Twin studies - investigations of the telomerase system and potential telomere-mitochondria interplay

Ph.D. Dissertation

Dóra Melicher

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor:
András Falus, Member of Hungarian Academy of Sciences

Official reviewers:
János Aradi, Ph.D.,
Gábor L. Petheő, M.D., Ph.D.

Head of the Final Examination Committee:
Zsuzsa Schaff, Member of Hungarian Academy of Sciences

Members of the Final Examination Committee:
András Hrabák, Ph.D.
József Prechl, M.D., Ph.D.

Budapest, 2018

Table of Contents

List of Abbreviations

*	$p < 0.05$
**	$p < 0.01$
****	$p < 0.001$
ALB	Albumin gene
ALT	Alternative Lengthening of Telomeres
ANOVA	Analysis of Variance
ApoA1	apolipoprotein A1
ApoB	apolipoprotein B
AS	absorbance of sample
AS,0	absorbance of heat- or RNase-treated sample
AS,IS	absorbance of Internal Standard (IS) of the sample
ATP	adenosine triphosphate
ATRX	α-thalassemia/mental retardation syndrome X-linked gene
ATS8	absorbance of Control template (TS8)
ATS8,0	absorbance of Lysis buffer
ATS8,IS	absorbance of Internal Standard (IS) of the Control template (TS8)
B*	unstandardized coefficient
BMI	body mass index
BPdias	diastolic blood pressure
BPsys	systolic blood pressure
CD11a	Integrin Subunit Alpha L (Antigen CD11A (P180)
CD70	Tumor Necrosis Factor Ligand Superfamily Member 7
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CI	95% confidence interval
CN	carbamide
CNV	copy number variation
Cr	creatinine
CRP	C-reactive protein
Ct	cycle threshold

CV	coefficient variation
DAXX	death domain-associated protein gene
DC	Dyskeratosis Congenita
DKC1	dyskerin
DM	diabetes mellitus
DMSO	dimethyl sulfoxide 10%
DNMT1	DNA (Cytosine-5-)-Methyltransferase 1
DNMT3a	DNA (Cytosine-5-)-Methyltransferase 3 Alpha
DZ	dizygotic
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
Geo mean	Geometric mean channel values of fluorescence (MFI)
HDL	high-density lipoprotein cholesterol
hip circ.	hip circumference
hTERT	human telomerase reverse transcriptase
hTERT	hTERT protein level in the PBMC gate
hTERTTc	hTERT protein level in cytotoxic T cells
hTERTTh	hTERT protein level in helper T cells
hTERTTly	hTERT protein level in T cells,
hTERTTreg	hTERT protein level in regulatory T cells
ICC	intraclass correlation
IFN-γ	Interferon Gamma
IS	Internal Standard
KIR2DL4	Killer Cell Immunoglobulin Like Receptor, Two Ig Domains And Long Cytoplasmic Tail 4
LDL	low-density lipoprotein cholesterol
LDL/HDL	high-density lipoprotein cholesterol- low-density lipoprotein cholesterol ratio
LipA	lipoprotein A
LTL	leukocyte telomere length
MM6	Monomac 6 human leukemia cell
MT-CYB	Cytochrome b gene

mtDNA	mitochondrial DNA
mtDNAcn	mitochondrial DNA copy number
MZ	monozygotic
n	number of subjects
NGS	Next-Generation Sequencing
NHP2	NHP2 Ribonucleoprotein
NKRs	Expression of NK cell Receptors
NOP10	NOP10 Ribonucleoprotein
NS	not significant
P	significance level
p53	cellular tumor antigen p53
PBMC	peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGC-1β	Peroxisome proliferator-activated receptor gamma coactivator 1-beta
PHA	phytohemagglutinin
PI	Propidium Iodide
POT1	protection of telomeres 1
Pr	pulse rate
qPCR	quantitative polymerase chain reaction
r^2	coefficient of determination in linear regression
RAP1 or TERF2IP	TRF2-interacting telomeric protein 1
ROS	Reactive Oxygen Species
SD	standard deviation
SE	standard error
SNF2	*helicase/ATPase and chromatin remodeler*
SNP	single nucleotide polymorphism
t	coefficient
T-loop	Telomere loop

TA	telomerase activity
TAMRA	tetramethylrhodamine
TC	total cholesterol
Tc	CD3+/CD8+ T-cells, cytotoxic T-cells
TC/HDL	total cholesterol-high-density lipoprotein ratio
TERC	telomerase RNA component
TERRA	telomeric repeat-containing RNA
TG	triglyceride
Th	CD3+/CD4+ T-cells, helper T-cells
TIN2 or TINF2	TRF1-interacting nuclear protein 2
TINF2	TERF1 Interacting Nuclear Factor 2
TL	telomere length
Tly	CD3+ T-cells
TPP1 or ACD	adrenocortical dysplasia protein homolog
TRAP	Telomeric Repeat Amplification Protocol
Treg	CD3+/CD4+/Foxp3+ T-cells, regulatory T-cells
TRF1 or TERF1	telomere repeat binding factor 1
TRF2 or TERF2	telomere repeat binding factor 2
waist circ.	waist circumference
β	standardized coefficient

1. Introduction

1.1. The value and relevance of twin studies

Twin studies have unique potential to address crucial questions in epigenetics such as to what extent epigenetic changes are heritable, how much variation can be in epigenetic heritability across the genome and to what extent epigenetic factors contribute to complex phenotypes (Bell and Spector, 2011; Melicher et al., 2015). Studies of twins and families can be used in an attempt to quantify the contribution of genetic variation to inter-individual differences, and large-scale twin studies frequently address the question of heritability (i.e. the proportion of phenotypic variation in a population that is attributable to genetic variation among individuals).

Twin studies, including the classical twin design and the discordant MZ twin design have received great interest in recent years as special methods for studying molecular biology (van Dongen et al., 2012). Monozygotic and dizygotic twins are uniquely valuable subjects for studies aiming to control over genetic background and early environmental influences (van Dongen et al., 2012). MZ twins are derived from a single zygote, after the fertilized egg splits, and are therefore matched for genetic background, they share (nearly) 100 percent of their segregating alleles (van Dongen et al., 2012). DZ twins arise after a double ovulation, they are derived from two distinct zygotes and share the same amount of genetic material as normal siblings, on average 50 percent (Bell and Saffery, 2012; van Dongen et al., 2012). Both MZ and DZ twins share prenatal and part of their postnatal environment (Boomsma, 2013).

Alterations in gene expression due to global epigenetic changes triggered by genetic, environmental and stochastic effects accumulate over time, and it was famously found that younger identical twins are epigenetically more similar than older ones, suggesting aging-related epigenetic modifications, induced by accumulated stimulation caused by internal and external influences including environmental exposure (Fraga et al., 2005; Tan et al., 2013).

1.1.1. Classical twin design

Classical twin studies have served as a powerful tool in biomedical, psychiatric and behavioural research for decades, the method compares the phenotypic similarity of monozygotic and dizygotic twins to estimate the importance of heritable and environmental influences on complex trait variation, using concordance rates or intra-class correlation (van Dongen et al., 2012).

The comparison between MZ and DZ twins allows an estimation of the heritability of traits, which describes the proportion of the total variance in a trait that is attributable to genetic factors (Bell and Saffery, 2012). Environmental influences comprise those that are shared by family members, termed as common environment, and also comprise influences that are unique to each individual, referred to as unique environment (van Dongen et al., 2012). Structural equation models estimate heritability by decomposing phenotypic variance of a parameter into genetic, common environmental and unique environmental effects (Neale and R. Cardon, 1992). The common environment involves all environmental factors that make a certain twin pair similar for a given trait, such as shared womb, childhood experiences and early socialization or parental socioeconomic status. The term, unique environment, includes all environmental factors and experiences to which only one member of the twin pair was exposed to, making co-twins dissimilar, such as certain viral infections, accidents, individual life events, etc. (van Dongen et al., 2012). Genetic variance can be further decomposed into additive genetic variance and variance due to non-additive genetic effects. Additive genetic factors (A) refer to the sum of the allelic effects on the phenotype over all susceptible loci. The percentage of variance in a trait that is explained by additive genetic factors (A) equals the narrow-sense heritability (h^2) of a trait, which can be estimated by taking twice the difference between the MZ and DZ twin correlations: $h^2 = 2(rMZ - rDZ)$. When rMZ > rDZ, there is evidence for a contribution of non- additive genetic influences, also referred to as genetic dominance (D), and the variance explained by A and D together is referred to as the broad-sense heritability (H^2) (Boomsma, 2013). Multivariate twin studies address the causes of association among phenotypes. Associations can be among different phenotypes or across age and are explained by common genetic or environmental influences (van Dongen et al., 2012).

1.1.2. Monozygotic twin design

A unique advantage of the monozygotic twin design is the ability to study biological discordance against an equivalent genetic background (van Dongen et al., 2012). The discordant twin model, by comparing the molecular profiles of phenotypically discordant monozygotic twin pairs, is a powerful method to identify molecular characteristics associated with complex traits, including point mutations and genomic structural variation, differentially expressed and differentially methylated genes as well as metabolic profiles (van Dongen et al., 2012). Several studies have reported phenotypic differences between individual members of monozygotic twin pairs (Zwijnenburg et al., 2010). It was established that non-genetic variation could contribute to disease penetrance and etiology (Bell and Saffery, 2012). Earlier assumptions considering MZ twins to be genetically totally identical had been disproven, as specific point mutations (Kondo et al., 2002; Robertson et al., 2006; Sakuntabhai et al., 1999), uniparental disomy (Smith et al., 2006), triplet repeat expansion (Helderman-van den Enden et al., 1999; Kruyer et al., 1994), chromosomal mosaicism (Bonilla et al., 1990), chromosomal aneuploidies (Zwijnenburg et al., 2010) and heteroplasmy for mitochondrial-encoded mutations (Biousse et al., 1997; Blakely et al., 2004) have all been linked to specific phenotypic differences in monozygotic twins (Bell and Saffery, 2012). Notably, differences in both telomere length (Bakaysa et al., 2007; Slagboom et al., 1994) and DNA copy number (Bruder et al., 2008) have also been reported in phenotypically discordant and concordant MZ twin pairs as well. It was suggested that these rare genetic differences, mosaicism, as well as non-genetic (epigenetic) variation within MZ twin pairs could underpin the majority of observed phenotypic discordance (Bell and Saffery, 2012). Monozygotic twins show phenotypic discordance for several traits, including a wide variety of complex diseases (Boomsma et al., 2002; Castillo-Fernandez et al., 2014). Identical twins can be relatively often discordant for such common complex diseases (Castillo-Fernandez et al., 2014), as type 1 diabetes mellitus (DM) (estimated to 61 percent) (Condon et al., 2008), type 2 DM (41 percent) (Lehtovirta et al., 2010), autism (58-60 percent) (Hallmayer et al., 2011), schizophrenia (58 percent) (Beckmann and Franzek, 2000), and different types of cancer (0-16 percent) (Castillo-Fernandez et al., 2014; Lichtenstein et al., 2000).

Epigenetics was proposed to be one of the main mediators of the interplay between genes and environment (Castillo-Fernandez et al., 2014; Jaenisch and Bird, 2003). Measurement of epigenetic differences in phenotypically discordant MZ pairs could provide a unique opportunity to identify genes sensitive to the environmental factors that are associated with complex diseases (Bell and Saffery, 2012; Tarnoki et al., 2014). In addition, monozygotic twins reared apart provide an exclusive opportunity for studying genetic effects in different environmental settings (Segal, 2012).

Limitations of epigenetic studies should be noted as well. While epigenetics might explain many discordances between twins, but in some cases dissimilarities could come from other sources (Castillo-Fernandez et al., 2014). Although MZ twins were formed from the same zygote, post-zygotic mutations might occur and induce somatic mosaicism (Li et al., 2014), potentially causing developmental disorders or increased susceptibility to a disease in later life (Castillo-Fernandez et al., 2014). Another potential confounding factor could be twin chorionicity, considered to be influential in epigenetic status (Kaminsky et al., 2009), however most studies treated MZ twins as a uniform group, sub-classification depending on whether they shared the same placenta or not (monochorionic or dichorionic, respectively) would be desirable (Castillo-Fernandez et al., 2014). Additionally, even MZ twins might differ in their common environmental exposure, which could confound some assumption of classical twin studies (Sahu and Prasuna, 2016).

1.1.3. Future perspectives

Nevertheless, epigenetic comparisons of MZ twins and heritability studies of DNA methylation and other modifications highlight that most epigenetic variability might be unique to the individual, which makes epigenetics potentially valuable for personalized medicine (Castillo-Fernandez et al., 2014). Future implications include the role of epigenetics in P4 medicine (personalized, predictive, preventive, and participative medicine), as a stable yet potentially reversible molecular mechanism (Castillo-Fernandez et al., 2014).

In the coming years, extensive longitudinal phenotypic information coupled with biological material collected by worldwide twin registries will be a highly valuable

resource for large-scale molecular studies (van Dongen et al., 2012). Next-generation sequencing across multiple tissues and cell types will extremely speed up the detection of genome-wide SNPs, CNVs and epigenetic variation in discordant twins, suggesting that twins will continue to provide valuable insights into human genetics (van Dongen et al., 2012).

1.2. The telomere/telomerase system

Telomeres are special heterochromatic structures located at the end of linear chromosomes. Telomeres play a key role in preserving genomic stability, as a cap, they protect the integrity of chromosomes and ensure complete replication (Blackburn, 2001; van Steensel et al., 1998). Mammalian telomeres are formed by tandem repeats of the TTAGGG hexanucleotide sequences, coated by shelterin, a specialized six-protein complex, which has fundamental role in the protection of chromosomes and in the regulation of telomerase activity (Blasco, 2007; de Lange, 2005). Telomeres can fold back on themselves, the 3' single-stranded telomere overhangs can invade the double-stranded telomeric regions which results in the formation of a T-loop (telomere loop), resembling the replication D-loop (Griffith et al., 1999). This structure is maintained by the shelterin complex, comprising of a set of telomere-binding proteins, which are the following: telomere repeat binding factor 1 (TRF1 or TERF1), telomere repeat binding factor 2 (TRF2 or TERF2), protection of telomeres 1 (POT1), adrenocortical dysplasia protein homolog (TPP1 or ACD), TRF1-interacting nuclear protein 2 (TIN2 or TINF2), and TRF2-interacting telomeric protein 1 (RAP1 or TERF2IP) (de Lange, 2005; Palm and de Lange, 2008). POT1 binds the single-stranded telomeric DNA directly, while TRF1 and TRF2 bind to the double-stranded telomeric region, also directly. Through their interactions with other shelterin proteins, POT1, TPP1, TIN2 and RAP1 interact indirectly with the double-stranded telomeric DNA (Cesare and Reddel, 2010). TRF1 and the TRF1-interacting proteins were proposed to act as negative regulators of telomere length possibly by controlling the access of telomerase to the telomeric region (de Lange, 2005). TRF2 and POT1 were suggested to have additional roles in telomere

protection by preventing end-to-end fusions of chromosomes (Blasco, 2007; Hockemeyer et al., 2006).

Telomerase is a ribonucleoprotein enzyme complex that maintains telomere length by telomere synthesis. The human telomerase complex consists of a catalytic reverse transcriptase protein subunit (hTERT), and a telomerase RNA component (hTERC or hTR), which contains the RNA template for the telomere sequences, dyskerin (DKC1) (Cohen et al., 2007) and additional proteins (Venteicher et al., 2009). The telomerase ribonucleoprotein complex exerts RNA dependent DNA polymerase activity and can add telomere sequence repeats *de novo* after each cell division, correcting the incomplete end-replication events, solving the so called "end replication problem" (Chan and Blackburn, 2002; Collins and Mitchell, 2002). Composition and structure of the human telomerase system are illustrated by ***Figure 1***.

Telomerase activity was amply detected in germ cells, stem cells and in some progenitor cell types, however, telomerase level was undetectable in most somatic cell sorts (Kim et al., 1994; Morrison et al., 1996; Wright et al., 1996) in which telomeres shorten with each cell division until they finally reach a critically short length which leads to replicative senescence, crisis and ultimately apoptosis (Blackburn et al., 2006). Thereby telomere length represents a balance between the loss of terminal telomeric repeats, occurring during cell division with incomplete DNA replication, and the addition of telomeric repeats by telomerase (Hathcock et al., 2005). Both the hTERT and TERC components are indispensable and sufficient for the reconstitution of telomerase catalytic activity in cell free conditions, however, for the proper maintenance of telomere length *in vivo*, a holoenzyme, comprising diskeryn and additional associated proteins are also necessary (Cohen et al., 2007; Saretzki, 2014; Venteicher et al., 2009). TERC, dyskerin and telomere-associated proteins were both found abundant and constitutively expressed in cells regardless of telomerase enzymatic activity (Blasco et al., 1996; Feng et al., 1995; Greider, 1998). It was established that the level of hTERT protein was the rate-limiting factor for telomerase activity and telomere length homeostasis, it was suggested that hTERT serves as the major limiting agent and considered a key determinant for the control of telomerase activity *in vivo* (Cairney and Keith, 2008; Cong and Shay, 2008; Hara et al., 2015; Qian et al., 2014; Ramlee et al., 2016; Zhou et al., 2014). The expression of *hTERT* strongly depends on cell type and

tissue environment, transcription factors regulating *hTERT* expression were found partly tissue specific (Daniel et al., 2012). Although different splice products of the *hTERT* gene were described, the exact functions for most of them have not been fully elucidated yet (Saretzki, 2014).

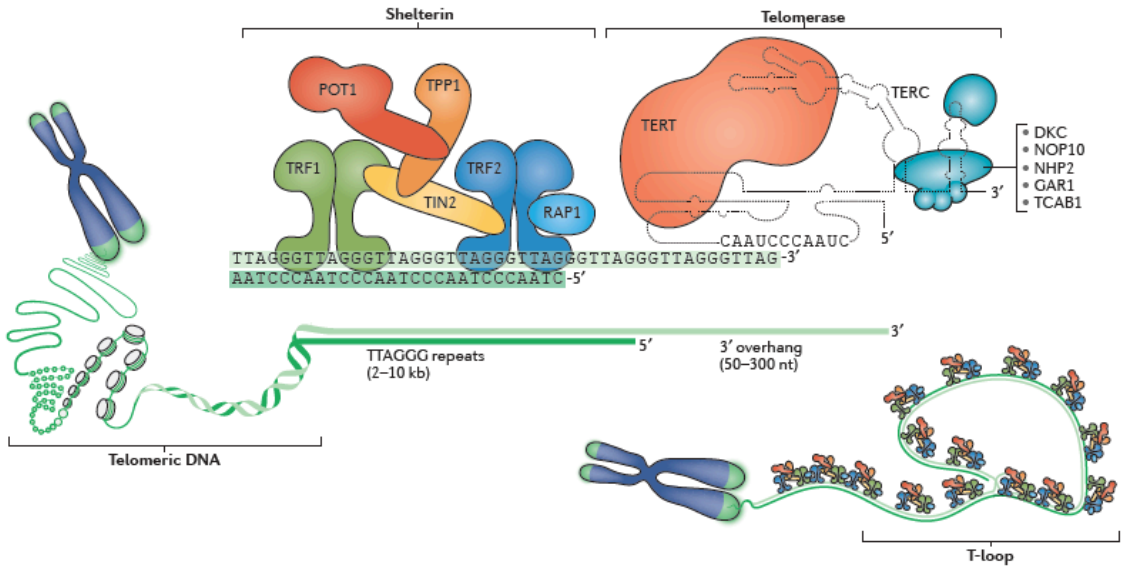

Figure 1. *Composition and structure of the human telomere system (Maciejowski and de Lange, 2017)*

Human telomeres comprise three components: telomeric DNA, the shelterin complex and the telomerase complex. Telomeric DNA consists of a long array of double-stranded TTAGGG repeats that culminates in a 50–300 nucleotide (nt) single-stranded 3′ overhang. This 3′ overhang invades double-stranded telomeric repeats to form a t-loop structure that is crucial for telomere function. Telomeric DNA protects chromosome ends through its association with the six-subunit shelterin complex. The length of telomeric repeats can be maintained by telomerase, which is composed of telomerase reverse transcriptase (TERT), telomerase RNA template component (TERC) and several accessory proteins (blue). TERT synthesizes telomeric DNA de novo using TERC as a template, whereas the accessory factors contribute to the biogenesis and nuclear trafficking of telomerase. DKC, dyskerin; NHP2, non-histone protein 2; NOP10, nucleolar protein 10; POT1, protection of telomeres 1; RAP1, repressor/activator protein 1; TCAB1, telomerase Cajal body protein 1; TIN2, TRF1-interacting nuclear factor 2; TRF, telomeric repeat-binding factor (Maciejowski and de Lange, 2017).

1.2.1. Telomeres and telomerase in human pathophysiology

Besides their role in the physiological cellular ageing process, telomerase activity and telomere length stability have critical part in the pathobiology of several human diseases. Telomere dysfunction and defects in telomere length maintenance contribute to a number of disease manifestations (Blasco, 2005).

Following mutations in telomerase subunits, different genetic disorders were shown to develop. Most phenotypic consequences of human telomerase deficiency were suggested to result from compromised telomere function that limits the proliferation of cells, including stem cells (Lansdorp, 2009). The first disease that was revealed to associate with telomerase deficiency was Dyskeratosis Congenita (DC). The name derives from the dyskerin gene which encodes a small nucleolar protein that binds to TERC and ribosomal RNA, affecting several cell functions, thus DC patients carry mutations in components of the telomerase complex, resulting in decreased telomerase stability and critically short telomeres (Calado and Young, 2009; Mitchell et al., 1999). In approximately 50 percent of DC patients, mutation in either *hTERT*, *TERC* or *DKC1* genes was found (Aubert and Lansdorp, 2008) while 50 percent showed mutation in other, with telomere maintenance associated genes, like *TINF2*, *NHP2* and *NOP10* (Lansdorp, 2009; Savage et al., 2008; Vulliamy et al., 2008; Walne et al., 2007).

hTERT and *hTERC* gene mutations were also detected in idiopathic pulmonary fibrosis (Armanios et al., 2007), liver disease (Calado et al., 2009; Carulli and Anzivino, 2014; Qazilbash et al., 1997), aplastic anaemia (Ly et al., 2005; Marrone et al., 2004; Vulliamy et al., 2005; Yamaguchi et al., 2003) and myelodysplastic syndrome (Yamaguchi et al., 2003). Association between telomere length and cardiovascular diseases were proposed, although with varying results across studies (Epel et al., 2009; Farzaneh-Far et al., 2008; Fitzpatrick et al., 2007; Satoh et al., 2009; Wang et al., 2011), and relations between the dysregulation of telomere length and diabetes were also reported (Al-Attas et al., 2010; Salpea and Humphries, 2010; Salpea et al., 2010).

Several ageing associated diseases including cancer and premature ageing syndromes, were likewise characterized by short telomeres, undermining cell viability (Blasco, 2005). It was shown by *in vitro* studies that progressive shortening of telomeres

during cellular replication can not only lead to loss of chromosomal stability, senescence and apoptosis, but also to changes in gene expression within the subtelomeric region. This indicated that that alterations affecting telomeres at the level of chromatin structure might also have a role in human diseases (Blasco, 2005). Telomere attrition was also associated with the risk of cancer by several reports, ranging from high rates of specific cancers to modest contributions to oncogenesis in general, while in some specific inflammatory and immune diseases, telomere shortening was suggested to be a major contributing factor to the promotion of tumour development (Calado and Young, 2009; Londono-Vallejo, 2008; Shay and Wright, 2011). Although the traditional view regards leukocyte telomere length as a passive biomarker of human ageing, interesting new evidence suggested a "trade-off" concept for specific health outcomes (Aviv and Shay, 2018), according to which an active role of telomere length in ageing-related diseases might occur, due to telomeres increasing the risk of certain type of pathologies related to restricted cell proliferation and tissue degeneration, such as cardiovascular diseases, whereas long telomeres might increase the risk of other types of diseases related to increased proliferative growth, such as major cancers (Aviv and Shay, 2018).

Besides, upregulated hTERT expression was observed in the majority of human cancer cells (>85%), and tumour cell lines were found to express telomerase constitutively (Armanios and Greider, 2005; Cao et al., 2008; Holt and Shay, 1999; Ju and Rudolph, 2006; Kyo and Inoue, 2002; Meyerson, 2000; Shin et al., 2006). It was suggested that the aberrant upregulation of telomerase was required for the sustained growth of the majority of advanced cancers (Shay and Wright, 2011).

1.2.2. Alternative Lengthening of Telomeres

A telomerase-independent manner of telomere length maintenance, termed as alternative lengthening of telomeres (ALT) was also uncovered (Bryan et al., 1997). The mechanism was unveiled by the observation that telomere length could be maintained in immortalised telomerase-negative human cells (Bryan et al., 1995), and it was shown that human cells lacking telomerase, could undergo rapid shortening and lengthening events (Cesare and Reddel, 2010; Murnane et al., 1994). ALT relies on homologous recombination dependent exchange and/or homologous recombination dependent synthesis of telomeric DNA, thus making the addition of telomere sequence repeats possible, without the involvement of telomerase (Dunham et al., 2000; O'Sullivan and Almouzni, 2014). Linear or circular extra-chromosomal telomeric fragments, observed in ALT cells could also serve as alternative templates for telomere synthesis by rolling circle amplification (Natarajan and McEachern, 2002).

Recent efforts of basic and translational research concentrate on revealing the molecular biology of how the ALT pathway is activated especially in cancer cells, and how it could potentially be targeted (O'Sullivan and Almouzni, 2014), however there are still many unknowns about how exactly telomere elongation occurs in ALT cells (Cesare and Reddel, 2010). Telomeric repeat-containing RNAs (TERRA) were also rendered role in telomere length homeostasis in ALT tumour cells, as the formation of RNA:DNA hybrid structures, known as telomeric R-loops were observed (Arora et al., 2014; Balk et al., 2013; Cusanelli and Chartrand, 2015).

Recent research has highlighted the importance of studying the epigenetic regulation of telomeres in the activation and regulation of ALT cells (O'Sullivan and Almouzni, 2014). Large-scale genome sequencing methods identified ALT associated mutations in the α-thalassemia/mental retardation syndrome X-linked gene (*ATRX*), which is a SNF2 helicase/ATPase and chromatin remodeler (Gibbons et al., 2000), and in death domain-associated protein gene (*DAXX*), a histone chaperone (Drane et al., 2010; Lewis et al., 2010). In light of the findings that telomere function was sensitive to changes in chromatin structure and post-translational histone modifications (Benetti et al., 2007; Schoeftner and Blasco, 2009), the comprehensive characterization of the ALT

telomeric chromatin structure and its relationship to chromatin dysfunction emerged as a new focus of ongoing research (O'Sullivan and Almouzni, 2014).

Interesting new findings pointed to the association between ALT and mitochondrial adaptive mechanism, as ALT positive tumour cells in mice were shown to express mitochondrial regulators, due to prevailing mitochondrial dysfunction (Hu et al., 2012). Investigations of the relationship between mitochondrial dynamics and ALT mechanisms have been underway (Hu et al., 2012; Melicher et al., 2015).

1.2.3. Telomere-independent activities of hTERT

Until recently, the vast majority of research on human TERT focused on the crucial function of the enzyme in adding telomeric (TTAGGG sequence) repeats, thus protecting against telomere shortening with cell replication. In the past few years, new discoveries shed light on many additional functions of hTERT that are independent of its reverse transcriptase activity and telomere maintenance, referred to as non-telomeric/non-canonical/extra-telomeric or extracurricular functions of telomerase. The various telomere-independent functions of hTERT include regulating gene expression, growth factors and cell proliferation, enhancing DNA repair, maintaining stem cells, besides anti-apoptotic function of nuclear as well as of mitochondrial hTERT were also observed (Bagheri et al., 2006; Cong and Shay, 2008; Hoffmeyer et al., 2012; Li et al., 2005; Maida et al., 2009; Smith et al., 2003).

