA content in MEF cells (Kulawiec et al. 2009). Consistent with the mtDNA CN reduction, p53 null cells show a decrease in mitochondrial membrane potential and reduction in mitochondrial mass, linking p53 to promotion of normal mitochondrial function (Lebedeva et al. 2009). P53 improves the accuracy of mtDNA synthesis, possibly by providing a proofreading function for Poly γ (Achanta et al. 2005; Bakhanashvili et al. 2008). P53 regulates mitochondrial respiration directly by interacting with synthesis of cytochrome c oxidase 2 (SCO2) and affects COX complex in the mitochondria (Matoba et al. 2006). In addition, p53 can act as a pro-oxidant meaning that the loss of p53 results in reduced mitochondrial superoxide and disrupted cellular ROS homeostasis (Lebedeva et al. 2009). Safdar et al. have shown that p53 translocates to the mitochondria and facilitates mtDNA mutation repair and mitochondrial biogenesis in response to endurance exercise in mice. These results suggest that in cells under certain conditions, more p53 is located in

mitochondria than in nuclei, and in this way, p53 helps to maintain healthier cells, longer telomeres, less mtDNA mutations and prevents apoptosis (Safdar et al. 2016). Alternatively, another study has found that overexpression of p53 negatively affects the mtDNA abundance in HepG2 cells. They showed that overexpression of p53 negatively affects normal mitochondrial homeostasis, decreases mtDNA abundance, and enhances sensitivity to nucleoside reverse transcriptase inhibitors that deplete mtDNA (Koczor et al. 2012). These studies show how complicated is p53 pathways in a cell and how much is still to find out how it influences telomeres and mtDNA (reviewed in Park et al. 2016). Pieters et al. showed that SIRT1 also plays a role in the telomere-mitochondrial ageing axis. In their work, TL was positively correlated with SIRT1, which deacetylates and inactivates p53 protein, and NRF1 (involved in regulation of mtDNA transcription and replication) and negatively correlated with p53 expression, while mtDNA content was positively correlated with SIRT1 and NRF1 expression (Pieters et al. 2015). SIRT1 is involved in glucose homeostasis as a modulator of PGC-1α, and it deacetylates p53, leading to reduced apoptosis (Vaziri et al. 2001; Rodgers et al. 2005); extra expression of SIRT1 enhances mitochondrial function by upregulating mitochondrial biogenesis and degradation, thus improving oocyte development (Sato et al. 2014).

The first observation of TL and mtDNA CN correlation in peripheral leukocytes in population studies comes from studies of patients with diseases linked to alterations of these two cell components such as type 2 diabetes (Monickaraj et al. 2012). A study about depression found no correlation: the authors reported shortened telomeres but no reduced mtDNA CN in leukocytes in patients with depressive symptomology (Verhoeven et al. 2018), while childhood adversity and lifetime psychopathology were each linked to shorter telomeres but higher mtDNA CN in leukocytes (Tyrka et al. 2016). In a study about cognitive dysfunction in individuals over 75 years of age authors saw positive relationship between TL and mtDNA in blood samples after adjustment for age and gender; and for individuals with conative dysfunction both high TL and high mtDNA CN amount were observed, speculating that it might be due to increased oxidative stress and inflammation (Lee et al. 2017a).

Data from different tissues are also available. Patients with Fuchs' endothelial corneal dystrophy (FECD) had higher mtDNA levels but shorter telomeres in endothelial cells (Gendron et al. 2016) while in patients suffering mental disorders had shorter telomeres and

lower mtDNA CN in post-mortem brains of people who had committed suicide (Otsuka et al. 2017). For people with colorectal carcinogenesis, a positive correlation depended on the stage of the cancer in tubular adenoma and serrated polyp tissue. In normal and cancer tissues, the correlation was positive, but in precancerous legions, the correlation was not observed, suggesting that the disturbance of the telomere-mitochondrial axis by ageing or other factors may be important in the development of carcinogenesis (Lee et al. 2017b). Jung et al. also showed a sign of dynamic TL and mtDNA CN relationship. They found a positive correlation in normal tissues and the intestinal type of gastric cancer but not in the diffuse type of the disease (Jung et al. 2017). In addition, not only was mtDNA CN correlated with TL, but mtDNA non-silent mutations had a negative correlation with TL in bone marrow and oral epithelial cells in aplastic anaemia patients (Cui et al. 2014).

As in diverse pathologies and tissues the telomere-mitochondrial relationship can vary, it is important to look if this connection is evident in general populations during the healthy ageing process. The use of peripheral blood leukocytes for these studies could be argued as not the ideal tissue as discussed above. However, this approach is widely used as the evidence suggests that TL measurement in easily accessible tissues such as blood could serve as a surrogate parameter for the relative TL in other tissues (Friedrich et al. 2000), and general population peripheral blood mtDNA content is significantly associated with sex and age (Knez et al. 2016). One of the first population studies in healthy humans came from Kim et al. who showed a positive association between leucocyte TL and mtDNA CN in elderly women a with an average age of 73 years, suggesting that telomere function might influence mitochondrial function in humans (Kim et al. 2013). Other researchers have confirmed this association in individuals aged 18 to 64 years (Tyrka et al. 2015) and individuals aged 60 to 80 years, suggesting that telomeres and mitochondria are co-regulated in humans (Pieters et al. 2015). Additionally, Qiu and colleagues observed a positive correlation between TL and mtDNA CN in leukocytes of pregnant women after adjusting for age and plasma vitamin B12 (Qiu et al. 2015). A positive association was found in children ages 6–12 (Alegría-Torres et al. 2016). While the exact molecular mechanism underlying the telomeres-mtDNA associations is not clear and more studies are required, it is even less know how it is in nonagenarians (people between 90 and 100 years old) and centenarians (over 100 years old). Telomere shortening might influence mtDNA amount or vice versa differently; these findings could be partly explained by different polymorphisms in the mtDNA sequences (e.g., Niemi

et al. 2005; Takasaki 2008; Guney et al. 2014), the diverse nuclear DNA SNPs and the healthier lifestyle noted in these individuals compared with individuals who do not live over 90 years of age (e.g., Yashin et al. 2000; Debrabant et al. 2014; Gierman et al. 2014; Govindaraju et al. 2015).

2. MATERIAL AND METHODS

2.1. Description of samples from each publication separately

I. "*Dynamics of telomere length in different age groups in a Latvian population*". Blood samples were collected from healthy individuals, without any disorders considered as telomere affecting, in the Latvian population aged from 20 to 100 years old. In total, 121 individuals were enrolled in this study. These individuals belonged to mitochondrial haplogroups, as follows: H (45%), U (25%), Y chromosomal N1c (40%) and R1a1 (40%). All participants provided appropriate written informed consent to use their phenotypic and genetic data, which were voluntarily provided via detailed health and heredity questionnaires. The samples were divided into five age groups: 20–40 years (control group, n=32, there is no statistically significant difference between 20-30 and 31-40 years, P= 0.4209), 60-70 years (n=21), 71-80 years (n=24), 81-90 years (n=19) and over 90 years (centenarians, n=25). Groups 41-50 years and 51-60 years were not selected, because in the current study our focus of interest was elderly individuals with age above 60 years due to the highest mortality rate among the representatives of this age group in the Latvian population. The mean gender ratios for this study was 64:36 (%) for females:males. The gender ratios (females:males, expressed as percent) by group were 53:47, 67:33, 54:46, 79:21 and 72:28 for the 20–40 years, 60-70 years, 71-80 years, 81-90 years, and over 90 years groups, respectively. For PBMC study 21 sample were obtained and divided in two age groups (20-40 years and 65-85 years).

II. "*Comparison of telomere length between population-specific mitochondrial haplogroups among different age groups in a Latvian population*". Blood samples were collected from healthy individuals, without any disorders considered as TL affecting, in the Latvian population aged from 20 to over 90 years old. In total, 772 individuals were enrolled in this study. All participants provided appropriate written informed consent to use their phenotypic and genetic data, which were voluntarily provided via detailed health and heredity questionnaires. The samples for mitochondrial hgs were divided into three age groups: 20–45 years (control group, n=378), 55-89 years (n=271), and over 90 years (centenarians, n=128). For 128

samples TL was measured. These samples were divided into the same age groups: 20–45 years (control group, n=37), 55-89 years (n=67) and over 90 years (centenarians, n=25). A group 45-55 year was not selected, because in the current study our focus of interest was elderly individuals with age above 60 years due to the highest mortality rate among the representatives of this age group in the Latvian population.

III. "*Linkage between mitochondrial genome alterations, telomere length and ageing population*".

Blood samples were collected from healthy individuals from Latvian population ranging ages from 20 to 100 years. In total, 210 samples were divided into three age groups: 20–59 years (n=70, mean age=32 years, females=65%), elderly group (60-89 years, n=70, mean age=73 years, females=73%) and nonagenarians (90-100 years, n=70, mean age=93 years, females=76%). All individuals were Caucasian. All participants provided appropriate written informed consent to use of their phenotypic and genetic data, which were voluntarily and anonymously provided via health and heredity questionnaires. The samples and information about samples were obtained from the Genome Database of the Latvian Population (VIGDB, bmc.biomed.lu.lv/lv/par-mums/saistitas-organizacijas/vigdb/). None of the selected individuals had reported any severe diseases during the medical examination. Some individuals from the elderly group and nonagenarians had vision problems, hearing loss, dizziness, arthritis, osteochondrosis and joint pain, fatigue and sleep disorders, minor urinary tract or digestive tract disorders. None of the study participants had Alzheimer's disease, Parkinson disease or cancer – diseases associated with TL or mtDNA alterations. No information of smoking and drinking habits was available.

2.2. Extraction of genomic DNA

Genomic DNA was extracted from the peripheral white blood cells using the standard phenol–chloroform method as described in (Sambrook et al. 1989).

2.3. Southern blots of terminal restriction fragments (TRFs)

The method described in Kimura et al. work was used, with some modifications, to determine TL. Briefly, a Southern blot of TRFs was conducted using a Telo TAGGG

Telomere Length Assay kit (Roche, UK). Concentrated DNA (~1 μg) was digested with restriction endonucleases *Hinf*I (10 U) and *Rsa*I (10 U) (Kimura et al. 2010). Digested DNA samples, a DNA size marker (GeneRuler 1 Kb DNA ladder, Thermo Scientific, Lithuania), and the DIG Molecular weight marker (Roche, UK) were loaded into a 0.8% agarose gel and run for 20 hours (19 V and 25 mA) to resolve fragment sizes. The DNA in the gel was then depurinated in 0.25 M HCl for 10 min. Further, the gel with the samples was denatured in 0.5 L of 0.5 M NaOH and 1.5 M NaCl for two 20-min washes. The samples were neutralized in 1 L of 0.5 M Tris-OH containing 3 M NaCl (pH 7.5) for two 20-min washes. The DNA was transferred to a positively charged nylon membrane (Amersham Hybond^{TM}-N^{+}, GE Healthcare Life Sciences, UK) for 2 hours using a vacuum blotter (VacuGene Pump, Pharmacia Biotech, Sweden) with a 20x SSC transfer buffer solution that contained 0.3 M sodium citrate and 3 M NaCl (pH 7.0). DNA was fixed to a membrane using a 30-sec UV exposure, and the membrane was briefly washed in 2x SSC solution. The subsequent steps were performed using the manufacturer's protocol for the Telo TAGGG Telomere Length Assay kit (Roche, UK). The membrane was visualized on a high performance chemiluminescence film (GE Healthcare Life Sciences, UK). The film was scanned, and the TRF signal was detected. DNA migration distances were measured using the Kodak Digital Science D1 program (Kodak, US); the DIG ladder was used for molecular size reference. The optical density of the DNA fragments was measured using the ImageJ software (Rasband, 1997-2014). TL was calculated using the following equation: mean TRF length=Σ(ODi)/Σ(ODi/Li), where ODi = optical density at position i and Li = TRF length at position i.

2.4. Relative qPCR SYBR green telomere length quantification assay

Relative TL was measured as telomere repeat copy number relative to single gene copy number (T/S ratio) using real-time polymerase chain reaction (qPCR) with the Maxima SYBR green qPCR Master Mix (2X) (Thermo Scientific, USA). The forward and reverse primers of telomeres for one reaction were as follows: Telo1 (200 nM), 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′, and Telo2 (200 nM), 5′-CCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′. After a denaturation step at 95 °C for 10 min, DNA samples were incubated for 40 cycles at 95 °C for 10 s and 58 °C for 1 min. TL was normalized using the following forward and reverses primers for the β-globin

gene in a separate run: Beta-glob1 (300 nM), 5′-GCTTCTGACACAACTGTGTTCACTAGC-3′, and Beta-glob2 (500 nM), 5′-CACCAACTTCATCCACGTTCACC-3′. After a denaturation step at 95 °C for 10 min, DNA samples were incubated for 40 cycles at 95 °C for 10 s and 56 °C for 20 s (Kim et al. 2013). The concentration of the DNA samples was 10 ng/μL in a 10 μL reaction. Each sample was run in triplicate. A no-template control and duplicate calibrator samples were used in all runs to allow comparisons of the results across all runs. A melting curve analysis was performed to verify the specificity and identity of the PCR products. TL was calculated using threshold cycle values and the following equation: relative TL=2ΔCt(ΔCt=Ctβ-globin−Ct telomeres).

2.5. Relative qPCR TaqMan mtDNA copy number quantification assay

Relative mtDNA copy number was measured using qPCR with the Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Scientific, USA). MtDNA copy number amount was normalized by simultaneous measurements of the nuclear gene Gapdh and the mitochondrial D-loop. The forward and reverse primers for the Gapdh reaction (1250 nM each) were GapdhF, 5′-GAAGGTGAAGGTCGGAGT-3′, and GapdhR, 5′-GAAGATGGTGATGGGATTTC-3′, respectively, and the TaqMan probe was GapdhTqM (250 nM), 5′-CAAGCTTCCCGTTCTCAGCC-3′. The forward and reverse primers (50 nM each) for the mitochondrial D-loop were FmtMinArc, 5′-CTAAATAGCCCACACGTTCCC-3′, and RmtMinArc, 5′-AGAGCTCCCGTGAGTGGTTA-3′, respectively, and the TaqMan probe was PmtMinArc (250 nM) - 5′-CATCACGATGGATCACAGGT-3′ (Phillips et al. 2014). The DNA concentration for the samples was 10 ng/μL in a 15 μL reaction. After a denaturation step at 95 °C for 10 min, the DNA samples were incubated for 40 cycles at 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s. Each sample was run in triplicate. A no-template control and duplicate calibrator samples were used in all runs to allow for comparison of results across the runs. MtDNA copy number was calculated using threshold cycle values and the following equation: relative copy number = 2ΔCt(ΔCt=CtGapdh−CtD-loop).

2.6. Detection of heteroplasmy

The presence of mtDNA heteroplasmy was determined using the SURVEYOR™ Mutation Detection Kit, which is based on the use of a mismatch-specific endonuclease, SURVEYOR Nuclease (Transgenomic, USA) (Bannwarth et al. 2006). This kit detects

heteroplasmy as low as 5% and was our choice having been tested it in our previous work (Pliss et al. 2011) although there are certain limitations using this kit (Yen et al. 2014). Due to restricted amount of DNA available for this study only HVS-I region was tested. This region was chosen due to the possible presence of a higher number of heteroplasmic polymorphisms (Vigilant et al. 1989, Ngili et al. 2012). The DNA fragment encompassing the mtDNA HVS-I between nucleotide positions (nps) 16024–16390 was amplified and sequenced in all samples using forward (F-HVS-I) and reverse (R-HVS-I) primers (200 nM each) (Vigilant et al. 1989) in final volume of 25.5 µL containing 10–50 ng DNA, 1.5 mM MgSO4, 1× Optimase reaction buffer, 10 mM each dNTP, 1.25 U/µL Optimase polymerase (Transgenomic, USA). The amplification conditions: denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s, and final elongation at 72 °C for 5 min. Negative and positive controls were prepared for amplification. The PCR products were heated to denature the DNA and were then slowly cooled to room temperature to allow for reannealing of the DNA strands using the following protocol: initial denaturation at 95 °C for 2 min, followed by a 95 °C to 85 °C (−2 °C/s) gradual temperature decrease, and then a 85 °C to 25 °C (−0.10 °C/s) temperature decrease. Afterwards, products were incubated with Surveyor Enhancer S 0.5 µl and Surveyor nuclease S 0.5 µL at 42 °C for 20 min; 1 µL of stop solution was added at the end of reaction. The samples were analysed by a 6% polyacrylamide gel electrophoresis.

2.7. Mitochondrial genotyping

To confirm the hg affiliation of mitochondrial sequences, hierarchical PCR–RFLP analysis was performed using 17 restriction endonucleases: *Alu*I, *Ava*II, *Dde*I, *Bsh*1236I, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Rsa*I, *Nla*III, *Acc*I, *BstO*I, *Mse*I, *Alw*44I, *Ssp*I, *Eco*47I, and *Bsu*RI (van Oven and Kayser 2009). The classification of hgs was based on their position in the hierarchy of the mitochondrial phylogenetic tree (www.phylotree.org, van Oven and Kayser 2009).

2.8. Statistical analysis

The statistical significance of the differences between the observed distributions of mitochondrial hgs in three age groups was evaluated with the G-test using the R 3.1.1 (R Core Team, 2014) software program. Linear regression and correlation, unpaired two-tailed t-test

and Analysis of variance (ANOVA) was performed using GraphPad Prism version 5 for Windows, GraphPad Software (La Jolla California USA, www.graphpad.com). Data were expressed as means ± SEM (standard error of the mean) ±SD (standard deviation) and differences of $P<0.05$ were considered significant.

3. RESULTS

I Dynamics of telomere length in different age groups in a Latvian population

Highlights:

Part of the results from this publication was used in these theses.

1. Telomere length shorten with age as it was expected, and the smallest variability of telomere length was absorbed in nonagenarian (in this paper referred as centenarian) group.

2. 20% of the variation in telomere length was associated with ageing.

3. Telomere length was not significantly longer for any of gender.

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Dynamics of Telomere Length in Different Age Groups in a Latvian Population

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Abstract: The shortening of telomeres with ageing is a well-documented observation; however, the reported number of nucleotides in telomeres varies between different laboratories and studies. Such variability is likely caused by ethnic differences between the populations studied. Until now, there were no studies that investigated the variability of telomere length in a senescent Latvian population of the most common mitochondrial haplogroups, defined as H (45%), U (25%), Y chromosomal N1c (40%) and R1a1 (40%). Telomere length was determined in 121 individuals in different age groups, including a control group containing individuals of 20-40 years old and groups of individuals between 60-70 years old, 71-80 years old, 81-90 years old, and above 90 years old. Telomere length was determined using the Southern blot telomeric restriction fragment assay (TRF). Decreased telomere length with ageing was confirmed, but a comparison of centenarians and individuals between 60-90 years of age did not demonstrate a significant difference in telomere length. However, significant variability in telomere length was observed in the control group, indicating probable rapid telomere shortening in some individuals that could lead up to development of health status decline appearing with ageing. Telomere length measured in mononuclear blood cells (MNC) was compared with the telomere length measured in whole peripheral white blood cells (WBC) using TRF. Telomere length in MNC was longer than in WBC for the control group with individuals 20 to 40 years old; in contrast, for the group of individuals aged 65 to 85 years old, measured telomere length was shorter in MNC when compared to WBC.

