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Review

The telomere world and aging: Analytical challenges and future perspectives

Emanuela Mensà[a], Silvia Latini[a], Deborah Ramini[a], Gianluca Storci[b,c], Massimiliano Bonafè[b,c,d], Fabiola Olivieri[a,e,*]

[a] Department of Clinical and Molecular Sciences, DISCLIMO, Università Politecnica delle Marche, Ancona, Italy
[b] Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy
[c] Interdepartmental Centre "L. Galvani" (CIG), University of Bologna, Bologna, Italy
[d] Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), IRCCS, Biosciences Laboratory, Meldola, Italy
[e] Center of Clinical Pathology and Innovative Therapy, IRCCS INRCA, Ancona, Italy

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ABSTRACT

Telomeres, the terminal nucleoprotein structures of eukaryotic chromosomes, play pleiotropic functions in cellular and organismal aging. Telomere length (TL) varies throughout life due to the influence of genetic factors and to a complex balancing between "shortening" and "elongation" signals. Telomerase, the only enzyme that can elongate a telomeric DNA chain, and telomeric repeat-containing RNA (TERRA), a long non-coding RNA involved in looping maintenance, play key roles in TL during life. Despite recent advances in the knowledge of TL, TERRA and telomerase activity (TA) biology and their measurement techniques, the experimental and theoretical issues involved raise a number of problems that should carefully be considered by researchers approaching the "telomere world". The increasing use of such parameters – hailed as promising clinically relevant biomarkers – has failed to be paralleled by the development of automated and standardized measurement technology. Consequently, associating given TL values to specific pathological conditions involves on the one hand technological issues and on the other clinical-biological issues related to the planning of clinically relevant association studies. Addressing these issues would help avoid major biases in association studies involving TL and a number of outcomes, especially those focusing on psychological and bio-behavioral variables. The main challenge in telomere research is the development of accurate and reliable measurement methods to achieve simple and sensitive TL, TERRA, and TA detection. The discovery of the localization of telomeres and TERRA in cellular and extracellular compartments had added an additional layer of complexity to the measurement of these age-related biomarkers. Since combined analysis of TL, TERRA and TA may well provide more exhaustive clinical information than a single parameter, we feel it is important for researchers in the various fields to become familiar with their most common measurement techniques and to be aware of the respective merits and drawbacks of these approaches.

1. Introduction

Telomeres, the terminal nucleoprotein structures of eukaryotic chromosomes, have a key role in protecting chromosomal DNA ends (Blackburn, 1990). Mounting evidence suggests that they play pleiotropic functions that are not simply related to the maintenance of chromosome homeostasis, but also to the regulation of gene expression and the modulation of stress-related signaling pathways (Blackburn, 2005). Telomere length (TL) is species-specific and heritable (Chiang et al., 2010); therefore, its value at any time depends on genetic characteristics and on the balance between "shortening" and "elongation" signals (Honig et al., 2015). Shortening signals come from a variety of stressors like repeated cell division, nuclease activation, oxidative damage, DNA replication, and transcriptional stress (Blackburn et al., 2015). Elongation signals are capable of activating telomerase or alternative lengthening of telomeres (ALT); the latter mechanism relies on recombination-mediated telomere elongation and can be induced by telomere-specific DNA damage (Hu et al., 2016). Telomerase is an RNA-protein complex that extends telomeric DNA at the 3′ ends of chromosomes through telomerase reverse transcriptase (TERT) and integral template-containing telomerase RNA (TER) (Jiang et al., 2018). Telomerase is responsible for the preservation of replicative potential in most eukaryotic cells and is involved in pleiotropic functions that range from TL maintenance and genome stability to tissue renewal and

* Corresponding author at: Department of Clinical and Molecular Sciences (DISCLIMO), Università Politecnica delle Marche, Via Conca 70, Ancona, Italy.
E-mail address: f.olivieri@univpm.it (F. Olivieri).