It was reported that hTERT can shuttle dynamically between different cellular locations, as under increased oxidative stress hTERT was excluded from the nucleus and translocated to the mitochondria (Ahmed et al., 2008; Haendeler et al., 2009; Haendeler et al., 2003; Santos et al., 2004; Santos et al., 2006). A protective role of telomerase within mitochondria was proposed (Ahmed et al., 2008; Haendeler et al., 2009; Indran et al., 2011; Saretzki, 2009), while it has also been reported that the inability of TERT shuttling leaded to increased cellular stress (Sharma et al., 2012). It was suggested that by shuttling into the organelles, hTERT not only protected mitochondria, but by decreasing mitochondrial superoxide production, the protein also indirectly protected the nucleus from DNA damage (Singhapol et al., 2013). Telomere dysfunction was associated with impaired mitochondrial biogenesis, function and increased level of reactive oxygen species as well (Sahin et al., 2011). Besides, regardless of the presence of its RNA component hTERC, hTERT was shown to function as an RNA-dependent RNA-polimerase (Maida et al., 2009). Additionally, TERT was found to bind to mitochondrial DNA, increase mitochondrial membrane potential and interact with mitochondrial tRNAs (Saretzki, 2014).

1.2.4. The emerging role of epigenetics

Epigenetic regulation of hTERT

Recently new research findings were published about the diverse factors playing role in the regulation of the *hTERT* gene. Telomerase is known to be regulated at various molecular levels, including genetic, mRNA, protein levels and subcellular localization (Ramlee et al., 2016). Among them transcription modulation is regarded to be the most important one (Ramlee et al., 2016). Epigenetic modifications of *hTERT* have also recently gained new attention. Trans-acting factors, like transcription factors and epigenetic modifiers were shown to collectively contribute to the transcriptional regulation of *hTERT* gene (Ramlee et al., 2016), DNA methylation, histone methylation, and histone acetylation were considered as basic epigenetic regulations, besides non-coding RNAs were also suggested to be involved in the epigenetic control (Avin et al., 2016; Lewis and Tollefsbol, 2016). Regulation of *hTERT* can also occur through unique pattern of CpG promoter methylation and alternative splicing (Avin et al., 2016). In addition to expression level changes, changes in promoter binding was also reported to affect alternative splicing (Avin et al., 2016). Alternative splicing of *hTERT* could produce either the full length transcript that could form the active telomerase enzyme complex with TERC, or numerous inactive isoforms (Avin et al., 2016). Both regulation strategies could be exploited in cancers to activate telomerase, however, the exact mechanisms are unknown (Avin et al., 2016). Despite the wealth of knowledge in the roles that transcription factors and epigenetic modifiers play in the regulation of *hTERT* expression, it was also pointed out that it was still unclear how *hTERT* was silenced during stem cell differentiation as well as reactivated during somatic cell reprogramming and cellular transformation, respectively (Ramlee et al., 2016). It was emphasized that these processes were likely to involve a host of these transcription regulators in a cell context-dependent manner, in addition, they were likely to be controlled by epigenetic changes accompanying these cellular events (Ramlee et al., 2016). It was proposed that given the complexity of the regulatory network, it could perhaps be more meaningful to approach the issue from a wider perspective, and it was alongside suggested that the epigenetic-based regulation of *hTERT* might serve as a crucial contributing mechanism to the reversibility of *hTERT* control in different

biological states, such as aging or the pathology of cancer (Avin et al., 2016; Lewis and Tollefsbol, 2016).

Epigenetics of telomeres and subtelomeres

Key links between telomere length regulation and epigenetic status were proposed (Blasco, 2007) as emerging data indicated the existence of functional connections between telomere length homeostasis and epigenetic marks (Garcia-Cao et al., 2002; Garcia-Cao et al., 2004; Gonzalo et al., 2006). The chromatin region of telomeres in mammals was characterized with several properties resembling the heterochromatin at pericentromeric regions, including the exertion of telomere position effect, which is the ability to transcriptionally silence nearby genes (Baur et al., 2001; Koering et al., 2002). Telomeric chromatin contains nucleosomes with slightly altered spacing compared with non-telomeric chromatin (Makarov et al., 1993; Tommerup et al., 1994). Telomeric and subtelomeric chromatin were shown to be enriched in epigenetic marks, such as histone modifications of telomeres (Garcia-Cao et al., 2004; Gonzalo et al., 2005; Gonzalo et al., 2006), and DNA methylation at subtelomeres (Bellon et al., 2010) were found. Telomere length deregulation and disruption of telomeric silencing were reported in states, characterised by the loss of heterochromatic marks at telomeres and subtelomeres, while loss of either histone methylation or DNA methylation leaded to de-repression of telomere recombination (Blasco, 2007). Changes in the epigenetic status of telomeric and subtelomeric chromatin were observed in critically shortened telomeres, decreased histone and DNA methylation and increased histone acetylation were also reported (Blasco, 2007). It was alongside suggested that the changed epigenetic marks could have led to the observed preferential elongation of short telomeres by telomerase (Hemann et al., 2001; Samper et al., 2001). Epigenetic marks, such as increased H3 and H4 acetylation in the states of accelerated telomere loss (Benetti et al., 2007) could be recognized preferentially by the telomerase complex (Blasco, 2007).

Telomeric repeat-containing RNAs (TERRA)

Despite the heterochromatic state, mammalian telomeres can be transcribed into long non-coding RNAs, called TERRA (telomeric repeat-containing RNA) (Azzalin et al., 2007; Schoeftner and Blasco, 2008). TERRA was shown to be transcribed from CpG dinucleotide containing promoters, methylated by DNMT1 and DNMT3b, which promoters were found to be located at least on half of human subtelomeres (Arora et al., 2014; Nergadze et al., 2009). TERRA bounded to human telomerase in cells *in vitro*, and it was suggested that TERRA transcripts could play a role in the regulation of telomerase activity (Redon et al., 2010; Schoeftner and Blasco, 2008). Recent research pointed out that TERRA molecules could play critical roles in telomere biology, including the regulation of telomerase activity and heterochromatin formation at chromosome ends, although there are still a lot of unknowns to be revealed in order to understand the role of TERRA regulation *in vivo* (Cusanelli and Chartrand, 2015).

1.3. Telomere-telomerase functioning in the human immune system

The human immune system is an ample example of a highly dynamic cellular network with unique characteristics, one of which is the regulation of the telomere/telomerase system (Melicher et al., 2015). Telomerase is repressed in most human somatic cells, but lymphocytes are unique in the sense that they retain the ability to upregulate telomerase upon activation, which allows the intense clonal expansion and proliferation required to generate effector cells and long lived memory cells while maintaining telomere length (Chou and Effros, 2013; Maini et al., 1999). High levels of telomerase activity were measured in germinal center B-cells and thymocytes and telomerase activity was found to be highly regulated during T-cell development and B-cell differentiation (Weng et al., 1997). In the capacity for expressing detectable amount of telomerase, lymphocytes resembled the characteristics of stem cells (Weng, 2008).

1.3.1. Activation of telomerase in lymphocytes

For telomerase activity to be induced in human peripheral T-cells, signalling via the T-cell receptor (TCR) and other co-stimulatory molecules such as CD28 were required (Antonio Moro-Garcia et al., 2012). *In vitro* activated T-cells could increase both the levels of *hTERT* transcripts and telomerase activity. Several *in vitro* studies found that after repeated stimulation, T lymphocytes progressively lost the ability to induce telomerase activity, and it was proposed that this caused telomere erosion and eventually replicative senescence (Hooijberg et al., 2000; Plunkett et al., 2005; Valenzuela and Effros, 2002; Weng et al., 1996).

In human lymphocytes, besides the quantitative level of *hTERT* mRNA, which is considered the major limiting factor for telomerase activity (Daniel et al., 2012), additional transcriptional regulations were also proposed in determining the activity of the enzyme (Liu et al., 2001). hTERT protein and *hTERT* mRNA were reported to be present in all subsets of lymphocytes (thymocytes, tonsillar- and peripheral blood T- and B-cells) despite the substantial differences in telomerase activity of these cells (Deville et al., 2009; Liu et al., 2001; Liu et al., 1999). It was also found that activation of telomerase in peripheral blood resting CD4+ T-cells after *in vitro* stimulation did not require an increase in hTERT protein level (Liu et al., 2001). Two posttranslationally regulated events, phosphorylation and nuclear translocation of hTERT, were identified and proposed to be associated with telomerase activation in CD4+ T lymphocytes, which were reported to be independent of total levels of the hTERT protein (Liu et al., 2001). The details of the *in vivo* regulation of telomerase activity in lymphocytes still remains to be established.

1.3.2. Telomere length in lymphocytes

Lineage-specific telomere shortening was observed in CD4+, CD8+ T lymphocytes, B lymphocytes, granulocytes, monocytes and NK-cell populations with different kinetics (Kaszubowska, 2008). Investigating telomere length and telomerase activity in four different lymphocyte subgroups from the same individual, B-cells were found to have the highest telomerase activity followed by CD4+ T helper- and CD8+CD28+ T-cells, while the replicatively senescent CD8+CD28- T-cells showed the

lowest telomerase activity (Lin et al., 2010). Among the same cell types, the longest telomere length was measured in B-cells, followed by CD4+ and CD8+CD28+ T-cells with approximately equal measures, then by CD8+28- T-cells with the shortest observed telomere length (Lin et al., 2010). It was also found that a higher percentage of CD8+CD28- T-cells correlated with shorter total telomere length in peripheral blood mononuclear cells (PBMCs) (Lin et al., 2010). Further studies confirmed that among T lymphocytes the relatively undifferentiated T-cells (CD27+CD28+) showed the longest telomeres, followed by the intermediate populations (CD27-CD28+ in CD4+ T-cells and CD27+CD28- in CD8+ T-cells) with telomere lengths between those of the undifferentiated and highly differentiated lymphocytes, and highly differentiated T-cells (CD27-CD28-) presented the shortest telomere length (Antonio Moro-Garcia et al., 2012). The ratio of proliferation in human T-cells was found to be much higher in highly differentiated cells (CCR7-CD27-CD28-) with shorter telomeres (Antonio Moro-Garcia et al., 2012; Macallan et al., 2004).

1.3.3. In vivo and ex vivo experiments

Few studies investigated the telomere/telomerase system in human lymphocytes in the frame of *in vivo* or *ex vivo* experiments. Telomerase activation, associated with telomere length maintenance was reported in Epstein-Barr virus-specific CD8+ T lymphocytes during acute infectious mononucleosis (Hathcock et al., 2003; Maini et al., 1999; Plunkett et al., 2001). Despite the high telomerase activity and facilitated telomere preservation in EBV-specific CD8+ T-cells, significant telomere shortening was observed in EBV-specific CD8+ T lymphocytes isolated from the same donor 15 month and 14 years later (Plunkett et al., 2001). Shortening of telomeres in memory T-cells with age and during chronic HIV infection was also reported (Effros and Pawelec, 1997; Son et al., 2000; Valenzuela and Effros, 2002). These findings indicated that telomerase induction was not sufficient to maintain telomeres in repeatedly activated memory T-cells *in vivo* (Akbar and Vukmanovic-Stejic, 2007).

Further evidence that hTERT could play a crucial role in the proliferative capacity of human T-cells comes from the study of Wolf et al, investigating telomere length and telomerase activity in regulatory T-cells (Treg) isolated from peripheral

blood of cancer patients (Wolf et al., 2006). CD4+CD25+ Treg were found to have retained telomere length, suggesting that the induction of telomerase *in vivo* helped in preventing replicative senescence. However, in the *in vitro* stimulated regulatory T-cells, although telomerase activity was readily inducible, but was insufficient to prevent further telomere shortening and replicative senescence. The authors noted that they had no data available on the regulation of *hTERT* expression and telomerase function in primary human Treg and concluded that their findings indicated a central role of telomerase induction for regulatory T-cell expansion, albeit under conditions of extensive *in vitro* stimulation, induced telomerase activity was obviously not sufficient to maintain telomere length in the proliferating cell fraction (Wolf et al., 2006).

It should be noted that several potential confounding factors were addressed regarding the *in vivo* or *ex vivo* experiments. Valenzuela and Effros pointed out that *in vivo* experiments investigating the telomere-telomerase system were a huge challenge to interpret on sequentially derived peripheral blood samples from human donors because of several potential confounding issues (Valenzuela and Effros, 2002). Among others various localization of effector memory cells (Masopust et al., 2001), undetermined number of naive T-cells freshly leaving the thymus were mentioned, which could not ensure that the same populations of immune cells were actually evaluated over a longitudinal study (Valenzuela and Effros, 2002). It was also proposed that the reason for the earlier, seemingly contradictory results according to which the capacity for activation-induced telomerase activity in polyclonal T- and B-cells did not decrease with donor age (Son et al., 2000), might be that donor age may not reveal the differences in replication of the entire CD4+ or CD8+ T lymphocyte population (Roth et al., 2003). It was suggested that variations in telomerase expression might be more evident between T-cell populations within the same donor (Roth et al., 2003).

1.3.4. Immunosenescence and epigenetics

Experiments showed that the regulation of telomerase activation in T lymphocytes was changed during progressive differentiation, the ability to induce telomerase was getting lost as cells aged, at the same time it was also suggested that while endogenous hTERT alone could not prevent overall telomere shortening, it had a major influence on the longevity of human T-cells (Roth et al., 2003). It was proposed that the loss of telomerase inducibility in T lymphocytes after repeated stimulation might be an intrinsic protective mechanism against excessive proliferation, possible mutations or transformations (Valenzuela and Effros, 2002). The question was also addressed about whether the decreased enzymatic activity (telomerase down-regulation) in highly differentiated $CD8^{+}CD28^{-}CD27^{-}$ T-cells was more likely related to decreased hTERT synthesis or to posttranslational regulation of the enzyme (Akbar and Vukmanovic-Stejic, 2007). The underlying mechanisms that inhibit telomerase activity during T-cell differentiation remain to be revealed (Akbar and Vukmanovic-Stejic, 2007).

Shortened telomeres and reduced telomerase activity were identified as key features of T-cell senescence *in vitro* and has been associated with many pathological conditions *in vivo* (Chou and Effros, 2013). Increased telomere shortening could contribute to defective immune responses in elder people, accelerated T-cell aging combined with telomere attrition may predispose for autoimmune responses and could thereby explain the increased susceptibility for chronic inflammatory diseases in the elderly (Kaszubowska, 2008). Repetitively stimulated T-cells were characterised with shortened telomeres and decreased capacity of telomerase induction, which could contribute to T-cells entering the path of replicative senescence (Bellon et al., 2010; Scheuring et al., 2002). This process could be followed by the accumulation of highly differentiated T-cells with newly acquired functional capacities, resulting from aberrant expression of genes, normally suppressed by epigenetic mechanisms in CD4+ or CD8+ T-cells (Antonio Moro-Garcia et al., 2012). Genes (such as CD11a, perforin, CD70, IFN-γ and the NKRs family), normally suppressed by DNA methylation, were overexpressed in senescent T-cells (Y. Liu et al., 2009; Lu et al., 2003). Demethylation of the CD70, perforin, and KIR2DL4 promoters and a decrease in DNMT1 and DNMT3a levels in CD4+CD28- T-cells were also observed (Chen et al., 2010). It was

suggested that due to a lifetime of exposure to and proliferation against a variety of antigens, highly differentiated T-cells suffer molecular modifications which alter their cellular homeostasis mechanisms (Antonio Moro-Garcia et al., 2012).

1.3.5. hTERT as a potential therapeutic tool

Forced expression of hTERT was shown to immortalize cells, and several studies reported that hTERT alone was sufficient to restore telomerase activity resulting in tumorigenesis *in vitro* (Bodnar et al., 1996; Burns et al., 2000; Halvorsen et al., 1999; Liu et al., 1999; Mitchell et al., 1999; Pan et al., 1997; Rufer et al., 1998; Son et al., 2000; Vaziri and Benchimol, 1998; Vaziri et al., 1993). Subsequently, inhibiting cancer cell proliferation by targeting telomerase has become one of the focus points of anti-cancer therapeutics research for years. It is important to highlight that although telomerase down-regulation in lymphocytes might protect against tumorigenesis, yet it could also lead to replicative senescence of repeatedly activated memory T-cells.

The idea of telomerase-based therapeutics emerged as potential tool for extending the replicative lifespan of normal cells and for limiting the growth of malignant tumour cells. The perspectives of influencing the replicative senescence of T cells, inspired *in vitro* investigations studying the effect of reintroducing hTERT to lymphocytes. Several groups transduced human T-cells with hTERT and found that hTERT overexpression protected human T lymphocytes from replicative senescence in long-term cultures *in vitro* (Barsov, 2011). Transduced T-cells that constitutively expressed hTERT were found to maintain characteristics of primary lymphocytes, such as specific recognition and response to cognate antigens and maintenance of natural effector functions (Barsov, 2011; Hooijberg et al., 2000; Migliaccio et al., 2000; Rufer et al., 2001). Despite the considerably increased survival, most studies found that hTERT-transduced cells eventually lost the ability to expand after activation, due to cumulative oxidative damage or loss of genomic stability (Akbar and Vukmanovic-Stejic, 2007; Hooijberg et al., 2000; Röth et al., 2005; von Zglinicki, 2000). The most advanced use of hTERT-immortalized T lymphocytes could be their application for adoptive immunotherapy. However, adoptively transferred immortalized T-cells may potentially behave differently *in vivo* than in long-term *in vitro* cultures, in light of that,

further studies are indispensable to establish long-term survival and fate of hTERT-immortalized T-cells *in vivo* (Barsov, 2011).

It was proposed that one of the reasons why the efforts to develop novel anticancer therapies using hTERT as a molecular target could not entirely succeed yet, might be the overlooking of the non-canonical functions of hTERT, and it was suggested that targeting the novel functions of hTERT would facilitate the development of new cancer therapies (Maida and Masutomi, 2015). Further experiments investigating telomerase activity and hTERT protein combined with research on non-canonical functions of the enzyme, specifically in different lymphocytes subpopulations, could help yield novel insight into the immune regulation in health and disease.

1.4. mtDNA copy number and telomere length

1.4.1. LTL and mtDNAcn as potential markers of cellular aging

In the last decades, telomere length and mitochondrial DNA copy number have both been suggested as important markers of cellular aging (Hjelmborg et al., 2015; Mengel-From et al., 2014).

Mitochondria are regarded as the power generators of the cell as they produce most of the ATP, fundamental for cellular functions, and have an integral role in both cell growth, differentiation, replication and death. Furthermore, reactive oxygen species (ROS) are produced in mitochondria as a result of energy metabolism. The aging process is characterized by increases of mitochondrial ROS production, accumulating mutations in mtDNA, dysfunction of mitochondrial biogenesis and an overall decline in mitochondrial function (Bratic and Larsson, 2013; Kazachkova et al., 2013). Mitochondrial DNA copy number (mtDNAcn), that is the number of circular mitochondrial DNA per cell, provides an indirect measure for assessing mitochondrial biogenesis, which process could compensate for the increasing energy demand and compromised mitochondrial function (Sahin and DePinho, 2012).

During each somatic cell division, telomeric DNA was estimated to shorten at a rate of 22 – 46 basepairs per year, telomere attrition was reported to be accelerated by environmental stressors, oxidative stress, proinflammatory mediators and by autonomic and endocrine dysfunctions as well (Perseguini et al., 2015; Revesz et al., 2014).

Large-scale population studies investigated leukocyte telomere length or mtDNA copy number separately and associated them with ageing and various disease manifestations and suggested them as candidate biomarkers.

Focusing on telomere length, epidemiological studies have principally relied on measurements of telomere length in leukocytes, since earlier it was found that leukocyte telomere length (LTL) reflected telomere length in other somatic cells as well, and could serve as a surrogate parameter for the relative TL in other tissues (Aviv and Shay, 2018; Friedrich et al., 2000). Leukocyte telomere length has been associated with chronological age (Valdes et al., 2005) as well as with aging-related diseases, such as cardiovascular diseases (Brouilette et al., 2003; Fitzpatrick et al., 2007), neurological

diseases (Thomas et al., 2008) and cancer (Artandi and DePinho, 2010; Shay and Wright, 2011). Several studies found an association between telomere length and life span in humans (Bakaysa et al., 2007; Berglund et al., 2016; Deelen et al., 2014; Gomes et al., 2011; Heidinger et al., 2012; Kimura et al., 2008; Muezzinler et al., 2013; Sahin and Depinho, 2010; Steenstrup et al., 2017), a meta-analysis proposed that LTL could serve as a biomarker of somatic redundancy, the capacity of the body to absorb damage (Boonekamp et al., 2013). As LTL was proposed to predict mortality (Cawthon et al., 2003; Kimura et al., 2008) and longevity (Vera et al., 2012), it was suggested as a potential biomarker of biological aging (Mather et al., 2011; Zierer et al., 2016). Besides its inverse association with age, leukocyte telomere length was reported to be influenced by genetic (Codd et al., 2013), as well as environmental factors (Valdes et al., 2005), and telomeres in men were found shorter than in women (Benetos et al., 2001; Berglund et al., 2016; Möller et al., 2009; Okuda et al., 2002).

Regarding mitochondrial DNA copy number addressed by population studies, Ashar et al investigated the association of mtDNAcn with frailty and all-cause mortality (Ashar et al., 2015). Utilizing data from a total of 16.401 participants from two multi-center, multi-ethnic, community-based, prospective studies, a significant inverse association of mtDNA copy number with age, and higher mtDNAcn in women relative to men were found (Ashar et al., 2015). Ashar et al observed a significant association between lower mtDNAcn and prevalent frailty, and reported that mtDNAcn was a strong independent predictor of all-cause mortality in an age and sex-adjusted, race-stratified analysis (Ashar et al., 2015). Ding et al carried out a large-scale population analysis of mtDNA dynamics in lymphocytes of ~2.000 Sardinians, applying tailored sequencing analysis tools (Ding et al., 2015). Their data on mtDNAcn showed correlations with gender, age, and metabolic traits. According to their findings mtDNAcn averaged ~110 copies/lymphocyte and females on average had slightly but significantly higher mtDNAcn than males (6.7 copies) (Ding et al., 2015). Although several studies concluded that mtDNAcn decline seemed to be a valid marker of cellular aging, it has to be noted that inconsistencies in the literature can also be found (Revesz et al., 2018; Zole and Ranka, 2018). Picard et al suggested that a higher mtDNAcn might be a marker of poor mitochondrial condition or mitochondrial allostatic load (Picard et al., 2014), and mtDNAcn increase was proposed to compensate for DNA

damage or mitochondrial dysfunction by Yu-Wai-Man et al (Revesz et al., 2018; Yu-Wai-Man et al., 2011).

1.4.2. Links between telomere shortening and mitochondrial dysfunction

Recently key molecular links have been revealed between telomere shortening and mitochondrial dysfunction, two mechanisms that are both considered to play central role in the aging process (Sahin et al., 2011; Sahin and DePinho, 2012).

Telomere shortening was reported to be associated with impaired mitochondrial biogenesis and function, as well as increased ROS level, and specific molecular mechanisms were suggested about how these processes altogether could promote decline in stem cells, progenitor cells and post-mitotic tissues (Kim et al., 2013; Sahin et al., 2011). Sahin et al demonstrated that telomere dysfunction-induced p53 repressed PGC-1α and PGC-1β and thereby linked telomeres to mitochondrial biology, oxidative defense, and metabolism (Sahin et al., 2011). It was proposed that telomere-p53-PGC axis could expand our understanding about how telomere dysfunction might contribute to organ and metabolic failure and diminished organismal fitness (Sahin et al., 2011). It was alongside reported that increased mitochondrial dysfunction could result in increased ROS concentrations and subsequent activation of the DNA damage response pathway, including telomere damage and erosion and the consequent enhanced p53 activation (J. Liu et al., 2009; Zhu et al., 2017). It was also shown that improvement of mitochondrial function resulted in less telomeric damage and slower telomere shortening, while telomere-dependent growth arrest was associated with increased mitochondrial dysfunction (Passos et al., 2007). It should be noted that studies mostly on cell lines demonstrated how telomere erosion and mitochondrial stress could be interrelated (Correia-Melo et al., 2014; Passos et al., 2007).

In a recently published study, different T-cell subsets of healthy human donors were analysed and it was indicated that accumulating ROS with age could contribute to telomere shortening in primary memory T lymphocytes (Sanderson and Simon, 2017). The authors of the report demonstrated for the first time a correlation between age and increased mitochondrial ROS specifically in CD8+ T-cell subgroups, and suggested a strong link between age and telomere length, mitochondrial ROS and health (Sanderson and Simon, 2017). However, it was also concluded that the detailed mechanism of

association between telomere status and mitochondrial function in humans still remained to be determined (Sanderson and Simon, 2017).

Besides revealing the molecular mechanisms underlying the associations of telomere dysfunction and mitochondrial functions, investigations of mtDNAcn and TL in association with healthy and disease human phenotypes have likewise begun. Up to now, only a modest number of research have been published that measured mitochondrial mtDNAcn and telomere length in humans simultaneously and assessed the relationship between them, but recently a growing number of population studies examined specifically the relationship of mtDNAcn and TL (Alegria-Torres et al., 2016; Kim et al., 2013; Li et al., 2015; Monickaraj et al., 2012; Otsuka et al., 2017; Pieters et al., 2015; Qiu et al., 2015; Revesz et al., 2018; Tyrka et al., 2015; Tyrka et al., 2016; Zhu et al., 2017; Zole et al., 2017). Among them most studies claimed positive association between mtDNAcn and TL both in healthy individuals (Kim et al., 2013; Revesz et al., 2018; Tyrka et al., 2015), in elderly people (Pieters et al., 2015; Zole et al., 2017), in children (Alegria-Torres et al., 2016), pregnant women (Qiu et al., 2015) and colorectal carcinomas (H. Lee et al., 2017). Negligible but significant negative correlation between mtDNAcn and TL was also reported (Zhu et al., 2017), and inverse association was found in patients with psychiatric disorders (Li et al., 2015; Otsuka et al., 2017; Tyrka et al., 2016). Two recent studies investigated the association between telomere length and mtDNA copy number in subjects without clinical condition, both of which found positive correlation. In a Korean sample of community-dwelling elderly women a correlation of r=0.39 was reported (Kim et al., 2013), while in a community sample of healthy younger adults a correlation of r=0.120 between mtDNAcn and LTL was detected (Tyrka et al., 2015), and the authors suggested co-regulation of telomeres and mitochondrial function (Tyrka et al., 2015). A recent report, investigating the combined impact of telomere length and mtDNAcn on cognitive function of community-dwelling very old adults, suggested that the two parameters might be useful for monitoring cognitive decline in older people (J. Y. Lee et al., 2017).

1.4.3. LTL and mtDNAcn in twin studies

As studies highlighted that telomere length can be modified by genetic and epigenetic factors, sex hormones, reactive oxygen species and inflammatory reactions (Kaszubowska, 2008), it has become clear that in a quest to find out more about the potential factors influencing the rate of telomere attrition *in vivo*, genetic, as well as epigenetic mechanisms are crucial to be further explored, for which twin studies could provide great potential (Melicher et al., 2015).

The earliest twin studies in telomere research investigated the genetic determination of telomere length (TL) by estimating its heritability, and a twin study attempted to analyse the heritability of telomerase inducibility (Kosciolek and Rowley, 1998). Subsequent twin studies examined the relationship between age, mortality and TL (Bakaysa et al., 2007; Bischoff et al., 2006; Kimura et al., 2008), and others tried to decipher the relative influence of genetic and environmental factors influencing telomere length (Huda et al., 2007) and telomere attrition (Hjelmborg et al., 2015).