Keywords: Ageing, mononuclear cells, telomere length, TRF.

INTRODUCTION

Telomeres are the specialised chromosomal DNA–protein structures that cap and protect the terminal regions of eukaryotic chromosomes. The sequence of telomeric DNA is (TTAGGG)n, and in humans its length ranges from 5 to 20 kb. Telomeres are dynamic structures that become shorter with every division of a cell until a critical stage is reached when the cell cannot divide anymore, which is thought to be a consequence of ageing [1].

However, population studies of blood cells have demonstrated that telomere length in one age group can be quite diverse among different populations. Differences have been observed between the French and the Italian populations, with the French population containing longer telomeres in comparison to the Italian population [2]. Analysis of telomere length in different populations has shown that individuals from southern Europe possess shorter telomeres than individuals from the Baltic region and that individuals from the middle of Europe contain the longest telomeres [3]. However, another report that included 14 populations from Europe showed that telomere length varied among populations, with the shortest telomeres measured in an Italian population (5100 bp) and the longest telomeres measured in a Belgian population (18640 bp). Telomeres were 7340 bp in an Estonian population, a geographically proximal population to the Latvian population studied in this work. Similar results were obtained for two subpopulations in Finland: in an Oulu-based population the mean telomere length was 7620 bp, and in a Helsinki-based population the mean telomere length was 12280 bp [4]. However, until now, there were no studies concerning the variability of telomere length in ageing in a Latvian population; the most common mitochondrial haplogroups were defined as H (44.5%), U (25.4%), Y chromosomal N1c (~38.6%) and R1a1 (~39.6%). Estonians: H (43.5%), U (26.9%), Y chromosomal N1c (30.6%) and R1a1 (33.5%). Finns: H (40.5%), U (27.9%), Y chromosomal N1c (30.6%) and R1a1 (33.5%) [*e.g.* 5, 6].

Peripheral white blood cells (WBC) consist of two cell types, including mononuclear cells (MNC – T and B cells, Plasma cells, NK (Natural killer) cells, Dendritic cells and monocytes) and cells with segmented or granulated nuclei, also known as granulocytes. WBC lifespan ranges from a couple of hours, to days, to years for lymphocyte memory cells, whereas the lifespan of granulocytes can be from a couple of hours to several days [7]. Previous studies have reported that telomere length in MNC is longer than in WBC (9.4% longer) and bone marrow cells (2.7% longer) [8, 9].

The aims of this study were to determine telomere length and observe any variation in telomere length in a senescent Latvian population of the most common mitochondrial haplogroups, defined as H (45%), U (25%), Y chromosomal N1c (40%) and R1a1 (40%). Analysis of telomere length in WBC and MNC in different age groups within the Latvian

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population enabled a comparison of the variability of telomere length in the control group *versus* the age group containing individuals who were at least 90 years old.

MATERIALS AND METHODS

Samples

Blood samples were collected from healthy individuals, without any disorders considered as telomere affecting, in a Latvian population aged from 20 to 100 years old. In total, 121 individuals were enrolled in this study. These individuals belonged to mitochondrial haplogroups, as follows: H (45%), U (25%), Y chromosomal N1c (40%) and R1a1 (40%). All participants provided appropriate written informed consent to use their phenotypic and genetic data, which were voluntarily provided *via* detailed health and heredity questionnaires. The samples were divided into five age groups: 20–40 years (control group, n=32, there is no statistically significant difference between 20-30 and 31-40 years, P= 0.4209), 60-70 years (n=21), 71-80 years (n=24), 81-90 years (n=19) and over 90 years (centenarians, n=25). Groups 41-50 years and 51-60 years were not selected, because in the current study our focus of interest was elderly individuals with age above 60 years due to the highest mortality rate among the representatives of this age group in a Latvian population. The mean gender ratio for this study was 64:36 (%) for females: males. The gender ratios (females: males, expressed as percent) by group were 53:47, 67:33, 54:46, 79:21 and 72:28 for the 20–40 years, 60-70 years, 71-80 years, 81-90 years, and over 90 years groups, respectively.

Acquisition of Mononuclear Blood Cells (MNC)

Mononuclear blood cells (MNC – T and B cells, Plasma cells, NK cells, Dendritic cells and monocytes) were obtained from 21 samples in two age groups (20-40 years and 65-85 years) using ACCUSPIN System-HISTOPAQUE-1077 tubes based on the manufacturer's protocol (Sigma-Aldrich, Germany). Five ml of fresh whole blood was poured into the upper chamber of each pre-filled tube, and then centrifuged at 1000 x g, at 18–26°C, for 50 minutes. After centrifugation, the plasma layer was carefully aspirated with a pipet to within 0.5 cm of the opaque interface containing the mononuclear cells. The mononuclear band was carefully transferred with a pipet into a clean centrifuge tube and was washed by adding 10 ml of isotonic PBS. The cells were resuspended by gentle aspiration with a pipet and were then centrifuged at 250 x g, at 18–26°C, for 10 minutes. Samples were stored at -20 to -70°C.

Extraction of Genomic DNA

Genomic DNA was extracted from the peripheral blood white cells (WBC) and from MNC using the standard phenol–chloroform method as described in [10].

Southern Blots of Terminal Restriction Fragments (TRFs)

Telomere length was detected *via* a Southern blot of terminal restriction fragments (TRFs) using the Telo TAGGG Telomere Length Assay kit (Roche, UK) according to the method of Kimura *et al*. (2010) with some modifications. Seven µl of concentrated DNA (~1 µg) was digested with restriction endonucleases Hinf I (10 U) and *Rsa* I (10 U) [8]. Digested DNA samples, a DNA size marker (GeneRuler 1 Kb DNA ladder, Thermo Scientific, Lithuania), and the DIG Molecular weight marker (Roche, UK) were loaded into a 0.8% agarose gel and run for 20 hours with 19 V or 25 mA to resolve fragment sizes. Afterwards, the DNA in the gel was depurinated in 0.25 M HCl for 10 min. The gel with the samples were then denatured in 0.5 M NaOH and 1.5 M NaCl for 20 min and washed 2x with 0.5 L. Samples were neutralised in 0.5 M Tris-OH containing 3 M NaCl (pH 7.5) for 20 min and washed 2X with 1 L. The DNA was transferred to a positively charged nylon membrane (Amersham, UK) for 2 hours using a vacuum blotter (VacuGene Pump, Pharmacia Biotech, Sweden) with a 20x SSC transfer buffer solution that contained 0.3 M sodium citrate dehydrate and 3 M NaCl (pH 7.0). DNA was fixed to a membrane using a 30 sec UV exposure, and then the membrane was washed in 2x SSC solution. The subsequent steps were performed based on the protocol for the Telo TAGGG Telomere Length Assay kit (Roche, UK). The membrane was visualised on a high performance chemiluminescence film (Amersham, UK). The film was scanned, and the TRF signal was digitised. DNA migration distances were measured using a Kodak Digital Science D1 program using the DIG ladder for molecular size reference. The optical density of the DNA fragments was measured using the ImageJ program. Telomere length was calculated by the following equation: mean TRF length = (Σ(ODi)/Σ(ODi/Li), where ODi=optical density at position i and Li is TRF length at position i.

Statistical Analysis

Linear regression and t-tests were performed using GraphPad Prism version 5 for Windows, GraphPad Software (La Jolla California USA, www.graphpad.com).

RESULTS

Telomere Length Variation and Distribution in Different Age Groups in the Latvian Population

Statistically significant differences between the control group (individuals aged 20-40 years old) and age groups, containing individuals who were above 60 and 90 years old, were observed; the results demonstrated an approximate 1037 bp difference in length (14.5%) between the control group and these age groups. Comparison of the telomere length variability within an individual age group demonstrated that that the distribution of telomere length values was greater in the control group (individuals 20-40 years old) than in older age groups. The smallest variability of telomere length values was observed among the centenarian group (Fig. **1**). The same trend was observed in previously reported studies [11-13]. However, the approximate difference (1.9%) between the centenarian group (with individuals over 90 years old) and groups with individuals aged from 60 to 90 years old was not significant. Similar observations were shown in previous studies [14, 15]. There were no statistically significant differences observed between females and males among different age groups under the study (Fig. **2**). Among the 121 analysed samples, the mean telomere lengths for each group were as follows: 7132 bp (control group),

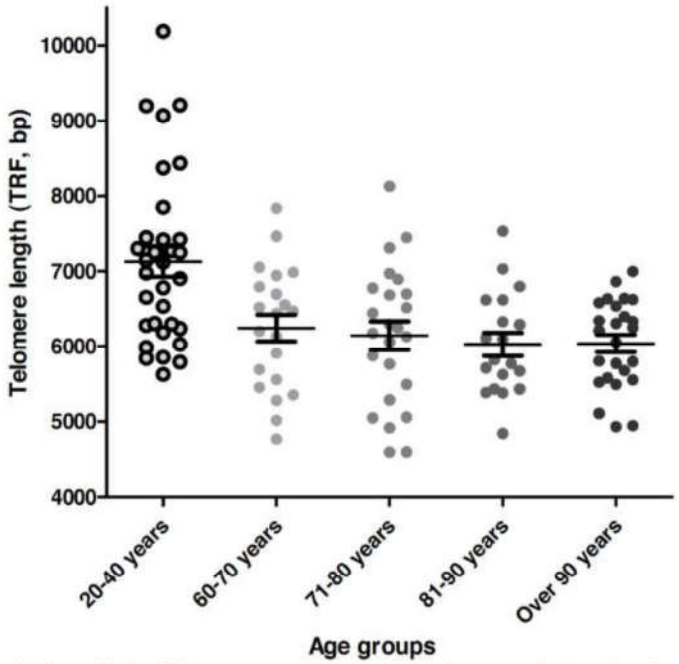

Fig. (1). Telomere length and distribution of it in different age groups (with grouping procedure). The absolute value of the telomere length of each individual sample and the mean ± SD (standard deviation) are provided for each age group. The P-value of the comparison between the control group (individuals 20-40 years old) and the 60-90-year-old group is <0.0001. The P-value of the comparison between the 60-70-year-old age group and 71-80-year-old age group is 0.7015; 60-70 and 81-90 years groups P=0.3684; 60-70 years and centenarian groups P=0.3299. The P-value of the comparison between the 71-80-year-old age group and the 81-90-year-old age group is 0.6472; 71-80 years and centenarian groups P=0.6389. The P-value of the comparison between the 81-90-year-old age group and the centenarian group is 0.9476.

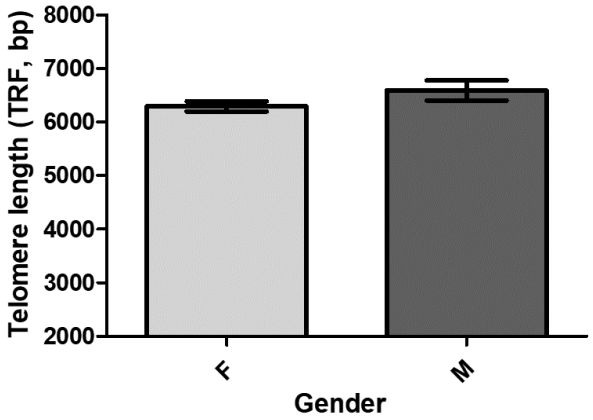

Fig. (2). Comparison of telomere length between females and males. F-female, M-male. The telomere length values are given as the mean ± SD, P= 0.1193.

6243 bp (60-70-year-old group), 6142 bp (71-80-year-old group), 6025 bp (81-90-year-old group), and 6038 bp (centenarians).

In the current study there was observed a correlation between age and telomere length (Fig. **3**). Linear regression analysis demonstrated this correlation with P<0.0001, R^2= 0.1979. These results confirm that telomeres shorten during ageing in a Latvian population. The R^2 value indicates that 20% of the variation in telomere length was associated with ageing; however, there are also other genetic and/or non-genetic factors that may have influenced the measured telomere length.

Comparison of Telomere Lengths Between MNC and WBC

Analysis of telomere length for 21 samples derived from WBC and MNC demonstrated that telomeres from MNC were longer than telomeres from WBC by 11.5% (Fig. **4**). The results of a t-test showed that the difference between telomeres from WBC and MNC was statistically significant (P<0.0001). Similar observations were reported by Sakoff *et al.* 2002 [9].

Comparison of telomeres from these cell types in two different age groups, *i.e.*, the control group with individuals aged 20-40 years and the group with individuals aged 65-85

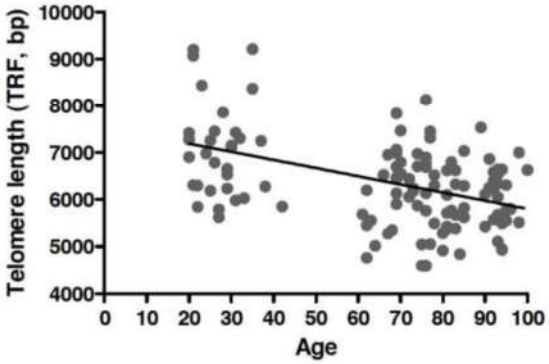

Fig. (3). Correlation between telomere length and age without grouping procedure (P <0.0001).

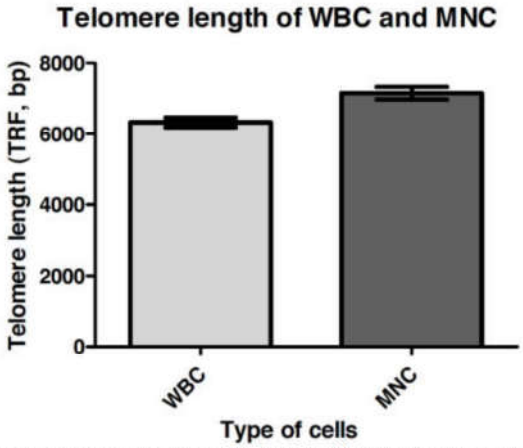

Fig. (4). Differences in telomere length between the peripheral white blood cells (WBC) and the mononuclear blood cells (MNC). The telomere length values are given as the mean ± SD, P<0.0008.

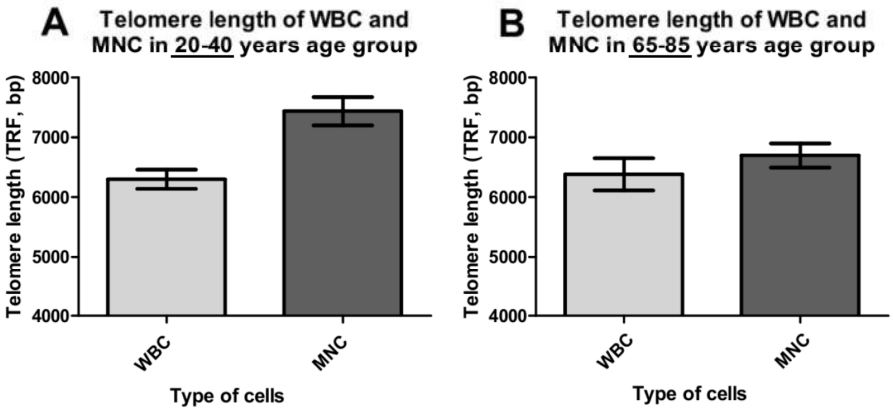

Fig. (5). Differences in telomere length between the peripheral white blood cells (WBC) and the mononuclear blood cells (MNC) in two different age groups. The telomere length values are given as the mean ± SD. A: P< 0.0001; B: P=0.1890.

years old. The group with age 65-85 years was selected due to the highest mortality rate observed in it suggested that after 65 years of age telomere length decreases by a smaller degree in MNC than in WBC (P=0.1890). This difference was not statistically significant, and in some cases telomere length in MNC samples was shorter than in samples from WBC after 65 years (Fig. **5**). To confirm or refute the presence of a statistically significant difference in telomere length separately in the MNC and separately in the WBC between the control group (20-40 years old) and the group with individuals aged 65-85 years old, a t-test was performed (Fig. **6**). The results demonstrated a statistically significant difference in MNC telomere length by age groups (P=0.0428), but there was no significant difference in WBC telomere length by age groups (P=0.7887).

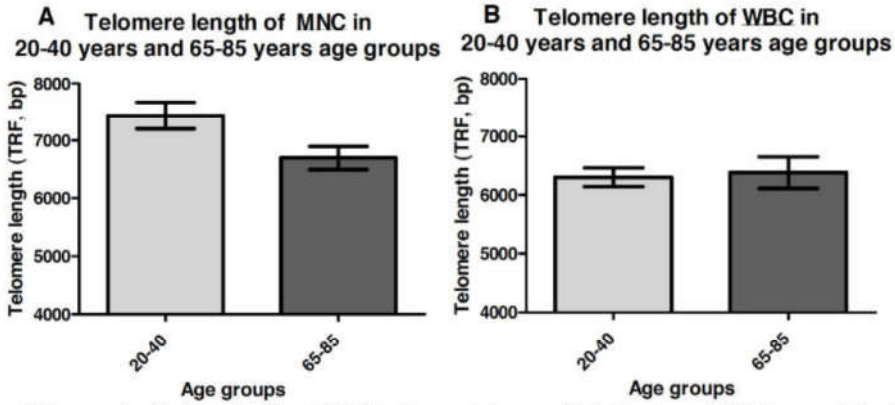

Fig. (6). Comparison of telomere length from MNC and WBC in the control group (individuals aged 20-40 years old) and 65-85-year-old age groups. The telomere length values are given as the mean ± SD. A: P=0.0428 indicates that there was a statistically significant difference between the 20-40-year-old and 65-85-year-old age groups for telomere length in MNC. B: P=0.7887 indicates that there was no statistically significant difference between the telomere lengths in WBC for the 20-40-year-old and 65-85-year-old age groups.

DISCUSSION

The average telomere length in the Latvian population is 6381 bp, and this study confirmed telomeric shortening with increasing age. Many studies have shown that telomeres shorten during ageing [*e.g.*, 16, 17], and the results of the current research showed the same relationship. Shortened telomeres due to ageing are associated with higher rates of mortality from different age-related pathologies, including heart and vascular diseases, diabetes mellitus, Parkinson's disease, and Alzheimer's disease, and other disorders [*e.g.* 18, 19]. The observation that centenarians had almost the same telomere length as individuals from the 60-90-year-old age groups may be explained by the assumption that at certain age telomere maintenance is more critical than the exact telomere length [11, 17, 20]. In some studies were mentioned that females possess longer telomeres than males but in the current study this tendency was not observed [*e.g.* 21]. Our results showed statically insignificant longer telomeres for males that could be explained by sample ratio between the genders, where male individuals formed a smaller proportion in comparison to females.

Variability in telomere length within an individual age group may be explained by the "selection hypothesis", which proposed that the centenarians harboured very long telomeres, and variation in telomere length reduces with age due to the selective loss of individuals with shorter telomeres [11].

The telomere length of geographically proximal populations to Latvia, such as Estonian and Finish populations, varied from 7340 bp to 12280 bp [4]. These differences among populations are poorly understood. The evidence could be explained by differences in genetics, nutrition, life expectancies, economic and social status or stress level. This phenomenon (telomere variation) could be elucidated by these population differences, as telomere length is only one important factor that influences ageing, which is a complicated senescence process in humans. Most likely, every population has its own specific determinants of telomere length and cellular homeostasis [4, 22]. In Latvia, the average life expectancy is 72-73 years (78.4 years for females and 68.8 years for males). In Estonia and Finland, the average life expectancies are 75 and 80 years, respectively [23, 24]. Heart and circulatory diseases are associated with shortened telomeres [25], and there is a high mortality rate from those diseases in Latvia; for every 100,000 inhabitants, 254.5 cases of heart disease(s) and 479.5 cases of circulatory diseases are diagnosed. In Estonia 204.8 cases of heart disease(s) and 423.6 cases of circulatory diseases are diagnosed per 100,000 people, and in Finland, 122.5 cases of heart disease(s) and 218.1 cases of circulatory diseases are diagnosed per 100,000 people. Health care and socioeconomic status are lower in Latvia in comparison to Estonia and Finland [26]. Telomere length can be affected by lifestyle choices including smoking and alcohol consumption.

It is not much studied how different haplogroups affect telomere length but it is shown that there is association with mitochondrial DNA (mtDNA) halplogrops and longevity [27]. According to Fernández-Moreno *et al.* (2011) study, individuals with haplogroup J have longer telomeres than non-J carriers, with explanation that haplogroup J carriers are less prone to suffer oxidative stress that can affect telomere length [28].

In this study, as in others by [29, 30], a difference between the telomere lengths found in WBC *versus* MNC cells was reported (Fig. **4**). It has been demonstrated that telomerase is active in activated T and B lymphocytes. Telomerase becomes active when these cells perceive antigen. This function provides for their survival for several years, as well as their ability to undergo clonal expansion [29, 31].

It is already known that the immune system loses its effectiveness during ageing. There is clear evidence that leucocytes lose telomere length with age, as noted by Slagboom *et al.* (1994) and Takubo *et al.* (2010) [12, 13]. In the present study, the telomere length of MNC (including T and B, NK cells) shortened more rapidly with age than telomeres in WBC (including all cells: granulocytes and T, B, NK cells etc.) cells (Fig. **6**). One possible explanation could be age-associated thymic involution theory. Weng *et al.* (1996) demonstrated that the most active telomerase is in found within thymocites (T cells in the thymus gland), and medium-activity telomerase was found in T cells in the tonsil gland; however, low or undetectable telomerase activity was

found in quiescent peripheral blood T cells [32]. At birth, when the thymus gland is fully developed, it weighs approximately 10 g. By the age of 50, fat accounts for more than 80% of the total thymic volume. In childhood, tonsillar involution proceeds at a rate of approximately 3% per year until middle age; afterwards, it slows to 1% per year, according to reports by [33-35]. With increasing age, thymic output decreases, but as naïve T cells are long-lived, this decreased output does not affect immune system functions for some time [36]. During ageing, the capacity to maintain telomere length decreases due to the increasingly rapid rate of telomeric shortening in MNC, which results in a reduction of T cells clonal expansion.

Naïve T cells have longer telomeres than memory and effector T cells. During the ageing process, the number of naïve T cells decreases, but the number of memory T cells stays more or less stable in peripheral blood [37, 38]. Therefore, it may also influence telomere length in MNC cell group.

With increasing age, NK cells lose telomere length and telomerase activity [39]. Ageing can affect the rate of telomeric shortening. Telomere length is longer in immature NK cells than in mature NK cells. The ratio of immature to mature NK cells in peripheral blood during ageing does not change dramatically, but a higher number of immature NK cells are present than mature NK cells [30]. Therefore, we could suppose that by loosing telomere length during ageing, NK cells could influence telomere length in MNC group.

Naïve B cells and memory B cells have similar telomerase activities and similar telomere length in individuals of the same age. However, during ageing the number of naïve B cells present in peripheral blood decrease, in contrast to the number of memory B cells, which increase with age [31]. This implies that B cells do not affect the rate of telomere length shortening during ageing in MNC group.

CONCLUDING REMARKS

Analysis of samples from a Latvian population confirmed that during the ageing process, telomere lengths shorten but that this process does not necessarily occur instantaneously or at a specific age. Among centenarians, the variability in telomere length is not significantly different from the other age groups included in this study. The variability in telomere length observed in the younger control age group may be explained by the "selection hypothesis", and this theory should be further examined, taking into account the ethical

Comparison of two haematopoietic cell types, MNC and WBC samples, demonstrated that the telomere length is longer in MNC samples. However, the telomere lengths measured in the MNC and WBC appear increasingly similar to individuals within the age group at least 65 years old. In comparison between two age-groups within a one type of cells (MNC or WBC) the MNC group shows more rapid telomere length shortening during ageing than WBC group. It shows that there are molecular mechanisms that affect telomere length in mononuclear immune blood cells more strongly than other immune cell types during ageing.

All those mechanisms that are associated with telomere elongation are lost during ageing in MNC cells, but the MNC cells are proliferating also when individuals become older; therefore, telomeres are getting shorter more rapidly, in addition, the amount of those cells is getting smaller. Other circulating immune cells possess more or less stable telomere length during ageing because they are not specifically activated after hematopoiesis for fission (as T and B lymphocytes) [40], and it keeps telomere length more constant, which reflects to WBC group.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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PATIENT'S CONSENT

Declared none.

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II Comparison of telomere length between population-specific mitochondrial haplogroups among different age groups in a Latvian population

Highlights:

1. There was no disparity among mitochondrial haplogroups in different age groups. Hg H was slightly more abundant among nonagenarians (in this paper referred as centenarians).

2. There was no significant difference of telomere length among H, U, T, J, V and W mitochondrial haplogroups.

3. There was also no difference of telomere length among mitochondrial haplogroups after samples were divided into three age groups (20-45, 55-90 and 90-100).

4. There was no significant correlation among mitochondrial haplogroups, telomere length and age in the sample cohort.

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Comparison of telomere length between population-specific mitochondrial haplogroups among different age groups in a Latvian population

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ABSTRACT

Population studies have demonstrated that telomere length (TL) displays great diversity among different populations. Previously described controversial findings associated longevity with specific mitochondrial DNA haplogroups (hgs) (e.g., *J* and *U*). These observations may be influenced by population diversity, geographic location, and/or specific historic background. The aims of this study were to identify a specific hg which correlates with aging in a Latvian populating and to evaluate the possible association of TL variability with specific mitochondrial hgs. The results show no significant correlation between TL, mitochondrial DNA hgs and longevity. A slight increase in frequency was observed among centenarians of hg *H*; however, these findings were not statistically significant. TL did not show any statically significant difference, only hg *W* had slightly longer telomeres among others. An insignificant increase in TL was observed in the 55–89 age group of hg *W* but in the <90 age group for hg *J* which also had the longest TL in the 20–45 age group. In conclusion this study indicates that specific mitochondrial DNA hgs do not have a significant, if any, influence on the variation of TL in Latvians.

1. Introduction

Mitochondrial DNA (mtDNA) is maternally inherited, and many inherited variants of mtDNA, i.g. hgs do exist, that are geographically distributed. Previous studies have shown that some of hgs are associated with common complex traits and a possible connection between age-related diseases, longevity, mitochondrial haplogroup background and population divergences (e.g., Tanaka et al., 1998; Czarnecka and Bartnik 2011). A human mitochondrial hg defines differences in human mtDNA by SNPs (single nucleotide polymorphisms) which lead to amino acid changes within the OXPHOS (oxidative phosphorylation) respiratory complexes. There are 9 major hgs found in Europe (H, I, J, M, T, U, V, W, and X) (Torroni et al., 1997; Kenney et al., 2014). Some researches suggest that human adoption to chronic cold and irregular caloric availability due to seasonal changes could influence evolution by disrupting mitochondrial hgs and also longevity (Wallace 2005; Robine et al., 2012). Resent findings support the hypothesis that different mtDNA hgs lineages from different geographic origins might take a part in diverse susceptibilities to age-related diseases. The large accumulation of SNPs can cause amino acid and functional changes, while others cause changes in the rates of replication and transcription of the mtDNA. Progressive loss of mitochondrial function in several tissues is a common feature of aging believed to be influenced by life-long production of reactive oxygen species (ROS) as by-products of oxidative metabolism leads to the accumulation of DNA and protein damages (Shigenaga et al., 1994; Bellizzi et al., 2006; Kenney et al., 2014).

Several conflicting studies have also evaluated the possible association of various hgs with healthy aging. Beckstead et al. indicates that hg *H* individuals may live longer when compared to hg *U* individuals, under calorie restriction (Beckstead et al., 2009). Numerous studies have observed that hg *J* is more abundant among centenarians, while hg *U* decreases among centenarians (de Benedictis et al., 1999; Rose et al., 2001). Conversely, Pinós et al. have refuted the observation that hg *J* is associated with longevity and have

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suggested that longevity is population-specific (Pinós et al., 2012). On the other hand several other studies have failed to find an association of longevity with hgs *H* and *U* (de Benedictis et al., 1999; de Benedictis et al., 2000; Pinós et al., 2012). A study by Benn et al. also concludes that there are no hg associations with mortality and longevity (Benn et al., 2008). It has also been shown, in a population from Finland, that hgs H and HV are less frequent among centenarians than hgs *U*, *J* and *U*8 (Niemi et al., 2003). Beside that, defined mutations in the genes of mtDNA associated with hgs *D*, *D*1, *H*1 have been described and are more frequently found among centenarians. These haplogroup-defining mutations may affect ATP (adenosine triphosphate) synthesis, suggesting that specific mitochondrial variants are associated with biochemical differences (Tanaka et al., 1998; Tanaka et al., 2000). Numerous studies have described specific hgs as being associated with healthy aging and having a protective or opposite effect on the occurrence of some diseases and tumors (e.g., Czarnecka and Bartnik, 2011). In particular, different hgs were associated with Leber's hereditary optic neuropathy, ichemic stroke, coronary artery disease and diabetic retinopathy and osteoarthritis (OA) (Torroni et al., 1997; Hudson et al., 2007; Kofler et al., 2009; Rego-Pérez et al., 2008). Other studies have found an effect of some hgs against ischemic stroke, Alzheimer disease and Parkinson's disease (Carrieri et al., 2001; van der Walt et al., 2003; Ghezzi et al., 2005; Gaweda-Walerych et al., 2008; Rosa et al., 2008).

Telomere shortening is thought to be a major theory of aging. Telomeres are specialized chromosomal DNA–protein structures that cap and protect the terminal regions of eukaryotic chromosomes. Telomeres are dynamic structures that become shorter with every division of a cell. Once a critical length is no longer maintained, the cell is not able to divide; this halt in cell division is thought to be a consequence of aging (Blackburn 2001). However, to date, only few studies have addressed the possible association of mitochondrial hgs with TL. Fernández-Moreno et al. examined TL in hg *J* individuals and showed that they have significantly longer telomeres than non-J carriers (Fernández-Moreno et al., 2011). Considering that both cell elements are involved in the process of aging and longevity, there could be a possible association between mitochondrial inherited polymorphisms and the dynamics of TL. The aim of this study was to identify correlations between distribution frequencies among different age groups of the most prevalent mitochondrial variants (hgs *H*, *U*, *T*, *J*, *V* and *W*) in a Latvian population and to investigate possible associations of these hgs with TL.

2. Materials and methods

2.1. Samples

Blood samples were collected from healthy individuals, without any disorders that are known to affect TL, in a Latvian population from age 20 to over 90 years old. In total, 772 individuals were enrolled in this study. All participants provided appropriate written informed consent for the use of their phenotypic and genetic data that were voluntarily provided via detailed health and heredity questionnaires. All samples were obtained from genome database of the Latvian population (VIGDB, bmc.biomed.lu.lv/lv/par-mums/saistitas-organizacijas/vigdb/). Samples from participants in the mitochondrial hg studies were divided into three age groups: 20–45 years old (control group, $n = 374$), 55–89 years old (middle group, $n = 271$), and over 90 years old (centenarians, $n = 127$). As only small part of the samples of DNA was obtained with enough high concentration and quality for TRF (terminal restriction fragments) assay, the TL was measured and hgs *H*, *U*, *T*, *J*, *V* and *W* were detected in 221 samples. Samples were selected with similar percentage frequency of hgs among age groups as in the whole sample cohort (Table 1). These samples were divided into the same age groups: 20–45 years old (control group, $n = 61$), 55–89 years old ($n = 80$) and over 90 years old (centenarians, $n = 80$). A 45–55 year old group was not included because this study focuses on elderly individuals, ages 60 and above. This elderly population has the highest mortality rate among Latvians.

2.2. Extraction of genomic DNA

Genomic DNA was extracted from the peripheral white blood cells (WBC) using the standard phenol–chloroform method as previously described (Sambrook et al., 1989).

2.3. Southern blots of terminal restriction fragments (TRFs)

The method described in Kimura et al. (2010) was used, with some modifications, to determine TL. Briefly, a Southern blot of TRFs was conducted using a Telo TAGGG telomere length assay kit (Roche, UK). Concentrated DNA (~1 μg) was digested with restriction endonucleases Hinf I (10 U) and Rsa I (10 U) (Kimura et al., 2010). Digested DNA samples, a DNA size marker (GeneRuler 1 Kb DNA ladder, Thermo Scientific, Lithuania), and the DIG molecular weight marker (Roche, UK) were loaded into a 0.8% agarose gel and run for 20 h (19 V and 25 mA) to resolve fragment sizes. The DNA in the gel was then depurinated in 0.25 M HCl for 10 min. Further, the gel with the samples was denatured in 0.5 L of 0.5 M NaOH and 1.5 M NaCl for two 20-min washes. The samples were neutralized in 1 L of 0.5 M tris-OH containing 3 M NaCl (pH 7.5) for two 20-min washes. The DNA was transferred to a positively charged nylon membrane (Amersham Hybond^{TM}-N^{+}, GE Healthcare Life Sciences, UK) for 2 h using a vacuum blotter (VacuGene Pump, Pharmacia Biotech, Sweden) with a 20× SSC transfer buffer solution that contained 0.3 M sodium citrate and 3 M NaCl (pH 7.0). DNA was fixed to a membrane using a 30-s UV exposure, and the membrane was briefly washed in 2× SSC solution. The subsequent steps were performed using the manufacturer's protocol for the Telo TAGGG telomere length assay kit (Roche, UK). The membrane was visualized on a high performance chemiluminescence film (GE Healthcare Life Sciences, UK). The film was scanned, and the TRF signal was detected. DNA migration distances were measured using the Kodak digital science D1 program (Kodak, US); the DIG ladder was used for molecular size reference. The optical density of the DNA fragments was measured using the ImageJ software (Rasband and ImageJ, 1997–2014). TL was calculated using the

Table 1
Comparison of the all haplogroups found in a Latvian population in the three age groups.

Age groups, years (No)	Haplogroups number (%)								
	H	*U*	*T*	*J*	*V*	*W*	*I*	HV	*X*
20–45 (374)	41.7 (156)	27.3 (102)	9.9 (37)	6.7 (25)	3.5 (13)	4.0 (15)	4.3 (16)	2.1 (8)	0.5 (2)
55–89 (271)	42.4 (115)	28.8 (78)	6.3 (17)	6.3 (17)	4.8 (13)	4.1 (11)	1.1 (3)	4.8 (13)	1.5 (4)
<90 (127)	48.8 (62)	21.3 (27)	10.2 (13)	4.7 (6)	6.3 (8)	3.9 (5)	1.6 (2)	2.3 (3)	0.8 (1)
Total	44.6 (333)	25.7 (207)	8.7 (67)	5.9 (48)	4.4 (22)	4.0 (31)	3.3 (21)	2.6 (24)	0.9 (7)

For hgs *H*, *U*, *T*, *J*, *V* and *W*. Hgs – mitochondrial haplogroups, TL– telomere length, df – degrees of freedom, F – fixation indices.

following equation: mean TRF length = Σ (ODi)/Σ (ODi/Li), where ODi = optical density at position i and Li = TRF length at position i.

2.4. Mitochondrial genotyping

To confirm the hg affiliation of mitochondrial sequences, hierarchical PCR–RFLP analysis was performed using 17 restriction endonucleases: *Ava*II, *Dde*I, *Bsh*1236I, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Rsa*I, *Nla*III, *Acc*I, *Bst*OI, *Mse*I, *Alw*44I, *Ssp*I, *Eco*47I, and *Bsu*RI (van Oven and Kayser, 2009) The classification of hgs was based on their position in the hierarchy of the mitochondrial phylogenetic tree (www.phylotree.org, van Oven and Kayser, 2009).

2.5. Statistical analysis

All statistical analyses were performed using the R 3.1.1 (Core Team, 2014) software program. The statistical significance of the differences between the observed distributions of mitochondrial hgs in three age groups was evaluated with the *G*-test. Analysis of variance (ANOVA) was used to test differences of mean TL values for age groups and mitochondrial hgs. In this analysis, only hgs *H*, *U* and *T* were included because they had replicates for each combination within each age group. A *P* value equal to or less than 0.05 was considered significant.

3. Results

3.1. Distribution of mitochondrial haplogroups among different age groups

Mitochondrial hgs were analyzed among 772 individuals of three age groups (age 20–45, 55–89, over 90 years) in a Latvian population (Table 1). Hg *H* represents 7.1% more of the population among centenarians than in the control group. Only those individuals bearing hg *V* showed a gradual growth with increasing age (1.3% more in the age group 55–89 when compared to the control group; 2% more in the group >90 age group when compared to the 55–89 group). Interestingly, hg *U*, despite being the second most abundant hg among centenarians, shows a 7.5% decrease compared to the 55–89 age group. Hg *T* is equally represented both in the control and centenarian groups but less in the middle group. Hg *I* shows an obvious decrease, averaging 2.9%, in both the 55–89 and >90 age groups. Other hgs do not demonstrate a noteworthy gradual decrease or increase in distribution frequencies among the analyzed age groups. A *G*-test ($G = 20.53$, df = 16 un $P = 0.1971$) emphasizes that none of the hgs investigated show a significant association with longevity.

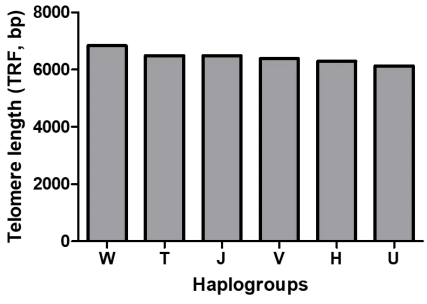

Fig. 1. Variation of telomere length for the hgs *H*, *U*, *T*, *J*, *V* and *W*. TRF-terminal restriction fragments, bp – base pairs.

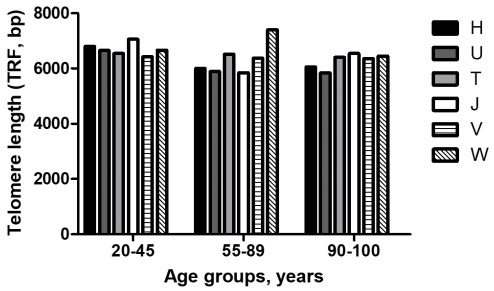

Fig. 2. Comparison of telomere length for the hgs *H*, *U*, *T*, *J*, *V* and *W* in three different age groups. 20–45, $n = 61$; 55–89, $n = 80$; <90 $n = 80$. TRF – terminal restriction fragments, bp-base pairs.

Table 2
Results of dispersion analysis (ANOVA).