Twin- and other population-based studies assumed the heritability of telomere length between 36–86% (Andrew et al., 2006; Atzmon et al., 2010; Broer et al., 2013; Jeanclos et al., 2000; Kosciolek and Rowley, 1998; Slagboom et al., 1994; Vasa-Nicotera et al., 2005), a meta-analysis reported a heritability of 0.70 (Broer et al., 2013). A recent longitudinal study extended the findings by estimating how telomere attrition rates might vary between twins, and found that genetic background contributed to the overall LTL, but not to its rate of change (Berglund et al., 2016). Besides, the genetic effect on LTL attrition was found equal in both men and women and the importance of individual predisposition to LTL attrition was emphasized (Berglund et al., 2016). It should be noted that, the genetic basis of telomere length still remains elusive (Kim et al., 2012) and there are also many controversies about the extent to which genetic and/or environmental factors might influence telomere length during later stages of the human lifespan (Huda et al., 2007).

With regard to mtDNA copy number, Mengel-From et al investigated the association between mtDNAcn and aging, and a number of health parameters involving 1.067 Danish twins and singletons, besides they also examined the differences in mtDNAcn between males and females and twin zygosity (Mengel-From et al., 2014).

According to their findings the decrease in mtDNAcn initiated in middle age, approximately at age 50. The group reported the first longitudinal data in mtDNAcn in blood cells among healthy subjects, in consistency with their cross-sectional data, the longitudinal data also demonstrated that mtDNAcn declined at the individual level (Mengel-From et al., 2014). Mengel-From et al also found a consistent direction of association between low mtDNAcn and higher mortality and poorer condition in terms of both self-rated health, cognitive and physical performance, and suggested that mtDNAcn might serve as a biomarker for general health in combination with other markers of health and disease among the elderly (Mengel-From et al., 2014). The heritability of mtDNA copy number measured in lymphocytes and buccal swaps was also reported to be relatively high (Curran et al., 2007; Ding et al., 2015; Mengel-From et al., 2014; Reiling et al., 2010; Xing et al., 2008), strong genetic regulation (estimated to 54%) of mtDNA level was proposed (Ding et al., 2015).

2. Objectives

Both telomerase activity, hTERT protein level, telomere length and mitochondrial DNA copy number are important markers in reflecting the replicative capacity of cells. We aimed to contribute to the deeper understanding of the telomerase system and telomere-mitochondria interplay by simultaneously investigating these four variables in a genetically controlled study design, and being the first research group to study them in monozygotic twins.

We alongside aimed to help in deciphering the possible contribution of genes and environment by comparing the degree of phenotypic similarities of monozygotic and dizygotic twins. Applying the unique research potential of twin studies, we approach the issue of possible genetic contribution, by calculating intraclass correlation coefficients for mtDNAcn and TL among MZ and DZ twins in estimating co-twin similarity. Studying possible associations of mtDNAcn and TL firstly in twins, also belonged to our main objectives.

We further detail our specific objectives below:

Analysis of the distribution of T lymphocyte subgroups in peripheral blood mononuclear cells of monozygotic twins of different age and gender by applying multi-parametric flow cytometry.

- Comparison and assessment of co-twin similarities of T-cell subgroup distribution in monozygotic twins.

Measurement of the catalytic reverse transcriptase protein subunit of telomerase (hTERT) in T lymphocyte subsets.

- Analysis and comparison of hTERT protein level in T lymphocytes, helper-, cytotoxic-, and regulatory T-cells.
- Comparison and assessment of co-twin similarities of hTERT protein level of different T-cell subgroups of MZ twins.

Detection of telomerase activity in PBMCs, utilizing the Telomeric Repeat Amplification Protocol (TRAP).
- Comparison and assessment of co-twin similarities of telomerase activity of MZ twins.

Absolute telomere length measurement, using quantitative polymerase chain reaction standard curve method.
- Comparison and assessment of co-twin similarities of telomere length of MZ and DZ twins.

Absolute mitochondrial DNA copy number measurement, using quantitative polymerase chain reaction standard curve method.
- Comparison and assessment of co-twin similarities of mtDNA copy number of MZ and DZ twins.

Assessment of co-twin similarities of mitochondrial DNA copy number, telomere length, hTERT protein level and telomerase activity in PBMCs of MZ twins, calculation of intraclass correlation coefficients and assessment of within-pair similarity measures with increasing age.

Bivariate association analysis and regression model of mtDNA copy number, telomere length and clinical data of MZ and DZ twins.

Co-twin similarity assessment and comparison of MZ and DZ twins in mtDNA copy number and telomere length.

3. Methods

3.1. Subjects

We examined in total 142 adult volunteers (mean age 50.54 ± 15.43 years ± standard deviation /SD/, range 20-75 years), including 48 monozygotic pairs and 23 dizygotic twin pairs ***(Table 1)***.

Table 1. *Basic sample characteristics of the studies*

	Monozygotic twin study	mtDNAcn-TL study
Subjects, n	32	142
Monozygotic- Dizygotic, n	32 - 0	96 - 46
Male-female, n	10 - 22	42 - 100
Age, years (mean ± SD)	46.0 ± 12.88	50.54 ± 15.42

3.1.1. Sample of Monozygotic twin study

32 subjects, comprising of 16 monozygotic twin pairs were included in the Monozygotic twin study consisting of combined telomerase activity measurements, flow cytometric detection of hTERT protein level and analysis of the distribution of various T-cell subpopulations, mtDNA copy number and telomere length measurements. The mean age of participants was 46.0 ± 12.88 (years ± SD), with an age-range of 21 – 69 years. Eleven pairs of the twin subjects were female and five pairs were male (***Table 1***).

3.1.2. Sample of mtDNA copy number and telomere length study

142 subjects, comprising of 96 monozygotic- (48 complete pairs) and 46 dizygotic twins (23 complete pairs) were analysed in the mtDNA copy number and telomere

length measurement study. The mean age of participants was 50.54 ± 15.43 (years ± SD), with an age-range of 20 – 75 years. 50 pairs of the twin subjects were female and 21 pairs were male (***Table 1***).

3.2. Study design

The study participants were recruited from the members of the Hungarian Twin Registry (Littvay et al., 2012).

The subjects - one twin pair at a time - attended a full-night polysomnography in the frame of an ongoing clinical study attending the Sleep Laboratory of, the Department of Pulmonology, Semmelweis University, Budapest, Hungary. The following morning, within an hour after awakening, blood pressure and heart rate were measured and fasting venous blood was drawn. Venous blood was drawn always at the same time for both member of a twin pair. After blood draw laboratory test was done by the Central Laboratory Buda, Semmelweis University for serum glucose, renal function, lipid profile and C-reactive protein (CRP) measurements, while venous blood was also collected for molecular biology analysis purposes separately.

The blood collection for further molecular biology studies, was facilitated by the author, within half an hour after blood draw, from its site at the Department of Pulmonology, Semmelweis University. Right after that, the samples were transported to the Department of Genetics, Cell- and Immunobiology, Semmelweis University, where the aliquoting, storing and peripheral blood mononuclear cells (PBMCs) isolation protocols were carried out by the author in case of each sample, collected on a weekly basis.

In order to assess past medical history and personal habits (including socio-demographic, anthropometric data, and data on lifestyle, as diet, smoking, alcohol consumption, and history of physical activity), all study subjects were requested to complete an on-site questionnaire.

The subjects in the selected sample were in generally good health, none of the participants had any acute clinical condition and none of them had reported any severe diseases during the medical examination. None of the selected participants were

diagnosed with Alzheimer's disease, Parkinson disease or any type of cancer. Further exclusion criteria included pregnancy, acute or severe chronic diseases or foreseeable lack of compliance with test procedures.

The study was approved by the Ethical Community of Semmelweis University according to the Helsinki Declaration and was also authorized by Ethic Committee of Scientific Research of Hungary (ETT-TUKEB) (Semmelweis University TUKEB 30/2014, 3583/2015/EKU and 30/2014, 3583-3/2015/EKU).

All volunteers gave their written informed consent.

3.3. Blood collection and PBMC isolation

Venous blood samples were collected in 9 ml ethylenediamine tetraacetic acid (EDTA) tubes (EDTA tube, Greiner Bio-One). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation at 830 g for 20 minutes at 20 °C by layering the blood over the Ficoll–Paque (Sigma, St. Louis, USA). After centrifugation, the obtained buffy coat was aspired and washed once with phosphate-buffered saline (PBS). The pelleted PBMC were resuspended in fetal calf serum (FCS) containing 10% dimethyl sulfoxide (DMSO) and were frozen at –80 °C until analysis.

3.4. Flow cytometry

The identification of lymphocyte subpopulations and the detection of intracellular hTERT protein level, in subpopulations of lymphocytes were carried out using the method of concurrent staining of cell surface antigen, multicolor flow cytometry was applied for the analysis, using the reagents as indicated in ***Table 2***.

***Table 2**. Reagents for immunophenotyping analysis of lymphocyte subpopulations*

Antibody	Reactivity	Host/Isotype	Dilutions	Manufacturer	Clone
hTERT-FITC	Human	Rabbit	1:100	Novus Biologicals	polyclonal
FoxP3-Pe	Human	Mouse / IgG1, kappa	1:50	eBioscience	236A/E7
CD8-Pe	Human	Mouse / IgG1	1:50	cytognos	143-44
CD4-PerCP-Cy5.5	Human	Mouse / IgG2b, kappa	1:50	eBioscience	OKT4
CD3-APC	Human	Mouse / IgG2a	1:50	cytognos	33-2A3

3.4.1. Identification of lymphocyte subpopulations

T lymphocytes were defined on the basis of their CD3 expression. Helper T-cells were identified by CD3+/CD4+ double positivity, while cytotoxic T-cells were described by the co-expression of CD3 and CD8. Regulatory T lymphocytes were characterized by the intracellular presence of Foxp3 transcription factor in CD3+/CD4+ cells.

The identification of T cell subpopulations is depicted on ***Figure 2***. The representative dot plot on ***Figure 2 A)*** shows the distribution of the PBMCs by relative size (FSC-Height) and relative granulation (SSC-Height). Based on size and granulation, the smaller and less granular population corresponded to lymphocytes.

The representative dot plot on ***Figure 2 B)*** demonstrates cells exhibiting CD3 positivity inside the lymphocyte gate, detectable after APC-conjugated anti-CD3 labeling. Defining the CD3+ T-cells by gating, T-cells were distinguished from other cells, also present inside the lymphocyte gate based on their size and granulation (B cells, NK cells and basophil granulocytes).

The representative dot plot on ***Figure 2 C)*** demonstrates cells exhibiting CD4 positivity inside the lymphocyte gate, detectable after PerCP-conjugated anti-CD4 labeling. Defining CD4+ T-cells by gating was used to identify helper T-cells (Th) as follows: cells that simultaneously exhibited CD3 and CD4 expression (CD3+/ CD4+ double positive cells) inside the lymphocyte gate were considered Th.

The representative dot plot on ***Figure 2 D)*** demonstrates cells exhibiting CD8 positivity inside the lymphocyte gate, detectable after Pe-conjugated anti-CD8 labeling. Defining CD8+ T-cells by gating was used to identify cytotoxic T-cells (Tc) as follows: cells that simultaneously exhibited CD3 and CD8 expression (CD3+/ CD8+ double positive cells) inside the lymphocyte gate were considered Tc.

The representative dot plot on ***Figure 2 E)*** demonstrates cells exhibiting Foxp3 positivity inside the lymphocyte gate, detectable after Pe-conjugated anti-Foxp3 labeling. Defining Foxp3+ cells by gating was used to identify regulatory T-cells (Treg) as follows: cells that simultaneously exhibited CD3 and CD4 and intracellular Foxp3 expression (CD3+/ CD4+/ Foxp3 triple-positive cells) inside the lymphocyte gate were considered Treg.

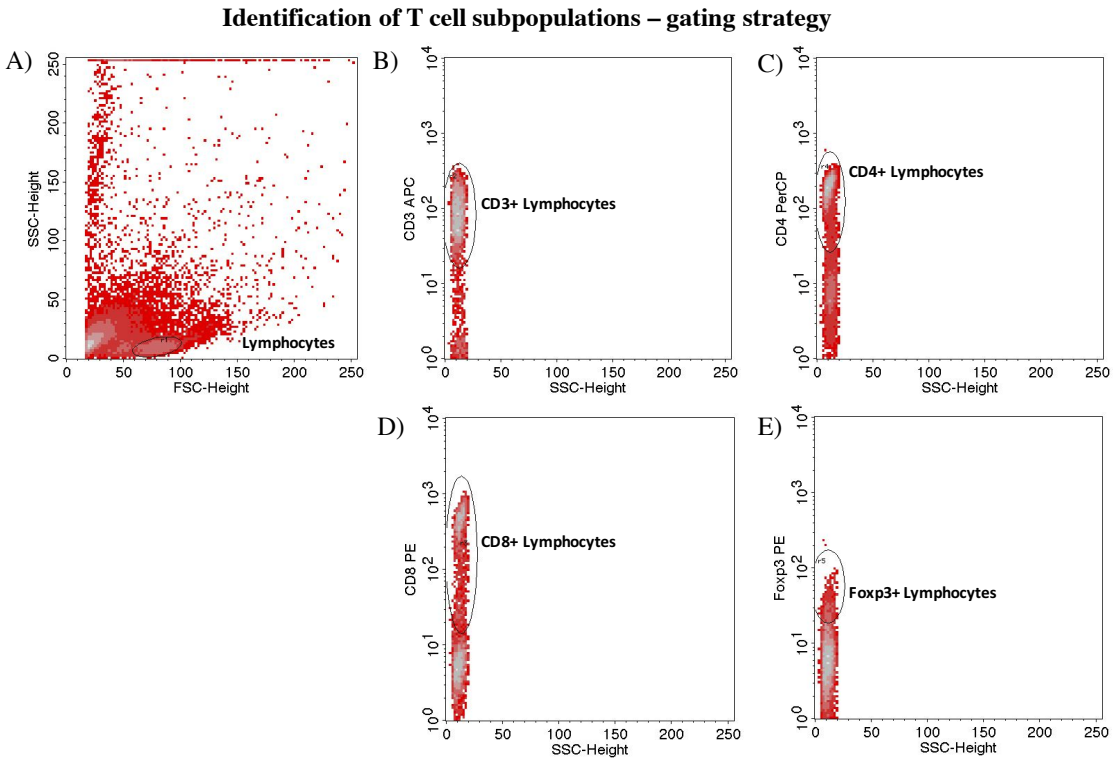

Figure 2. *Identification of T cell subpopulations*

A) *Distribution of the PBMCs by relative size (FSC-Height) and relative granulation (SSC-Height).*

B) *CD3+ cells inside the lymphocyte gate, detectable after APC-conjugated anti-CD3 labeling.*

C) *CD4+ cells inside the lymphocyte gate, detectable after PerCP-conjugated anti-CD4 labeling.*

D) *CD8+ cells inside the lymphocyte gate, detectable after Pe-conjugated anti-CD8 labeling.*

E) *Foxp3+ cells inside the lymphocyte gate, detectable after Pe-conjugated anti-Foxp3 labeling.*

3.4.2. Analysis of cell viability

Propidium iodide uptake assay was used for the determination of the viability of PBMCs before staining. 10 μL of Propidium Iodide (PI) solution was added to 1 mL of cell suspension. Cells were analyzed by flow cytometry. Dead cells could be distinguished by showing positivity for PI, while viable cells could be included into the analysis.

3.4.3. Validation procedures of TERT antibody

Our preliminary testing procedures showed consistency with the published results (Handa et al., 2010) about the reliability of the reactivity of hTERT antibody. Both direct and indirect antibody labeling methods (isotype control and secondary antibody) were used, and methods were carefully tested in preliminary experiments on samples of subjects with various clinical conditions in peripheral blood, and testing was also performed on Monomac 6 (MM6) human leukemia cell lines and on Jurkat cells (D'Hautcourt J, 2002). TERT antibody (NB110-89471, Novus Biologicals) was also validated using telomerase negative neutrophil granulocytes as negative controls ***(Figure 3).***

Validation of hTERT antibody using neutrophil granulocytes as negative controls is further detailed and depicted on ***Figure 3***.

Figure 3 A) displays a representative dot plot showing the distribution of cells in peripheral venous blood by relative size (FSC-Height) and relative granulation (SSC-Height). Based on size and granulation, the large and largely granular cells corresponded to the neutrophil granulocyte population. Neutrophils identified by size and granulation were defined by gating (circumcision).

Figure 3 B) depicts the validation of the purity of the neutrophil granulocyte gate identified on FS-SS dot plot, by CD15 labeling. CD15 (3-fucosyl-N-acetyl-lactosamine) is a cluster of differentiation antigen which is physiologically expressed on the surface of circulating neutrophils. The representative overlay histogram shows the intensity of the fluorescence signals detected after isotype control (anti-mouse IgG Pe) and anti-human CD15 antibody labeling within the neutrophil granulocyte gate. After Pe-

conjugated anti-human CD15 antibody labeling, a two-magnitude higher fluorescence intensity signal could be detected compared to the fluorescence signal of the isotype control, which clearly demonstrated the CD15 expression of cells, thus validating that within the applied gate, indeed neutrophil granulocytes could be found.

Figure 3 C) depicts a representative overlay histogram demonstrating the intensity of the fluorescence signals, detected after isotype control and FITC-conjugated anti-human hTERT antibody labeling within the neutrophil granulocyte gate. Given that the intensity signals, detectable after either isotype control (rabbit IgG FITC) or anti-human hTERT antibody labeling were comparable, it could be stated that the FITC-labeled anti-hTERT antibody did not stain the neutrophil granulocytes specifically. The lack of hTERT expression in neutrophil granulocytes correlated with previous findings which had generally shown that telomerase activity was not present in those cells (Hiyama et al., 1995) (Counter et al., 1995).

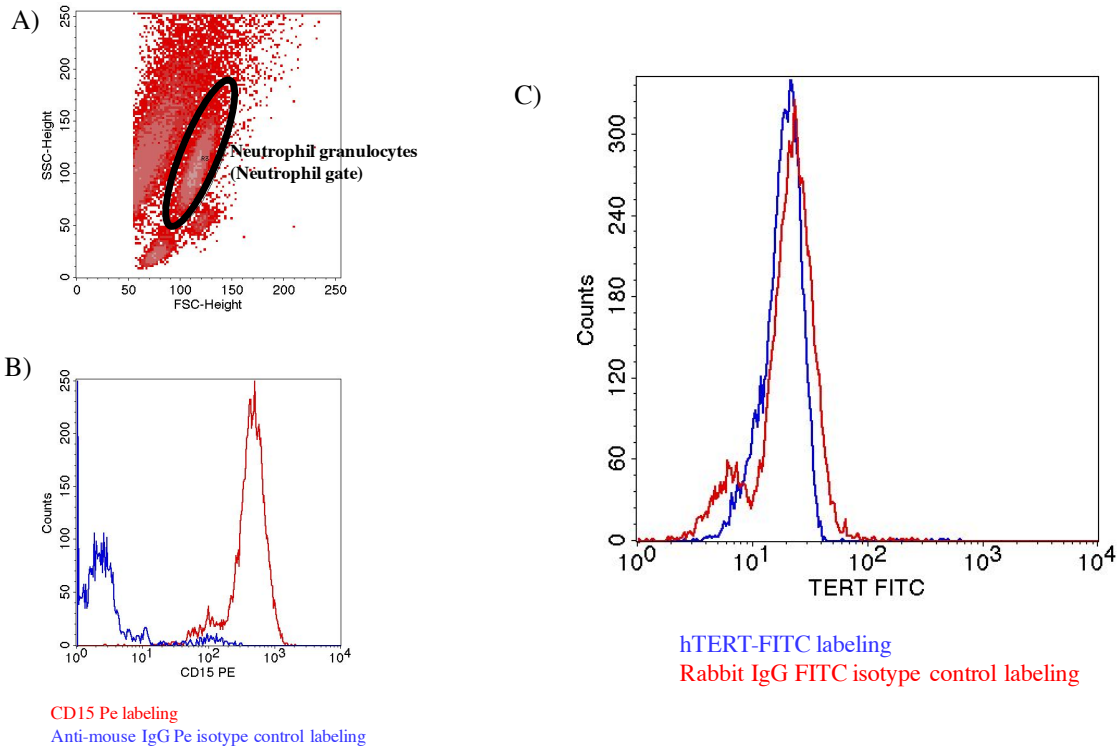

Figure 3. *Validation of hTERT antibody using neutrophil granulocytes as negative controls*

A) *Distribution of cells in peripheral venous blood by relative size (FSC-Height) and relative granulation (SSC-Height).*

B) *Validation of the purity of the neutrophil granulocyte gate, identified on FS-SS dot plot by CD15 labeling.*

C) *Intensity of the fluorescence signals, detected after isotype control and FITC-conjugated anti-human hTERT antibody labeling within the neutrophil granulocyte gate.*

3.4.4. Detection of hTERT protein level

The assay procedure for the detection of hTERT protein level was carried out as follows: $5x10^5$ PBMC suspensions were incubated with fluorochrome-conjugated antibodies for exofacial staining in the dark for 15 minutes, at room temperature. During washing steps the supernatant of PBS diluted cell suspension was removed by centrifugation, allowing a minor volume of liquid (ca. 100 μL) to remain. Cell pellet was fixed by 4% paraformaldehyde solution for 10 minutes, at room temperature. After washing in PBS, the cells were permeabilized by 0.1% saponin solution for subsequent staining for intracellular Foxp3 and hTERT protein. Intracellular staining lasted for 15 minutes. Unbound antibodies were removed by washing with 0.1% saponin solutions. All antibodies were carefully titrated in earlier experiments.

Measurements were carried out using a FACSCalibur flow cytometer (Becton Dickinson San Jose, CA, USA) on the day of the staining, collecting $1x10^5$ cells/tube. CellQuest-Pro software (Becton Dickinson San Jose, CA, USA) was used for the acquisition and analysis. Lymphocyte subpopulations were determined inside the lymphocyte gate on the basis of their exofacial protein pattern. The relative amounts of intracellular hTERT protein were analyzed by the comparison of geometric mean channel values of fluorescence (MFI).

The phases of hTERT protein detection are depicted on ***Figure 4***, where ***Figure 4 A)*** displays a representative dot plot showing the distribution of the peripheral blood mononuclear cells (PBMC) by relative size (FSC-Height) and relative granulation (SSC-Height). Based on size and granulation, the smaller and less granular population corresponded to lymphocytes, while the larger and more granular population corresponded to monocytes. Cells identified by size and granulation were defined by gating (circumcision). The gating not only served to identify the lymphocyte and monocyte cell populations, but also to exclude dead cells, that were previously identified by propidium iodide viability staining.

Figure 4 B) depicts a representative dot plot showing the fluorescence signal detected following FITC fluorochrome conjugated anti-hTERT antibody labeling, as a function

of granulation. The figure presents fluorescence signals detected inside the lymphocyte and monocyte gates (lymphocyte gate and monocyte gate together). The continuous line shown in the figure demonstrates the separation of fluorescence signals from the background. The value of the fluorescent (aspecific) background was determined by isotype control labeling.

Figure 4 C) demonstrates the fluorescence signal detected after the isotype control antibody was attached. On the scatter plot (similarly to ***Figure 4 B)***, fluorescence is depicted as a function of granulation. Also similarly to ***Figure 4 B)***, isotype control staining is depicted inside the lymphocyte and monocyte gates.

hTERT protein detection

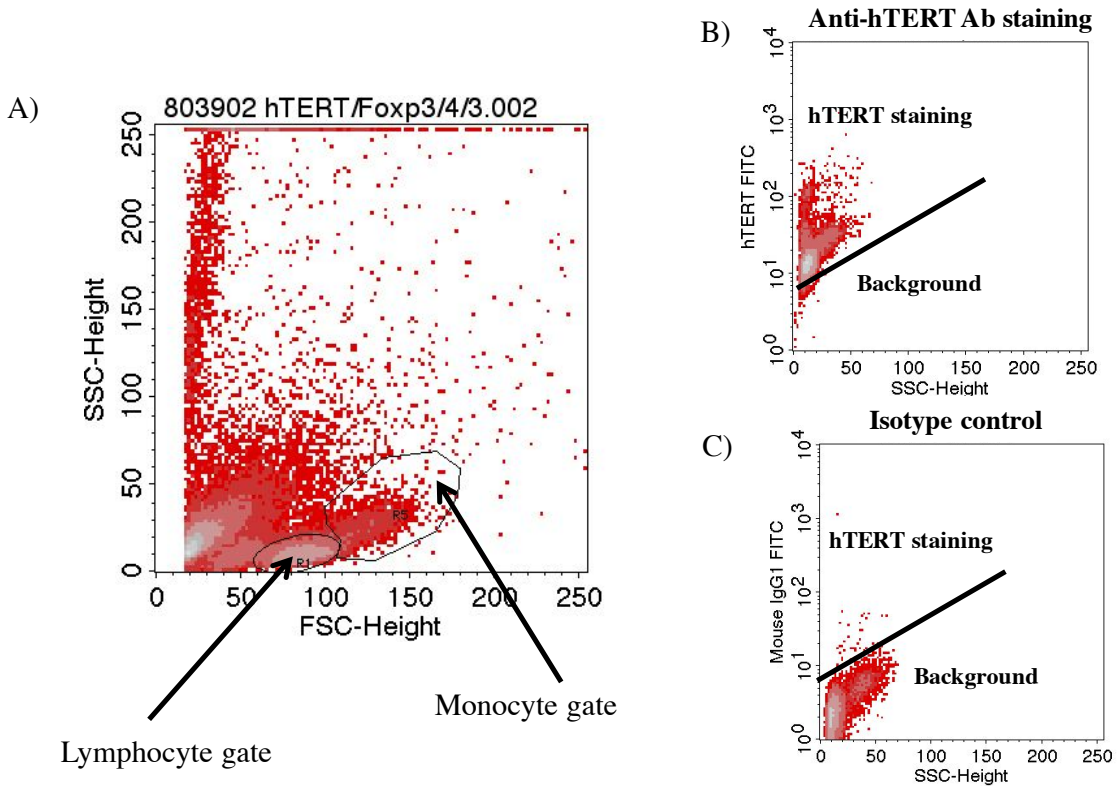

Figure 4. *hTERT protein detection*

A) *Distribution of the peripheral blood mononuclear cells by relative size (FSC-Height) and relative granulation (SSC-Height).*

B) *Fluorescence signals detected following FITC fluorochrome conjugated anti-hTERT antibody labeling, as a function of granulation.*

C) *Fluorescence signals detected after the isotype control antibody was attached.*

The gating strategy is further detailed on ***Figure 5***, where ***Figure 5 A)*** displays a representative dot plot showing the distribution of the PBMCs by relative size (FSC-Height) and relative granulation (SSC-Height). Based on size and granulation, the smaller and less granular population corresponded to lymphocytes.

Figure 5 B) depicts an overlay histogram showing the intensity of the fluorescence signal detected after isotype control and hTERT antibody labeling inside the lymphocyte gate.