TL	Hierarchical method				
	df	Sum of squares	Mean square	*F*	*P* value
Hgs	5	7840991	1568198	1.637	0.151748
Hgs: age	10	7888462	788846	0.823	0.606487
Residuals	203	194486331	958061	–	–

3.2. Association of telomere length with mitochondrial haplogroups and aging

A comparison of TL among different mitochondrial hgs in all together studied age groups is shown in Fig. 1. The average TL for hgs is: *H* (~6290 bp), *U* (~6130 bp), *T* (~6490 bp), *J* (~6480 bp), *V* (~6390 bp) and ~6840 bp). Consequently, there were a limited number of samples that were available for TL testing for hgs *I*, HV and *X*. There are no statically significant differences of average TL among hgs (ANOVA, $P = 0.1780$; $F = 1.542$; $R^2 = 0.03461$). Individuals belonging to hg *W* have the longest average TL, while those of hg *U* have the shortest average TL. Fig. 2 shows a comparison of the TL of individuals with hgs *H*, U, T, J, V and W in the three age groups separately. Hg *J* shows the longest TL in the control group and among centenarians. In the middle group hg *W* has the longest TL. Hgs *T* and *V* do not have obvious difference of TL among age groups. Results of the ANOVA analyses (Table 2) show that there are no significant correlations among TL, hgs and age in the Latvian population.

4. Discussion

Our previous study showed that the most common mitochondrial hgs in the Latvian population are *H* (44.5%), *U* (25.4%) and *T* (9.4%) (Pliss et al., 2006). In the current study none of hgs show statistically significant correlation with aging and the percentage varies slightly. The observed frequencies of hgs *H*, *U*, *T*, *J*, *V*, *I* and *X* were in stark contrast to those reported in a study from Finland (Niemi et al., 2003). Although Finland is geographically close to Latvia, hg *U* and cluster TJ were found to be more frequent than hg *H* among centenarians. The climate in Finland is only slightly colder than in Latvia and is considered to be similar. In one study, it was described that climate has an influence on human longevity (Robine et al., 2012). Although the climate is warmer, studies from South Europe showed a similar distribution of hg *J* to the Finnish population; however, the current study, along with other studies, did not support this finding (de Benedictis et al., 1999; de Benedictis et al., 2000; Pinós et al., 2012). Another study had similar observation regarding to hg *U* frequency in different age groups as it was in the current study (de Benedictis et al., 1999). One hypothesis describes a connection between population migration waves

to the North Europe and hg frequency that could be contributed to a necessary adaptation to the chronic cold and irregular caloric availability by varying mitochondrial metabolism (Wallace 2005). Hgs may play a protective role in colder climates by generating greater amounts of heat through higher electron transport rates and looser coupling or partially uncoupled OXPHOS. For the cold-adapted hgs, uncoupling mutations would produce less ATP per calorie consumption, which can be used for heat generation; therefore, more oxidized and less ROS would be formed (Mishmar et al., 2003; Baudouin et al., 2005; Wallace 2005). Mitochondrial hgs have different coupling efficiency (the percentage of oxygen consumption used for ATP synthesis rather than heat generation) and mitochondrial ROS production (Wallace, 2005). Previous observation suggests that hg *H* may not be as suitable as hg *U* for longevity in cold environments; however, this study contradicts this hypothesis. The Beckstead' research group showed that due to historic caloric restriction, longevity is observed in individuals with hg *H* compared to hg *U*, and that this longevity has not been changed during periods of caloric abundance (Beckstead et al., 2009). There are many parameters that can influence the survival of specific hgs in different environments. Differences of hgs frequencies during aging in various populations might be explained with diverse historical events and climate changes. As previously mentioned certain hgs have varying influences on diverse age-related diseases and tumors and therefore may slightly affect the frequency of specific hgs in older age groups.

Sahin et al. have proposed a theory connecting/linking telomere length with mitochondrial genetics. They have shown that short telomeres, which are sensed by cells as double-strand breaks and DNA instability, suppress PGC-1α/β (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and beta) action via *p53* gene activation. These molecular interactions lead to mitochondrial dysfunction, cell senescence, growth arrest and apoptosis (Chin et al., 1999; Sahin and Depinho, 2010; Sahin et al., 2011; Rufini et al., 2013). PGC-1α/β regulates a vast amount of mitochondrion functions, including mitochondrial replication/transcription, OXPHOS, oxidative stress and gluconeogenesis, etc. (e.g., Wu et al., 1999; Lin et al., 2005). These mitochondrial outcomes, due to reduced electron transport chain efficiency due to chemical or genetic causes, lead to ROS production that cause a drop in ATP levels, leading to aging, which may limit lifespan as well as effect telomere length (Balaban et al., 2005; Moiseeva et al., 2009).

This study/current study demonstrates that the average TL in all mitochondrial hgs is similar and does not show statically significant differences among the hgs, however, some similarities with other studies can be observed. Martínez-Redondo et al. have shown that hg *H* has higher mitochondrial oxidative damage than hg *J*. In their study, hg *H* had higher oxygen uptake and therefore more ROS production than other hgs. The next highest level of oxygen uptake is found in hg *V* followed by hgs *T* and *U*, with the lowest level found in hg *J* (Martínez-Redondo et al., 2010). It has been shown that oxidative stress is associated with increased telomere attrition (von Zglinicki 2002; Kawanishi and Oikawa 2004). This means that TL should be shorter for hgs that produce more ROS, such as hg *H* compared to hg *U*. Similar interrelationship can be observed in our study for hg *H* (Fig. 1), however, it is not statistically significant. This hypothesis also might explain slightly longer telomeres for hg *J* among centenarians in the current/present study, due to less ROS accumulation during aging. Also previously, Fernández-Moreno et al. have shown that individuals with hg *J* have significantly longer telomeres than non-J carriers (Fernández-Moreno et al., 2011). However, Pinós et al. have found that hg *J* and longevity are not related and have proposed that different findings in previous reports could be due to a population-specific background (Pinós et al., 2012). Previously reported conflicting results related to TL may also be attributed to population-specific differences (Zole et al., 2013; Eisenberg et al., 2011; Salpea et al., 2008; Canela et al., 2007).

5. Highlights

Mitochondrial hgs are not significantly associated with longevity or longer telomere length in a Latvian population. Only hg *J* had slightly longer TL among centenarians. None of the hgs correlated with age either. Only hg *H* shows an insignificant increase of frequency among centenarians compared to the control group. Assuming that longevity and TL are either population-specific or geographically or historically specific, their association can be influenced by many factors during an individual's lifespan, environmental and genetic background.

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

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III Linkage between mitochondrial genome alterations, telomere length and ageing population

Highlights:

1. MtDNA copy number content got slightly higher in nonagenarian group in comparison to the elderly group (60-89 year age group).
2. Positive correlation between telomere length and mtDNA copy number was significant only for individuals up to 90 years, but not for nonagenarians.
3. HVS-I region heteroplasmy did not influence telomere length and mtDNA copy number in the sample cohort.
4. SNPs that determine mitochondrial haplogroups did not influence mtDNA copy number in the sample cohort.

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RESEARCH ARTICLE

Linkage between mitochondrial genome alterations, telomere length and aging population

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ABSTRACT

We studied telomere length (TL) and mitochondrial DNA (mtDNA) copy number variations in individuals from Latvian Caucasian population in different age groups. We showed a positive correlation between TL and mtDNA copy number in individuals of up to 90 years of age; however, this correlation was not observed in the 90–100 years age group. While TL shortened with age and mtDNA content decreased with increasing age, in this study it was observed that mtDNA copy number in nonagenarians was slightly higher than in the 60–89 years age group. The presence of heteroplasmy in the mtDNA HVS-I control region did not correlate with TL and mtDNA copy number. TL and mtDNA values also did not differ between mitochondrial haplogroups. In conclusion, while both TL and mtDNA are involved in the aging process and link between these cell components exists, nonagenarians may have differences in senescence-related pathways and systems, which may function as a protective mechanism that allows them to live longer.

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Introduction

There are many hypotheses that describe aging, but the focus of this study is on two theories that are thought to be central to the cellular senescence process; one involves telomere length (TL) shortening, and the second is associated with mitochondrial DNA (mtDNA) alterations (reviewed in Lipsky & King 2015). As there are contradicting results about these theories, there is a need for more studies addressing the aging processes (Viña et al. 2013).

Telomeres are specialized chromosomal DNA-protein structures that cap and protect the terminal regions of eukaryotic chromosomes. Telomeres are dynamic structures that become shorter with every division of a cell until a critical stage is reached, when the cell can no longer divide and it enters a phase of senescence; the telomere shortening also causes genomic instability and is associated with a higher risk of age-related diseases and cancer (Harley et al. 1990; reviewed in Blackburn et al. 2015). Telomere maintenance and shortening is a complex process which can be affected by different factors, such as stress and genetic background, and is different in diverse tissues; furthermore, the exact relationship between telomere length and aging is not fully understood (reviewed in Blackburn et al. 2015).

There are nine major mitochondrial haplogroups (hgs) found in the human population of Europe (H, I, J, M, T, U, V, W, and X) (Torroni et al. 1994). A human hg is defined by differences in human mtDNA called single nucleotide polymorphisms (SNPs). Accumulation of certain mutations or SNPs can cause amino acid and functional changes, while others cause changes in the rates of replication and transcription of the mtDNA (Bellizzi et al. 2006; Kenney et al. 2014). Another example of accumulation of mutations or SNPs is heteroplasmy which is the presence of multiple mtDNA variants in a single cell or among cells within an individual (Potter et al. 1975). The highest degree of polymorphisms is concentrated within two hypervariable segments of the control region: hypervariable segment I (HVS-I) and hypervariable segment II (HVS-II) (Wilkinson-Herbots et al. 1996). There have been studies claiming that the heteroplasmy of certain polymorphisms within the control region is associated with longevity, whereas heteroplasmy of some other polymorphisms is not (e.g. Sondheimer et al. 2011).

Each mammalian cell contains hundreds to more than a thousand mitochondria, and each organelle harbors 2–10 copies of mtDNA (Robin & Wong 1988). There have been controversial results from different studies regarding how mtDNA copy number changes during human aging. A substantial age-related decline in the abundance of mtDNA has been shown in various tissues, organs and organisms (Cree et al. 2008; Mengel-From et al. 2014). In contrast, other studies did not observe any changes (Miller et al. 2003; Frahm et al. 2005). However, He et al. (2014) recently showed that mtDNA content is higher in healthy centenarians than in younger age groups. High mtDNA copy numbers were associated with better general health, including higher cognition and lower mortality (Kim et al. 2011; Mengel-From et al. 2014). In addition, there are associations between higher mtDNA copy

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number amount and some cancers like head, neck and prostate cancers (Jiang et al. 2005; Zhou et al. 2014).

Passos et al. (2007) has proposed a theory that connects TL with mitochondrial genetics and reactive oxygen species (ROS). It was previously shown that short telomeres, which are sensed by cells as double-strand breaks and DNA instability, suppress peroxisome proliferators-activated receptor gamma, coactivator 1 alpha and beta (PGC-1 α/β) action via p53 transcription factor (Chin et al. 1999; Sahin et al. 2011). Population studies have also shown a connection between TL and mitochondria. One of the first *in vivo* studies in humans came from Kim et al. who demonstrated a positive association between TL and mtDNA copy number in elderly women of an average age of 73 years (Pieters et al. 2015). Other researchers have confirmed this association in individuals aged 18–64 (Kim et al. 2013), and aged 60–80 that telomeres and mitochondria are co-regulated in humans (Tyrka et al. 2015).

To our knowledge, so far there have been no studies about TL and mtDNA copy number correlation in the Caucasian nonagenarians (individuals that are 90–100 years old). Literature contains evidence that centenarians have shorter telomeres but higher mtDNA copy number than persons in the younger age groups, although for the groups under 90 years of age both the TL and mtDNA copy number decreased with age. The aim of the present study was to examine the association between mtDNA alterations and TL in aging process in Latvian population. First, we explored if TL shortening and mtDNA alterations come together in nonagenarians in the same way as in individuals up to 90 years of age. Second, we looked if different mitochondrial hgs influence mtDNA copy number amount.

Materials and methods

Samples

Blood samples were collected from healthy individuals from Latvian population ranging ages from 20 to 100 years. In total, 210 samples were divided into three age groups: 20–59 years ($n = 70$, mean age = 32 years, females = 65%), elderly group (60–89 years, $n = 70$, mean age = 73 years, females = 73%) and nonagenarians (90–100 years, $n = 70$, mean age = 93 years, females = 76%). All individuals were Caucasian. All participants provided appropriate written informed consent to use of their phenotypic and genetic data, which were voluntarily and anonymously provided via health and heredity questionnaires. The samples and information about samples were obtained from the Genome Database of the Latvian Population (VIGDB, bmc.biomed.lu.lv/lv/par-mums/saistitas-organizacijas/vigdb/). None of the selected individuals had reported any severe diseases during the medical examination. Some individuals from the elderly group and nonagenarians had vision problems, hearing loss, dizziness, arthritis, osteochondrosis and joint pain, fatigue and sleep disorders, minor urinary tract or digestive tract disorders. None of the study participants had Alzheimer's disease, Parkinson disease or cancer – diseases associated with TL or mtDNA alterations. No information of smoking and drinking habits was available.

Extraction of genomic DNA

Genomic DNA for all samples was extracted from the peripheral white blood cells (WBC) using the standard phenol–chloroform method as previously described (Sambrook et al. 1989).

Relative qPCR SYBR green telomere length quantification assay

The ΔC_T method using a reference gene was used to measure TL in the DNA samples. Although quantitative real-time polymerase chain reaction (qPCR) will not determine TL in kb (kilobases) but only the abundance of telomeric DNA in a sample, it is a faster method and requires less DNA than the Southern blot telomeric restriction fragment assay (TRF) which is considered the golden standard. qPCR was performed using Maxima SYBR green qPCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA). The forward and reverse primers of telomeres for one reaction were as follows: Telo1 (200 nM), 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAG-GGT-3′, and Telo2 (200 nM), 5′-CCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′. After a denaturation step at 95 °C for 10 min, DNA samples were incubated for 40 cycles at 95 °C for 10 s and 58 °C for 1 min. Efficiency = 92%. TL was normalized using the following forward and reverses primers for the β-globin gene in a separate run: beta-glob1 (300 nM), 5′-GCTTCTGACACAACTGTGTTCACTAGC-3′, and beta-glob2 (500 nM), 5′-CACCAACTTCATCCACGTTCACC-3′. After a denaturation step at 95 °C for 10 min, DNA samples were incubated for 40 cycles at 95 °C for 10 s and 56 °C for 20 s (Kim et al. 2013). Efficiency = 94%. The concentration of the DNA was 10 ng/μl in a 10 μl reaction. Each sample was run in triplicate. A no-template control and duplicate calibrator samples were used in all runs to allow comparisons of the results across all runs. A melting curve analysis was performed to verify the specificity and identity of the PCR products. TL was calculated using threshold cycle or C_T values and the following equation: relative TL $\text{ratio}_{(\text{test/reference})} = 2^{\text{Ct}(\beta\text{-glo bin}) - \text{Ct}(\text{telomeres})}$.

Relative qPCR TaqMan mtDNA copy number quantification assay

Relative mtDNA copy number was measured by qPCR using the Maxima Probe/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA). mtDNA copy number amount was normalized by simultaneous amplifications of the nuclear gene Gapdh and the mitochondrial D-loop DNA fragments. The forward and reverse primers for the Gapdh reaction (1250 nM each) were GapdhF 5′-GAAGGTGAAGGTCGGAGT-3′ and GapdhR 5′-GAAGATGGTGATGGGATTTC-3′, respectively, and the TaqMan probe (250 nM) GapdhTqM 5′-CAAGCTTCCCGTTCTCAGCC-3′. Efficiency = 98%. The forward and reverse primers (50 nM each) for the mitochondrial D-loop were FmtMinArc 5′-CTAAATAGCCCA CACGTTCCC-3′ and RmtMinArc 5′-AGAGCTCCCGTGAGTGGTTA-3′, respectively, and

the TaqMan probe (250 nM) PmtMinArc 5′-CATCACGATGGATCACAGGT-3′ (Phillips et al. 2014). Efficiency = 95%. The DNA concentration was 10 ng/μl in a 15 μl reaction. After a denaturation step at 95 °C for 10 min, the DNA samples were incubated for 40 cycles at 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s. Each sample was run in triplicate. A no-template control and duplicate calibrator samples were used in all runs to allow for the comparison of results across the runs. mtDNA copy number was calculated using threshold cycle values and the following equation: relative copy number $\text{ratio}_{(\text{test/reference})}=2^{\text{Ct(Gapdh)}-\text{Ct(D-loop)}}$.

Detection of heteroplasmy

The presence of mtDNA heteroplasmy was determined using the SURVEYOR™ Mutation Detection Kit, which is based on the use of a mismatch-specific endonuclease, SURVEYOR Nuclease (Transgenomic, USA) (Bannwarth et al. 2006). This kit detects heteroplasmy as low as 5%, and was our choice having been tested it in our previous work (Pliss et al. 2011) although there are certain limitations using this kit (Yen et al. 2014). Due to restricted amount of DNA available for this study, only HVS-I region was tested. This region was chosen due to the possible presence of a higher number of heteroplasmic polymorphisms (Vigilant et al. 1989; Ngili et al. 2012). The DNA fragment encompassing the mtDNA HVS-I between nucleotide positions (nps) 16,024–16,390 was amplified and sequenced in all samples using forward (F-HVS-I) and reverse (R-HVS-I) primers (200 nM each) (Vigilant et al. 1989) in final volume of 25.5 μl containing 10–50 ng DNA, 1.5 mM $MgSO_4$, 1 × Optimase reaction buffer, 10 mM each dNTP, 1.25 U/μl Optimase polymerase (Transgenomic, USA). The amplification conditions: denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s, and final elongation at 72 °C for 5 min. Negative and positive controls were prepared for amplification. The PCR products were heated to denature the DNA and were then slowly cooled to room temperature to allow for reannealing of the DNA strands using the following protocol: initial denaturation at 95 °C for 2 min, followed by a 95 °C to 85 °C (−2 °C/s) gradual temperature decrease, and then a 85 °C to 25 °C (−0.10 °C/s) temperature decrease. Afterwards, products were incubated with Surveyor enhancer S 0.5 μl and Surveyor nuclease S 0.5 μl at 42 °C for 20 min; 1 μl of stop solution was added at the end of reaction. The samples were analyzed by a 6% polyacrylamide gel electrophoresis.

Mitochondrial haplogroup determination

To confirm the hg affiliation of mitochondrial sequences, hierarchical PCR–RFLP analysis was performed using 17 restriction endonucleases: *Alu*I, *Ava*II, *Dde*I, *Bsh*1236I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Rsa*I, *Nla*III, *Acc*I, *Bst*OI, *Mse*I, *Alw*44I, *Ssp*I, *Eco*47I, and *Bsu*RI (van Oven & Kayser 2009). The classification of hgs was based on their position in the hierarchy of the mitochondrial phylogenetic tree (www.phylotree.org 2016; van Oven & Kayser 2009).

Statistics

Linear regression and correlation, unpaired two-tailed t-test and analysis of variance (ANOVA) were performed using GraphPad Prism version 5 for Windows (La Jolla, CA, www.graphpad.com 2016). Data was expressed as means ± SEM (standard error of the mean) and differences of $p < .05$ were considered significant. For mtDNA copy number assay the power was 37%, $\alpha = 0.05$, $\beta = 0.62943$, SM (standard deviation of means) = 0.05, SD (standard deviation) = 0.39, $n = 70$ in each of the three groups. The low power could be due to small sample cohort and high variance between sample data which may lead to lower r^2 and higher p values for the mtDNA copy data. For TL assay the power was 100%, $\alpha = 0.05$, $\beta = 0.00288$, SM= 0.08, SD = 0.22, $n = 70$ in each of the three groups (Hintze 2008).

Results

Relative mtDNA copy number and telomere length in the three different age groups

The results show that TL shortens with increasing age, in accordance with previous studies (Figure 1). The 60–89 years olds had 18.8% shorter telomeres than in the 20–59 years age group ($p = .0042$); 90–100 years old individuals had 10.3% shorter telomeres than the 60–89 year age group ($p = .0397$) and 27.2% shorter than the 20–59 years age group ($p = .0001$) (Figure 1(A)). The mtDNA copy numbers reduced with increasing age but surprisingly were slightly higher for nonagenarians; however, the difference was not statistically significant. The 60–89 age group had 12.4% less mtDNA than the 20–59 age group ($p = .0337$); the 90–100 age group had 7.6% more mtDNA than the 60–89 age group ($p = .5144$) but 5.7% less mtDNA than the 20-59 year age group ($p = .2956$) (Figure 1(B)).

Correlation between telomere length and mtDNA copy number

The results showed significant positive correlation between mtDNA copy numbers and TL ($p = .0335$, $r^2 = 0.0215$) (Figure 2). This correlation means that individuals with longer telomeres have more mtDNA copies. However, when the correlation between TL and mtDNA copy amount in three different age groups was analyzed, the results showed that this correlation was significant only in individuals of up to 90 years of age (Figure 3). Correlation for mtDNA copy number and TL was significant both in the 20–59 years age group ($p = .0195$, $r^2 = 0.0799$) and the 60-89 years age group ($p = .0352$, $r^2 = 0.0636$) (Figure 3(A,B)). For nonagenarians, no correlation was observed between mtDNA copy number and TL ($p = .6029$, $r^2 = 0.0040$) (Figure 3(C)).

mtDNA copy number and telomere length in samples with HVS-I heteroplasmy

The percentage of HVS-I mtDNA heteroplasmy in our sample cohort showed a different trend to that observed previously.

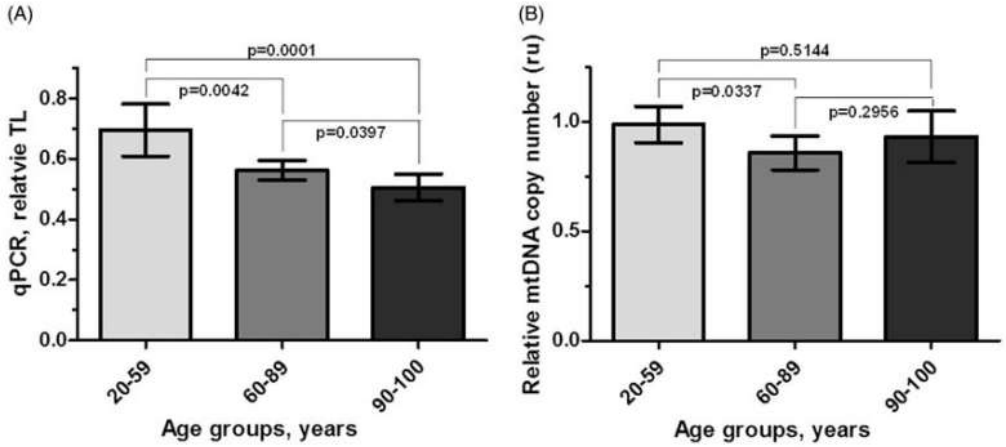

Figure 1. Telomere length and mtDNA copy number in three different age groups. (A) Telomere length; (B) mtDNA relative copy number. 29–59 year age group, $n = 70$; 60–89 years age group, $n = 70$; 90–100 years age group, $n = 70$. TL: telomere length; ru: relative units. A p-value equal to or less than .05 was considered to be significant; data were expressed as means ± SEM.

For younger individuals (20–59 years) heteroplasmy was positive in 17% (12/70) of samples. As expected, the occurrence of the heteroplasmy increased with increasing age; in our study, an 18% increase was observed for the 60–89 years age group, where heteroplasmy was positive in 34% (24/70) of samples. In contrast, for nonagenarians (90–100 years old), the proportion of individuals with heteroplasmy decreased by ~11% in comparison with the middle-age group: 16 of 70 nonagenarian samples were heteroplasmy-positive by our used assay in HVS-I region (23% of the cohort). The results show that heteroplasmy of the mtDNA control region HVS-I was not linked to TL in any of the age groups. When heteroplasmy-positive and heteroplasmy-negative samples were compared no statistically significant differences were observed for the mean TL values (Figure 4(A)). Similarly, relative mtDNA copy number did not differ significantly between the heteroplasmy-positive and -negative samples (Figure 4(B)).

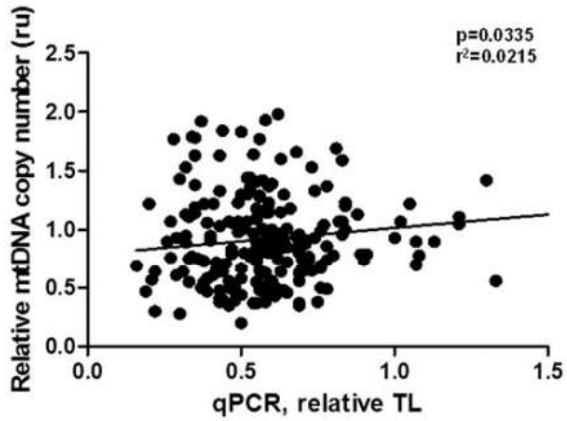

Figure 2. Correlation between telomere length and mtDNA copy numbers in all the samples grouped together. 20–100 years old individuals, $n = 210$. TL: telomere length; ru: relative units. A p-value equal to or less than .05 was considered to be significant.

Variation of mtDNA copy numbers in different mitochondrial haplogroups

A comparison of mtDNA copy number among different mitochondrial hgs across all age groups is shown in Figure 5(A). There were limited numbers of samples available for testing for hgs J, V, and W; more samples should be tested to reach a final conclusion; however, trends in mtDNA copy number can be seen. No hg had a significantly higher or lower mtDNA copy numbers than any other, ANOVA $p = .5788$ (Figure 5(A)). A similar observation was made when mtDNA copy number was compared between different hgs grouped by age (20–59 years age group: ANOVA $p = .2443$; 60–89 year age group: ANOVA $p = .6689$; and 90-100 year age group: ANOVA $p = .0939$) (Figure 5(B)).

Discussion

TL is important for a longer lifespan and healthier life and for capability of reaching nonagenarians age. There are several known causes for telomere shortening, such as stress and the presence of ROS, environment and genetic background (Von Zglinicki 2002; Blackburn et al. 2015). TL is usually the shortest in nonagenarians but, as previously shown, TL variability is smallest in this age group (Zole et al. 2013). This probably means that individuals who reach the nonagenarians or centenarians age may harbour very long telomeres at birth, and that variation in TL is reduced with increasing age due to the selective loss of individual cells with shorter telomeres (Haussmann & Mauck 2008). However, it was shown that with increasing age telomeres shorten at a slower pace (Salomons et al. 2009).

It has been shown that mtDNA copy numbers are higher in nonagenarians and centenarians than in the Chinese elderly people aged between 50 and 70 (He et al. 2014). In the current study of Caucasian subjects, similar trend was observed. Our results show that nonagenarians in Latvian population did not have significantly lower mtDNA copy numbers in comparison to the 20–59 year age group; in contrast, individuals in the 60–89 year age group had significantly

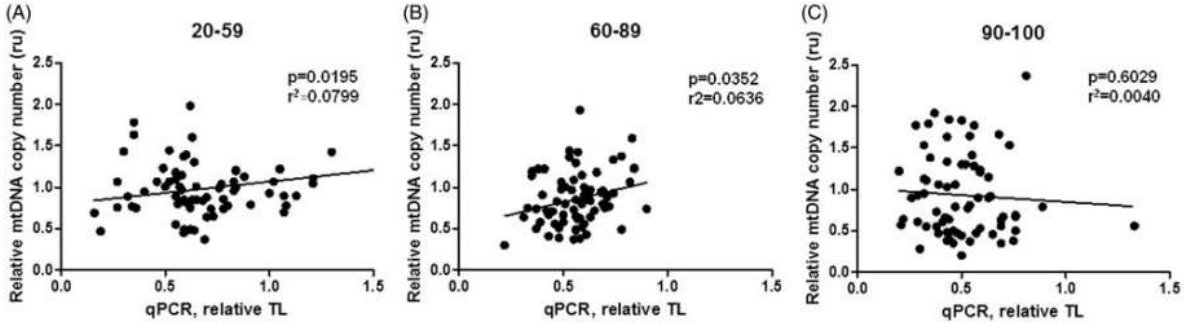

Figure 3. Correlation between telomere length and mtDNA copy number in three different age groups. (A) 20–59 years old individuals. (B) 60–89 years old individuals. (C) 90–100 years old individuals. 29–59 years age group $n = 70$; 60–89 years age group $n = 70$; 90–100 year age group $n = 70$. TL: telomere length; ru: relative units. A p-value equal to or less than .05 was considered to be significant.

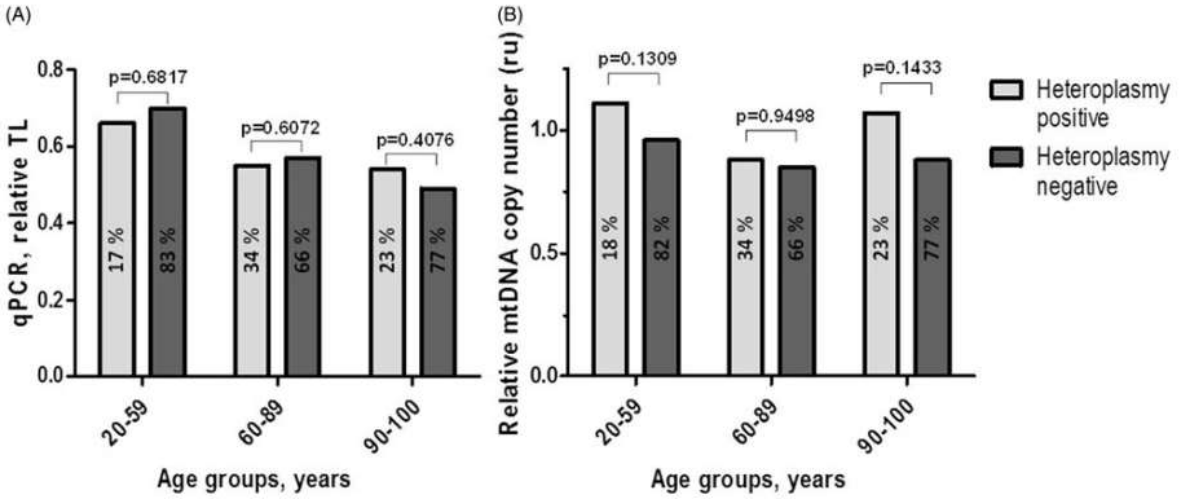

Figure 4. Comparison of mean TL and mtDNA copy number in mtDNA HVS-I region heteroplasmy positive and negative samples. (A) Telomere length. (B) mtDNA relative copy numbers. Percentage of heteroplasmy in the each age group: 20–59 years= 17% positive, 83% negative (total $n = 70$); 60–89 years= 34% positive, 66% negative (total $n = 70$); 90–100 years = 23% positive, 77% negative (total $n = 70$). TL: telomere length; ru: relative units. A p-value equal to or less than .05 was considered to be significant; data were expressed as means ± SEM.

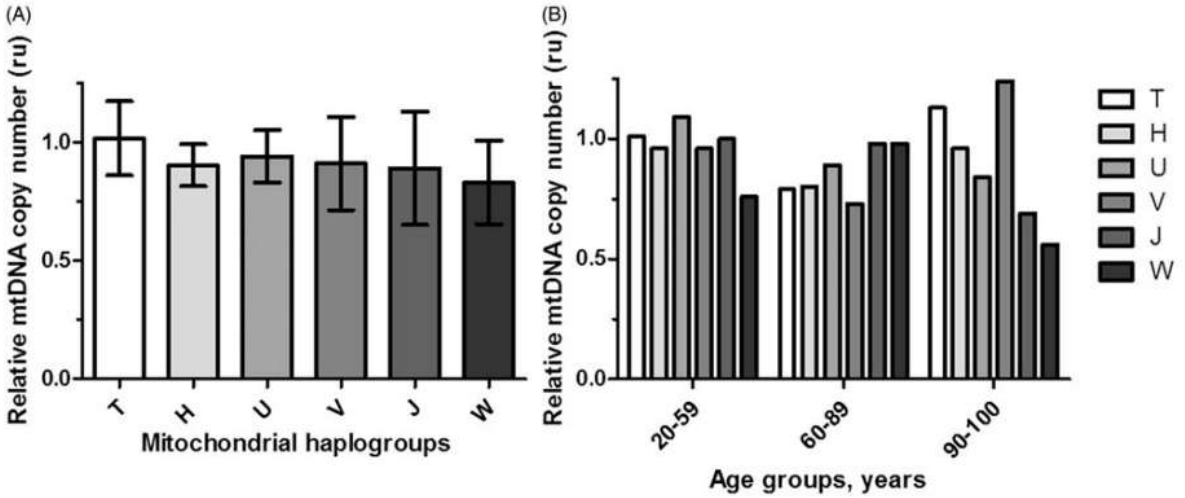

Figure 5. Variations of mtDNA copy amount in diverse mitochondrial haplogroups. (A) mtDNA relative copy number for hgs T, h, U, V, J, W. ANOVA $p = .5788$. (B) The samples are divided in the three age groups. For 20–59 age group ANOVA $p = .2443$ ($n = 70$); for 60–89 age group $p = .6689$ ($n = 70$); for 90–100 age group $p = .0939$ ($n = 70$). Ru: relative units. A p-value equal to or less than .05 was considered to be significant; data were expressed as means ± SEM.

less mtDNA than those in the 20–59 years age group. Often, but not always, high mtDNA copy number was associated with better physical and mental health (Kim et al. 2011; Mengel-From et al. 2014). Higher mtDNA copy numbers in centenarians have been linked to higher adenosine triphosphate (ATP) levels and it has been suggested that normal levels of ATP allow a cell to progress through the G1 phase of the cell cycle to proliferate and regenerate (Mandal et al. 2005; Owusu-Ansah et al. 2008; Sgarbi et al. 2014). This could indicate that individuals who have had high mtDNA copy numbers at birth can live longer and healthier lives. This most likely serves as a 'selection' process for individuals with many mtDNA to survive to nonagenarian and centenarian age.

The absence of differences in TL and mtDNA copy number between HVS-I heteroplasmy-positive and -negative individuals in this study advocates that there are no mtDNA molecules with polymorphisms in the HVS-I region which more likely than others may promote mtDNA replication and/or influence TL. Similarly, in another study, there was no difference in mtDNA copy number in samples with heteroplasmy (Szuhai et al. 2001). However, in diabetic patients, a correlation between the mtDNA copy number and SNPs in mtDNA has been observed (Chien et al. 2012). In the current study on nonagenarians, the amount of mtDNA in the heteroplasmy-positive samples was slightly higher than in the 60–89 years age group heteroplasmy-positive samples. Possibly, some mtDNA polymorphisms were selected with age, and such variants can compensate the defects induced by various types of mtDNA mutations, helping an individual to survive longer (Ono et al. 2001; Sondheimer et al. 2011). Of the cohorts in the current study, nonagenarians have smaller percentage of heteroplasmy than the 60–89 years age group (23% versus 34%, respectively). Altogether these results lead us to propose that individuals without mtDNA heteroplasmy or individuals with heteroplasmic SNPs that promote mtDNA replication are more likely to survive until old age. However, to draw general conclusions more samples among nonagenarians must be analyzed and the possible molecular mechanisms needs to be deciphered. As the heteroplasmy was detected only in HVS-I region in future, it would be useful to detect heteroplasmy in the whole mitochondrial genome and to test its effect on these two cell components because HVS-I represents only a small part of mtDNA and it may not show the whole picture of the effect of heteroplasmy.

Mitochondrial hgs affect neither mtDNA copy number, as can be seen in this work, nor TL, as was shown in our previous work (Zole et al. 2015). This means that even if hgs are determined by SNPs within the HVS-I control region, as for hgs H, V and J, these changes do not influence mtDNA replication. However, the work of Suissa et al. demonstrated that in hg-J hybrids, mtDNA content was increased compared with hg-H *in vitro* (Suissa et al. 2009). Although some researchers have found association between hg and longevity (Tanaka et al. 1998; Rose et al. 2001), other studies failed to support these associations (Raule et al. 2014; Zole et al. 2015). These observations indicate that the SNPs which determine hgs are not strongly associated with longevity or maintenance of mtDNA replication, but rather are population- and geographic region-specific (Santoro et al. 2006).

The connection between telomeres and mitochondria via p53, SIRT1 and PCG-1 α/β explains the observed positive correlation indicating that individuals with longer telomeres have more mtDNA copies, as well as how this correlation is linked to biological aging (Sahin et al. 2011; Pieters et al. 2015; Tyrka et al. 2015). The same is shown in the present study. It is unclear, however, why this correlation was not observed in nonagenarians. As nonagenarians have more mtDNA copies but shorter telomeres than the 60–89 age group – and there is no significant correlation between those two cell components – nonagenarians and centenarians may have different and balanced mechanisms of protection against premature death. Mei and coworkers have shown that at least in cancer cells increased mtDNA copy amount can prevent cells from apoptosis (Mei et al. 2015). Leading to an assumption that cells with more mtDNA copies can probably maintain themselves and these cells do not go into apoptosis although they have short telomeres and they are in a senescence state. It appears that telomere shortening might not influence mtDNA amount or vice versa in centenarians as strongly as in younger individuals, which might result from the different polymorphisms in mtDNA sequences (Niemi et al. 2004; Takasaki 2008), the diverse nuclear DNA SNPs and the healthier lifestyles noted in these individuals compared with individuals who do not live to be 90 years of age (Debrabant et al. 2014; Govindaraju et al. 2015).