Gating strategy

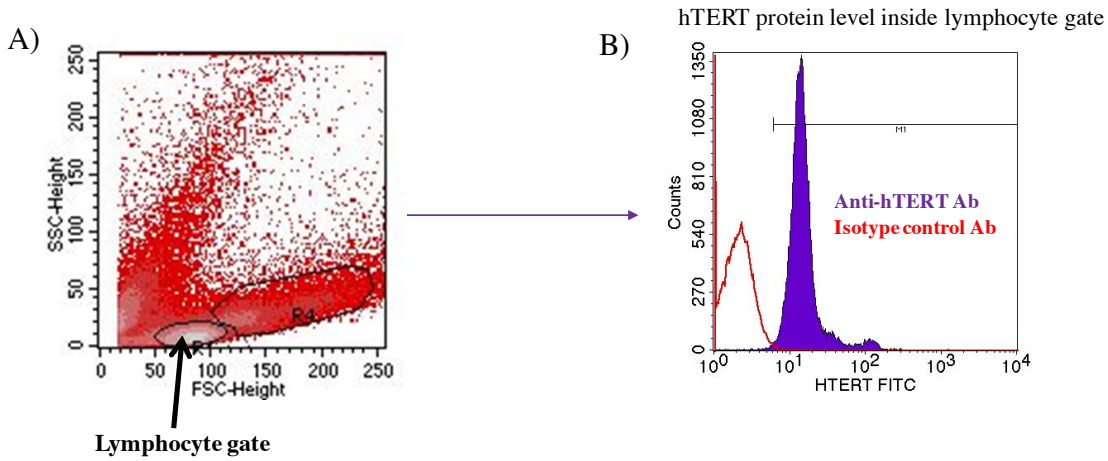

Figure 5. *Gating strategy*

A) *Distribution of the PBMCs by relative size (FSC-Height) and relative granulation (SSC-Height).*

B) *Intensity of the fluorescence signal detected after isotype control and hTERT antibody labeling inside the lymphocyte gate.*

3.5. Measurement of telomerase activity with TRAP-ELISA assay

For the detection of telomerase activity, the Photometric Enzyme Immunoassay for Quantitative Determination of Telomerase Activity, utilizing the Telomeric Repeat Amplification Protocol (TRAP) was applied. To determine telomerase activity $2x10^5$ cells were used for the assay. The cells were lysed in CHAPS lysis buffer, and the TRAP assay was performed using the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Roche Diagnostics GmbH) following the manufacturer's instruction. Briefly, the PCR based assay was carried out in a reaction mixture (biotinylated telomerase substrate P1-TS, optimized anchor-primer P2 nucleotides and Taq DNA polymerase) with the use of an internal standard (IS) and negative control (heat inactivation of telomerase-associated RNA) for each sample as well. After 20 minutes of incubation at 25 °C to allow the telomerase mediated extension of the TS primer, the reaction was performed in thermocycler (GeneAmp PCR System 9700, ABI) after the following steps: the reaction mixture was subjected to 94 °C for 5 minutes after which, 30 PCR cycles followed at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 90 seconds. After the cycling process, the samples were incubated at 72 °C for 10 minutes. To quantitate the cellular telomerase activity, the PCR product was denatured and then hybridized with a digoxigenin-labeled telomeric repeat-specific probe. The PCR product was visualized using peroxidase-conjugated antibodies against digoxigenin and measured at 450 nm, respectively at 690 nm using an ELISA reader (Labsystems Multiscan MS, Thermo). Relative telomerase activity (RTA) was calculated following the manufacturer's instructions: in the first step the absorbance readings of the negative samples were subtracted from the absorbance readings of the samples. When the difference in absorbance was higher than 0.2 A450nm-A690nm units, samples were considered telomerase positive.

As given in the manufacturer's instruction, relative telomerase activities (RTA) was calculated using the following formula:

$$RTA = \frac{(AS - AS,0)/AS,IS}{(ATS8 - ATS8,0)/ATS8,IS} \times 100$$

AS: absorbance of sample
AS,0: absorbance of heat- or RNase-treated sample
AS,IS: absorbance of Internal Standard (IS) of the sample
ATS8: absorbance of Control template (TS8)
ATS8,0: absorbance of Lysis buffer
ATS8,IS: absorbance of Internal Standard (IS) of the Control template (TS8)

3.6. Measurement of telomere length and mtDNA copy number

DNA was extracted from the previously isolated cells, using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA) as appropriate. Each DNA sample was quantified and qualified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), in case the 260/280 ratio was higher than 1.8, and the 260/230 ratio was in the range of 2.0-2.2, the DNA sample was qualified as pure. Absolute telomere length and absolute mitochondrial DNA copy number were measured using quantitative polymerase chain reaction (qPCR) as previously described (O'Callaghan et al., 2008) with minor modifications. First the mitochondrial DNA copy number was calculated based on the ratio between the amount of mtDNA (Cytochrome b [*MT-CYB*]) and that of a single-copy gene (albumin [*ALB*]). Telomere length was measured by calculating the number of telomere repeats relative to that of a single-copy gene (*ALB*) used as a quantitative control, relative to a reference sample. All qPCR reactions were performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems, Foster City, CA). Three qPCRs were performed to quantitate copy numbers for telomeres, mitochondrial genomes (Cytochrome b [*MT-CYB*]), and the albumin gene [*ALB*] as a single-copy standard.

The primer sequences for telomeres (O'Callaghan et al., 2008) and primers and TaqMan probe for *MT-CYB* (Hakonen et al., 2007) had been previously reported. The primers and probe of the *ALB* gene, and also probe for telomere were designed by using Primer3 program (Untergasser et al., 2012). The primer and probe sequences were described in ***Table 3***. The probe had a fluorophore at 5'-end, 6-carboxyfluorescein (FAM) and a quencher at 3'-end, tetramethylrhodamine (TAMRA). Primer concentrations were raised to 100 nM and cycling conditions were as follows: 10 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds, 60°C for 1 minutes. Each sample was run in triplicate using 20 ng of DNA and accepted only in case the standard deviation of the Ct values were <1Ct (CV >5%). Amplification of telomeres, *MT-CYB* and *ALB* were performed in separate runs, using the same reference samples in the same well positions. For the standard curve method, 6-point serial-dilution of cloned amplicons with no template control was applied and the same calibrator sample was used in all runs to allow comparison of results across runs. Data were analyzed by StepOne Software v2.3 (Applied Biosystems, Foster City, CA). All standard curve met the criteria that the correlation coefficient (R^2) was greater than 0.95, and the PCR efficiency was in the range of 90-100%.

Table 3. *Primer and probe sequences for qPCR standard curve method*

Locus	**Primers**		**Probes**
Telomere	Forward	CGGTTTGTTTGGGTTTGGGTTTG GGTTTGGG TTTGGGTT	TTAGGGTTAGGGTTAG GGTTAGGG
	Reverse	GGCTTGCCTTACCCTTACCCTTA CCCTTACCCTTACCCT	
mtDNA (*MT-CYB*)	Forward	GCCTGCCTGATCCTCCAAAT	CACCAGACGCCTCAAC CGCCTT
	Reverse	AAGGTAGCGGATGATTCAGCC	
single-copy gene (*ALB*)	Forward	TGTTGCATGAGAAAACGCCA	AAGTGACAGAGTCACC AAATGCTGCACAG
	Reverse	GTCGCCTGTTCACCAAGGAT	

3.7. Statistical analysis

Statistical analysis was carried out by applying linear mixed effects regression models (Pinheiro and Bates, 2009) using the lme4 package (Bates et al., 2015) of the software (R Core Team, 2016). Statistical significance level was set at $p<0.05$. Continuous variables were expressed as mean ± standard deviation (SD) unless otherwise specified.

Unpaired Student's t-test and analysis of variance (ANOVA) was applied to analyze the quantitative variables. Logarithmically transformed data were used in case of mtDNAcn and TL in order to approximate a normal distribution.

Intraclass correlation coefficients (ICCs), estimating the intra-individual stability of a variable across conditions, were computed for the twin pairs to estimate the level of co-twin similarity. ICCs were calculated using the residual variance proportion of a baseline and age and sex corrected mixed effects model. Confidence intervals were derived using a family based bootstrap with 1000 draws.

To assess the bivariate associations between mtDNAcn, TL and available predictors, standard coefficients from a full information maximum likelihood estimated regression were applied that also controlled for age and sex. The interpretation of the models is analogous to Pearson's correlations but account for the lack of independence between twin pair and incidental missing data on the predictors. P values were calculated using cluster corrected standard errors. To test the relationship between mtDNAcn and TL, the aforementioned strategy was used, and both standardized and unstandardized coefficients were presented. Additionally, regression model controlling for all significant predictors based on the bivariate analysis was used. The analyses were conducted using Mplus 8 (Muthén and Muthén, 2010). Statistics data was assessed using GraphPad PRISM v6.05 (GraphPad SoftwareInc., California, USA).

4. Results

4.1. Monozygotic twin study

We assessed the lymphocyte subpopulations of 16 monozygotic twin pairs of different age and gender, characteristics including birth order, birth weight and birth week of pregnancy are detailed in ***Table 4***.

Table 4. Characteristics of monozygotic twin subjects

Code	*Age (years)*	*Sex (1=male, 2=female)*	*BMI*	*Birth weight (g)*	*Birth week of pregnancy*
01A	21	2	19,22	2230	37
01B	21	2	20,78	1870	37
02A	25	1	20,23	1500	28
02B	25	1	19,81	1500	28
03A	27	1	25,35	2500	NA
03B	27	1	28,41	2900	NA
04A	31	1	28,69	NA	NA
04B	31	1	29,61	NA	NA
05A	37	2	20,83	NA	NA
05B	37	2	20,03	2500	NA
06A	39	2	20,90	2500	NA
06B	39	2	19,75	2250	NA
07A	40	2	22,31	2400	37
07B	40	2	21,95	2450	37
08A	45	2	27,04	2300	36
08B	45	2	29,05	1900	36
09A	51	2	23,92	NA	NA
09B	51	2	24,35	NA	NA
10A	53	1	26,26	NA	NA
10B	53	1	25,11	NA	NA
11A	55	2	18,07	NA	36
11B	55	2	17,26	NA	NA
12A	59	2	27,41	2500	39
12B	59	2	29,00	2550	39
13A	60	2	17,21	1960	28
13B	60	2	18,73	1950	28
14A	62	2	34,81	2500	36
14B	62	2	27,82	2500	36
15A	62	1	26,54	2700	40
15B	62	1	25,88	3100	40
16A	69	2	25,71	3500	39
16B	69	2	27,82	3500	39

4.1.1. T lymphocyte distribution and hTERT protein level in T-cell subgroups

We analysed the distribution of lymphocyte subgroups present inside the lymphocyte gate of peripheral blood mononuclear cells, and determined the level of the catalytic reverse transcriptase protein subunit of telomerase, hTERT in the different subgroups, using concurrent staining of surface antigens, intracellular hTERT protein and multi-parametric flow cytometry. ***Table 5*** summarises our results of hTERT protein levels and percentages of T-cells gated inside the lymphocyte gate. Multicolor flow cytometric detection of intracellular hTERT protein level can be referred to as a unique study method since by applying this technique, it was possible to measure the relative amount of hTERT protein on a single cell level in different lymphocyte subgroups, without requiring the separation of the individual cell types. The level of hTERT protein in the different subgroups could then be compared.

Table 5. hTERT protein levels and percentages of T-cells gated inside the lymphocyte gate

Abbreviations: hTERT: hTERT protein level, Geo mean: Geometric mean channel values of fluorescence (MFI), Tly: CD3+ T cells, Th: CD3+/CD4+ T cells, Treg: CD3+/CD4+/Foxp3+ T cells, Tc: CD3+/CD8+ T cells

Code	*hTERT CD3+ (Geo mean)*	*hTERT CD3+/CD4+ (Geo mean)*	*hTERT CD3+/CD4+/ FoxP3+ (Geo mean)*	*hTERT CD3+/CD8+ (Geo mean)*	*hTERT in PBMC gate (Geo mean)*	*CD3+ (% Gated)*	*CD3+/CD4+ (% Gated)*	*CD3+/CD4+ /FoxP3+ (% Gated)*	*CD3+/CD8+ (% Gated)*
01A	20,27	20,82	53,12	21,52	36,41	74,60	54,59	1,18	24,98
01B	22,04	22,33	50,68	18,73	34,61	68,21	48,08	1,02	20,12
02A	16,59	16,29	29,90	22,05	22,40	74,17	48,44	0,54	34,95
02B	13,59	13,45	21,88	13,86	17,03	82,67	53,41	0,22	36,07
03A	12,24	11,80	15,04	12,23	16,89	78,53	53,97	0,15	24,66
03B	11,36	11,10	15,61	13,16	17,60	72,65	50,14	0,12	25,42
04A	5,98	5,78	8,23	6,35	5,71	80,16	52,44	0,24	27,77
04B	5,84	5,67	7,09	6,54	6,05	78,41	50,36	0,31	26,52
05A	7,78	7,75	7,75	7,48	7,37	72,63	49,93	0,72	19,49
05B	10,76	10,80	10,80	8,32	7,89	75,10	54,24	0,14	18,90
06A	15,68	15,57	25,72	16,11	18,96	80,95	63,22	0,21	24,44
06B	17,34	17,25	26,21	16,09	20,73	71,95	50,30	0,52	27,03
07A	38,82	38,64	112,09	40,88	47,07	79,82	49,29	1,33	26,06
07B	37,28	36,37	109,12	50,37	52,64	75,85	50,87	0,80	23,60
08A	6,84	6,74	10,61	7,75	7,70	66,08	45,34	0,13	20,50
08B	7,15	7,03	10,47	7,48	7,21	70,76	51,01	0,23	15,91
09A	12,83	12,56	18,72	13,81	17,21	75,50	42,20	0,67	29,74
09B	14,49	14,16	20,43	8,28	15,37	76,12	41,24	1,40	35,89
10A	9,87	9,43	11,96	6,94	8,08	73,65	43,94	0,64	35,31
10B	9,65	9,26	13,49	27,90	6,85	74,16	44,64	0,30	37,72
11A	19,38	19,98	48,87	24,24	46,10	78,89	54,52	2,17	33,17
11B	16,54	16,56	28,41	19,85	27,05	84,50	57,19	1,00	35,81
12A	15,31	14,93	21,17	17,05	19,20	83,38	57,65	0,15	34,53
12B	14,81	14,34	21,21	13,95	16,31	76,77	57,45	0,25	25,29
13A	14,78	14,64	28,74	13,56	17,23	61,76	53,26	0,23	8,89
13B	12,83	12,76	28,77	13,10	15,94	66,65	59,19	0,15	8,86
14A	13,19	12,65	22,57	9,24	17,88	63,48	44,17	0,44	18,80
14B	8,97	8,73	12,46	11,94	15,93	78,67	59,71	0,37	20,22
15A	14,66	14,40	25,93	9,82	18,59	65,92	36,62	0,67	31,78
15B	16,50	16,34	28,38	13,09	NA	71,10	42,42	0,72	26,96
16A	6,99	6,86	10,04	7,19	7,73	70,84	53,26	0,12	18,46
16B	6,61	6,56	8,91	7,51	8,47	77,31	54,09	0,10	19,82

4.1.2. ICCs for lymphocyte subgroups and hTERT protein levels

We intended to characterise the similarity level of twins with regard to their hTERT protein level in different T lymphocyte subgroups and the respective T-cell distributions by calculating intraclass correlation coefficients (ICCs). Intraclass correlations enumerate the proportion of variation across the families of the twins, as such, estimating co-twin similarity. Intraclass correlation coefficients were calculated for hTERT protein level in T lymphocytes (Tly) and subgroups, including helper T-cells (Th), cytotoxic T-cells (Tc), and regulatory T-cells (Treg), ICCs for the T lymphocyte subsets of the twin subjects were alongside calculated. We found very high intraclass correlation for hTERT protein levels in T lymphocytes (0.891) and both in the Th (0.896), Treg (0.885) and Tc (0.798) subgroups. Analysing the similarity of the distribution of T lymphocyte subpopulations, high ICCs were detected in Treg (0.728) and Tc (0.827) (***Table 6***).

Table 6. Intraclass correlations for hTERT protein levels and lymphocyte subgroups

Intraclass correlation coefficients enumerate exactly what percent of the variation in the sample was across the families of the twins. (Both uncorrected (ICC column) and age and gender corrected (Cor. ICC column) models were used to calculate the intraclass correlations and their corresponding 95% confidence intervals (CI).) It can be claimed with statistical confidence that twins were highly similar to each other for hTERTTly, hTERTTh, hTERTTreg, hTERTTc, Treg and Tc variables.

Incomplete pairs were also included in the intraclass correlation analysis with Full Information Maximum Likelihood estimation leading to potential mismatches. Incomplete pairs were a function of an aggressive outlier detection system where outliers two standard deviation from mean were eliminated in two rounds. This ensured normal distribution of the data better than other transformation techniques.

hTERTTly: hTERT protein level in T-cells, hTERTTh: hTERT protein level in helper T-cells, hTERTTreg: hTERT protein level in regulatory T-cells, hTERTTc: hTERT protein level in cytotoxic T-cells, Tly: T-cells, Th: helper T-cells, Treg: regulatory T-cells, Tc: cytotoxic T-cells, ICC: intraclass correlation, Cor. ICC: corrected intraclass correlation, n (Pairs): number of pairs, n (Ind.): number of individuals

Variable	ICC	CI Low	CI High	Cor. ICC	CI Low	CI High	n (Pairs)	n (Ind.)
hTERTTly	0.891	0.705	0.962	0.9	0.756	0.975	15	29
hTERTh	0.896	0.708	0.963	0.903	0.758	0.973	15	29
hTERTTreg	0.885	0.712	0.963	0.899	0.737	0.969	14	27
hTERTTc	0.798	0.507	0.927	0.799	0.51	0.944	15	29
Tly	0.388	0	0.724	0.451	0	0.802	16	30
Th	0.435	0	0.766	0.46	0	0.81	16	30
Treg	0.728	0.366	0.909	0.744	0.413	0.922	16	29
Tc	0.827	0.567	0.938	0.806	0.498	0.934	15	30

4.1.3. Average level of hTERT protein in T lymphocytes

We compared the level of hTERT protein in the different T-cell subgroups. Regulatory T-cells showed significantly higher level of hTERT protein (27.01 ± 4.42, [mean ± SE]) compared to T lymphocytes (14.28 ± 1.34), helper- (14.11 ± 1.34) or cytotoxic T-cell subgroups (15.20 ± 1.74) (***Figure 6)***.

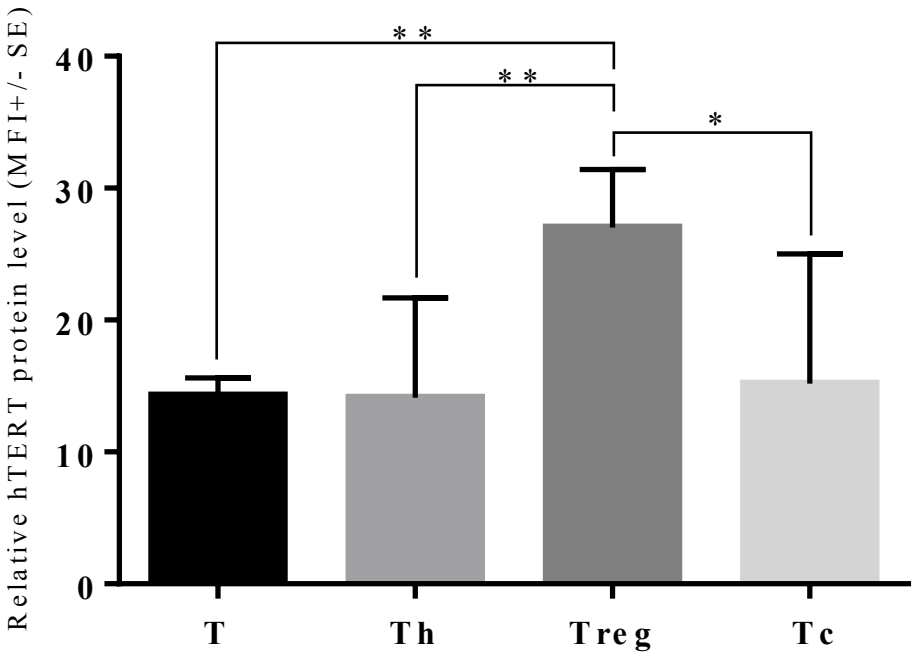

Figure 6. The average level of hTERT protein in different lymphocyte subgroups

Treg showed significantly higher level of hTERT protein compared to T lymphocytes, helper- or cytotoxic T cell subgroups.

The Y axis shows hTERT protein levels (Geo mean). The black bars represent standard error.

*T: T-cells, Th: helper T-cells, Treg: regulatory T-cells, Tc: cytotoxic T-cells, MFI: mean fluorescence intensity, SE: standard error, **p < 0.01, *p < 0.05*

4.1.4. Co-twin similarity for mtDNAcn, TL, hTERT protein level and TA

We defined and compared mitochondrial DNA copy number, telomere length, level of the catalytic reverse transcriptase protein subunit of telomerase (hTERT), and telomerase activity in PBMCs of monozygotic twins. Telomerase activity (TA) was measured, applying the TRAP-ELISA assay, telomere length (TL) and mitochondrial DNA copy number (mtDNAcn) were determined using qPCR standard curve method. In order to gain comparable results, we measured hTERT protein level (hTERT) present inside the PBMC gate.

Aiming to examine the similarity level within twin pairs, the first-born twins were plotted against the second-born twins, and the correlation coefficients were measured in the case of all four variables. We observed that co-twin correlations for mtDNAcn, TL and hTERT protein level were significantly strong (mtDNAcn: $r=0.70$, $p < 0.01$, TL: $r=0.84$, $p < 0.0001$, hTERT: $r=0.84$, $p < 0.0001$). In contrast, regarding telomerase activity (TA) lower similarity with no significance could be detected (TA: $r=0.11$, p = ns) (***Figure 7***).

We note that on ***Figure 7*** it can also be observed that telomerase activity was found to be very different not only between members of twin pairs but also among twin pairs.

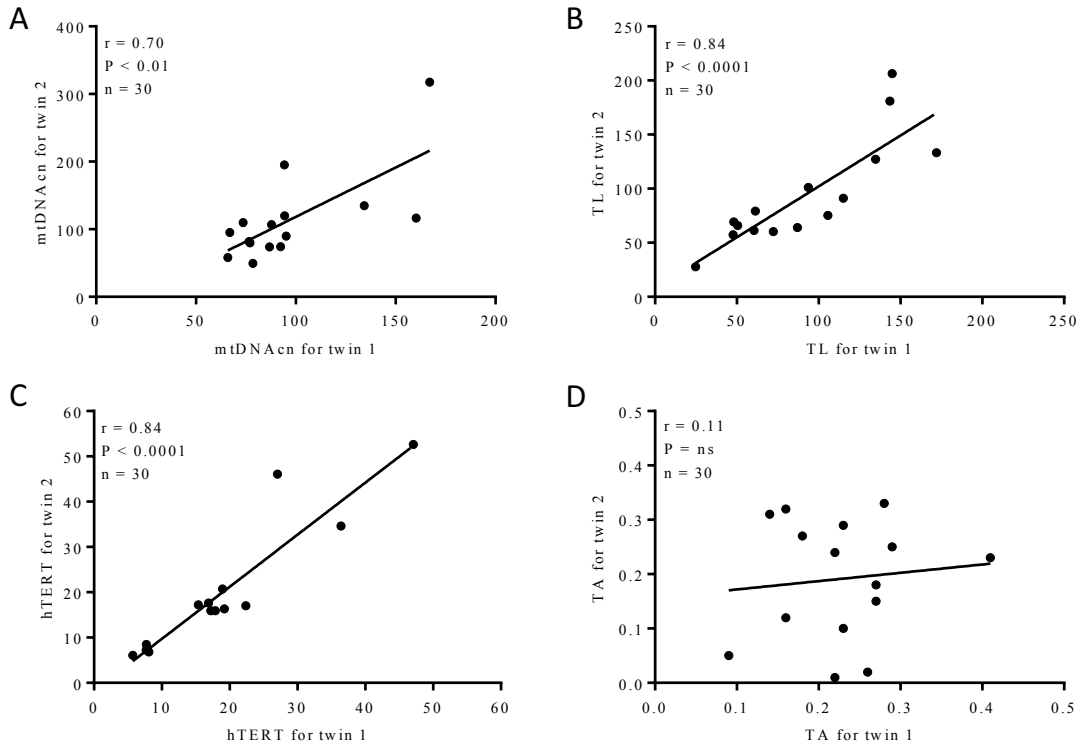

Figure 7. Level of similarity for mtDNAcn, TL, hTERT protein level and TA

A) *mtDNA copy number (mtDNAcn),* ***B)*** *telomere length (TL),* ***C)*** *hTERT protein level in PBMCs (hTERT) and* ***D)*** *telomerase activity (TA). Both in* ***A) B) C)*** *and* ***D)*** *first-born twins (Twin 1) are plotted against second-born twins (Twin 2). Correlation coefficients (r), significance level (p) and number of twin subjects (n) are included.*

4.1.5. Intraclass correlations for mtDNAcn, TL, hTERT and TA

Strong correlations of mtDNAcn, TL and hTERT were further supported by intraclass correlation coefficient (ICC) values. We found very high ICC for hTERT protein level (0.946), it was also high for TL (0.815) and considerable for mtDNAcn (0.524). While in case of TA intraclass correlation coefficient equalled 0 (***Table 7***).

Table 7. Intraclass correlations for mtDNAcn, TL, hTERT and TA

Intraclass correlation coefficients enumerate the proportion of variation in the sample across the families of the twins, as such, co-twin similarity. Both uncorrected (ICC column) and age and gender corrected (Cor. ICC column) models were used to calculate the intraclass correlations and their corresponding 95% confidence intervals (CI) for mtDNAcn, TL, hTERT and TA. As it is shown with statistical confidence, twins were highly similar to each other in their TL and hTERT values.

(In case of one twin pair (displayed under code 15 in 5.) data about telomerase activity was not available for the second twin due to missing data.)

mtDNAcn: mitochondrial DNA copy number, TL: telomere length, hTERT: hTERT protein level inside the PBMC gate, TA: telomerase activity, ICC: intraclass correlation, Cor. ICC: corrected intraclass correlation, n (Pairs): number of pairs, n (Ind.): number of individuals

Variable	ICC	CI Low	CI High	Cor. ICC	CI Low	CI High	n (Pairs)	n (Ind.)
mtDNAcn	0.524	0	0.816	0.535	0,167	0.838	16	30
TL	0.815	0.532	0.930	0.829	0.607	0.949	16	29
hTERT	0.946	0.838	0.983	0.954	0.872	0.987	14	26
TA	0	0	0.585	0	0	0.672	16	27

4.1.6. Within-pair similarity with increasing age

The tendency of within-pair similarity with increasing age was also assessed. We selected study subjects of different age groups, the 16 pairs of identical twins recruited to our study equally represented an age range of 21 to 69 years. ***Figure 8*** shows that despite increasing age, within-pair ratios remained high for mtDNA copy number, telomere length and hTERT protein level, while telomerase activity was very different between co-twins (***Figure 8***).

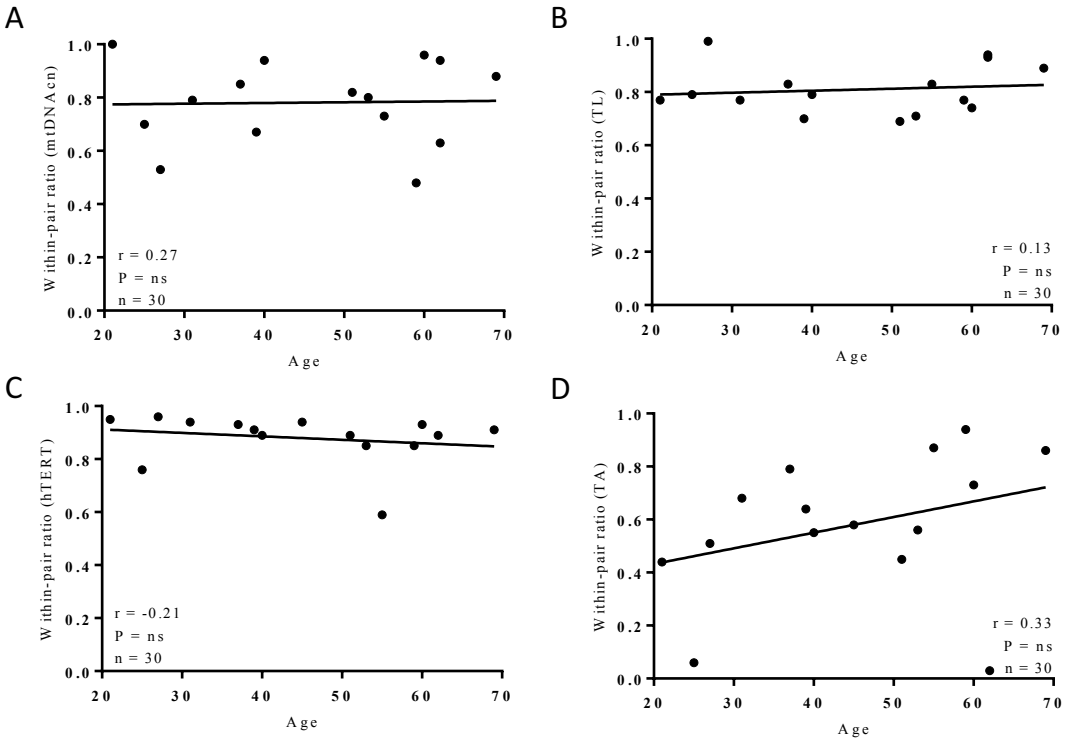

Figure 8. Within-pair similarity of mtDNAcn, TL, hTERT protein level and TA in twins aged 21-69

A) *mtDNA copy number (mtDNAcn),* ***B)*** *telomere length (TL),* ***C)*** *hTERT protein level (hTERT) and* ***D)*** *telomerase activity (TA). The Y axis shows within-pair ratios, the X axis represents age. Dots represent twin pairs. Correlation coefficients (r), significance level (p) and number of twins (n) are included.*

Within-pair ratio was calculated for each twin pair, in each case by dividing the value of the lower scoring twin by the value of his/her co-twin. The more the values approximate 1.0, the more similar were the twins of the given pair.