Conclusions

In summary, there is no doubt that both TL and mtDNA are involved in the senescence process and healthy aging, and that the two theories of aging are connected. Since the present study has demonstrated that the positive correlation between TL and mtDNA copy numbers is lost in nonagenarians, it might mean that nonagenarians and also centenarians may have some differences in pathways and systems, which may function as a protective mechanism allowing them to live longer. Better understanding of the interactions between telomere maintenance and mitochondrial processes might lead to new therapeutic approaches to prevent aging diseases and improve self-sustaining in the elderly. It is also important to repeat this type of studies between different populations and laboratories to make more reliable conclusions as the results often are conflicting between different researches.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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4. DISCUSSION

In the Latvian population, TL is, on average, similar or slightly shorter than in populations of closely neighbouring countries such as Estonia and Finland but differs from other European populations where the observed TL is very diverse among different countries. It is very hard to determine the precise TL in different populations as measurements are usually performed in different laboratories by using different methods. However, trends can be identified, and these trends show that TL is very variable and can be influenced by different factors as described above. Additionally, TL is not a determinant by life span as shown; for example, Italians, who have longer life spans, on average, have shorter TL in comparison with other populations in which the life span is not as long (Eisenberg et al. 2011). Rather, it could be more important how fast telomeres shorten or how active the telomerase is (Spivak et al. 2016). The dynamics or shortening of TL with age in our study is as expected compared with other studies. TL does not differ between genders in our cohort, but TL does differ in other studies, suggesting that on average, females have longer telomeres, but this finding has not been confirmed in some studies (Gardner et al. 2014).

In Latvia, we have all the common mitochondrial hgs as there is in Europe (Richards et al. 1998). We did not find any of the hgs that are linked with longer life span, although some researchers claim that there is a connection, such as with hgs J, U and H (Robine et al. 2012; Beckstead et al. 2009; de Benedictis et al. 1999). There are many hypotheses about what could influence the distribution and connection between life span and hgs. It could be population specific, different climate and geographical location, life style or historical events, and of course SNPs could determine whether an hg affects OXPHOS efficiency, ATP synthesis, ROS production and health and diseases as described above. As a consequence, these diseases that are associated with some hgs can reduce amount or frequency of theses hgs in elderly people population.

We showed that mtDNA copy number decreases until a certain age, and then it slightly increases again in very old individuals or nonagenarians. High mtDNA CN is inherited from parents to offspring in families with longevity, which might act as a favourable factor for longevity by guaranteeing adequate energy supply (He et al. 2016a). This result could indicate that individuals who have had high mtDNA copy numbers from birth can live

longer and healthier lives. This most likely serves as a "selection" process for individuals with many mtDNA copies to survive to centenarian age. It appears to be that mtDNA contributes equally to longevity for both females and males (He et al. 2016b). As our sample cohort is made of individuals who are not related, we cannot say if they have inherited high mtDNA CN, and we also cannot identify dynamic change during ageing as the DNA samples were taken only once from each individual, which means there is a necessity for longitudinal studies of mtDNA CN dynamics during ageing.

We did not observe that any of the hgs influenced TL or mtDNA copy number at any age in our cohort. Although, one study claims that individuals with hg J have longer telomeres because of a low level of ROS produced (Fernández-Moreno et al. 2011). Based on another study, hg H produces the most ROS and therefore, should have the shortest telomeres (Martínez-Redondo et al. 2010); our study for hg H found one of the shortest telomeres, though the results were nonsignificant. In vitro hg J has more mtDNA than hg H, which was explained by increased TFAM binding that regulates mtDNA CN in mammals (Suissa et al. 2009; Ekstrand et al. 2004); however, in our cohort, none of the hgs had significantly more mtDNA than others. We did not find that mitochondrial heteroplasmy influences TL or mtDNA amount. Additionally, in contrast to other studies (Rose et al. 2007; Ding et al. 2015), the frequency of heteroplasmy in our cohort in the HVS-1 region determined by the SURVEYOR™ mutation detection method did not increase in nonagenarians. All these results may show that mutations that determine hgs or heteroplasmy do not strongly influence parameters such as TL and mtDNA that can influence life span.

TERT deficiency and telomere dysfunction induces disturbed mitochondrial biogenesis and function in various tissues (Sahin et al. 2011), and the longer telomeres we have, the more mtDNA CN we have (Kim et al. 2013; Tyrka et al. 2015; Pieters et al. 2015; Qui et al. 2015; Alegría-Torres et al. 2016). Not only telomeres influence mitochondrial function but also actions and dysfunction of mitochondria influences length and "health" of telomeres (reviewed in Gonzales-Ebsen et al. 2017). We also confirmed a correlation between TL and mtDNA CN, but this correlation disappeared in the very old or nonagenarian age group. For nonagenarians and centenarians, can speculate that cells with more mtDNA copies can maintain themselves and that the cells do not go into apoptosis, although they have short telomeres and are in a senescent state (Mei et al. 2015). It seems that telomere shortening

might not influence mtDNA amount or vice versa as strongly in nonagenarians as in younger individuals. As there has not been this kind of study in nonagenarian and centenarians, this hypothesis has yet to be confirmed by other laboratories, who may also determine the reason for this difference between very old and younger age groups.

It is important to maintain healthy life style to have long telomeres and fit mitochondria, and different factors can influence those two cell components very differently through lifetime. For example, considerable weight gain in the middle-life may increase late-life TL shortening, whereas mtDNA CN is likely to be reduced constantly by adiposity over the lifetime (Hang et al. 2018).

As in diverse pathologies and tissues the telomere-mitochondrial relationship can vary, it is important to look if this connection is evident in general populations during the healthy ageing process. MtDNA dynamics vary among studies, but in general population mtDNA content in peripheral blood was significantly associated with both sex and age; thus, the possibility was proposed to use it as a biological ageing marker in population studies and senescence (Knez et al. 2016). Also, variation of TL in different studies is an issue that makes it difficult to use it as an age and longevity marker for people. Additionally, both for mtDNA and TL methods and approach used among laboratories differ, which makes it hard to compare different studies and their results. Studied tissue also affects the results – stem cells would offer better representation for the ageing research as these cells are responsible for tissue renewal. Decline in tissue regenerative capacity is associated with age and is believed to be a result from an exhaustion, and a loss of function of adult stem cells (reviewed in Sui et al. 2016). Albeit, TL shortening in leukocytes reflects telomere shortening in hematopoietic stem cells – faster shortening of telomeres in leukocytes during adulthood suggests a faster telomere shortening in hematopoietic stem cells (Shepherd et al. 2004; Sidorov et al. 2009). Studies of human population ageing are mostly conducted on easily accessible peripheral blood leukocytes. Although, TL in peripheral blood leukocytes is not the most representative indicator of ageing mechanisms in our bodies but there is vast amount of papers that have proven a strong correlation between age and TL in leukocytes and the possibility to monitor dynamic changes of TL during ageing in this cell type (e.g. Ehrlenbach et al. 2009). TL is also synchronised among tissue, meaning that if for one individual telomeres are long (or short) in

one tissue they are as also long (or short) in other tissues, and TL attrition rate is alike if compared within an individual (Wilson et al. 2008; Daniali et al. 2013).

There is a lot of work to be done to fully understand ageing in people especially if we want to expand our health-span and maybe even our lifespan. And we have to continue research of ageing in populations as it is an opportunity to understand ageing in human bodies and not only in vitro cells or animal models as there is a great difference among them.

5. CONCLUSIONS

1. Telomere length, mtDNA amount and distribution of mitochondrial haplogroups differs between populations.

2. Homogeneity of telomere length and higher mtDNA content for nonagenarians might explained by “selection hypothesis” – bearing longer telomeres or more mtDNA at birth or they can have protective mechanisms against telomere attrition and mtDNA loss.

3. There is an interaction between telomeres and mtDNA, but for nonagenarians and centenarians this interaction weakens, or different mechanisms exist.

4. Benign SNPs in mtDNA, like haplogroups or heteroplasmy, do not influence telomere length, mtDNA amount and longevity as strongly in the tested sample cohort as in other studies.

5. Optimal mitochondrial maintenance might be more important for healthy ageing than telomere length loss at nonagenarian and centenarian age.

6. THESIS

1. Telomere length, mtDNA amount and distribution of mitochondrial haplogroups are population specific.

2. Telomere length and mtDNA CN age-related changes are interconnected during human ageing, this connection of telomere length and mtDNA CN alterations could be different in people who reach the nonagenarian or centenarian age.

3. Mitochondrial haplogroups and mtDNA heteroplasmy directly do not influence telomere length, mtDNA amount and population ageing.

7. PUBLICATIONS

Original work:

1. **Zole E**, Zadinane K, Pliss L, Ranka R. Linkage between mitochondrial genome alterations, telomere length and aging population. Mitochondrial DNA A DNA Mapp Seq Anal. 2018 Apr;29(3):431-438. Epub 2017 Mar 24.

2. **Zole E**, Elferts D, Kimsis J, Krumina A, Narels K, Pole I, Ranka R, Pliss L. Comparison of telomere length between population-specific mitochondrial haplogroups among different age groups in a Latvian population. Mech Ageing Dev. 2015 Feb 7;145C:13-17.

3. **Zole E**, Pliss L, Ranka R, Krumina A, Baumanis V. Dynamics of telomere length in different age groups in a Latvian population. Curr Aging Sci. 2013 Dec;6(3):244-250.

Review article:

4. **Zole E**, Ranka R. Mitochondria, its DNA and telomeres in ageing and human population. Biogerontology. 2018 Jul;19(3-4):189-208.

8. APPROBATION OF RESEARCH

1. Zadinane K, **Zole E**, Pole I, Ranka R. Links between mitochondrial DNA and ageing (2016) Abstract Book, Health Sciences, Riga: RSU, p. 108.

2. **Zole E**, Ranka R, Narels K, Pliss L. Correlation between mitochondrial DNA (mtDNA) copy number and telomere length in different age groups (2015) The FEBS Journal, vol. 282 (Suppl. 1), p. 362.

3. **Zole E**, Narels K, Pliss L, Ranka R. Molecular connection between telomeres and mtDNA during ageing (2015) 73rd conference of University of Latvia, Riga, oral performance.

4. **Zole E**, Narels K, Pliss L, Ranka R. hTR (hTERC) and Tankyrase-1 mRNA expression in a human primary fibroblast-like stem cell line culture after treating with hydrogen peroxide (2014) The FEBS Journal, vol. 281 (Suppl. 1), p. 252.

5. **Zole E**, Narels K, Pliss L, Ranka R. Dynamics of telomere length in diverse types of leucocytes of different age groups of people (2014) 72nd conference of University of Latvia, Riga, oral performance.

6. **Zole E**, Pliss L, Ranka R, Krumina A, Baumanis V. Telomere length between mononuclear blood cells (MNC) and peripheral white blood cells (WBC) in context with population-specific mitochondrial (MT) lineages in a Latvian population ageing (2013) The FEBS Journal, vol. 208 (Suppl. 1), p. 16.

7. Pliss L, **Zole E**, Ranka R, Kimsis J, Aitullina A, Krumina A, Baumanis V. Telomeres length variation in different age groups in Latvian population. 5th International School of Molecular genetics “Genome Instability” (2012) Moscow-Zvenigorod, Russia.

8. Ranka R, Pliss L, **Zole E**, Aitullina A, Gustina A, Krumina A, Baumanis V. Telomere length and mtDNA heteroplasmy in different age groups (2011) The FEBS Journal, vol. 278 (Suppl. 1), p. 248.

9. Aitullina A, Pliss L, **Zole E**, Ranka R, Krūmiņa A, Baumanis V. Increased mitochondrial DNA (mtDNA) heteroplasmy in aged person groups and approaches of its analysis (2011) Abstracts of IMBG conference. Molecular Biology advances and perspectives. Kiiv, Ukraine.

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Appendix I

Mitochondria, its DNA and telomeres in ageing and human population

Review paper

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REVIEW ARTICLE

Mitochondria, its DNA and telomeres in ageing and human population

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Abstract In the last decades, studies about ageing have become more essential as our population grows older. The incidence of age-related diseases increases, which pose challenges both for societies and individuals in terms of life quality and economic impact. Understanding ageing and ageing-related processes will help us to slow down or even prevent these diseases and provide opportunities for healthy ageing; additionally, we all want to live longer. Ageing is a consequence of the interaction between processes that occur over time and genetics interacting with various disease states and an individual's lifestyle. There are several hallmarks of ageing that are generally accepted, but neither of the theories appears to be fully satisfactory. The focus of this article is on two theories of ageing: telomere shortening and mitochondrial DNA (mtDNA) alterations and dysfunction. We discuss characteristic molecular features such as mitochondrial haplogroups, telomere length, mtDNA copy number and heteroplasmy, and how all these traits come together in the ageing population. The recent evidence shows the existence of a strong linkage between these two theories suggesting common molecular mechanisms and a complicated telomere-mitochondria interplay during the humans' ageing. However, this relationship is still not completely understood, which is why it needs more attention.

Keywords Population ageing · Mitochondrial haplogroups · Heteroplasmy · mtDNA copy number · Telomere length

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Introduction

Ageing is a consequence of the interaction between molecular processes that occur over lifespan and genetics interacting with various disease states and an individual's lifestyle. We can view ageing as an unavoidable passage of time and as a cell replicative senescence, which is a process when cells stop dividing and undergo distinctive phenotypic alterations. Evidence shows that the effects of cellular senescence are continuously acquired and that senescent cells exist in tissues even early in life (Eisenberg 2011; Dimri et al. 1995).

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As the human population ages, it is essential to think about how to increase the quality and years of healthy life (Peel et al. 2005). Deciphering the molecular senescence processes and the complicated interplay of various factors may lead us towards effective strategies of healthy ageing and long life in the future. There are nine hallmarks of ageing that are generally accepted: genomic instability (nuclear, mitochondrial DNA (mtDNA) and nuclear architecture alterations); telomere attrition; epigenetic alterations (alterations in DNA methylation patterns, posttranslational modification of histones, and chromatin remodelling and transcriptional alterations); loss of proteostasis (chaperone-mediated protein folding and stability, proteolytic systems); deregulated nutrient sensing (the insulin- and IGF-1-signalling pathways, mTOR, AMPK, and sirtuins); mitochondrial dysfunction (reactive oxygen species (ROS), mitochondrial integrity and biogenesis, mitohormesis); cellular senescence (telomere loss, INK4a/ARF locus and p53); stem cell exhaustion; and altered intercellular communication (inflammation and other types of intercellular communication) (reviewed in López-Otín et al. 2013). The emphasis of this review is on two of the theories of ageing: one involves telomere length (TL) shortening, which leads to cellular replicative senescence, and the second involves mtDNA alterations and progressive mitochondrial malfunction and apoptosis. While these hypotheses were initially separated, the evidence suggests the existence of direct molecular links between telomere and mitochondrial dysfunction (reviewed in Jin 2010; Lipsky and King 2015; Sergiev et al. 2015; Sahin and Depinho 2010).

Thus far, to our knowledge, there is no review article available with a focus on population studies analysing mitochondrial and telomeric hallmarks such as mitochondrial haplogroups (hgs), mitochondrial DNA copy number (mtDNA CN), heteroplasmy and TL.

Telomeres

Telomeres are the specialized chromosomal DNA–protein structures that cap and protect the terminal regions of eukaryotic chromosomes. The sequence of telomeric DNA in vertebrates is $(TTAGGG)_n$; in humans, its length ranges from 5 to 15 kb and varies between different tissues. Human telomeres typically terminate in a 35–600 nt single-strand 3′ overhang of the G-rich sequence, which are bound by a specific protein complex termed 'shelterin' (proteins connected with telomeres). Telomeres are dynamic structures that become shorter with every cell division until a critical stage is reached when the cell can no longer divide and enters a phase of senescence. Telomere attrition causes genomic instability, which is associated with a higher risk of age-related diseases and cancer, leads to potentially maladaptive cellular changes, and blocks cell division (Harley et al. 1990; Blackburn 2001; de Lange et al. 2006; reviewed in Samassekou et al. 2010; Blackburn et al. 2015). Telomeres are essential for stabilizing eukaryotic chromosomes in different ways. The main functions of telomeres are to prevent the end of the linear chromosomal DNA from being recognized as a broken end; telomeres are also the "first responders" to threats to genomic stability and problems with DNA maintenance. Telomeres are located at the nuclear envelope, and their specific association with the spindle pole body is required for normal recombination, protecting cells from nonhomologous recombination and broken DNA ends ensuring normal mitosis and meiosis (Blackburn 2001).

Each time a cell divides the telomeres get shorter due to the chromosome end-replication problem. By removal of the RNA primer, which initiates the last Okazaki fragment, a single-stranded G-rich 3′-overhang is formed when a replication fork has reached the end of the chromosome's lagging strand (Makarov et al. 1997). The telomeric 3′-overhangs are also observed at the end of the leading strand, although of different size (Chai et al. 2006). As the leading-strand replication generates a blunt-ended telomere, 5′-end resection by Apollo exonuclease is required to form the single-stranded 3′-overhang to protect telomeres. Thereby, the telomeric 3′-overhangs are formed by shelterin-controlled multistep process which is slightly different for each strand (Wu et al. 2012; reviewed in Higa et al. 2017). Telomerase is a specific enzyme that extends telomeres, but it is significantly active only in special cells such as stem cells, gametes, some types of blood cells and most cancer cells (Greider and Blackburn 1985; Kim et al. 1994; Wright et al. 1996; Kaszubowska 2008; Wu et al. 2017). The catalytic subunit of telomerase, the telomerase reverse transcriptase (TERT), is also found in mitochondria

and binds to mtDNA to protect it from oxidative damage (Santos et al. 2004; Haendeler et al. 2009). Telomere maintenance and shortening is a complex process that can be affected by different factors, such as stress and genetic background, and occurs slightly differently in diverse tissues; furthermore, the relationship between TL and ageing is not fully understood (reviewed in Blackburn et al. 2015). It is widely accepted that TL gradually decreases with age in human cells (except for the terminally differentiated tissues such as brain and myocardium) and for women, telomeres shorten more slowly due to oestrogen effects on telomerase (Kyo et al. 1999; Takubo et al. 2010). For men, in sperm cells, telomeres elongate with age (Kimura et al. 2008). There are claims that TL is paternally inherited (Nordfjäll et al. 2005), although some studies suggest a strong maternal or an X-linked inheritance pattern of TL (Nawrot et al. 2004; Broer et al. 2013). Under the hypothesis that inheritance of TL is stronger linked through the maternal than paternal lineage is association with the mitochondrial genome during embryonic and fetal development and TL (reviewed in Aviv 2018). In addition, it seems that TL depends more on the age of parents at which a child has been conceived and that older fathers pass on longer telomeres, possibly because of the paternal age-dependent germ stem cell selection process, where sperm cells with longer telomeres are more successfully selected because they are more age-resistant (Njajou et al. 2007; Hjelmborg et al. 2015). Telomeric DNA is a target of persistent DNA damage which can induce cellular ageing and stress-induced senescence (Hewitt et al. 2012). Shortened blood cells' telomeres in population studies were associated with higher rates of mortality from different age-related pathologies, including heart and vascular diseases, diabetes mellitus, Parkinson's disease and Alzheimer's disease, among others (reviewed in Blasco 2007; Jiang et al. 2007; van der Harst et al. 2007). Several studies have reported that telomeres do not shorten as quickly in older individuals and TL does not predict mortality in the very oldest. This observation presumably may be related to a gradual reduction in cell turnover with advancing years. However, the opinion exists that, at a certain age, telomere maintenance is more critical than the exact TL (Mondello et al. 1999; Jiang et al. 1992; Shay and Wright 2007; Haussmann and Mauck 2008; Ehrlenbach et al. 2009; reviewed in Simons 2015). Our studies confirmed that telomeres do not necessarily shorten instantaneously or at a specific age; the higher variability in TL among nonagenarians (90–100 years old individuals) is not as pronounced as in the age group between 60 and 90 years old and in younger age groups (Zole et al. 2013). The variability in TL for the group of younger individuals may be explained by the "selection hypothesis", which proposes that the nonagenarians and centenarians harboured very long telomeres, and variation in TL reduces with age due to the selective loss of individuals with shorter telomeres (Haussmann and Mauck 2008; Halaschek-Wiener et al. 2008).