4.1.7. Twin correlation analysis for mtDNAcn, TL, hTERT and TA levels

We aimed to assess similarity data of monozygotic twin pairs, whereby we found positive association between telomere length and mitochondrial DNA copy number ($r = 0.81$, $p < 0.001$). However, telomere length showed no association with either TA or hTERT protein level. No correlation could be observed between telomerase activity and hTERT protein level, either (***Table 8***).

Table 8: Correlation analysis of mtDNAcn, TL, hTERT and TA levels

Within-pair ratio is calculated for each twin pair, in each case by dividing the value of the lower scoring twin by the value of his/her co-twin for the following variables separately: mtDNAcn, TL, hTERT and TA. The within-pair ratios were used to calculate Pearson correlation among the four variables (mtDNAcn, TL, hTERT and TA) by applying logarithmic transformation, n=30. (In case of one twin pair (displayed under code 15 in Table 5) data about telomerase activity was not available for the second twin due to missing data.)

TL: telomere length, mtDNAcn: mitochondrial DNA copy number, TA: telomerase activity, hTERT: hTERT protein level inside the PBMC gate, ns: not significant

	mtDNAcn	TL	hTERT	TA
mtDNAcn	.	0.81 (p<0.001)	-0.09 (p=ns)	-0.13 (p=ns)
TL	.	.	-0.14 (p=ns)	-0.19 (p=ns)
hTERT	.	.	.	0.06 (p=ns)
TA	.	.	.	.

4.2. mtDNA copy number – telomere length study

A total of 142 asymptomatic adult twins, comprising of 96 monozygotic- (48 complete pairs) and 46 dizygotic twins (23 complete pairs) were included in the analysis. The mean age of participants was 50.54 ± 15.42 years (range 20 – 75 years). All subjects completed detailed health history questionnaire and provided blood sample. The subjects in the selected sample were in generally good health, without any acute medical conditions.

DNA was isolated from peripheral blood mononuclear cells, absolute telomere length (kilobase per diploid cell) and absolute mitochondrial DNA copy number (number of circular DNA per cell) were analysed by qPCR standard curve method. During TL and mtDNAcn measurement, age and twin status of participants were completely blinded for all samples. By measuring mtDNAcn and TL, mtDNA content in the cohort was 201.57 ± 103.73, and TL was 167.35 ± 84.74 (mean ± SD). ***Table 9*** details demographic and metabolic characteristics of participants, as well as mtDNAcn and TL values, stratified to MZ and DZ twin groups ***(Table 9).***

Table 9. Demographic and metabolic characteristics of participants

Data is shown as means and ± standard deviation.

Abbreviations: mtDNAcn: mitochondrial DNA copy number, TL: telomere length, BMI: body mass index, HDL: HDL-cholesterol, TC/HDL: TC and HDL ratio, LDL: LDL-cholesterol, LDL/HDL: LDL and HDL ratio, TG: Triglyceride

Abbreviations: mtDNAcn: mitochondrial DNA copy number, TL: telomere length, BMI: body mass index, HDL: high-density lipoprotein cholesterol, LDL: low-density lipoprotein cholesterol, ApoA1: apolipoprotein A1, ApoB: apolipoprotein B, n: number of individuals

	Monozygotic twins		Dizygotic twins	
	mean ± SD	n	mean ± SD	n
Age	47.71 ± 14.94	96	56.43 ± 15.06	46
mtDNAcn	199.60 ± 106.10	96	203.80 ± 101.10	46
TL	166.30 ± 84.92	96	171.90 ± 85.33	46
BMI	24.96 ± 4.47	96	27.92 ± 6.22	46
Glucose	4.99 ± 1.51	73	5.05 ± 0.77	28
Cholesterol	5.57 ± 1.18	92	5.49 ± 1.16	43
HDL	1.68 ± 0.80	92	1.75 ± 0.68	41
LDL	3.27 ± 1.14	92	3.20 ± 1.18	41
LDL/HDL	2.31 ± 1.24	92	2.14 ± 1.13	41
Cholesterol/HDL	3.79 ± 1.56	92	3.51 ± 1.33	41
Triglyceride	1.48 ± 0.92	92	1.50 ± 0.90	43
ApoA1	1.48 ± 0.40	89	1.60 ± 0.27	43
ApoB	1.21 ± 0.42	92	1.18 ± 0.39	43
Lipoprotein A	0.41 ± 0.51	90	0.19 ± 0.22	40
Carbamide	5.09 ± 1.37	92	5.30 ± 1.56	43
Creatinine	71.74 ± 10.52	91	69.33 ± 11.29	43
Systolic blood pressure	123.30 ± 15.02	81	133.90 ± 20.35	37
Diastolic blood pressure	76.17 ± 8.78	81	80.70 ± 10.85	37
Pulse rate	76.83 ± 8.22	80	77.92 ± 8.75	37
CRP	3.39 ± 8.47	74	2.16 ± 1.61	31
Hip circumference (cm)	88.24 ± 14.07	37	90.50 ± 15.48	22
Waist circumference (cm)	98.57 ± 9.03	37	101.50 ± 9.74	22
Weight (kg)	70.42 ± 15.33	96	75.75 ± 16.06	46
Height (cm)	167.6 ± 9.91	96	165.00 ± 6.63	46

4.2.1. Association of mtDNAcn, TL and clinical data

We carried out bivariate analysis and presented age and sex corrected standardized regression coefficients. The results of the bivariate analysis indicated that mtDNA copy number and telomere length were positively associated ($p < 0.01$). ***Table 10*** also shows that lower levels of HDL cholesterol were significantly associated with shorter telomere length ($p < 0.001$), while lower pulse rate and carbamide levels were associated with higher TL ($p < 0.001$ and $p < 0.01$ respectively). As for mtDNA copy number, lower hip-and waist circumference were correlated with higher mtDNAcn ($p < 0.05$) while higher ApoB and CRP levels were positively associated with mtDNAcn ($p < 0.05$) (***Table 10)***.

We confirmed positive association between mtDNAcn and TL (see ***Table11*** for age and sex corrected results). Not displayed in the tables, after correcting the estimates with the additional significant predictors these were modified from 0.277 ($p < 0.01$) to 0.25 ($p < 0.05$) in case of mtDNAcn predicted by TL (and controls); and from 0.278 ($p < 0.01$) to 0.239 ($p < 0.01$) in case of TL predicted by mtDNA (and controls).

Table 10. Bivariate association. Age and sex corrected standardized regression coefficients

	mtDNA copy number Estimate	Telomere length Estimate
Birth order	0.092*	0.037
Birth week	-0.099	0.042
Birth week of pregnancy	0.042	0.022
BMI	-0.019	-0.203
Glucose	0.021	-0.064
Cholesterol	-0.032	0.007
HDL	-0.001	0.300***
LDL	-0.030	-0.194
LDL/HDL	0.034	-0.236
Cholesterol/HDL	0.056	-0.201
Triglyceride	0.007	-0.041
ApoA1	-0.173	-0.119
ApoB	0.153*	-0.041
Lipoprotein A	0.130	-0.026
Carbamide	-0.041	-0.304**
Creatinine	-0.125	0.057
Systolic blood pressure	0.143	-0.095
Diastolic blood pressure	0.134	-0.062
Pulse rate	-0.025	-0.427***
DM	0.008	0.081
Alcohol	-0.004	0.007
Smoking (present)	0.090	0.183**
Smoking (past)	-0.098	-0.110
Hypertonia	0.145	-0.069
CRP	0.126*	0.047
Sport	0.042	-0.085
Hip circumference	-0.370*	-0.120
Waist circumference	-0.373*	-0.280
Weight	-0.090	-0.210
Height	-0.208	0.033
mtDNA copy number	-	0.278**
Telomere length	0.277**	-

**** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$*

Table 11 shows the regression model controlling only for age and sex (bivariate columns) and for all significant predictors (multivariate columns) that were found significantly associated with mtDNAcn or TL based on the bivariate analysis of ***Table 10***. The association of mtDNAcn and TL was confirmed, which do not depend on relevant controls.

Table 11. Regression unstandardized coefficients

	mtDNA copy number		Telomere length	
	Bivariate	Multivariate	Bivariate	Multivariate
Telomere length	0.257**	0.23*		
mtDNA copy number			0.3**	0.264**
Birth order		0.033		
ApoB		0.017		
CRP		0.014		
Waist circumference		-0.005		
HDL				0.062**
Carbamide				-0.036*
Pulse rate				-0.001***
Smoking				0.096*
Age	0.002	0.002	-0.001	0.002
Female	-0.003	0.014	-0.048	-0.099^{+}

**** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, $^{+}$ $p < 0.1$*

4.2.2. Intraclass correlation for mtDNAcn and TL of MZ and DZ twins

We approached the issue of possible genetic and environmental contribution, by calculating intraclass correlation coefficients (ICCs) for mtDNAcn and TL among monozygotic and dizygotic twins. ICC enumerates the proportion of variation across the families of the twins, as such estimating co-twin similarity. Intraclass correlation coefficients for mtDNAcn and TL were calculated for both MZ and DZ pairs respectively, to be able to compare their co-twin similarity values.

In the case of telomere length, strong ICC values were measured for both MZ (ICC=0.794) and DZ twins (ICC=0.785). ICC values for mtDNA copy number were also strong, with MZ twins (ICC=0.758) presenting slightly higher results compared to DZ twins (ICC= 0.641) ***(Table 12).***

Table 12. Intraclass correlations for mtDNAcn and TL by zygosity

The results of 96 MZ twin subjects (48 complete pairs) and 46 DZ twin subjects (23 complete pairs) are presented.

Intraclass correlation coefficient enumerate the proportion of variation in the sample across the families of the twins, as such, estimating co-twin similarity. The corresponding 95% confidence intervals are included.

Abbreviations: MZ: monozygotic twins, DZ: dizygotic twins, mtDNAcn: mitochondrial DNA copy number, TL: telomere length, Corr.ICC: age and sex corrected intraclass correlation coefficient, CI: confidence interval

		Corr.ICC	CI Low	CI High
TL	MZ	0.794	0.672	0.885
	DZ	0.785	0.568	0.905
mtDNAcn	MZ	0.758	0.614	0.862
	DZ	0.641	0.353	0.845

4.2.3. Illustration of within-pair similarity

We note that our studied 71 pairs of MZ and DZ twins belonged to different age groups, equally representing an age range of 20 to 75 years. To highlight the dynamics of co-twin similarity with advancing age, within-pair ratios were calculated. Within-pair ratio is gained when in case of a given twin pair, the value of the twin, scoring lower than his/her co-twin is divided by the value of the other twin, thus, the within-pair ratio expresses within-pair similarity. The more the value approximates 1.0, the greatest is the similarity between members of a given twin pair.

We observed that despite increasing age, within-pair ratios remained high for mtDNA copy number, telomere length for both MZ and DZ co-twins that further illustrated our age and sex corrected intraclass correlation coefficient calculation results (***Figure 9***).

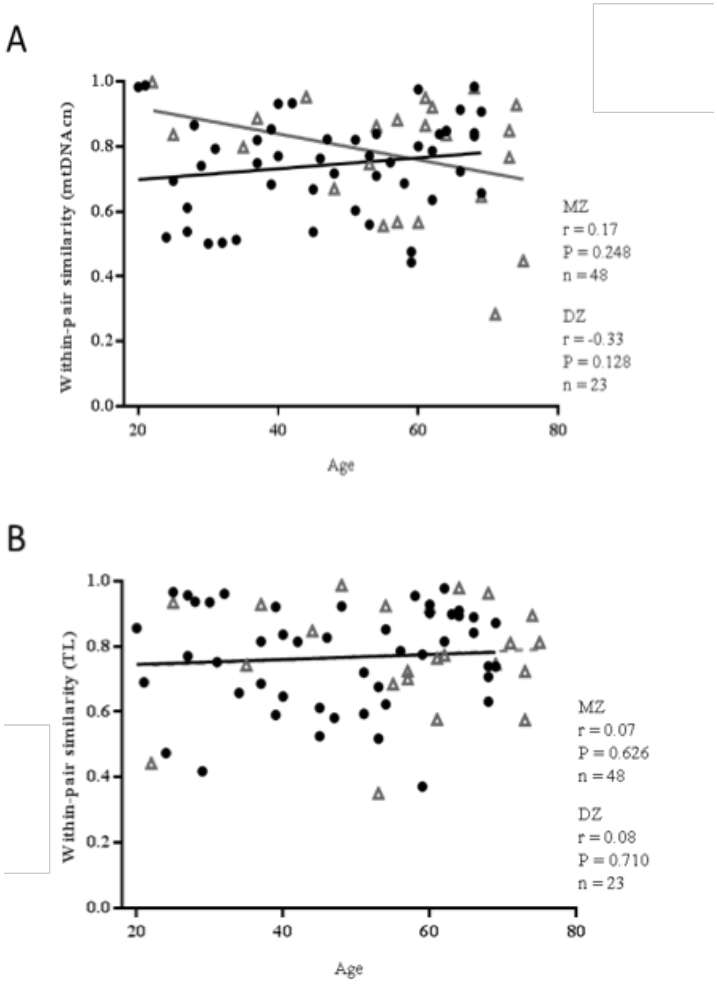

Figure 9. Within-pair similarity of mtDNAcn and TL in twins aged 20-75

The Y axis shows within-pair similarity, the X axis represent age. Black dots represent MZ within-pair similarity values with corresponding black trendline, grey triangles represent DZ within-pair similarity values with corresponding grey trendline. Correlation coefficients (r), significance level (P) and number of twins (n) are included. Within-pair similarity is calculated for each twin pair, in each case by dividing the value of the lower scoring twin by the value of his/her co-twin. The more the values approximate 1.0, the more similar are the twins of the give pair. A) shows within-pair similarity for mtDNAcn in MZ and DZ twins with increasing age. B) shows within-pair similarity for TL in MZ and DZ twins with increasing age.

Abbreviations: MZ: monozygotic twin, DZ: dizygotic twins, mtDNAcn: mitochondrial DNA copy number, TL: telomere length

To better illustrate female and male similarities, we divided our cohort by sex, and compared female and male within-pair similarity measures for mtDNAcn an TL. ***Table 13*** displays female and male mean within-pair similarity values of MZ and DZ twins, presenting no significant difference between female and male twin pairs in either mtDNAcn or TL.

Table 13. Within-pair similarity of mtDNAcn and TL in female and male twin pairs

Within-pair similarity is calculated for each twin pair, in each case by dividing the value of the lower scoring twin by the value of his/her co-twin. The more the values approximates 1.0, the more similar are the twins of a given pair. No significant difference was found between the female and male subgroups in the average value of within-pair ratios of mtDNAcn and TL.

Abbreviations: mtDNAcn: mitochondrial DNA copy number, TL: telomere length, n: number of individuals

	n	mtDNAcn (mean ± SD)	TL (mean ± SD)
Female	50	0.77 ± 0.17	0.75 ± 0.17
Male	21	0.71 ± 0.13	0.82 ± 0.13
Female MZ	31	0.77 ± 0.16	0.73 ± 0.16
Male MZ	18	0.71 ± 0.13	0.82 ± 0.13
Female DZ	20	0.78 ± 0.19	0.77 ± 0.17
Male DZ	3	0.70 ± 0.17	0.79 ± 0.17

5. Discussion

5.1. Implications of the results from the monozygotic twin study

Both telomerase activity, hTERT protein level, telomere length and mitochondrial DNA copy number are important markers in reflecting the replicative capacity of cells. We aimed to contribute to the deeper understanding of the telomerase system and telomere-mitochondria interplay by simultaneously investigating four variables in a genetically controlled study design. In the frame of the monozygotic twin study, we analyzed lymphocyte subpopulations of 32 twins and compared telomerase activity, hTERT protein level, telomere length and mtDNA copy number measured from PBMCs. In addition, we assessed the distribution of different T-cell subpopulations and measured hTERT protein level in T lymphocytes, helper-, cytotoxic- and regulatory T-cell subgroups. In our study design, we approached the subject of possible genetic contribution by calculating intraclass correlations among identical twins. Intraclass correlation coefficients enumerate the proportion of variation across the families of the twins, as such, estimating co-twin similarity. For our investigations, we worked with several techniques. Multi-parametric flow cytometry was applied for the characterisation of lymphocyte subsets and assessment of hTERT protein level, telomerase activity was measured using TRAP-ELISA assay, while telomere length and mitochondrial DNA copy number were determined by qPCR standard curve method.

Assessing hTERT protein level and distribution of different T-cell subgroups, hTERT protein level was demonstrated with very high ICCs in T lymphocytes (0.891) and in both helper-, (0.896), cytotoxic-, (0.798) and regulatory T-cell (0.885) subsets. Regarding the distribution of T-cell subgroups, high ICCs were found in regulatory T-cells (0.728) and cytotoxic T-cells (0.827) (***Table 6***). Analyzing the results of our four simultaneously measured parameters from PBMCs, high intraclass correlation was calculated for telomere length (0.815), considerable for mtDNA copy number (0.524) and again, exceptionally high ICC was detected in hTERT protein levels (0.946), while in contrast, telomerase activity showed no co-twin similarity (ICC=0) (***Table 7***). Considering the very high co-similarity between twins for the rate-limiting telomerase

subunit, hTERT protein, and also in case of telomere length and mtDNA copy number, the fact that monozygotic twins were highly different in their telomerase activity, can be described as a rather striking result. High intraclass correlations seem to be relevant for MZ twin pairs, suggesting that identical twins were very similar in the given variable, due to their shared genetics, maternal and further epigenetic effects as well. For the differences between identical twins, individual specific non-genetic (potential epigenetic) factors could be responsible, which should be further investigated by expanding the limitations, while building on and utilizing the results of our presented studies. We also note that we found telomerase activity to be very different not only between members of twin pairs but also among twin pairs (***Figure 7***). We further remark that the 16 pairs of identical twins belonged to different age groups, equally representing an age range of 21 to 69 years. The fact that despite increasing age, within-pair ratios remained high for mtDNA copy number, telomere length and hTERT protein level, while telomerase activity was very different between co-twins (***Figure 8***), is also of notice and should be further investigated and confirmed on larger twin samples as well.

We carried out our measurements for the characterization of lymphocyte subgroups applying concurrent staining of surface antigens, intracellular hTERT protein and multi-parametric flow cytometry. With the help of this technique, we could compare the level of hTERT protein in different lymphocyte subgroups, since by applying multicolor flow cytometric detection of intracellular hTERT protein level, it was possible to measure the relative amount of hTERT protein on a single cell level in different lymphocyte subsets, without requiring the separation of the individual cell types (Melicher et al., 2018). We detected that regulatory T-cells contained significantly higher level of hTERT protein compared to other T lymphocytes, cytotoxic- or helper T-cells (***Figure 6***), in light of which, further investigations could help provide deeper insights into the regulation of the activation of regulatory T-cells. Multi-parametric flow cytometry could be the bridge between proteomics and cytomics, since it can characterize the functional and morphological properties of single cells at the same time.

Telomerase plays a crucial role in the pathology of aging and cancer through maintaining genome integrity, controlling cell proliferation, and regulating tissue

homeostasis (Zhou et al., 2014). The human immune system is an ample example of a highly dynamic cellular network with unique characteristics, one of which is the regulation of the telomere/telomerase system (Melicher et al., 2015). Telomerase is repressed in most human somatic cells, but lymphocytes are unique in the sense that they retain the ability to upregulate telomerase upon activation, which allows the intense clonal expansion and proliferation, required to generate effector cells and long lived memory cells while maintaining telomere length (Chou and Effros, 2013; Maini et al., 1999). Telomerase activity is induced in T and B lymphocytes upon antigen stimulation, it was established that T-cells depend on telomerase activity for their proliferation after being activated, but lose the capacity to up-regulate the enzyme during senescence, eventually resulting in telomere shortening (Effros et al., 2003). Earlier research also revealed that in T-cells telomerase function is regulated via phosphorylation and nuclear translocation (Liu et al., 1999). Capacity for cellular replication is vital for lymphocyte function, and telomerase activity is commonly referred to as a marker of the proliferative activity of cells. Telomerase down-regulation in lymphocytes may protect against tumorigenesis, yet also leads to replicative senescence of repeatedly activated memory T-cells (Maida and Masutomi, 2015). The human telomerase holoenzyme is essentially composed of an RNA component, hTERC, which serves as a template for telomeric DNA synthesis, and the catalytic core subunit, telomerase reverse transcriptase (hTERT). The canonical function of TERT was described as the synthesis of telomeric DNA repeats, and the maintenance of telomere length. It was established that hTERT protein level was the rate-limiting factor for reconstituting telomerase activity and for telomere length homeostasis *in vivo* (Cong et al., 2002; Ramlee et al., 2016). However, accumulating evidence has demonstrated that TERT could also have some fundamental functions that are independent of its enzymatic activity (Zhou et al., 2014). These activities were referred to as non-telomeric/extra-telomeric/extra-curricular/non-canonical or telomere-independent functions of telomerase. These newly discovered activities of TERT influenced various essential cellular processes, such as gene expression, signaling pathways, mitochondrial function as well as cell survival and stress resistance (Saretzki, 2014). Furthermore, it was revealed that telomere-independent functions of telomerase could be performed in various cellular compartments besides the nucleus, including the mitochondria (Saretzki, 2014).

Recently a growing number of research papers reported about the non-canonical functions of telomerase in human cells, however limited data exists on human immune cells as only few studies investigated hTERT specifically in lymphocytes (Saretzki, 2014). A recent review (Saretzki, 2014) summarized findings about extra-telomeric functions of human telomerase, whereby noted that the scenario about the shuttling of hTERT from the nucleus to mitochondria seemed to be typical for non-lymphocytic cells, whereas in T lymphocytes telomerase seemed to reside in the cytoplasm of unstimulated cells and actively transported to the nucleus upon activation (Akiyama et al., 2003; Akiyama et al., 2004; Kawauchi et al., 2005; Liu et al., 2001; Saretzki, 2014). The idea emerged that one of the reasons why the efforts to develop novel therapeutic strategies using TERT as a molecular target for anticancer treatment could not entirely succeed yet, might be the overlooking of the non-canonical functions of hTERT and it was suggested that targeting those might facilitate the development of novel cancer treatments (Maida and Masutomi, 2015).

With respect to the above findings and as more and more information has been revealed about hTERT taking part in several, crucial processes for the cell integrity, further research investigating the role of this protein should be carried out specifically focusing on human lymphocytes as well. Taking into account our findings, that regulatory T-cells contained significantly more hTERT protein than other T-cell subgroups, regulatory T-cells deserve special attention. Continuing research for *in vivo* elucidation of extra-telomeric functions and tracking potential subcellular localisation of hTERT in lymphocytes could further help in the better understanding of the immune regulation, as it can be hypothesized that crucial non-canonical functions of hTERT might also contribute to the proliferative capacity or integrity of cells, among others possibly by altering the relative survival and clonal amplification of hTERT expressing cells. We suggest that investigations using human lymphocyte samples should address the above questions integrally, preferably in the frame of twin studies.

The relationship of telomere length and telomerase activity in human lymphocytes still holds many unanswered questions, partly because *in vivo* studies involving human subjects are complicated to design and among others, various physiological and pathological conditions as well as undisclosed epigenetic mechanisms might influence the findings, which are often contradictory. Despite the potential

confounders, we are convinced that in the effort to gain deeper insights about the inter-relationship of telomere length and telomerase activity, and about underlying regulatory processes, more studies experimenting with human lymphocytes should be published. We alongside propose that for the investigation of potential genetic and epigenetic factors, more extensive research should be carried out with enlarged sample sizes, carefully selected participants, while applying sophisticated experimental setting. In the frame of our monozygotic twin study, our investigations involving 16 pairs of identical twins can be interpreted as a pilot study, and the results are calling for further research with larger sample size and with the involvement of disease discordant twins. Given the complex nature of common diseases, epigenetic studies applying the discordant twin design should be highly encouraged. The powerful case co-twin analysis combined with multi-parametric research tools should help to identify important environmental risk factors responsible for disease or health end points (Tan et al., 2015).

5.2. Implications of the results of the mtDNAcn-TL study

Mitochondria are essential organelles of the cell, generating most of the ATP, fundamental for cellular functions, and have an integral role in both cell growth, differentiation, replication and death. The aging process is characterized by increases of mitochondrial reactive oxygen species production, accumulating mutations in mtDNA, dysfunction of mitochondrial biogenesis and an overall decline in mitochondrial function (Bratic and Larsson, 2013; Kazachkova et al., 2013). Mitochondrial DNA copy number, the number of circular mitochondrial DNA per cell, provides an indirect way for measuring mitochondrial biogenesis, the process that could compensate for the increasing energy demand and compromised mitochondrial function (Sahin and DePinho, 2012). Lately, key links have been revealed between telomere shortening and mitochondrial dysfunction, two mechanisms that are both considered to play central role in the aging process (Sahin et al., 2011; Sahin and DePinho, 2012). Studies mostly on cell lines demonstrated that mitochondrial stress could contribute to telomere attrition and vice versa (Correia-Melo et al., 2014; Passos et al., 2007). Telomere shortening was reported to be associated with impaired mitochondrial biogenesis and function, as well as increased ROS level, and following experiments with a telomere dysfunction mouse

model, it was proposed that the telomere-p53-peroxisome proliferator-activated receptor gamma coactivator axis could have a direct connection with telomere dysfunction and mitochondrial compromise (Kim et al., 2013; Sahin et al., 2011). Until now, there have been limited number of studies that experimented with human lymphocyte samples while researching the topic. A recent publication, analyzing different T-cell subsets from healthy human donors, reported that ROS that accumulated with age could contribute to telomere shortening in primary memory T lymphocytes (Sanderson and Simon, 2017). The study demonstrated for the first time a correlation between age and increased mitochondrial reactive oxygen species in CD8+ T-cell subsets and suggested a strong link between age and telomere length, mitochondrial ROS and health (Sanderson and Simon, 2017). The authors of the report highlighted that oxidative damage contributing to telomere attrition could be true under physiological conditions in humans as well (Sanderson and Simon, 2017).

Common mechanisms and complicated telomere-mitochondria interplay during human ageing were suggested, however the relationship is still not completely understood (Zole and Ranka, 2018). Besides experimental studies, population studies have also started to highlight the dynamic nature and complexity of this interaction (Zole and Ranka, 2018). In addition to molecular studies, as an alternative approach of investigating the potential association between telomere status and mitochondrial function, research groups started to examine specifically the relationship of mtDNAcn and TL in the frame of population studies (Alegria-Torres et al., 2016; Kim et al., 2013; Li et al., 2015; Monickaraj et al., 2012; Otsuka et al., 2017; Pieters et al., 2015; Qiu et al., 2015; Revesz et al., 2018; Tyrka et al., 2015; Tyrka et al., 2016; Zhu et al., 2017; Zole et al., 2017). Most of the publications reported positive association between mtDNAcn and TL such as in healthy subjects (Kim et al., 2013; Revesz et al., 2018; Tyrka et al., 2015), in elderly people (Pieters et al., 2015; Zole et al., 2017), in children (Alegria-Torres et al., 2016), pregnant women (Qiu et al., 2015) and colorectal carcinomas (H. Lee et al., 2017). Negligible but significant negative correlation between mtDNAcn and TL was also found (Zhu et al., 2017), and inverse association was detected in patients with psychiatric disorders (Li et al., 2015; Otsuka et al., 2017; Tyrka et al., 2016). A recent report investigating the combined impact of telomere length and mtDNAcn on cognitive functions of community-dwelling very old adults

suggested that the two parameters might be useful for monitoring cognitive decline in older people (J. Y. Lee et al., 2017).

The detailed mechanism of association between telomere status and mitochondrial function in humans still remains to be determined, and demands more focus and attention (Zole and Ranka, 2018).

The promise of using telomere length and mtDNA copy number as combined biomarkers of health and disease prompted us to investigate the relationship of these two important cellular markers, in the frame of a genetically controlled study design, at the same time assessing co-twin similarities of monozygotic- and dizygotic twin subjects. With our results, we extended the thus far limited number of studies investigating mtDNAcn and TL simultaneously in humans. Furthermore, we are the first to analyze the relationship between telomere length and mtDNAcn in MZ and DZ twin subjects. We involved a total of 142 asymptomatic twins into our study who represented different age groups ranging from 20 to 75 years.

The twin structure provides unique opportunities to disentangle genetic and environmental factors that contribute to the variance in a certain phenotype (Bell and Spector, 2011; Tan et al., 2013).

Classical twin studies have served as a powerful tool in biomedical, psychiatric and behavioural research for decades, the method compares the phenotypic similarity of monozygotic and dizygotic twins to estimate the importance of heritable and environmental influences on complex trait variation (van Dongen et al., 2012). The common environment involves all environmental factors that make a certain twin pair similar for a given trait, such as shared womb, childhood experiences and early socialization or parental socioeconomic status. The term, unique environment, includes all environmental factors and experiences to which only one member of the twin pair was exposed to, making co-twins dissimilar, such as certain viral infections, accidents, individual life events, etc. (van Dongen et al., 2012)..

Although the size of our sample was not sufficient for full fetched heritability analysis using the classical twin design and structural equation modeling, still, as this study is the first of its kind, we presented ICCs and consider it a pilot study. For our estimations regarding possible genetic or environmental contribution, intraclass correlation coefficients were calculated for TL and mDNAcn among MZ and DZ twins,

as ICC enumerates the proportion of variation across the families of the twins, as such estimating co-twin similarity. We found that twins were similar in their ICC measures irrespective of zygosity, suggesting a possibly more important role of common (shared) environmental factors compared to non-shared (unique) environmental and to a smaller degree also individual genetic influences. It should be noted that while conclusive inferences cannot be drawn due to the uncertainty of these estimates (mainly owing to the low, especially DZ, sample size), it appears that common environmental factors might play a considerable role for both TL and mtDNAcn.

We also aimed to investigate if any of the studied baseline clinical data could explain inter-individual variation in TL and mtDNAcn in subjects of different age and sex and with generally good health condition. Our age and sex corrected results confirmed significant positive association between TL and mtDNAcn. Following bivariate estimates and correction with significant predictors, the independent positive associations were further verified. Our findings of positive association between TL and mtDNAcn firstly shown in twin subjects, was in line with recent population based studies that also reported positive relationship in healthy subjects (Kim et al., 2013; Revesz et al., 2018; Tyrka et al., 2015).

We acknowledge a number of shortcomings of our study, in the first place, the still moderate sample size should be further increased. We note that for the regression analysis, in some cases clinical data was missing for certain twin subjects due to incidental technical issues of blood laboratory assessment or missing demographic data. However, these were treated using advanced statistical methods and without the discarding of any information, such as the elimination of the person from the analysis. We also remark that measuring mtDNAcn in DNA extracted from whole blood instead of from peripheral blood mononuclear cells may yield different results due to mtDNA present in platelets. In order to prevent this inconsistency, DNA was extracted from PBMCs in case of all the samples.

The results of our pilot studies should inspire further investigations with considerably increased sample sizes and by involving twins discordant for certain disease phenotypes, while longitudinal studies should also be encouraged.

As both intra- and interpair comparisons between twins are equally powerful tools, studying twins can make new contributions in revealing and understanding both the

genetic and epigenetic mechanisms (Tan et al., 2013) in the regulation of the immune system in vivo (Melicher et al., 2018).

In conclusion, we suggest that further studies should investigate the potential of using the combination of telomere length and mitochondrial DNA copy number as possible biomarkers in health, and in various specific disease conditions in the future.

6. Conclusions

To gain more insights about the telomerase system and telomere-mitochondria interplay, we simultaneously defined and compared mitochondrial DNA copy number, telomere length, level of the catalytic reverse transcriptase protein subunit of telomerase, hTERT, and telomerase activity in genetically matched subjects. Using PBMCs of 32 monozygotic twin subjects, we applied qPCR standard curve method, multi-parametric flow cytometry and TRAP-ELISA assay. Aiming to estimate the level of co-twin similarity, we pursued intraclass correlation coefficient measurements. Analyzing lymphocyte subgroups of identical twins, we were the first to show very high ICC for hTERT protein levels in T lymphocytes and both in the helper-, cytotoxic- and regulatory T-cell subgroups, while analysing the similarity of the distribution of T lymphocyte subpopulations, high ICC was detected in regulatory- and cytotoxic T-cells. Assessing the results of four simultaneously measured parameters, high intraclass correlation was calculated for telomere length, considerable for mtDNA copy number and again, exceptionally high ICC was detected in hTERT protein levels, while in contrast, telomerase activity showed no co-twin similarity.

We analysed the distribution of lymphocyte subgroups present inside the lymphocyte gate and determined the level of the catalytic reverse transcriptase protein subunit of telomerase, hTERT in the different subgroups, using concurrent staining of surface antigens, intracellular hTERT protein and multi-parametric flow cytometry. We presented novel information by firstly characterizing the relative amount of hTERT protein in different lymphocyte subpopulations, whereby we showed that regulatory T-cells contained significantly higher level of hTERT protein compared to other T lymphocytes, cytotoxic- or helper T-cells.

The relationship of telomere length and telomerase activity in human lymphocytes still remains elusive, as *in vivo* studies involving human subjects are complicated to design and various physiological and pathological conditions and undisclosed epigenetic mechanisms might influence the findings, which are often contradictory. Despite the potential confounders, our results emphasized that in the effort to gain more insights about the inter-relationship of telomere length and

telomerase activity, and about underlying regulatory processes, more extensive research based on studying human lymphocytes in twin subjects should be carried out.

We were the first to examine the relationship between mtDNAcn and telomere length in MZ and DZ twin subject. We aimed to gain further insights about the potential association between telomere status and mitochondrial function. In our extended analysis, we involved 142 asymptomatic twins, comprising of 96 monozygotic- (48 complete pairs) and 46 dizygotic twins (23 complete pairs), representing different age groups ranging from 20 to 75 years. We confirmed significant positive association between TL and mtDNAcn ($r=0.28$, $p<0.01$) in age and sex corrected analysis. Following bivariate estimates and correction with additional significant predictors, the independent positive relationships were further verified. Assessing co-twin similarities of monozygotic and dizygotic twin subjects, we found that twins were similar in their intraclass correlation measures irrespective of zygosity, suggesting a possibly more important role of common (shared) environmental factors compared to non-shared (unique) environmental and to a smaller degree also individual genetic influences. We propose that further studies should investigate the potential of using the combination of telomere length and mtDNA copy number as possible biomarkers in health and in various specific disease conditions in the future.

The results of our pilot studies should inspire further research, we propose that for the future investigation of potential genetic and epigenetic factors, higher number of twin studies should be carried out with increased sample sizes, carefully selected participants, and applying sophisticated research methods. The involvement of twins discordant for certain disease phenotypes, as well as longitudinal studies should also be encouraged. We are convinced that future twin studies can make new contributions in revealing and understanding both the genetic and epigenetic mechanisms in the regulation of the immune system *in vivo* (Melicher et al., 2018).

7. Summary

Aiming to contribute to the deeper understanding of the telomerase system and telomere-mitochondria interplay, we carried out the first study, whereby four variables were simultaneously assessed, applying twin study design. We pursued co-twin similarity measurements of hTERT protein level, telomerase activity, telomere length and mitochondrial DNA copy number in 16 pairs of monozygotic twins, applying multi-parametric flow cytometry, TRAP-ELISA assay and qPCR standard curve method on peripheral blood mononuclear cell samples. Analyzing co-twin similarity of lymphocyte subsets, we were the first to demonstrate very high intraclass correlation coefficients (ICC) of hTERT protein level in T lymphocytes (0.891) and in both helper T-cells (0.896), regulatory T-cells (0.885) and cytotoxic T-cells (0.798). In combined assessment of PBMCs, high intraclass correlation was calculated for telomere length (0.815), considerable for mtDNA copy number (0.524) and again, exceptionally high ICC was detected in hTERT protein level (0.946), while in contrast, telomerase activity showed no co-twin similarity (ICC=0). Besides, we provided novel information in characterizing the relative amount of hTERT protein in different lymphocyte subpopulations, whereby we showed that regulatory T-cells contained significantly higher level of hTERT protein compared to other T lymphocytes, cytotoxic- or helper T-cells. To further investigate the association of telomere length and mtDNA copy number, we substantially increased the sample size to a total of 142 healthy subjects, comprising of 96 monozygotic and 46 dizygotic twins, whereby significant positive association ($r=0.28$, $p<0.01$) was confirmed in age and sex corrected analysis. Following bivariate estimates and correction with significant predictors, the independent positive associations were further verified. In addition, analysing similarities of telomere length and mtDNA copy number of MZ and DZ twin subjects, we found that twins were similar in their ICC measures irrespective of zygosity, suggesting a possibly more important role of common environmental factors compared to non-shared (unique) environmental and to a smaller degree also individual genetic influences. We propose that further studies should investigate the potential of using the combination of telomere length and mtDNA copy number as possible biomarkers in health and in specific disease conditions in the future.

8. Összefoglalás

A telomeráz rendszer és a telomer-mitokondrium összefüggéseiről való ismeretek bővítése céljával az első olyan kísérletsorozatot végeztük, melyben négy változó egyidejű elemzése történt, iker kutatási módszer alkalmazásával. Iker hasonlósági méréseket végeztünk a hTERT fehérje szint, a telomeráz aktivitás, a telomer-hossz és a mitokondriális DNS kópiaszám tekintetében 16 egypetéjű ikerpárban. Kísérleteink során multiparaméteres áramlási citometria, TRAP-ELISA meghatározás, valamint qPCR standard görbe módszereket alkalmaztunk, perifériás vér mononukleáris sejt mintákon. Az egyes limfocita alcsoportok iker-hasonlóságának elemzése során elsőként mutattunk ki igen magas intraklassz-korrelációs együttható (ICC) értékeket a T-limfociták hTERT fehérje szintjére vonatkozólag (0.891), éppúgy a helper T-sejtek (0.896), a regulátoros T-sejtek (0.885), valamint a citotoxikus T-sejtek (0.798) esetében. A PBMC sejtek kombinált elemzése során magas intraklassz-korreláció értéket mutatott a telomer-hossz (0.815), számottevő mértékűt a mtDNS kópiaszám (0.524) és kifejezetten magasat a hTERT fehérje szintje (0.946), míg a telomeráz aktivitás mértéke rendkívül eltérőnek bizonyult az ikrek között (ICC=0). Emellett a hTERT fehérje relatív szintjét különböző limfocita alcsoportokban elemezve, új eredményként sikerült demonstrálnunk, hogy a regulátoros T-sejtek szignifikánsan magasabb hTERT protein szintet tartalmaztak más T limfociták, citotoxikus- és helper T-sejtekhez képest. A telomer-hosszúság és a mtDNS kópiaszám kapcsolatának további vizsgálata céljából a mintaszámot lényegesen bővítettük. 142 egészséges alany, köztük 96 egypetéjű és 46 kétpetéjű iker bevonásával szignifikáns pozitív asszociációt igazoltunk ($r=0.28$, $p<0.01$) életkor és nem korrigált analízist végezve. Bivariáns elemzés és további szignifikáns prediktorokkal történő korrigálást követően a független pozitív asszociáció további megerősítésre került. Emellett a MZ és DZ ikrek telomer-hossz és mtDNS kópiaszám hasonlóságát elemezve az találtuk, hogy az ikrek intraklassz-korrelációjának mértéke zigozitástól függetlenül hasonlóságot mutatott, mely a közös környezeti tényezők valószínűsíthetően nagyobb szerepét sugallja a nem-közös (egyéni) környezeti és kisebb mértékben az egyéni genetikai hatásokhoz képest. Javasoljuk további kutatások során vizsgálni a telomer-hossz és a mtDNS kópiaszám kombinációjának biomarkerként való alkalmazhatóságának lehetőségét egészségesekben és specifikus kórképek esetén.

9. Bibliography

Ahmed S, Passos JF, Birket MJ, Beckmann T, Brings S, Peters H, Birch-Machin MA, von Zglinicki T, Saretzki G. (2008). Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. J Cell Sci, 121(Pt 7), 1046-1053. doi:10.1242/jcs.019372

Akbar AN, Vukmanovic-Stejic M. (2007). Telomerase in T lymphocytes: use it and lose it? J Immunol, 178(11), 6689-6694.

Akiyama M, Hideshima T, Hayashi T, Tai YT, Mitsiades CS, Mitsiades N, Chauhan D, Richardson P, Munshi NC, Anderson KC. (2003). Nuclear factor-kappaB p65 mediates tumor necrosis factor alpha-induced nuclear translocation of telomerase reverse transcriptase protein. Cancer Res, 63(1), 18-21.

Akiyama M, Yamada O, Hideshima T, Yanagisawa T, Yokoi K, Fujisawa K, Eto Y, Yamada H, Anderson KC. (2004). TNFalpha induces rapid activation and nuclear translocation of telomerase in human lymphocytes. Biochem Biophys Res Commun, 316(2), 528-532. doi:10.1016/j.bbrc.2004.02.080

Al-Attas OS, Al-Daghri NM, Alokail MS, Alfadda A, Bamakhramah A, Sabico S, Pritlove D, Harte A, Tripathi G, McTernan PG, Kumar S, Chrousos G. (2010). Adiposity and insulin resistance correlate with telomere length in middle-aged Arabs: the influence of circulating adiponectin. European Journal of Endocrinology, 163(4), 601-607. doi:Doi 10.1530/Eje-10-0241

Alegria-Torres JA, Velazquez-Villafana M, Lopez-Gutierrez JM, Chagoyan-Martinez MM, Rocha-Amador DO, Costilla-Salazar R, Garcia-Torres L. (2016). Association of Leukocyte Telomere Length and Mitochondrial DNA Copy Number in Children from Salamanca, Mexico. Genet Test Mol Biomarkers, 20(11), 654-659. doi:10.1089/gtmb.2016.0176

Andrew T, Aviv A, Falchi M, Surdulescu GL, Gardner JP, Lu XB, Kimura M, Kato BS, Valdes AM, Spector TD. (2006). Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs. American Journal of Human Genetics, 78(3), 480-486. doi:Doi 10.1086/500052

Antonio Moro-Garcia M, Alonso-Arias R, Lopez-Larrea C. (2012). Molecular Mechanisms Involved in the Aging of the T-cell Immune Response. Current Genomics, 13(8), 589-602.

Armanios M, Greider CW. (2005). Telomerase and cancer stem cells. Cold Spring Harb Symp Quant Biol, 70, 205-208. doi:10.1101/sqb.2005.70.030

Armanios MY, Chen JJL, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie MY, Vulto I, Phillips JA, Lansdorp PM, Greider CW, Loyd JE. (2007). Telomerase mutations in families with idiopathic pulmonary fibrosis. New England Journal of Medicine, 356(13), 1317-1326. doi:Doi 10.1056/Nejmoa066157

Arora R, Lee Y, Wischnewski H, Brun CM, Schwarz T, Azzalin CM. (2014). RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells. Nature Communications, 5, 5220. doi:10.1038/ncomms6220

Artandi SE, DePinho RA. (2010). Telomeres and telomerase in cancer. Carcinogenesis, 31(1), 9-18. doi:10.1093/carcin/bgp268

Ashar FN, Moes A, Moore AZ, Grove ML, Chaves PHM, Coresh J, Newman AB, Matteini AM, Bandeen-Roche K, Boerwinkle E, Walston JD, Arking DE. (2015). Association of Mitochondrial DNA levels with Frailty and All-Cause Mortality. Journal of molecular medicine (Berlin, Germany), 93(2), 177-186. doi:10.1007/s00109-014-1233-3

Atzmon G, Cho M, Cawthon RM, Budagov T, Katz M, Yang XM, Siegel G, Bergman A, Huffman DM, Schechter CB, Wright WE, Shay JW, Barzilai N, Govindaraju DR, Suh Y. (2010). Genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians. Proceedings of the National Academy of Sciences of the United States of America, 107, 1710-1717. doi:DOI 10.1073/pnas.0906191106

Aubert G, Lansdorp PM. (2008). Telomeres and aging. Physiol Rev, 88(2), 557-579. doi:10.1152/physrev.00026.2007

Avin BA, Umbricht CB, Zeiger MA. (2016). Human telomerase reverse transcriptase regulation by DNA methylation, transcription factor binding and alternative splicing (Review). Int J Oncol, 49(6), 2199-2205. doi:10.3892/ijo.2016.3743

Aviv A, Shay JW. (2018). Reflections on telomere dynamics and ageing-related diseases in humans. Philos Trans R Soc Lond B Biol Sci, 373(1741). doi:10.1098/rstb.2016.0436

Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E, Lingner J. (2007). Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. Science, 318(5851), 798-801. doi:10.1126/science.1147182

Bagheri S, Nosrati M, Li S, Fong S, Torabian S, Rangel J, Moore DH, Federman S, LaPosa RR, Baehner FL, Sagebiel RW, Cleaver JE, Haqq C, Debs RJ, Blackburn EH, Kashani-Sabet M. (2006). Genes and pathways downstream of telomerase in melanoma metastasis. Proceedings of the National Academy of Sciences of the United States of America, 103(30), 11306-11311. doi:DOI 10.1073/pnas.0510085103

Bakaysa SL, Mucci LA, Slagboom PE, Boomsma DI, McClearn GE, Johansson B, Pedersen NL. (2007). Telomere length predicts survival independent of genetic influences. Aging Cell, 6(6), 769-774. doi:10.1111/j.1474-9726.2007.00340.x

Balk B, Maicher A, Dees M, Klermund J, Luke-Glaser S, Bender K, Luke B. (2013). Telomeric RNA-DNA hybrids affect telomere-length dynamics and senescence. Nat Struct Mol Biol, 20(10), 1199-1205. doi:10.1038/nsmb.2662

Barsov EV. (2011). Telomerase and primary T cells: biology and immortalization for adoptive immunotherapy. Immunotherapy, 3(3), 407-421. doi:10.2217/imt.10.107

Bates D, Mächler M, Bolker B, Walker S. (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software; Vol 1, Issue 1 (2015). doi:10.18637/jss.v067.i01

Baur JA, Zou Y, Shay JW, Wright WE. (2001). Telomere position effect in human cells. Science, 292(5524), 2075-2077. doi:DOI 10.1126/science.1062329

Beckmann H, Franzek E. (2000). The genetic heterogeneity of "schizophrenia". World J Biol Psychiatry, 1(1), 35-41.

Bell JT, Saffery R. (2012). The value of twins in epigenetic epidemiology. Int J Epidemiol, 41(1), 140-150. doi:10.1093/ije/dyr179

Bell JT, Spector TD. (2011). A twin approach to unraveling epigenetics. Trends Genet, 27(3), 116-125. doi:10.1016/j.tig.2010.12.005

Bellon M, Baydoun HH, Yao Y, Nicot C. (2010). HTLV-I Tax-dependent and -independent events associated with immortalization of human primary T lymphocytes. Blood, 115(12), 2441-2448. doi:DOI 10.1182/blood-2009-08-241117

Benetos A, Okuda K, Lajemi M, Kimura M, Thomas F, Skurnick J, Labat C, Bean K, Aviv A. (2001). Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. Hypertension, 37(2 Pt 2), 381-385.

Benetti R, Garcia-Cao M, Blasco MA. (2007). Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. Nat Genet, 39(2), 243-250. doi:Doi 10.1038/Ng1952

Berglund K, Reynolds CA, Ploner A, Gerritsen L, Hovatta I, Pedersen NL, Hägg S. (2016). Longitudinal decline of leukocyte telomere length in old age and the association with sex and genetic risk. Aging (Albany NY), 8(7), 1398-1407. doi:10.18632/aging.100995

Biousse V, Brown MD, Newman NJ, Allen JC, Rosenfeld J, Meola G, Wallace DC. (1997). De novo 14484 mitochondrial DNA mutation in monozygotic twins discordant for Leber's hereditary optic neuropathy. Neurology, 49(4), 1136-1138.

Bischoff C, Petersen HC, Graakjaer J, Andersen-Ranberg K, Vaupel JW, Bohr VA, Kolvraa S, Christensen K. (2006). No association between telomere length and survival among the elderly and oldest old. Epidemiology, 17(2), 190-194. doi:10.1097/01.ede.0000199436.55248.10

Blackburn EH. (2001). Switching and signaling at the telomere. Cell, 106(6), 661-673. doi:Doi 10.1016/S0092-8674(01)00492-5

Blackburn EH, Greider CW, Szostak JW. (2006). Telomeres and telomerase: the path from maize, Tetrahymena and yeast to human cancer and aging. Nat Med, 12(10), 1133-1138. doi:Doi 10.1038/Nm1006-1133

Blakely E, He L, Taylor R, Chinnery P, Lightowlers R, Schaefer A, Turnbull D. (2004). Mitochondrial DNA deletion in "identical" twin brothers. Journal of Medical Genetics, 41(2), e19-e19. doi:10.1136/jmg.2003.011296

Blasco MA. (2005). Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet, 6(8), 611-622. doi:10.1038/nrg1656

Blasco MA. (2007). The epigenetic regulation of mammalian telomeres. Nat Rev Genet, 8(4), 299-309. doi:10.1038/nrg2047

Blasco MA, Rizen M, Greider CW, Hanahan D. (1996). Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. Nat Genet, 12(2), 200-204. doi:Doi 10.1038/Ng0296-200

Bodnar AG, Kim NW, Effros RB, Chiu CP. (1996). Mechanism of telomerase induction during T cell activation. Experimental Cell Research, 228(1), 58-64. doi:10.1006/excr.1996.0299

Bonilla E, Younger DS, Chang HW, Tantravahi U, Miranda AF, Medori R, DiMauro S, Warburton D, Rowland LP. (1990). Partial dystrophin deficiency in monozygous twin carriers of the Duchenne gene discordant for clinical myopathy. Neurology, 40(8), 1267-1270.

Boomsma D, Busjahn A, Peltonen L. (2002). Classical twin studies and beyond. Nat Rev Genet, 3(11), 872-882. doi:10.1038/nrg932

Boomsma DI. (2013). Twin, association and current "omics" studies. J Matern Fetal Neonatal Med, 26 Suppl 2, 9-12. doi:10.3109/14767058.2013.830405

Boonekamp JJ, Simons MJ, Hemerik L, Verhulst S. (2013). Telomere length behaves as biomarker of somatic redundancy rather than biological age. Aging Cell, 12(2), 330-332. doi:10.1111/acel.12050

Bratic A, Larsson NG. (2013). The role of mitochondria in aging. J Clin Invest, 123(3), 951-957. doi:10.1172/jci64125

Broer L, Codd V, Nyholt DR, Deelen J, Mangino M, Willemsen G, Albrecht E, Amin N, Beekman M, de Geus EJ, Henders A, Nelson CP, Steves CJ, Wright MJ, de Craen AJ, Isaacs A, Matthews M, Moayyeri A, Montgomery GW, Oostra BA, Vink JM, Spector TD, Slagboom PE, Martin NG, Samani NJ, van Duijn CM, Boomsma DI. (2013). Meta-analysis of telomere length in 19,713 subjects reveals high heritability, stronger maternal inheritance and a paternal age effect. Eur J Hum Genet, 21(10), 1163-1168. doi:10.1038/ejhg.2012.303

Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. (2003). White cell telomere length and risk of premature myocardial infarction. Arterioscler Thromb Vasc Biol, 23(5), 842-846. doi:10.1161/01.atv.0000067426.96344.32

Bruder CE, Piotrowski A, Gijsbers AA, Andersson R, Erickson S, Diaz de Stahl T, Menzel U, Sandgren J, von Tell D, Poplawski A, Crowley M, Crasto C, Partridge EC, Tiwari H, Allison DB, Komorowski J, van Ommen GJ, Boomsma DI, Pedersen NL, den Dunnen JT, Wirdefeldt K, Dumanski JP. (2008). Phenotypically concordant and discordant monozygotic twins display different DNA copy-number-variation profiles. Am J Hum Genet, 82(3), 763-771. doi:10.1016/j.ajhg.2007.12.011

Bryan TM, Englezou A, DallaPozza L, Dunham MA, Reddel RR. (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med, 3(11), 1271-1274. doi:Doi 10.1038/Nm1197-1271

Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. (1995). Telomere Elongation in Immortal Human-Cells without Detectable Telomerase Activity. Embo Journal, 14(17), 4240-4248.

Burns JB, Lobo ST, Bartholomew BD. (2000). In vivo reduction of telomere length in human antigen-reactive memory T cells. Eur J Immunol, 30(7), 1894-1901. doi:10.1002/1521-4141(200007)30:7<1894::aid-immu1894>3.0.co;2-n

Cairney CJ, Keith WN. (2008). Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. Biochimie, 90(1), 13-23. doi:10.1016/j.biochi.2007.07.025

Calado RT, Regal JA, Kleiner DE, Schrump DS, Peterson NR, Pons V, Chanock SJ, Lansdorp PM, Young NS. (2009). A Spectrum of Severe Familial Liver Disorders Associate with Telomerase Mutations. PLoS One, 4(11), e7926. doi:10.1371/journal.pone.0007926

Calado RT, Young NS. (2009). Telomere diseases. N Engl J Med, 361(24), 2353-2365. doi:10.1056/NEJMra0903373

Cao Y, Bryan TM, Reddel RR. (2008). Increased copy number of the TERT and TERC telomerase subunit genes in cancer cells. Cancer Science, 99(6), 1092-1099. doi:10.1111/j.1349-7006.2008.00815.x

Carulli L, Anzivino C. (2014). Telomere and telomerase in chronic liver disease and hepatocarcinoma. World Journal of Gastroenterology, 20(20), 6287-6292. doi:DOI 10.3748/wjg.v20.i20.6287

Castillo-Fernandez JE, Spector TD, Bell JT. (2014). Epigenetics of discordant monozygotic twins: implications for disease. Genome Medicine, 6. doi:10.1186/s13073-014-0060-z

Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA. (2003). Association between telomere length in blood and mortality in people aged 60 years or older. Lancet, 361(9355), 393-395. doi:10.1016/s0140-6736(03)12384-7

Cesare AJ, Reddel RR. (2010). Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet, 11(5), 319-330. doi:10.1038/nrg2763

Chan SW, Blackburn EH. (2002). New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. Oncogene, 21(4), 553-563. doi:10.1038/sj.onc.1205082

Chen YX, Gorelik GJ, Strickland FM, Richardson BC. (2010). Decreased ERK and JNK signaling contribute to gene overexpression in "senescent" CD4+CD28-T cells through epigenetic mechanisms. J Leukoc Biol, 87(1), 137-145. doi:Doi 10.1189/Jlb.0809562

Chou JP, Effros RB. (2013). T CELL REPLICATIVE SENESCENCE IN HUMAN AGING. Current Pharmaceutical Design, 19(9), 1680-1698.