Population studies have demonstrated that TL can be quite diverse among different populations. In a study of people over 60 years old, a French population (an average life expectancy (ALE) 82.4 years; the data of life expectancies for populations was taken from World Health Statistics 2015) contained longer telomeres than an Italian population (ALE 82.7 years) (Canela et al. 2007). Individuals with an average age of 22.7 years from Southern Europe (ALE 81.9 years) possessed shorter telomeres than individuals from one part of the Baltic region (ALE 79.3 years), and individuals from the Middle Europe (ALE 81.5 years) contained the longest telomeres (Salpea et al. 2008). However, another report that included fourteen European populations with individuals between ages 18-28 showed that TL varied among them drastically, with the shortest telomeres observed in an Italian population (5100 bp (base pairs)) (ALE 82.7 years) and the longest telomeres observed in a Belgian population (18,640 bp) (ALE 81.1 years). For an Estonian population (ALE 77.6 years), telomeres were 7340 bp long (Eisenberg et al. 2011). Similar results were obtained in a geographically proximal region: for a Latvian population (ALE 74.6), the average TL was 7132 bp for individuals up to 40 years old and 6112 bp for individuals older than 60 years of age (Zole et al. 2013). Pronounced differences in TL were observed for two subpopulations in Finland (ALE 81.38 years): in an Oulu-based population, the mean TL was 7620 bp, and in a Helsinki-based population, the mean TL was 12,280 bp (Eisenberg et al. 2011). The work of Hansen and colleagues also proved that TL between populations differs. They stated that Europeans (ALE 71.3–83.4 years) have much shorter telomeres than sub-Saharan Africans (Tanzania) (ALE 61.8 years) and Afro-Americans (ALE 75.6 years) in a long age span of 20–80 years (Hansen et al. 2016). In

comparison the TL in the USAs population of age range 19–93 years were longer in Afro-Americans (ALE 75.6 years) than in whites (ALE 79 years), although Afro-Americans showed a faster rate of TL shortening (Hunt et al. 2008; Chen et al. 2011; data for ALE in USA taken from Kenneth et al. 2016). In a similar study the same research group again demonstrated that black people have longer telomeres than white people, age range of 19–77 years (Daniali et al. 2013). These differences among populations and ethnicity are poorly understood. It seems that TL in leukocytes of the reviewed populations not always can be explained by life expectancies, while, there is a correlation between age and TL in peripheral blood leukocytes, with correlation coefficient ranged from − 0.088 to − 0.838 in different studies (reviewed in Müezzinler et al. 2013). In most studies, short telomeres in circulating leukocytes are also associated with high mortality (Cawthon et al. 2003; Rode et al. 2015). Though, telomere shortening may not affect mortality per se but could be controlled by progression of senescence that leads to mortality by other mechanisms (Cawthon et al. 2003). Life expectancy also depends on dynamics of TL or how fast telomeres shorten for each individual as a study on birds has shown (Bize et al. 2009). All these observations of different TL and life expectancies among populations could be explained by differences in genetics, sex, ethnicity, nutrition, economic and social status or stress level and health care systems. It can also be due to lifestyle choices including smoking, alcohol consumption, physical activities, body mass index, diet and supplement intake and environmental pollution that can influence the disparity (reviewed in Epel et al. 2004; Lin et al. 2012; Müezzinler et al. 2013; Vidacek et al. 2017). For example, Crous-Bou and colleagues showed that women who practised a Mediterranean diet had longer telomeres than those who did not (Crous-Bou et al. 2014). There is evidence that famine can also influence TL. For men who have experienced starvation, TL was shorter than for those who have not. It also seems that men whose parents had experienced recent starvation before conception had shorter telomeres (Kobyliansky et al. 2016).

As TL is only one important factor among others that influences ageing, it is not surprising that TL differs even among subpopulations. Variation is an issue that makes it difficult to use TL as an age and longevity marker for people. Additionally, methods used among laboratories differ, which makes it hard to compare different studies and their results. Studied tissue also affects the results—stem cells would offer better representation for the ageing research as these cells are responsible for tissue renewal. Decline in tissue regenerative capacity is associated with age and is believed to be a result from an exhaustion, and a loss of function of adult stem cells (reviewed in Sui et al. 2016). Albeit, TL shortening in leukocytes reflects telomere shortening in hematopoietic stem cells—faster shortening of telomeres in leukocytes during adulthood suggests a faster telomere shortening in hematopoietic stem cells (Shepherd et al. 2004; Sidorov et al. 2009). Studies of human population ageing are mostly conducted on easily accessible peripheral blood leukocytes. Although, TL in peripheral blood leukocytes is not the most representative indicator of ageing mechanisms in our bodies but there is vast amount of papers that have proven a strong correlation between age and TL in leukocytes and the possibility to monitor dynamic changes of TL during ageing in this cell type (e.g. Ehrlenbach et al. 2009). TL is also synchronised among tissue, meaning that if for one individual telomeres are long (or short) in one tissue they are as also long (or short) in other tissues, and TL attrition rate is alike if compared within an individual (Wilson et al. 2008; Daniali et al. 2013).

Mitochondria

A mitochondrion, a very essential organelle of an eukaryotic cell, has many functions that ensure differentiation, survival and control of death of cells. The mitochondrion works as a factory for ATP (adenosine triphosphate) and metabolite supplies for cell survival and releases cytochrome c to initiate cell death. It works as a signalling organelle by releasing specific proteins, ROS and metabolites, or by serving as a scaffold to configure signalling complexes (reviewed in Chandel 2014). It also regulates the bioenergetics of the cell through cytosolic calcium regulation (Rizzuto et al. 1993). By providing ATP and other vital components, mitochondria affect gene expression, transcriptional activation-related chromatin modification, transcription elongation and even alternative splicing processes (Guantes et al. 2016).

It has been shown that mitochondria are required for pro-ageing traits of the senescent cell phenotype

(Correia-Melo et al. 2016). Progressive loss of mitochondrial function in several tissues is a common feature of ageing believed to be influenced by the lifelong production of ROS as by-products of oxidative metabolism (Shigenaga et al. 1994; Bellizzi et al. 2006; Kenney et al. 2014). With age, mitochondria become less effective and even potentially toxic. Furthermore, they can induce apoptosis or necrosis that can lead to a decrease in the functions of tissues and the whole body. As oxidative phosphorylation (OXPHOS) occurs in the mitochondria, it leads to ROS production, which in turn oxidizes macromolecules and can lead to mtDNA mutations. During ageing, mutations in mtDNA accumulate and OXPHOS becomes less effective, leading to higher ROS production, which further leads to cell senescence and death (Wallace et al. 2010; Green et al. 2011; Mikhed et al. 2015). Mitochondria are also involved in processes of inflammation and autophagy or mitophagy. It was hypothesized that cells can adapt to stress by the induction of these processes, which can help to sustain a longer life span due to the elimination of dysfunctional mitochondria and damaged cells. During the time mitophagy and autophagy decreases, dysfunctional proteins accumulate and cells age (Levine and Kroemer 2008; Lipinski et al. 2010).

Mitochondrial DNA

The mitochondrion contains its own circular double-stranded DNA which is maternally inherited (Giles et al. 1980). mtDNA is ~ 16,569 bp long and encodes only 37 genes: 22 tRNAs, 13 subunits of the respiratory chain (79 subunits are encoded by the nuclear genome), and 12S and 16S rRNAs genes (Anderson et al. 1981). The 16S gene also encodes humanin peptide and is then transported into intra- and extra-cellular compartments and functions in mitochondrial-nuclear retrograde signalling (Hashimoto et al. 2001; Lee et al. 2013). Additionally, the *cox1* gene on the antisense strand possibly encodes a short protein named Gau (Gene Antisense Ubiquitous) (Faure et al. 2011). The genome structure of the two strands is distinguished by their nucleotide composition—the heavy strand (H-strand) is guanine-rich, compared with the cytosine-rich light strand (L-strand). mtDNA genes lack introns. Most genes are located very close to each other, separated by only one or two non-coding base pairs, and some genes, such as ATP6 and ATP8, have overlapping regions. mtDNA has only one major non-coding region (NCR), that contains the displacement loop (D-loop) and hypervariable (HVS) regions. The D-loop contains a site of mtDNA replication initiation (O_H—origin of heavy strand synthesis) and is also the location site of both H-strand transcription promoters (the first heavy-strand promoter (HSP1) and a potential, second distal heavy-strand promoter (HSP2). The origin of the light strand synthesis (O_L) is located two-thirds of the lengths from the O_H. mtDNA replication is not driven by the cell cycle or cell division and is continuously recycled (reviewed in Chinnery and Hudson 2013). Accumulation of certain mtDNA mutations or single nucleotide polymorphisms (SNPs) can cause amino acid and functional changes of OXPHOS respiratory complexes, while others cause changes in the rates of replication and transcription of the mtDNA (Shigenaga et al. 1994; Bellizzi et al. 2006; Kenney et al. 2014).

mtDNA copy number

Most of the mammalian cells contains hundreds to more than a thousand mitochondria each, and each organelle harbours 2–10 copies of mtDNA (Robin and Wong 1988). The mtDNA amount in a cell is heritable, implying genetic regulation of mtDNA levels, and the mtDNA CN in peripheral blood cells is higher for women than men (Ding et al. 2015; Knez et al. 2016). There are debated results from different studies regarding how mtDNA CN in blood cells changes during human ageing. A substantial age-related decline in the abundance of mtDNA has been shown between 17 and 93 years of age (Mengel-From et al. 2014; Ding et al. 2015; Zhang et al. 2017), and low mtDNA content in blood cells was associated with familial longevity in a study from the Netherlands. Based on their results, the authors discussed the necessity of preserving mitochondrial functions rather than enhancing mitochondrial biogenesis via the YY1 transcriptional repressor protein (van Leeuwen et al. 2014). It was reported that an increase in the mtDNA CN is associated with elevated oxidative stress in the human tissues of aged individuals (Barrientos et al. 1997a, b). In contrast, He et al. have shown that mtDNA content in blood samples was higher for healthy centenarians than for the younger age groups (He et al. 2014), while Moore and colleagues have

reported that association of mtDNA CN with age was not statistically significant among participants of the InCHIANTI study (age range 29–96) (Moore et al. 2018). Our recent findings corroborate that elderly group's individuals (60–90 years of age) had significantly lower mtDNA CN in blood cells in comparison with a younger age group; in contrast, nonagenarians did not have significantly less mtDNA CN in comparison with the younger age group (Zole et al. 2017). High mtDNA CN in leukocytes in many studies has been associated with better health, including higher cognition and lower mortality (Lee et al. 2010; Kim et al. 2011; Mengel-From et al. 2014; Ashar et al. 2015). In a recent study, higher mtDNA was associated with a significantly lower risk of both solid tumours and other diseases, independent of age and sex (Memon et al. 2017). On the other hand, high mtDNA CN was associated with breast, prostate, gastric and colorectal cancer risk (Thyagarajan et al. 2013; Zhou et al. 2014; Zhu et al. 2017; Kumar et al. 2017). Additionally, stress and depression were associated with increased mtDNA amounts in blood cells (Cai et al. 2015) as well as other health-related factors that were shown to have an influence on mtDNA quantity in human cells (reviewed in Malik and Czajka 2013). There are several molecular factors in a cell that can regulate mtDNA CN, such as p53, mitochondrial transcription factor A (TFAM), Twinkle helicase and ATP synthase (Kulawiec et al. 2009; Ekstrand et al. 2004; Tyynismaa et al. 2004; Fukuoh et al. 2014; Moraes 2001). It was also shown that mtDNA CN is regulated by expression of the only mtDNA polymerase γ subunit A (Polγ-A) in a cell-specific manner by nuclear DNA methylation (Kelly et al. 2012). Also, other factors like lifestyle can influence mtDNA amount. Obesity, weight gain and pack years of smoking were associated with reduced mtDNA CN (Meng et al. 2016). Reduced sleep duration and efficiency were associated with lower mtDNA amount (Wrede et al. 2015) but regular exercise for postmenopausal women increases mtDNA CN in leukocytes (Chang et al. 2016).

Centenarians have increased mitochondrial mass and higher ATP level in primary cultures of fibroblasts isolated from the skin (have defective mitochondria but a preserved bioenergetic competence) indicating that longevity is characterized by a preserved bioenergetic function likely attained by a successful mitochondrion remodelling that can compensate for functional defects through an increase in mass, i.e. a sort of mitochondrial "hypertrophy" (Sgarbi et al. 2014). One reason could be due to compensatory mechanisms in centenarians' cells both for higher mtDNA and ATP level as a normal level of ATP is necessary for a cell to progress through the G1 phase of the cell cycle to proliferate and regenerate, which could help the cells to be more fit during ageing by providing energy reserves (Mandal et al. 2005; Owusu-Ansah et al. 2008; Moiseeva et al. 2009). High mtDNA CN from birth for centenarians could also provide healthier lives and longer life span. This most likely serves as a "selection" process for individuals with many mtDNA CN to survive to centenarian age. Apart from possible heterogeneity of studied populations and analytical factors which may affect measurements of mtDNA CN, associations between mtDNA CN and ageing phenotypes are influenced by complicated interplay between unknown hereditary factors and exposures affecting biological pathways associated with mitochondrial function, DNA damage, oxidative stress and senescence. A considerable amount of research is still needed to fully understand mtDNA replication mechanisms and the regulation of mtDNA CN, as well as the possible roles of these processes in human ageing. Other interesting questions are whether the mtDNA CN is population-specific and whether mtDNA amounts can be inherited through the maternal line.

Heteroplasmy

mtDNA single nucleotide mutations and deletions as well as their accumulation (especially the 4977 bp deletion) were associated with a normal ageing process in several studies and have been proposed either as a useful marker of natural ageing in human subjects or as a factor affecting human longevity (Cortopassi et al. 1992; Meissner et al. 1997; Linnane et al. 1989; Raule et al. 2014; reviewed in DeBalsi et al. 2017). However, mtDNA mutation sites, accumulation rate and impact on cell functionality are affected differently in each tissue type and there is a great importance of the individuals' ethnic background (Samuels et al. 2013; Raule et al. 2014). Research on the mtDNA mutation signatures suggest a limited role for ROS-induced mutation (Kauppila and Stewart 2015), and accumulation of mtDNA mutations can be related to the down regulation of DNA

repair that occurs with cellular senescence. Mice with defective Polγ show aged phenotypes and increased mutational load in the mtDNA (Trifunovic et al. 2004). Somatic mutations can be unevenly distributed and accumulated clonally in cells, causing a mosaic pattern of respiratory chain deficiency in tissues, or accumulation can occur during germline, embryonic or foetal development, in which case, the distribution of mutations would be even between tissues (Trifunovic et al. 2004, Greaves et al. 2014).

Mitochondrial heteroplasmy is the presence of multiple mtDNA variants with mutations or SNPs in a single cell or among cells within an individual (Potter et al. 1975). The frequency of heteroplasmy is the same between women and men (de Camargo et al. 2011; Ding et al. 2015). The highest degree of polymorphisms is concentrated within two hypervariable segments of the control region: hypervariable segment I (HVS-I) and hypervariable segment II (HVS-II) (Wilkinson-Herbots et al. 1996). The results of studies looking on the association between mtDNA heteroplasmy and population ageing are not fully consistent. Blood cells' mtDNA heteroplasmy becomes more common with increasing age (Ding et al. 2015; Sondheimer et al. 2011; Zhang et al. 2017), and increased mtDNA heteroplasmy was associated with impaired functioning and increased risk of mortality (Tranah et al. 2017). mtDNA heteroplasmy at specific DNA sites in platelets was associated with reduced neurosensory and mobility functions in older people (Tranah et al. 2015). However, there is also possibility that some mtDNA polymorphisms are selected with age and can compensate for the defects induced by various types of mtDNA mutations, helping an individual to survive longer (Ono et al. 2001; Sondheimer et al. 2011; Rose et al. 2007). In our study, mtDNA heteroplasmy was observed more often in elderly individuals comparing with centenarians (Zole et al. 2017), and several studies have shown that the total heteroplasmy values and patterns are maintained in centenarian families (Rose et al. 2007; Giuliani et al. 2014). It was reported that heteroplasmy can be inherited from a mother with 30% possibility; however, in 70% of cases, a mother does not pass the mutated site to her children, and heteroplasmic SNP changes can accrue spontaneously during the lifetime (Sondheimer et al. 2011; Ding et al. 2015).

In diabetic patients, a correlation between the mtDNA CN and SNPs in mtDNA has been observed in blood vessels tissue (Chien et al. 2012). Measuring mtDNA CN by shotgun sequencing, Wachsmuth and collaborators found that there was a correlation between mtDNA CN with the total number of heteroplasmies in blood and skeletal muscle cells; in the other tissues, heteroplasmy was correlating with age, indicating that this correlation could be explained mostly by age and not as much by the increase in the number of heteroplasmic sites (Wachsmuth et al. 2016). Another study recorded lower mtDNA CN in individuals having higher numbers of heteroplasmies but posits that decrease of age-related mtDNA CN and increase of heteroplasmy are independent from one another (Zhang et al. 2017). In blood cells, no differences for mtDNA CN between HVS-I heteroplasmy-positive and heteroplasmy-negative individuals have been found (Zole et al. 2017). Altogether these results may indicate that individuals without mtDNA heteroplasmy or individuals with specific heteroplasmic SNPs or patterns are more likely to survive to old age. Similar to the differences in TL, some population-specific or health-related factors may exist as well. However, in order to draw general conclusions, more studies should be conducted.