Codd V, Nelson CP, Albrecht E, Mangino M, Deelen J, Buxton JL, Hottenga JJ, Fischer K, Esko T, Surakka I, Broer L, Nyholt DR, Leach IM, Salo P, Hagg S, Matthews MK, Palmen J, Norata GD, O'Reilly PF, Saleheen D, Amin N, Balmforth AJ, Beekman M, de Boer RA, Bohringer S, Braund PS, Burton PR, de Craen AJM, Denniff M, Dong YB, Douroudis K, Dubinina E, Eriksson JG, Garlaschelli K, Guo DH, Hartikainen AL, Henders AK, Houwing-Duistermaat JJ, Kananen L, Karssen LC, Kettunen J, Klopp N, Lagou V, van Leeuwen EM, Madden PA, Magi R, Magnusson PKE, Mannisto S, McCarthy MI, Medland SE, Mihailov E,

Montgomery GW, Oostra B, Palotie A, Peters A, Pollard H, Pouta A, Prokopenko I, Ripatti S, Salomaa V, Suchiman HED, Valdes AM, Verweij N, Vinuela A, Wang XL, Wichmann HE, Widen E, Willemsen G, Wright MJ, Xia K, Xiao XJ, van Veldhuisen DJ, Catapano AL, Tobin MD, Hall AS, Blakemore AIF, van Gilst WH, Zhu HD, Erdmann J, Reilly MP, Kathiresan S, Schunkert H, Talmud PJ, Pedersen NL, Perola M, Ouwehand W, Kaprio J, Martin NG, van Duijn CM, Hovatta I, Gieger C, Metspalu A, Boomsma DI, Jarvelin MR, Slagboom PE, Thompson JR, Spector TD, van der Harst P, Samani NJ, Consortium C. (2013). Identification of seven loci affecting mean telomere length and their association with disease. Nat Genet, 45(4), 422-427. doi:Doi 10.1038/Ng.2528

Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. (2007). Protein composition of catalytically active human telomerase from immortal cells. Science, 315(5820), 1850-1853. doi:10.1126/science.1138596

Collins K, Mitchell JR. (2002). Telomerase in the human organism. Oncogene, 21(4), 564-579. doi:DOI 10.1038/sj.onc.1205083

Condon J, Shaw JE, Luciano M, Kyvik KO, Martin NG, Duffy DL. (2008). A study of diabetes mellitus within a large sample of Australian twins. Twin Res Hum Genet, 11(1), 28-40. doi:10.1375/twin.11.1.28

Cong Y, Shay JW. (2008). Actions of human telomerase beyond telomeres. Cell Res, 18(7), 725-732. doi:10.1038/cr.2008.74

Cong YS, Wright WE, Shay JW. (2002). Human telomerase and its regulation. Microbiology and Molecular Biology Reviews, 66(3), 407-+. doi:Doi 10.1128/Mmbr.66.3.407-425.2002

Correia-Melo C, Hewitt G, Passos JF. (2014). Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence? Longevity & Healthspan, 3, 1-1. doi:10.1186/2046-2395-3-1

Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S. (1995). Telomerase activity in normal leukocytes and in hematologic malignancies. Blood, 85(9), 2315-2320.

Curran JE, Johnson MP, Dyer TD, Goring HH, Kent JW, Charlesworth JC, Borg AJ, Jowett JB, Cole SA, MacCluer JW, Kissebah AH, Moses EK, Blangero J.

(2007). Genetic determinants of mitochondrial content. Hum Mol Genet, 16(12), 1504-1514. doi:10.1093/hmg/ddm101

Cusanelli E, Chartrand P. (2015). Telomeric repeat-containing RNA TERRA: a noncoding RNA connecting telomere biology to genome integrity. Front Genet, 6, 143. doi:10.3389/fgene.2015.00143

D'Hautcourt J L. (2002). Quantitative flow cytometric analysis of membrane antigen expression. Curr Protoc Cytom, Chapter 6, Unit 6.12. doi:10.1002/0471142956.cy0612s22

Daniel M, Peek GW, Tollefsbol TO. (2012). Regulation of the human catalytic subunit of telomerase (hTERT). Gene, 498(2), 135-146. doi:10.1016/j.gene.2012.01.095

de Lange T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev, 19(18), 2100-2110. doi:10.1101/gad.1346005

Deelen J, Beekman M, Codd V, Trompet S, Broer L, Hägg S, Fischer K, Thijssen PE, Suchiman HED, Postmus I, Uitterlinden AG, Hofman A, de Craen AJM, Metspalu A, Pedersen NL, van Duijn CM, Wouter Jukema J, Houwing-Duistermaat JJ, Samani NJ, Eline Slagboom P. (2014). Leukocyte telomere length associates with prospective mortality independent of immune-related parameters and known genetic markers. International Journal of Epidemiology, 43(3), 878-886. doi:10.1093/ije/dyt267

Deville L, Hillion J, Segal-Bendirdjian E. (2009). Telomerase regulation in hematological cancers: A matter of stemness? Biochimica Et Biophysica Acta-Molecular Basis of Disease, 1792(4), 229-239. doi:DOI 10.1016/j.bbadis.2009.01.016

Ding J, Sidore C, Butler TJ, Wing MK, Qian Y, Meirelles O, Busonero F, Tsoi LC, Maschio A, Angius A, Kang HM, Nagaraja R, Cucca F, Abecasis GR, Schlessinger D. (2015). Assessing Mitochondrial DNA Variation and Copy Number in Lymphocytes of ~2,000 Sardinians Using Tailored Sequencing Analysis Tools. Plos Genetics, 11(7), e1005306. doi:10.1371/journal.pgen.1005306

Drane P, Ouararhni K, Depaux A, Shuaib M, Hamiche A. (2010). The death-associated protein DAXX is a novel histone chaperone involved in the replication-

independent deposition of H3.3. Genes & Development, 24(12), 1253-1265. doi:Doi 10.1101/Gad.566910

Dunham MA, Neumann AA, Fasching CL, Reddel RR. (2000). Telomere maintenance by recombination in human cells. Nat Genet, 26(4), 447-450.

Effros RB, Dagarag M, Valenzuela HF. (2003). In vitro senescence of immune cells. Exp Gerontol, 38(11-12), 1243-1249.

Effros RB, Pawelec G. (1997). Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion? Immunol Today, 18(9), 450-454.

Epel ES, Merkin SS, Cawthon R, Blackburn EH, Adler NE, Pletcher MJ, Seeman TE. (2009). The rate of leukocyte telomere shortening predicts mortality from cardiovascular disease in elderly men. Aging (Albany NY), 1(1), 81-88.

Farzaneh-Far R, Cawthon RM, Na B, Browner WS, Schiller NB, Whooley MA. (2008). Prognostic value of leukocyte telomere length in patients with stable coronary artery disease - Data from the heart and soul study. Arteriosclerosis Thrombosis and Vascular Biology, 28(7), 1379-1384. doi:Doi 10.1161/Atvbaha.108.167049

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, et al. (1995). The RNA component of human telomerase. Science, 269(5228), 1236-1241.

Fitzpatrick AL, Kronmal RA, Gardner JP, Psaty BM, Jenny NS, Tracy RP, Walston J, Kimura M, Aviv A. (2007). Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. American Journal of Epidemiology, 165(1), 14-21. doi:Doi 10.1093/Aje/Kw346

Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M. (2005). Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci U S A, 102(30), 10604-10609. doi:10.1073/pnas.0500398102

Friedrich U, Griese E, Schwab M, Fritz P, Thon K, Klotz U. (2000). Telomere length in different tissues of elderly patients. Mech Ageing Dev, 119(3), 89-99.

Garcia-Cao M, Gonzalo S, Dean D, Blasco MA. (2002). A role for the Rb family of proteins in controlling telomere length. Nat Genet, 32(3), 415-419. doi:Doi 10.1038/Ng1011

Garcia-Cao M, O'Sullivan R, Peters AHFM, Jenuwein T, Blasco MA. (2004). Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. Nat Genet, 36(1), 94-99. doi:Doi 10.1038/Ng1278

Gibbons RJ, McDowell TL, Raman S, O'Rourke DM, Garrick D, Ayyub H, Higgs DR. (2000). Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. Nat Genet, 24(4), 368-371.

Gomes NMV, Ryder OA, Houck ML, Charter SJ, Walker W, Forsyth NR, Austad SN, Venditt C, Pagel M, Shay JW, Wright WE. (2011). Comparative biology of mammalian telomeres: hypotheses on ancestral states and the roles of telomeres in longevity determination. Aging Cell, 10(5), 761-768. doi:10.1111/j.1474-9726.2011.00718.x

Gonzalo S, Garcia-Cao M, Fraga MF, Schotta G, Peters AHFM, Cotter SE, Eguia R, Dean DC, Esteller M, Jenuwein T, Blasco MA. (2005). Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. Nature Cell Biology, 7(4), 420-U452. doi:Doi 10.1038/Ncb1235

Gonzalo S, Jaco I, Fraga MF, Chen TP, Li E, Esteller M, Blasco MA. (2006). DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nature Cell Biology, 8(4), 416-U466. doi:Doi 10.1038/Ncb1386

Greider CW. (1998). Telomerase activity, cell proliferation, and cancer. Proceedings of the National Academy of Sciences of the United States of America, 95(1), 90-92.

Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T. (1999). Mammalian telomeres end in a large duplex loop. Cell, 97(4), 503-514.

Haendeler J, Drose S, Buchner N, Jakob S, Altschmied J, Goy C, Spyridopoulos I, Zeiher AM, Brandt U, Dimmeler S. (2009). Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From

Damage. Arteriosclerosis Thrombosis and Vascular Biology, 29(6), 929-U400. doi:Doi 10.1161/Atvbaha.109.185546

Haendeler J, Hoffmann J, Brandes RP, Zeiher AM, Dimmeler S. (2003). Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Molecular and Cellular Biology, 23(13), 4598-4610. doi:Doi 10.1128/Mcb.23.13.4598-4610.2003

Hakonen AH, Isohanni P, Paetau A, Herva R, Suomalainen A, Lonnqvist T. (2007). Recessive Twinkle mutations in early onset encephalopathy with mtDNA depletion. Brain, 130(Pt 11), 3032-3040. doi:10.1093/brain/awm242

Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, Miller J, Fedele A, Collins J, Smith K, Lotspeich L, Croen LA, Ozonoff S, Lajonchere C, Grether JK, Risch N. (2011). Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry, 68(11), 1095-1102. doi:10.1001/archgenpsychiatry.2011.76

Halvorsen TL, Leibowitz G, Levine F. (1999). Telomerase Activity Is Sufficient To Allow Transformed Cells To Escape from Crisis. Molecular and Cellular Biology, 19(3), 1864-1870.

Handa H, Matsushima T, Nishimoto N, Inoue M, Saitoh T, Yokohama A, Tsukamoto N, Mitsui T, Nakahashi H, Toyama K, Karasawa M, Ogawara H, Nojima Y, Murakami H. (2010). Flow cytometric detection of human telomerase reverse transcriptase (hTERT) expression in a subpopulation of bone marrow cells. Leuk Res, 34(2), 177-183. doi:10.1016/j.leukres.2009.06.010

Hara T, Mizuguchi M, Fujii M, Nakamura M. (2015). Krüppel-like Factor 2 Represses Transcription of the Telomerase Catalytic Subunit Human Telomerase Reverse Transcriptase (hTERT) in Human T Cells. The Journal of Biological Chemistry, 290(14), 8758-8763. doi:10.1074/jbc.M114.610386

Hathcock KS, Jeffrey Chiang Y, Hodes RJ. (2005). In vivo regulation of telomerase activity and telomere length. Immunol Rev, 205, 104-113. doi:10.1111/j.0105-2896.2005.00267.x

Hathcock KS, Kaech SM, Ahmed R, Hodes RJ. (2003). Induction of telomerase activity and maintenance of telomere length in virus-specific effector and memory CD8+ T cells. J Immunol, 170(1), 147-152.

Heidinger BJ, Blount JD, Boner W, Griffiths K, Metcalfe NB, Monaghan P. (2012). Telomere length in early life predicts lifespan. Proceedings of the National Academy of Sciences of the United States of America, 109(5), 1743-1748. doi:10.1073/pnas.1113306109

Helderman-van den Enden AT, Maaswinkel-Mooij PD, Hoogendoorn E, Willemsen R, Maat-Kievit JA, Losekoot M, Oostra BA. (1999). Monozygotic twin brothers with the fragile X syndrome: different CGG repeats and different mental capacities. J Med Genet, 36(3), 253-257.

Hemann MT, Strong MA, Hao LY, Greider CW. (2001). The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell, 107(1), 67-77.

Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, Shay JW, Ishioka S, Yamakido M. (1995). Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol, 155(8), 3711-3715.

Hjelmborg JB, Dalgard C, Moller S, Steenstrup T, Kimura M, Christensen K, Kyvik KO, Aviv A. (2015). The heritability of leucocyte telomere length dynamics. J Med Genet, 52(5), 297-302. doi:10.1136/jmedgenet-2014-102736

Hockemeyer D, Daniels JP, Takai H, de Lange T. (2006). Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres. Cell, 126(1), 63-77. doi:DOI 10.1016/j.cell.2006.04.044

Hoffmeyer K, Raggioli A, Rudloff S, Anton R, Hierholzer A, Del Valle I, Hein K, Vogt R, Kemler R. (2012). Wnt/β-Catenin Signaling Regulates Telomerase in Stem Cells and Cancer Cells. Science, 336(6088), 1549-1554. doi:10.1126/science.1218370

Holt SE, Shay JW. (1999). Role of telomerase in cellular proliferation and cancer. J Cell Physiol, 180(1), 10-18. doi:10.1002/(sici)1097-4652(199907)180:1<10::aid-jcp2>3.0.co;2-d

Hooijberg E, Ruizendaal JJ, Snijders PJ, Kueter EW, Walboomers JM, Spits H. (2000). Immortalization of human CD8+ T cell clones by ectopic expression of telomerase reverse transcriptase. J Immunol, 165(8), 4239-4245.

Hu J, Hwang SS, Liesa M, Gan B, Sahin E, Jaskelioff M, Ding Z, Ying H, Boutin AT, Zhang H, Johnson S, Ivanova E, Kost-Alimova M, Protopopov A, Wang YA, Shirihai OS, Chin L, DePinho RA. (2012). Antitelomerase therapy provokes ALT and mitochondrial adaptive mechanisms in cancer. Cell, 148(4), 651-663. doi:10.1016/j.cell.2011.12.028

Huda N, Tanaka H, Herbert BS, Reed T, Gilley D. (2007). Shared environmental factors associated with telomere length maintenance in elderly male twins. Aging Cell, 6(5), 709-713. doi:10.1111/j.1474-9726.2007.00330.x

Indran IR, Hande MP, Pervaiz S. (2011). hTERT Overexpression Alleviates Intracellular ROS Production, Improves Mitochondrial Function, and Inhibits ROS-Mediated Apoptosis in Cancer Cells. Cancer Res, 71(1), 266-276. doi:Doi 10.1158/0008-5472.Can-10-1588

Jaenisch R, Bird A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet, 33. doi:10.1038/ng1089

Jeanclos E, Schork NJ, Kyvik KO, Kimura M, Skurnick JH, Aviv A. (2000). Telomere length inversely correlates with pulse pressure and is highly familial. Hypertension, 36(2), 195-200.

Ju Z, Rudolph KL. (2006). Telomeres and telomerase in cancer stem cells. European Journal of Cancer, 42(9), 1197-1203. doi:10.1016/j.ejca.2006.01.040

Kaminsky ZA, Tang T, Wang SC, Ptak C, Oh GH, Wong AH, Feldcamp LA, Virtanen C, Halfvarson J, Tysk C, McRae AF, Visscher PM, Montgomery GW, Gottesman, II, Martin NG, Petronis A. (2009). DNA methylation profiles in monozygotic and dizygotic twins. Nat Genet, 41(2), 240-245. doi:10.1038/ng.286

Kaszubowska L. (2008). Telomere shortening and ageing of the immune system. J Physiol Pharmacol, 59 Suppl 9, 169-186.

Kawauchi K, Ihjima K, Yamada O. (2005). IL-2 increases human telomerase reverse transcriptase activity transcriptionally and posttranslationally through

phosphatidylinositol 3'-kinase/Akt, heat shock protein 90, and mammalian target of rapamycin in transformed NK cells. J Immunol, 174(9), 5261-5269.

Kazachkova N, Ramos A, Santos C, Lima M. (2013). Mitochondrial DNA Damage Patterns and Aging: Revising the Evidences for Humans and Mice. Aging Dis, 4(6), 337-350. doi:10.14336/AD.2013.0400337

Kim J-H, Kim HK, Ko J-H, Bang H, Lee D-C. (2013). The Relationship between Leukocyte Mitochondrial DNA Copy Number and Telomere Length in Community-Dwelling Elderly Women. Plos One, 8(6), e67227. doi:10.1371/journal.pone.0067227

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science, 266(5193), 2011-2015.

Kim S, Parks CG, Xu ZL, Carswell G, DeRoo LA, Sandler DP, Taylor JA. (2012). Association between Genetic Variants in DNA and Histone Methylation and Telomere Length. PLoS One, 7(7). doi:ARTN e40504

DOI 10.1371/journal.pone.0040504

Kimura M, Hjelmborg JVB, Gardner JP, Bathum L, Brimacombe M, Lu XB, Christiansen L, Vaupel JW, Aviv A, Christensen K. (2008). Telomere length and mortality: A study of leukocytes in elderly Danish twins. American Journal of Epidemiology, 167(7), 799-806. doi:Doi 10.1093/Aje/Kwm380

Koering CE, Pollice A, Zibella MP, Bauwens S, Puisieux A, Brunori M, Brun C, Martins L, Sabatier L, Pulitzer JF, Gilson E. (2002). Human telomeric position effect is determined by chromosomal context and telomeric chromatin integrity. Embo Reports, 3(11), 1055-1061. doi:DOI 10.1093/embo-reports/kvf215

Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, Howard E, de Lima RL, Daack-Hirsch S, Sander A, McDonald-McGinn DM, Zackai EH, Lammer EJ, Aylsworth AS, Ardinger HH, Lidral AC, Pober BR, Moreno L, Arcos-Burgos M, Valencia C, Houdayer C, Bahuau M, Moretti-Ferreira D, Richieri-Costa A, Dixon MJ, Murray JC. (2002). Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nat Genet, 32(2), 285-289. doi:10.1038/ng985

Kosciolek BA, Rowley PT. (1998). Human lymphocyte telomerase is genetically regulated. Genes Chromosomes Cancer, 21(2), 124-130.

Kruyer H, Mila M, Glover G, Carbonell P, Ballesta F, Estivill X. (1994). Fragile X syndrome and the (CGG)n mutation: two families with discordant MZ twins. Am J Hum Genet, 54(3), 437-442.

Kyo S, Inoue M. (2002). Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy? Oncogene, 21(4), 688-697. doi:10.1038/sj.onc.1205163

Lansdorp PM. (2009). Telomeres and disease. Embo j, 28(17), 2532-2540. doi:10.1038/emboj.2009.172

Lee H, Cho JH, Park WJ, Jung SJ, Choi IJ, Lee JH. (2017). Loss of the Association between Telomere Length and Mitochondrial DNA Copy Number Contribute to Colorectal Carcinogenesis. Pathol Oncol Res. doi:10.1007/s12253-017-0245-z

Lee JY, Kim JH, Lee DC. (2017). Combined Impact of Telomere Length and Mitochondrial DNA Copy Number on Cognitive Function in Community-Dwelling Very Old Adults. Dementia and Geriatric Cognitive Disorders, 44(3-4), 232-243.

Lehtovirta M, Pietilainen KH, Levalahti E, Heikkila K, Groop L, Silventoinen K, Koskenvuo M, Kaprio J. (2010). Evidence that BMI and type 2 diabetes share only a minor fraction of genetic variance: a follow-up study of 23,585 monozygotic and dizygotic twins from the Finnish Twin Cohort Study. Diabetologia, 53(7), 1314-1321. doi:10.1007/s00125-010-1746-4

Lewis KA, Tollefsbol TO. (2016). Regulation of the Telomerase Reverse Transcriptase Subunit through Epigenetic Mechanisms. Front Genet, 7, 83. doi:10.3389/fgene.2016.00083

Lewis PW, Elsaesser SJ, Noh KM, Stadler SC, Allis CD. (2010). Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. Proceedings of the National Academy of Sciences of the United States of America, 107(32), 14075-14080. doi:DOI 10.1073/pnas.1008850107

Li R, Montpetit A, Rousseau M, Wu SY, Greenwood CM, Spector TD, Pollak M, Polychronakos C, Richards JB. (2014). Somatic point mutations occurring early

in development: a monozygotic twin study. J Med Genet, 51(1), 28-34. doi:10.1136/jmedgenet-2013-101712

Li S, Crothers J, Haqq CM, Blackburn EH. (2005). Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. Journal of Biological Chemistry, 280(25), 23709-23717. doi:DOI 10.1074/jbc.M502782200

Li Z, Hu M, Zong X, He Y, Wang D, Dai L, Dong M, Zhou J, Cao H, Lv L, Chen X, Tang J. (2015). Association of telomere length and mitochondrial DNA copy number with risperidone treatment response in first-episode antipsychotic-naïve schizophrenia. Sci Rep, 5, 18553. doi:10.1038/srep18553

Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K. (2000). Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med, 343(2), 78-85. doi:10.1056/nejm200007133430201

Lin J, Epel E, Cheon J, Kroenke C, Sinclair E, Bigos M, Wolkowitz O, Mellon S, Blackburn E. (2010). Analyses and comparisons of telomerase activity and telomere length in human T and B cells: Insights for epidemiology of telomere maintenance. Journal of Immunological Methods, 352(1-2), 71-80. doi:10.1016/j.jim.2009.09.012

Littvay L, Métneki J, Tárnoki ÁD, Tárnoki DL. (2012). The Hungarian Twin Registry. Twin Research and Human Genetics, 16(1), 185-189. doi:10.1017/thg.2012.76

Liu J, Cao L, Chen J, Song S, Lee IH, Quijano C, Liu H, Keyvanfar K, Chen H, Cao LY, Ahn BH, Kumar NG, Rovira, II, Xu XL, van Lohuizen M, Motoyama N, Deng CX, Finkel T. (2009). Bmi1 regulates mitochondrial function and the DNA damage response pathway. Nature, 459(7245), 387-392. doi:10.1038/nature08040

Liu K, Hodes RJ, Weng N. (2001). Cutting edge: telomerase activation in human T lymphocytes does not require increase in telomerase reverse transcriptase (hTERT) protein but is associated with hTERT phosphorylation and nuclear translocation. J Immunol, 166(8), 4826-4830.

Liu KB, Schoonmaker MM, Levine BL, June CH, Hodes RJ, Weng NP. (1999). Constitutive and regulated expression of telomerase reverse transcriptase

(hTERT) in human lymphocytes. Proceedings of the National Academy of Sciences of the United States of America, 96(9), 5147-5152. doi:DOI 10.1073/pnas.96.9.5147

Liu Y, Kuick R, Hanash S, Richardson B. (2009). DNA methylation inhibition increases T cell KIR expression through effects on both promoter methylation and transcription factors. Clinical Immunology, 130(2), 213-224. doi:DOI 10.1016/j.clim.2008.08.009

Londono-Vallejo JA. (2008). Telomere instability and cancer. Biochimie, 90(1), 73-82. doi:DOI 10.1016/j.biochi.2007.07.009

Lu QJ, Wu AL, Ray D, Deng C, Attwood J, Hanash S, Pipkin M, Lichtenheld M, Richardson B. (2003). DNA methylation and chromatin structure regulate T cell perforin gene expression. Journal of Immunology, 170(10), 5124-5132.

Ly H, Calado RT, Allard P, Baerlocher GM, Lansdorp PM, Young NS, Parslow TG. (2005). Functional characterization of telomerase RNA variants found in patients with hematologic disorders. Blood, 105(6), 2332-2339. doi:DOI 10.1182/blood-2004-09-3659

Macallan DC, Wallace D, Zhang Y, De Lara C, Worth AT, Ghattas H, Griffin GE, Beverley PC, Tough DF. (2004). Rapid turnover of effector-memory CD4(+) T cells in healthy humans. J Exp Med, 200(2), 255-260. doi:10.1084/jem.20040341

Maciejowski J, de Lange T. (2017). Telomeres in cancer: tumour suppression and genome instability. Nat Rev Mol Cell Biol, 18(3), 175-186. doi:10.1038/nrm.2016.171

Maida Y, Masutomi K. (2015). Telomerase reverse transcriptase moonlights: Therapeutic targets beyond telomerase. Cancer Science, 106(11), 1486-1492. doi:10.1111/cas.12806

Maida Y, Yasukawa M, Furuuchi M, Lassmann T, Possemato R, Okamoto N, Kasim V, Hayashizaki Y, Hahn WC, Masutomi K. (2009). An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. Nature, 461(7261), 230-U104. doi:Doi 10.1038/Nature08283

Maini MK, Soares MV, Zilch CF, Akbar AN, Beverley PC. (1999). Virus-induced CD8+ T cell clonal expansion is associated with telomerase up-regulation and

telomere length preservation: a mechanism for rescue from replicative senescence. J Immunol, 162(8), 4521-4526.

Makarov VL, Lejnine S, Bedoyan J, Langmore JP. (1993). Nucleosomal Organization of Telomere-Specific Chromatin in Rat. Cell, 73(4), 775-787. doi:Doi 10.1016/0092-8674(93)90256-P

Marrone A, Stevens D, Vulliamy T, Dokal I, Mason PJ. (2004). Heterozygous telomerase RNA mutations found in dyskeratosis congenita and aplastic anemia reduce telomerase activity via haploinsufficiency. Blood, 104(13), 3936-3942. doi:DOI 10.1182/blood-2004-05-1829

Masopust D, Vezys V, Marzo AL, Lefrancois L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science, 291(5512), 2413-2417. doi:10.1126/science.1058867

Mather KA, Jorm AF, Parslow RA, Christensen H. (2011). Is telomere length a biomarker of aging? A review. J Gerontol A Biol Sci Med Sci, 66(2), 202-213. doi:10.1093/gerona/glq180

Melicher D, Buzas EI, Falus A. (2015). Genetic and epigenetic trends in telomere research: a novel way in immunoepigenetics. Cell Mol Life Sci, 72(21), 4095-4109. doi:10.1007/s00018-015-1991-2

Melicher D, Illés A, Pállinger É, Kovács ÁF, Littvay L, Tárnoki ÁD, Tárnoki DL, Bikov A, Molnár MJ, Buzás EI, Falus A. (2018). Tight co-twin similarity of monozygotic twins for hTERT protein level of T cell subsets, for telomere length and mitochondrial DNA copy number, but not for telomerase activity. Cellular and Molecular Life Sciences, 75(13), 2447-2456. doi:10.1007/s00018-017-2738-z

Mengel-From J, Thinggaard M, Dalgård C, Kyvik KO, Christensen K, Christiansen L. (2014). Mitochondrial DNA copy number in peripheral blood cells declines with age and is associated with general health among elderly. Human Genetics, 133(9), 1149-1159. doi:10.1007/s00439-014-1458-9

Meyerson M. (2000). Role of telomerase in normal and cancer cells. Journal of Clinical Oncology, 18(13), 2626-2634. doi:10.1200/jco.2000.18.13.2626

Migliaccio M, Amacker M, Just T, Reichenbach P, Valmori D, Cerottini JC, Romero P, Nabholz M. (2000). Ectopic human telomerase catalytic subunit expression

maintains telomere length but is not sufficient for CD8+ T lymphocyte immortalization. J Immunol, 165(9), 4978-4984.

Mitchell JR, Wood E, Collins K. (1999). A telomerase component is defective in the human disease dyskeratosis congenita. Nature, 402(6761), 551-555.

Möller P, Mayer S, Mattfeldt T, Müller K, Wiegand P, Brüderlein S. (2009). Sex-related differences in length and erosion dynamics of human telomeres favor females. Aging (Albany NY), 1(8), 733-739.

Monickaraj F, Aravind S, Gokulakrishnan K, Sathishkumar C, Prabu P, Prabu D, Mohan V, Balasubramanyam M. (2012). Accelerated aging as evidenced by increased telomere shortening and mitochondrial DNA depletion in patients with type 2 diabetes. Molecular and Cellular Biochemistry, 365(1-2), 343-350. doi:10.1007/s11010-012-1276-0

Morrison SJ, Prowse KR, Ho P, Weissman IL. (1996). Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity, 5(3), 207-216. doi:Doi 10.1016/S1074-7613(00)80316-7

Muezzinler A, Zaineddin AK, Brenner H. (2013). A systematic review of leukocyte telomere length and age in adults. Ageing Res Rev, 12(2), 509-519. doi:10.1016/j.arr.2013.01.003

Murnane JP, Sabatier L, Marder BA, Morgan WF. (1994). Telomere Dynamics in an Immortal Human Cell-Line. Embo Journal, 13(20), 4953-4962.

Muthén LK, Muthén B. (2010). Mplus statistical analysis with latent variables : user's guide.

Natarajan S, McEachern MJ. (2002). Recombinational telomere elongation promoted by DNA circles. Molecular and Cellular Biology, 22(13), 4512-4521. doi:Doi 10.1128/Mcb.22.13.4512-4521.2002

Neale M, R. Cardon L. (1992). Methodology for Genetic Studies of Twins and Families.

Nergadze SG, Farnung BO, Wischnewski H, Khoriauli L, Vitelli V, Chawla R, Giulotto E, Azzalin CM. (2009). CpG-island promoters drive transcription of human telomeres. Rna, 15(12), 2186-2194. doi:10.1261/rna.1748309

O'Callaghan NJ, Dhillon VS, Thomas P, Fenech M. (2008). A quantitative real-time PCR method for absolute telomere length. Biotechniques, 44(6), 807-809. doi:10.2144/000112761

O'Sullivan RJ, Almouzni G. (2014). Assembly of telomeric chromatin to create ALTernative endings. Trends Cell Biol, 24(11), 675-685. doi:DOI 10.1016/j.tcb.2014.07.007

Okuda K, Bardeguez A, Gardner JP, Rodriguez P, Ganesh V, Kimura M, Skurnick J, Awad G, Aviv A. (2002). Telomere length in the newborn. Pediatr Res, 52(3), 377-381. doi:10.1203/00006450-200209000-00012

Otsuka I, Izumi T, Boku S, Kimura A, Zhang Y, Mouri K, Okazaki S, Shiroiwa K, Takahashi M, Ueno Y, Shirakawa O, Sora I, Hishimoto A. (2017). Aberrant telomere length and mitochondrial DNA copy number in suicide completers. Sci Rep, 7(1), 3176. doi:10.1038/s41598-017-03599-8

Palm W, de Lange T. (2008). How Shelterin Protects Mammalian Telomeres. Annual Review of Genetics, 42, 301-334. doi:DOI 10.1146/annurev.genet.41.110306.130350

Pan C, Xue BH, Ellis TM, Peace DJ, Diaz MO. (1997). Changes in telomerase activity and telomere length during human T lymphocyte senescence. Experimental Cell Research, 231(2), 346-353.

Passos JF, Saretzki G, von Zglinicki T. (2007). DNA damage in telomeres and mitochondria during cellular senescence: is there a connection? Nucleic Acids Res, 35(22), 7505-7513. doi:10.1093/nar/gkm893

Perseguini NM, Verlengia R, Milan JC, Minatel V, Rehder-Santos P, Takahashi AC, Santana-Lemos BA, Calado RT, Ferreira Filho P, Porta A, Catai AM. (2015). Cardiac autonomic modulation, C-reactive protein or telomere length: which of these variables has greater importance to aging? Int J Cardiol, 178, 79-81. doi:10.1016/j.ijcard.2014.10.123

Picard M, Juster RP, McEwen BS. (2014). Mitochondrial allostatic load puts the 'gluc' back in glucocorticoids. Nat Rev Endocrinol, 10(5), 303-310. doi:10.1038/nrendo.2014.22

Pieters N, Janssen BG, Valeri L, Cox B, Cuypers A, Dewitte H, Plusquin M, Smeets K, Nawrot TS. (2015). Molecular responses in the telomere-mitochondrial axis of

ageing in the elderly: a candidate gene approach. Mech Ageing Dev, 145, 51-57. doi:10.1016/j.mad.2015.02.003

Pinheiro J, Bates D. (2009). Mixed-effects models in S and S-plus. . NY Springer.

Plunkett FJ, Franzese O, Belaramani LL, Fletcher JM, Gilmour KC, Sharifi R, Khan N, Hislop AD, Cara A, Salmon M, Gaspar HB, Rustin MH, Webster D, Akbar AN. (2005). The impact of telomere erosion on memory CD8+ T cells in patients with X-linked lymphoproliferative syndrome. Mech Ageing Dev, 126(8), 855-865. doi:10.1016/j.mad.2005.03.006

Plunkett FJ, Soares MV, Annels N, Hislop A, Ivory K, Lowdell M, Salmon M, Rickinson A, Akbar AN. (2001). The flow cytometric analysis of telomere length in antigen-specific CD8+ T cells during acute Epstein-Barr virus infection. Blood, 97(3), 700-707.

Qazilbash MH, Liu JM, Vlachos A, Fruchtman S, Messner H, Zipursky A, Alter BP, Young NS. (1997). A new syndrome of familial aplastic anemia and chronic liver disease. Acta Haematologica, 97(3), 164-167.

Qian Y, Yang L, Cao S. (2014). Telomeres and telomerase in T cells of tumor immunity. Cell Immunol, 289(1-2), 63-69. doi:10.1016/j.cellimm.2014.03.009

Qiu C, Enquobahrie DA, Gelaye B, Hevner K, Williams MA. (2015). The association between leukocyte telomere length and mitochondrial DNA copy number in pregnant women: a pilot study. Clin Lab, 61(3-4), 363-369.

R Core Team. (2016). R: A language and environment for statistical computing. Retrieved from https://www.r-project.org/

Ramlee MK, Wang J, Toh WX, Li S. (2016). Transcription Regulation of the Human Telomerase Reverse Transcriptase (hTERT) Gene. Genes (Basel), 7(8), 50. doi:10.3390/genes7080050

Redon S, Reichenbach P, Lingner J. (2010). The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. Nucleic Acids Res, 38(17), 5797-5806. doi:10.1093/nar/gkq296

Reiling E, Ling C, Uitterlinden AG, Van't Riet E, Welschen LM, Ladenvall C, Almgren P, Lyssenko V, Nijpels G, van Hove EC, Maassen JA, de Geus EJ, Boomsma DI, Dekker JM, Groop L, Willemsen G, t Hart LM. (2010). The association of

mitochondrial content with prevalent and incident type 2 diabetes. J Clin Endocrinol Metab, 95(4), 1909-1915. doi:10.1210/jc.2009-1775

Revesz D, Verhoeven JE, Milaneschi Y, de Geus EJ, Wolkowitz OM, Penninx BW. (2014). Dysregulated physiological stress systems and accelerated cellular aging. Neurobiol Aging, 35(6), 1422-1430. doi:10.1016/j.neurobiolaging.2013.12.027

Revesz D, Verhoeven JE, Picard M, Lin J, Sidney S, Epel ES, Penninx B, Puterman E. (2018). Associations Between Cellular Aging Markers and Metabolic Syndrome: Findings From the CARDIA Study. J Clin Endocrinol Metab, 103(1), 148-157. doi:10.1210/jc.2017-01625

Robertson SP, Thompson S, Morgan T, Holder-Espinasse M, Martinot-Duquenoy V, Wilkie AO, Manouvrier-Hanu S. (2006). Postzygotic mutation and germline mosaicism in the otopalatodigital syndrome spectrum disorders. Eur J Hum Genet, 14(5), 549-554. doi:10.1038/sj.ejhg.5201586

Röth A, Baerlocher GM, Schertzer M, Chavez E, Dührsen U, Lansdorp PM. (2005). Telomere loss, senescence, and genetic instability in CD4(+) T lymphocytes overexpressing hTERT. Blood, 106(1), 43-50. doi:10.1182/blood-2004-10-4144

Roth A, Yssel H, Pene J, Chavez EA, Schertzer M, Lansdorp PM, Spits H, Luiten RM. (2003). Telomerase levels control the lifespan of human T lymphocytes. Blood, 102(3), 849-857. doi:10.1182/blood-2002-07-2015

Rufer N, Dragowska W, Thornbury G, Roosnek E, Lansdorp PM. (1998). Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. Nat Biotechnol, 16(8), 743-747. doi:10.1038/nbt0898-743

Rufer N, Migliaccio M, Antonchuk J, Humphries RK, Roosnek E, Lansdorp PM. (2001). Transfer of the human telomerase reverse transcriptase (TERT) gene into T lymphocytes results in extension of replicative potential. Blood, 98(3), 597-603.

Sahin E, Colla S, Liesa M, Moslehi J, Müller FL, Guo M, Cooper M, Kotton D, Fabian AJ, Walkey C, Maser RS, Tonon G, Foerster F, Xiong R, Wang YA, Shukla SA, Jaskelioff M, Martin ES, Heffernan TP, Protopopov A, Ivanova E, Mahoney JE, Kost-Alimova M, Perry SR, Bronson R, Liao R, Mulligan R, Shirihai OS, Chin L, DePinho RA. (2011). Telomere dysfunction induces metabolic and

mitochondrial compromise. Nature, 470(7334), 359-365. doi:10.1038/nature09787

Sahin E, Depinho RA. (2010). Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature, 464(7288), 520-528. doi:10.1038/nature08982

Sahin E, DePinho RA. (2012). Axis of ageing: telomeres, p53 and mitochondria. Nat Rev Mol Cell Biol, 13(6), 397-404. doi:10.1038/nrm3352

Sahu M, Prasuna JG. (2016). Twin Studies: A Unique Epidemiological Tool. Indian Journal of Community Medicine : Official Publication of Indian Association of Preventive & Social Medicine, 41(3), 177-182. doi:10.4103/0970-0218.183593

Sakuntabhai A, Ruiz-Perez V, Carter S, Jacobsen N, Burge S, Monk S, Smith M, Munro CS, O'Donovan M, Craddock N, Kucherlapati R, Rees JL, Owen M, Lathrop GM, Monaco AP, Strachan T, Hovnanian A. (1999). Mutations in ATP2A2, encoding a Ca2+ pump, cause Darier disease. Nat Genet, 21(3), 271-277. doi:10.1038/6784

Salpea KD, Humphries SE. (2010). Telomere length in atherosclerosis and diabetes. Atherosclerosis, 209(1), 35-38. doi:DOI 10.1016/j.atherosclerosis.2009.12.021

Salpea KD, Talmud PJ, Cooper JA, Maubaret CG, Stephens JW, Abelak K, Humphries SE. (2010). Association of telomere length with type 2 diabetes, oxidative stress and UCP2 gene variation. Atherosclerosis, 209(1), 42-50. doi:DOI 10.1016/j.atherosclerosis.2009.09.070

Samper E, Flores JA, Blasco MA. (2001). Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc(-/-) mice with short telomeres. Embo Reports, 2(9), 800-807. doi:DOI 10.1093/embo-reports/kve174

Sanderson SL, Simon AK. (2017). In aged primary T cells, mitochondrial stress contributes to telomere attrition measured by a novel imaging flow cytometry assay. Aging Cell, 16(6), 1234-1243. doi:10.1111/acel.12640

Santos JH, Meyer JN, Skorvaga M, Annab LA, Van Houten B. (2004). Mitochondrial hTERT exacerbates free-radical-mediated mtDNA damage. Aging Cell, 3(6), 399-411. doi:DOI 10.1111/j.1474-9728.2004.00124.x

Santos JH, Meyer JN, Van Houten B. (2006). Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage

and apoptosis. Hum Mol Genet, 15(11), 1757-1768. doi:Doi 10.1093/Hmg/Ddl098

Saretzki G. (2009). Telomerase, mitochondria and oxidative stress. Experimental Gerontology, 44(8), 485-492. doi:DOI 10.1016/j.exger.2009.05.004

Saretzki G. (2014). Extra-telomeric Functions of Human Telomerase: Cancer, Mitochondria and Oxidative Stress. Current Pharmaceutical Design, 20(41), 6386-6403.

Satoh M, Minami Y, Takahashi Y, Tabuchi T, Itoh T, Nakamura M. (2009). Effect of intensive lipid-lowering therapy on telomere erosion in endothelial progenitor cells obtained from patients with coronary artery disease. Clinical Science, 116(11-12), 827-835. doi:Doi 10.1042/Cs20080404

Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM, Alter BP. (2008). TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita. American Journal of Human Genetics, 82(2), 501-509. doi:DOI 10.1016/j.ajhg.2007.10.004

Scheuring UJ, Sabzevari H, Theofilopoulos AN. (2002). Proliferative arrest and cell cycle regulation in CD8(+)CD28(-) versus CD8(+)CD28(+) T cells. Hum Immunol, 63(11), 1000-1009.

Schoeftner S, Blasco MA. (2008). Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. Nature Cell Biology, 10(2), 228-236. doi:10.1038/ncb1685

Schoeftner S, Blasco MA. (2009). A 'higher order' of telomere regulation: telomere heterochromatin and telomeric RNAs. Embo Journal, 28(16), 2323-2336. doi:DOI 10.1038/emboj.2009.197

Segal NL. (2012). Reflections on twin relationships: twins reared apart and twins of opposite gender. Twin Res Hum Genet, 15(6), 788-792. doi:10.1017/thg.2012.92

Sharma NK, Reyes A, Green P, Caron MJ, Bonini MG, Gordon DM, Holt IJ, Santos JH. (2012). Human telomerase acts as a hTR-independent reverse transcriptase in mitochondria. Nucleic Acids Research, 40(2), 712-725. doi:Doi 10.1093/Nar/Gkr758

Shay JW, Wright WE. (2011). Role of telomeres and telomerase in cancer. Seminars in Cancer Biology, 21(6), 349-353. doi:DOI 10.1016/j.semcancer.2011.10.001

Shin JS, Hong A, Solomon MJ, Lee CS. (2006). The role of telomeres and telomerase in the pathology of human cancer and aging. Pathology, 38(2), 103-113. doi:10.1080/00313020600580468

Singhapol C, Pal D, Czapiewski R, Porika M, Nelson G, Saretzki GC. (2013). Mitochondrial Telomerase Protects Cancer Cells from Nuclear DNA Damage and Apoptosis. PLoS One, 8(1). doi:ARTN e52989

DOI 10.1371/journal.pone.0052989

Slagboom PE, Droog S, Boomsma DI. (1994). Genetic determination of telomere size in humans: a twin study of three age groups. Am J Hum Genet, 55(5), 876-882.

Smith AC, Rubin T, Shuman C, Estabrooks L, Aylsworth AS, McDonald MT, Steele L, Ray PN, Weksberg R. (2006). New chromosome 11p15 epigenotypes identified in male monozygotic twins with Beckwith-Wiedemann syndrome. Cytogenet Genome Res, 113(1-4), 313-317. doi:10.1159/000090847

Smith LL, Coller HA, Roberts JM. (2003). Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nature Cell Biology, 5(5), 474-479. doi:Doi 10.1038/Ncb985

Son NH, Murray S, Yanovski J, Hodes RJ, Weng N. (2000). Lineage-specific telomere shortening and unaltered capacity for telomerase expression in human T and B lymphocytes with age. J Immunol, 165(3), 1191-1196.

Steenstrup T, Kark JD, Verhulst S, Thinggaard M, Hjelmborg JVB, Dalgård C, Kyvik KO, Christiansen L, Mangino M, Spector TD, Petersen I, Kimura M, Benetos A, Labat C, Sinnreich R, Hwang S-J, Levy D, Hunt SC, Fitzpatrick AL, Chen W, Berenson GS, Barbieri M, Paolisso G, Gadalla SM, Savage SA, Christensen K, Yashin AI, Arbeev KG, Aviv A. (2017). Telomeres and the natural lifespan limit in humans. Aging (Albany NY), 9(4), 1130-1140. doi:10.18632/aging.101216

Tan Q, Christiansen L, Thomassen M, Kruse TA, Christensen K. (2013). Twins for epigenetic studies of human aging and development. Ageing Res Rev, 12(1), 182-187. doi:10.1016/j.arr.2012.06.004

Tan Q, Christiansen L, von Bornemann Hjelmborg J, Christensen K. (2015). Twin methodology in epigenetic studies. J Exp Biol, 218(Pt 1), 134-139. doi:10.1242/jeb.107151

Tarnoki DL, Tarnoki AD, Littvay L, Lazar Z, Karlinger K, Molnar AA, Melicher D, Garami Z, Berczi V, Horvath I. (2014). Transmission of second-hand smoke sensitivity and smoking attitude in a family. Ann Agric Environ Med, 21(4), 771-775. doi:10.5604/12321966.1129931

Thomas P, NJ OC, Fenech M. (2008). Telomere length in white blood cells, buccal cells and brain tissue and its variation with ageing and Alzheimer's disease. Mech Ageing Dev, 129(4), 183-190. doi:10.1016/j.mad.2007.12.004

Tommerup H, Dousmanis A, Delange T. (1994). Unusual Chromatin in Human Telomeres. Molecular and Cellular Biology, 14(9), 5777-5785.

Tyrka AR, Carpenter LL, Kao H-T, Porton B, Philip NS, Ridout SJ, Ridout KK, Price LH. (2015). Association of Telomere Length and Mitochondrial DNA Copy Number in a Community Sample of Healthy Adults. Experimental Gerontology, 66, 17-20. doi:10.1016/j.exger.2015.04.002

Tyrka AR, Parade SH, Price LH, Kao H-T, Porton B, Philip NS, Welch ES, Carpenter LL. (2016). Alterations of Mitochondrial DNA Copy Number and Telomere Length with Early Adversity and Psychopathology. Biological Psychiatry, 79(2), 78-86. doi:10.1016/j.biopsych.2014.12.025

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. (2012). Primer3--new capabilities and interfaces. Nucleic Acids Res, 40(15), e115. doi:10.1093/nar/gks596

Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. (2005). Obesity, cigarette smoking, and telomere length in women. Lancet, 366(9486), 662-664. doi:10.1016/s0140-6736(05)66630-5

Valenzuela HF, Effros RB. (2002). Divergent telomerase and CD28 expression patterns in human CD4 and CD8 T cells following repeated encounters with the same antigenic stimulus. Clinical Immunology, 105(2), 117-125.

van Dongen J, Slagboom PE, Draisma HH, Martin NG, Boomsma DI. (2012). The continuing value of twin studies in the omics era. Nat Rev Genet, 13(9), 640-653. doi:10.1038/nrg3243

van Steensel B, Smogorzewska A, de Lange T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell, 92(3), 401-413.

Vasa-Nicotera M, Brouilette S, Mangino M, Thompson JR, Braund P, Clemitson JR, Mason A, Bodycote CL, Raleigh SM, Louis E, Samani NJ. (2005). Mapping of a major locus that determines telomere length in humans. American Journal of Human Genetics, 76(1), 147-151. doi:Doi 10.1086/426734

Vaziri H, Benchimol S. (1998). Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol, 8(5), 279-282.

Vaziri H, Schächter F, Uchida I, Wei L, Zhu X, Effros R, Cohen D, Harley CB. (1993). Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. American Journal of Human Genetics, 52(4), 661-667.

Venteicher AS, Abreu EB, Meng Z, McCann KE, Terns RM, Veenstra TD, Terns MP, Artandi SE. (2009). A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science, 323(5914), 644-648. doi:10.1126/science.1165357

Vera E, Bernardes de Jesus B, Foronda M, Flores JM, Blasco MA. (2012). The rate of increase of short telomeres predicts longevity in mammals. Cell Rep, 2(4), 732-737. doi:10.1016/j.celrep.2012.08.023

von Zglinicki T. (2000). Role of oxidative stress in telomere length regulation and replicative senescence. Ann N Y Acad Sci, 908, 99-110.

Vulliamy T, Beswick R, Kirwan M, Marrone A, Digweed M, Walne A, Dokal I. (2008). Mutations in the telomerase component NHP2 cause the premature ageing syndrome dyskeratosis congenita. Proceedings of the National Academy of Sciences of the United States of America, 105(23), 8073-8078. doi:DOI 10.1073/pnas.0800042105

Vulliamy TJ, Walne A, Baskaradas A, Mason PJ, Marrone A, Dokal I. (2005). Mutations in the reverse transcriptase component of telomerase (TERT) in patients with bone marrow failure. Blood Cells Molecules and Diseases, 34(3), 257-263. doi:DOI 10.1016/j.bcmd.2004.12.008

Walne AJ, Vulliamy T, Marrone A, Beswick R, Kirwan M, Masunari Y, Al-Qurashi FH, Aljurf M, Dokal I. (2007). Genetic heterogeneity in autosomal recessive

dyskeratosis congenita with one subtype due to mutations in the telomerase-associated protein NOP10. Hum Mol Genet, 16(13), 1619-1629. doi:Doi 10.1093/Hmg/Ddm111

Wang YY, Chen AF, Wang HZ, Xie LY, Sui KX, Zhang QY. (2011). Association of shorter mean telomere length with large artery stiffness in patients with coronary heart disease. Aging Male, 14(1), 27-32. doi:Doi 10.3109/13685538.2010.529196

Weng N-p. (2008). Telomere and adaptive immunity. Mechanisms of Ageing and Development, 129(1-2), 60-66. doi:10.1016/j.mad.2007.11.005

Weng NP, Levine BL, June CH, Hodes RJ. (1996). Regulated expression of telomerase activity in human T lymphocyte development and activation. J Exp Med, 183(6), 2471-2479.

Weng NP, Palmer LD, Levine BL, Lane HC, June CH, Hodes RJ. (1997). Tales of tails: regulation of telomere length and telomerase activity during lymphocyte development, differentiation, activation, and aging. Immunol Rev, 160, 43-54.

Wolf D, Rumpold H, Koppelstatter C, Gastl GA, Steurer M, Mayer G, Gunsilius E, Tilg H, Wolf AM. (2006). Telomere length of in vivo expanded CD4(+)CD25 (+) regulatory T-cells is preserved in cancer patients. Cancer Immunol Immunother, 55(10), 1198-1208. doi:10.1007/s00262-005-0107-5

Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. (1996). Telomerase activity in human germline and embryonic tissues and cells. Dev Genet, 18(2), 173-179. doi:10.1002/(SICI)1520-6408(1996)18:2<173::AID-DVG10>3.0.CO;2-3

Xing J, Chen M, Wood CG, Lin J, Spitz MR, Ma J, Amos CI, Shields PG, Benowitz NL, Gu J, de Andrade M, Swan GE, Wu X. (2008). Mitochondrial DNA Content: Its Genetic Heritability and Association With Renal Cell Carcinoma. JNCI Journal of the National Cancer Institute, 100(15), 1104-1112. doi:10.1093/jnci/djn213

Yamaguchi H, Baerlocher GM, Lansdorp PM, Chanock SJ, Nunez O, Sloand E, Young NS. (2003). Mutations of the human telomerase RNA gene (TERC) in aplastic anemia and myelodysplastic syndrome. Blood, 102(3), 916-918. doi:DOI 10.1182/blood-2003-01-0335

Yu-Wai-Man P, Trenell MI, Hollingsworth KG, Griffiths PG, Chinnery PF. (2011). OPA1 mutations impair mitochondrial function in both pure and complicated dominant optic atrophy. Brain, 134(4), e164-e164. doi:10.1093/brain/awq288

Zhou J, Ding D, Wang M, Cong Y-S. (2014). Telomerase reverse transcriptase in the regulation of gene expression. BMB Reports, 47(1), 8-14. doi:10.5483/BMBRep.2014.47.1.284

Zhu X, Mao Y, Huang T, Yan C, Yu F, Du J, Dai J, Ma H, Jin G. (2017). High mitochondrial DNA copy number was associated with an increased gastric cancer risk in a Chinese population. Mol Carcinog. doi:10.1002/mc.22703

Zierer J, Kastenmüller G, Suhre K, Gieger C, Codd V, Tsai P-C, Bell J, Peters A, Strauch K, Schulz H, Weidinger S, Mohney RP, Samani NJ, Spector T, Mangino M, Menni C. (2016). Metabolomics profiling reveals novel markers for leukocyte telomere length. Aging (Albany NY), 8(1), 77-86.

Zole E, Ranka R. (2018). Mitochondria, its DNA and telomeres in ageing and human population. Biogerontology. doi:10.1007/s10522-018-9748-6

Zole E, Zadinane K, Pliss L, Ranka R. (2017). Linkage between mitochondrial genome alterations, telomere length and aging population. Mitochondrial DNA A DNA Mapp Seq Anal, 1-8. doi:10.1080/24701394.2017.1303490

Zwijnenburg PJ, Meijers-Heijboer H, Boomsma DI. (2010). Identical but not the same: the value of discordant monozygotic twins in genetic research. Am J Med Genet B Neuropsychiatr Genet, 153b(6), 1134-1149. doi:10.1002/ajmg.b.31091

10. Bibliography of own publications

10.1. Publications related to the current Ph.D. thesis

Melicher D, Illés A, Pállinger É, Kovács ÁF, Littvay L, Tárnoki ÁD, Tárnoki DL, Bikov A, Molnár MJ, Buzás EI, Falus A. (2018).

Tight co-twin similarity of monozygotic twins for hTERT protein level of T cell subsets, for telomere length and mitochondrial DNA copy number, but not for telomerase activity.

Cellular and Molecular Life Sciences, 75(13), 2447-2456. doi:10.1007/s00018-017-2738-z

Impact Factor: 5.788

Melicher D, Buzas EI, Falus A. (2015).

Genetic and epigenetic trends in telomere research: a novel way in immunoepigenetics.

Cellular and Molecular Life Sciences, 72(21), 4095-4109. doi:10.1007/s00018-015-1991-2

Impact Factor: 5.694

10.2. Publications not related to the current Ph.D. thesis

Tarnoki DL, Tarnoki AD, Littvay L, Lazar Z, Karlinger K, Molnar AA, Melicher D, Garami Z, Berczi V, Horvath I. (2014).

Transmission of second-hand smoke sensitivity and smoking attitude in a family.

Ann Agric Environ Med, 21(4), 771-775. doi:10.5604/12321966.1129931

Impact Factor: 1.126

Feith HJ, Melicher D, Máthé G, Gradvohl E, Füzi R, Darvay S, Hajdú Zs, Nagyné Horváth E, Soósné Kiss Zs, Bihariné Krekó I, Földvári-Nagy Lászlóné Lenti K, Molnár E, Szalainé Tóth T, Urbán V, Kassay A, Falus A. (2016).

Tapasztalatok és motiváltság: magyar középiskolások véleménye az egészségvédő programokról

ORVOSI HETILAP, 157(2), 65–69. DOI: 10.1556/650.2015.30338

Impact factor: 0.349

11. Acknowledgements

Hereby I would like to express my sincere gratitude and thanks to my supervisor, Prof. Dr. András Falus for his continuous support, mentorship and encouragement. It has been an honor to be his Ph.D. student, I appreciate all his contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating. I thank him wholeheartedly, not only for his tremendous academic support, but also for giving me the chance to be able to rely on him anytime both as a researcher but also as a person and friend.

I would also like to express my profound gratitude to Prof. Dr. Edit Irén Buzás for encouraging my research and for providing me the opportunity to become a member of the institute, where under her leadership she created such a caring atmosphere. I am very grateful to have the opportunity to collaborate in the MTA-SE Immunproteogenomics Extracellular Vesicle Research Group.

I would like to express special thanks and appreciation to Dr. Ádám Domonkos Tárnoki and Dr. Dávid László Tárnoki who contributed immensely to my research in every area of twin studies.

I am also very grateful to Dr. Éva Pállinger, for sharing her laboratory expertise, and being dedicated and supportive to me. I also thank Dr. Árpád Kovács and Nóra Fekete.

I am also thankful to Anett Illés as a friend and colleague.

I thank my beloved family especially my mother, father and grandmother for their caring, patience and love that helped me through every hurdle.

Finally, but by no means least, I would like to thank each and every colleague of the Department of Genetics, Cell-, and Immunobiology for accepting me into their remarkable community.