Mitochondrial haplogroups

The mtDNA sequence is diverse among populations and individuals. There are 9 major mitochondrial hgs found in the human population of Europe (H, I, J, M, T, U, V, W, and X) (Torroni et al. 1994; Richards et al. 1998). A human mtDNA hg is defined by differences in mtDNA sequences or SNPs. Previous studies showed that the mitochondrial genotype, or hg, can be associated with longevity and pathologies that can influence healthy ageing and mortality; however, the observed results are controversial. It has been detected that defined mutations in mtDNA which are associated with hgs D, D1, D4 (more abundant in Asian populations) and H1 (in European populations) are more frequently found among centenarians, while the frequencies of other hgs such as M9, N9 and B4a (more abundant in Asian populations) decrease in a centenarian group (Tanaka et al. 1998, 2000; Cai et al. 2009). Gender may also play a role in the distribution of hgs among centenarians (Fernández-Moreno et al. 2017). In Amish populations, the X hg was associated with successful ageing whilst hg J had opposite effects (Courtenay et al. 2012). Regarding European

populations, it has been observed that in Italy hg J is more abundant among centenarians, while hg U frequency decreases (de Benedictis et al. 1999; Rose et al. 2001). A study that has been performed in Finland showed that hgs H and HV were less frequent among centenarians than hgs U, J and U8 (Niemi et al. 2003). Nevertheless, in Spain, Pinós et al. have not identified such observations in relation to hg J and have suggested that longevity is population-specific (Dato et al. 2004; Pinós et al. 2012). In relation to hgs H and U, other researchers have not found significant associations with longevity (de Benedictis et al. 1999, 2000; Pinós et al. 2012). Similarly, our research in a Latvian population corroborates that certain hg abundance in older age groups is not associated with longevity and is population specific (Zole et al. 2015). Benn with colleagues also made an assumption that there are no associations of hgs with mortality and longevity (Benn et al. 2008). Beckstead et al. indicated that individuals with hg H may live longer in comparison to individuals with hg U under calorie restriction (Beckstead et al. 2009). Some researchers say that human adaptation to the chronic cold and irregular caloric availability, due to changes in seasons, could influence the evolution and distribution of mitochondrial hgs and longevity, especially in the North (Wallace 2005; Robine et al. 2012). In various studies, it has been revealed that hgs are definitely associated with healthy ageing and may have a protective effect on the occurrence of some diseases and tumours (e.g., Czarnecka and Bartnik 2011). For example, Hg J is associated with Leber's hereditary optic neuropathy (LHON), while hg H has a protective effect on LHON (Torroni et al. 1997; Hudson et al. 2007). In another study, there was no such observation; although, the lack of observation could be explained by the rarity of hg J in the population (Aitullina et al. 2013). Hg J shows a protective effect against the development of osteoarthritis (Fernández-Moreno et al. 2017). Rosa et al. found that hg H1 has a protective effect on ischaemic stroke while hgs HV and U have been involved in increased risk of it (Rosa et al. 2008). Hg U and its branch U8 could possess protective properties against Alzheimer's disease in patients with the ε4 allele (Carrieri et al. 2001). Furthermore, the results of two studies showed the protective properties of hgs U8 and J against Parkinson's disease in Italians and Poles, respectively (Ghezzi et al. 2005; Gaweda-Walerych et al. 2008). In contrast, a newer study from the UK did not find any linkage between hgs and Parkinson's disease (PD), Alzheimer's disease (AD), frontotemporal dementia–amyotrophic lateral sclerosis (FTD–ALS) or Creutzfeldt Jacob disease (CJD) (Wei et al. 2017). Hg T is associated with coronary artery disease (CAD) and diabetic retinopathy (Kofler et al. 2009). Moreover, among different types of tumours, some hgs may play a dual role of being either protective or not. Vulvar, prostate and renal cancers are associated with hg U, while hg H is more often represented in individuals suffering from head and neck cancers. However, hg H is underrepresented in other cancers such as vulvar, breast and endometrial cancers (Booker et al. 2006; Klemba et al. 2010).

The opinion exists that hgs might play a protective role for a cell against ROS because of greater heat generation (higher electron transport rates, looser coupling or partially uncoupled OXPHOS). Haplogroup-defining mutations may affect ATP synthesis because certain mitochondrial variants are biochemically different (Tanaka et al. 1998, 2000; Cai et al. 2009; Fernández-Moreno et al. 2017). Mitochondrial hgs have different coupling efficiencies (the percentage of oxygen consumption used for ATP synthesis rather than heat generation) or mitochondrial production of ROS. For the cold-adapted hgs, uncoupling mutations would produce less ATP per calorie consumption, which allows greater heat generation and more oxidization, and fewer ROS would be formed; for example, hg H has higher ATP production in comparison to hg J (Mishmar et al. 2003; Baudouin et al. 2005; Wallace 2005). Hgs J and U are uncoupled hgs, while hg H is tightly coupled and therefore produces more ROS and less heat. It has been proven that cybrids of hgs H and U had different amounts of mitochondria, where hg U had fewer mitochondria than hg H. Hg U had lower levels of cytochrome *b* mRNA, rRNA, protein synthesis and mitochondrial inner membrane potential (MIMP) than hg H (Martínez-Redondo et al. 2010; Gómez-Durán et al. 2010). In another case due to increased binding of TFAM cybrid cells harboring hg J had two-fold increase of mtDNA amount in comparison with cybrid cells containing hg H (Suissa et al. 2009). The H hg had higher peroxide and superoxide anion production and apoptosis but lower oxidative stress than the J hg (Fernández-Moreno et al. 2017). Martínez-Redondo et al. have also shown that hg H had higher

mitochondrial oxidative damage than hg J. In their study, hg H showed higher oxygen uptake than other hgs and therefore more ROS production was observed; the next highest ROS producer was hg V followed by hgs T and U; but the lowest was hg J (Martínez-Redondo et al. 2010). U3a and J2b subhaplogroups with a C150T SNP displayed lower ROS production than hgs without the C150T SNP, and this SNP was associated with longevity (Chen et al. 2012; Zhang et al. 2003). Other research groups found that hg H had more increased mitochondrial function than hg U in human skeletal muscle fibres (Larsen et al. 2014). Studying sperm mobility, scientists found that individuals with hg H had the highest activity of spermatozoids, compared to that of hg V and the lowest activity of spermatozoids has been detected for hg T, which is directly connected with the functionality of the OXPHOS system (Ruiz-Pesini et al. 2000).

By using shotgun sequencing, in recent studies no difference has been found among H, U, T, K and J hgs and mtDNA CN (Wachsmuth et al. 2016; Zhang et al. 2017), by using qPCR method, neither did we among H, U, T, J, V or W hgs, nor when compared among different age groups (Zole et al. 2017). Although in another research, it was found that hgs U5A1 and T2 were significantly associated with higher mtDNA CN by changing the *COXIII* and *COXI* amino acid sequences, respectively (Ridge et al. 2014). Additionally, the frequency of heteroplasmy among hgs did not differ in several studies (de Camargo et al. 2011; Ramos et al. 2013), but one study claimed that the HV hg had more frequent heteroplasmy while hgs J, T and U8 had less heteroplasmy (Ding et al. 2015). Previously, Fernández-Moreno et al. showed that individuals with hg J had significantly longer telomeres than non-J carriers (Fernández-Moreno et al. 2011), but our study showed that average TL is influenced very little by a hg and mitochondrial heteroplasmy (Zole et al. 2015).

There are many parameters that can influence the survival of individuals with specific hgs in different environments and populations, as well as the coexistence of other mutations that do not determine a hg, however, any of these parameters has the potential to be the most significant in particular settings. As there is the evidence that certain hgs have been associated with certain age-related diseases, this association may affect the frequency of specific hgs in the older age groups. In general, these studies showed that there is a possibility of some connection of mtDNA hgs with age-related diseases and longevity in humans, mitochondrial hg backgrounds and divergence of populations.

Interrelationship between mitochondria and telomeres

Heap of evidence suggests a crucial role for signalling from the nucleus to mitochondria in ageing (reviewed in Fang et al. 2016). Telomere theory declares telomere shortening as the main trigger and the best marker of cellular senescence and ageing, an adjacent theory is oxidative stress or mitochondrial theory (reviewed in Bernadotte et al. 2016). And for some time, scientists are trying to find a trade that links telomere attrition to metabolic compromise, which is central in cellular functional decline during ageing (Fig. 1). Mitochondrial dysfunction leads to perturbations on the electron transport chain resulting in increased ROS generation and reductions in ATP level (Balaban et al. 2005; Moiseeva et al. 2009). Additionally, increased mitochondrial density and biogenesis are associated with increased ROS production due to an increased number of sites where ROS generation can occur (Passos et al. 2007; Yoon et al. 2010). This, in turn, can further induce DNA damage, and it has been shown that oxidative stress is associated with increased telomere attrition (von Zglinicki 2002; Kawanishi and Oikawa 2004). One of the first studies that reported a connection between mtDNA mutations causing respiratory chain disorders and telomere shortening was about mutations in the mtDNA of LHON, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELS) syndrome patients; the researchers observed shorter telomeres in patients than in a control group (Oexle and Zwirner 1997). Liu et al., at the beginning of this century, demonstrated that mitochondrial dysfunction generates ROS and leads to chromosomal instability through telomere attrition (Liu et al. 2002). The study of Passos and colleagues also showed connections among TL with mitochondrial genetics and ROS in later years (Passos et al. 2007). On opposite side, an evidence exists that shortening of telomeres is a causal factor for mitochondrial dysfunction. Short telomeres, which are sensed as double-strand breaks and genomic DNA instability, suppress peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and

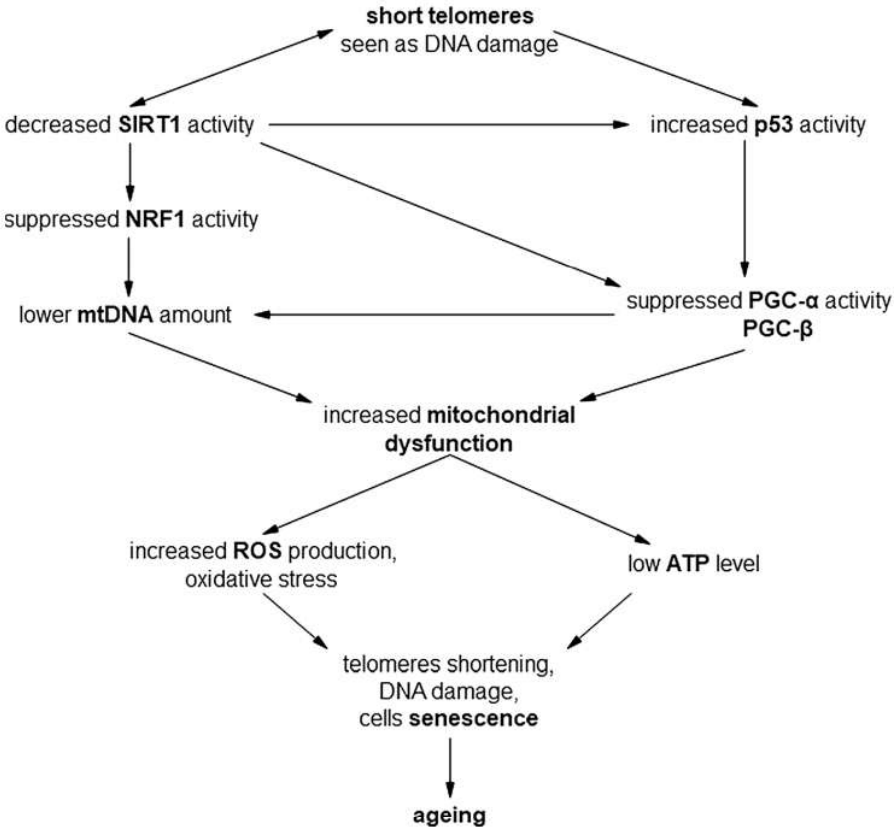

Fig. 1 Connection of telomere-mitochondrial axis of ageing. *SIRT1* NAD-dependent deacetylase sirtuin-1, *p53* tumor suppressor protein p53, *PGC-1α/β* peroxisome proliferator-activated receptor gamma coactivator 1-alpha/beta, *NRF1* nuclear respiratory factor 1, *ROS* reactive oxygen species, *ATP* adenosine triphosphate. The scheme adapted from Sahin and Depinho (2010), Moiseeva et al. (2009), Sahin et al. (2011), Pieters et al. (2015)

beta (PGC-1α/β, which regulate a vast number of mitochondrial functions, including mitochondrial replication/transcription, OXPHOS, oxidative stress and gluconeogenesis (e.g., Wu et al. 1999) action via the p53 transcription factor (Chin et al. 1999; Sahin and Depinho 2010; Sahin et al. 2011). Short telomeres cause increased p53 activity and high levels of apoptosis (Flores and Blasco 2009), as well as up-regulation of the mTOR and Akt survival pathways and down-regulation of cell cycle and DNA repair pathways (Schoeftner et al. 2009). Further work by Sahin and colleagues confirmed that telomere dysfunction is associated with reduced mtDNA content, while p53 deficiency partially rescues the transcriptional regulation of PGC-1α/β and mtDNA CN (Sahin et al. 2011). There are suggestions that p53 influences telomere DNA structure and promotes telomere DNA stability, and its binding can be induced by numerous stress conditions (Tutton et al. 2016). In contrast, in mitochondria, p53 plays a direct role in the mito-checkpoint response and positively regulates mtDNA CN; the loss of p53 leads to the reduction of mtDNA content in MEF cells (Kulawiec et al. 2009). Consistent with the mtDNA CN reduction, p53 null cells show a decrease in mitochondrial membrane potential and reduction in mitochondrial mass, linking p53 to promotion of normal mitochondrial function (Lebedeva et al. 2009). Safdar et al. have shown that p53 translocates to the mitochondria and facilitates mtDNA mutation repair and mitochondrial biogenesis in response to endurance exercise in mice. These results suggest that in cells under certain conditions, more p53 is located in mitochondria than in nuclei, and in this way, p53 helps to maintain healthier cells, longer telomeres, less mtDNA mutations and prevents apoptosis (Safdar et al. 2016). Alternatively, another study has found that overexpression of p53 negatively affects the mtDNA abundance in HepG2 cells (Koczor et al. 2012). Pieters et al. showed that SIRT1 also plays a role in the telomere-mitochondrial ageing axis. In their work, TL was positively correlated with SIRT1, which deacetylates and inactivates p53 protein, and NRF1 (involved in regulation of mtDNA transcription and replication) and negatively correlated with p53

expression, while mtDNA content was positively correlated with SIRT1 and NRF1 expression (Pieters et al. 2015). SIRT1 is involved in glucose homeostasis as a modulator of PGC-1α, and it deacetylates p53, leading to reduced apoptosis (Vaziri et al. 2001; Rodgers et al. 2005); extra expression of SIRT1 enhances mitochondrial function by upregulating mitochondrial biogenesis and degradation, thus improving oocyte development (Sato et al. 2014).

The first observation of TL and mtDNA CN correlation in peripheral leukocytes in population studies comes from studies of patients with diseases linked to alterations of these two cell components such as type 2 diabetes (Monickaraj et al. 2012). A study about depression found no correlation: the authors reported shortened telomeres but no reduced mtDNA CN in leukocytes in patients with depressive symptomology (Verhoeven et al. 2017), while childhood adversity and lifetime psychopathology were each linked to shorter telomeres but higher mtDNA CN in leukocytes (Tyrka et al. 2016). In a study about cognitive dysfunction in individuals over 75 years of age authors saw positive relationship between TL and mtDNA in blood samples after adjustment for age and gender; and for individuals with conative dysfunction both high TL and high mtDNA CN amount were observed, speculating that it might be due to increased oxidative stress and inflammation (Lee et al. 2017a).

Data from different tissues are also available. Patients with Fuchs' endothelial corneal dystrophy (FECD) had higher mtDNA levels but shorter telomeres in endothelial cells (Gendron et al. 2016) while in patients suffering mental disorders had shorter telomeres and lower mtDNA CN in post-mortem brains of people who had committed suicide (Otsuka et al. 2017). For people with colorectal carcinogenesis, a positive correlation depended on the stage of the cancer in tubular adenoma and serrated polyp tissue. In normal and cancer tissues, the correlation was positive, but in precancerous legions, the correlation was not observed, suggesting that the disturbance of the telomere-mitochondrial axis by ageing or other factors may be important in the development of carcinogenesis (Lee et al. 2017b). Jung et al. also showed a sign of dynamic TL and mtDNA CN relationship. They found a positive correlation in normal tissues and the intestinal type of gastric cancer but not in the diffuse type of the disease (Jung et al. 2017). In addition, not only was mtDNA CN correlated with TL, but mtDNA non-silent mutations had a negative correlation with TL in bone marrow and oral epithelial cells in aplastic anaemia patients (Cui et al. 2014).

As in diverse pathologies and tissues the telomere–mitochondrial relationship can vary, it is important to look if this connection is evident in general populations during the healthy ageing process. The use of peripheral blood leukocytes for these studies could be argued as not the ideal tissue as discussed above. However, this approach is widely used as the evidence suggests that TL measurement in easily accessible tissues such as blood could serve as a surrogate parameter for the relative TL in other tissues (Friedrich et al. 2000), and general population peripheral blood mtDNA content is significantly associated with sex and age (Knez et al. 2016). One of the first population studies in healthy humans came from Kim et al. who showed a positive association between leucocyte TL and mtDNA CN in elderly women from Korea with an average age of 73 years, suggesting that telomere function might influence mitochondrial function in humans (Kim et al. 2013). Other researchers have confirmed this association in individuals aged 18–64 years from the USA (Tyrka et al. 2015) and individuals aged 60–80 years from Belgium, suggesting that telomeres and mitochondria are co-regulated in humans (Pieters et al. 2015). Additionally, Qiu and colleagues observed a positive correlation between TL and mtDNA CN in leukocytes of pregnant women in the USA after adjusting for age and plasma vitamin B12 (Qiu et al. 2015). A positive association was found in a Mexican population for children ages 6–12 (Alegría-Torres et al. 2016). Our own study shows that nonagenarians from Latvia lost a correlation between TL and mtDNA CN in comparison with younger age groups, which indicates that an abundance of mtDNA does not mean that nonagenarians and centenarians might have longer telomeres, and, instead, they may have different and more balanced protection against premature death (Zole et al. 2017). While the exact molecular mechanism underlying the telomeres-mtDNA associations is not clear and more studies are required, it seems that in nonagenarians and centenarians, telomere shortening might not influence mtDNA amount or vice versa as strongly as in individuals who do not reach such old age; these findings could be partly explained by different polymorphisms in the mtDNA sequences

(e.g., Niemi et al. 2005; Takasaki 2008; Guney et al. 2014), the diverse nuclear DNA SNPs and the healthier lifestyle noted in these individuals compared with individuals who do not live over 90 years of age (e.g., Yashin et al. 2000; Debrabant et al. 2014; Gierman et al. 2014; Govindaraju et al. 2015).

In summary, the results of population studies have shown that TL is gradually shortening, and mtDNA amount is gradually decreasing during the humans' ageing process. However, the studies in centenarians have pointed out the possible existence of protection mechanisms which may affect the "standard" cellular senescence processes. Both population and experimental studies have highlighted the existence of telomere-mitochondria interplay, as well as a dynamic nature and complexity of this interaction. TL shortening is a generally accepted factor for causing cell replicative senescence and ageing, which also may have an effect on the mitochondria dysfunction. However, the evidence suggest that this process is not strait forward, and many factors, including mitochondria-related processes, can affect the TL in cells in turn. Only few studies have shown mitochondrial hgs as one of genetical markers which influences ageing, TL or mtDNA amount; while the possible mechanism beyond is not clear, the results suggest that specific hgs are prone or protective towards some age-related diseases. Also, when looking into mtDNA heteroplasmy, there are no clear answer if it is a cause or a consequence of ageing, while mtDNA damage per se could be causative of ageing process. One more ageing-related factor which is addressed in population studies is mtDNA CN. Different studies have shown that mtDNA amount decreases with age, while others have reported that for the very old it is similar or even higher comparing with people in a younger age groups. Depending on which population we come from and what heredity we have, our background can influence our longevity and health not only by social, health care or environmental factors, but also by genetic hallmarks that are diverse in different populations. There are a lot of controversial and unclear data about the topic, but it is important to understand and clarify, for example, at which point in our lifetime we should focus on maintaining the right cellular function. In a young age, it could be TL which could be positively influenced by exercise and stress-reduction, but with age it could be more important to convey more energy to mitochondrial maintenance for healthier and longer live. Meanwhile, before clear, science-based recommendations and strategies are available, it is important to continue research in the exciting field of bio-gerontology to improve human healthspan and prolong lives.

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Compilance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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