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mitochondrial protection (Li et al., 2018a, 2018b). TL is a marker of the metabolic activity and pluripotency state of embryonic stem cells (Wang et al., 2017a). In embryonic cell lines telomerase is activated and keeps TL constant; in adult stem cells its limited activity and provides only partial compensation for telomere shortening (TS); and in somatic cells it is usually not activated. As a result, during somatic cellular and organismal aging shortening outpaces elongation. Telomeres that have reached a critical length become dysfunctional and activate a DNA damage response which leads to a senescence phenotype characterized by reduced proliferative ability and the acquisition of a secretory proinflammatory phenotype (SASP) (d'Adda di Fagagna et al., 2003).

TL has long been known to be critically involved in cellular aging as a consequence of replication and/or the action of a wide range of stressors, but is also being extensively investigated in relation to organismal aging. Although cell senescence does not necessarily equate with aging of an organism, TS has been observed in both conditions (reviewed in Zhu et al., 2018), and TL has been investigated as a biomarker of aging *per se* and as a risk factor for the development and progression of the most common age-related diseases (ARDs) (Jose et al., 2017).

Recent insights into telomere biology and function indicate that telomeric repeat-containing RNA (TERRA), a long non-coding RNA, is involved in the telomere looping maintenance mechanism (Graf et al., 2017). An inverse correlation has been documented between TL and TERRA both *in vitro* and *in vivo*. According to two hypotheses that have been advanced to explain it, TERRA transcription could facilitate 5′-3′ nuclease activity at the chromosome ends (Pfeiffer and Lingner, 2012), or TS could induce TERRA expression to coordinate telomerase molecule recruitment and activity at the shortest telomeres (Cusanelli et al., 2013).

Notably, telomere DNA, TERRA, and telomerase are not independent molecules; in fact, their structures and temporal relationships are closely regulated by complexes such as shelterin, small nucleolar RNAs (snoRNAs), and small nucleolar ribonucleoproteins (snoRNPs), besides the transcription machinery (Blasco et al., 1999; Gomez et al., 2012). Even though the latter molecules are capable of affecting endpoint TL, the present work focuses on telomeres, TERRA, and telomerase activity (TA).

Despite recent advances, there are a number of outstanding issues related to the knowledge and measurement technology of TL, TERRA, and TA. The most important are: i) the accuracy of the methods employed to measure TL, TERRA, and TA in biomedical research; ii) their strengths and weaknesses; iii) the scope for their improvement; iv) the chemical-clinical variables to be correlated with TL in association studies of aging and ARDs; and v) the need for human longitudinal studies to evaluate the ability of specific approaches (pharmaceutical, nutraceutical) to reverse TS during aging.

Addressing these issues would help avoid major biases in association studies addressing TL and a wide range of outcomes (Montpetit et al., 2014).

This work describes the analytical caveats and criticisms regarding the various TL, TERRA, and TA measurement methods. Since not all protocols are appropriate (or recommended) for epidemiological and longitudinal ARD studies, and some approaches may be applicable only to tumor tissue, the paper focuses on the methods that are suitable for clinical and epidemiological applications.

We will also describe and discuss the localization of telomere and telomere-related sequences in the cellular and extracellular compartment, since localization adds an additional layer of complexity to telomere, TERRA, and TA measurement.

2. Localization of telomere and telomere-related sequences

Recent studies have highlighted a number of possible localizations of telomeric sequences, including the cellular and extracellular compartment (Fig. 1). In cells, telomere sequences can be detected in the nucleus, the chromosome ends and interstitial loci (Smith et al., 2018), and the cytoplasm (Cohen et al., 2010; Zhang et al., 2017a). Localizations in the extracellular compartment include exosomes (Li et al., 2008; Zinkova et al., 2017).

An increased number of telomeric sequences has been described in the cytoplasm of stressed cells in physiological as well as pathological conditions (Kuttler and Mai, 2007; Byrd et al., 2016). Telomeric circles (t-circles or c-circles) are extrachromosomal duplex or single-stranded circular DNA molecules composed of (CCCTAA)(n) sequences. Integration of t-circles in telomere repeats at the chromosome ends results in telomere elongation; circular DNA forms can also be involved in the rapid elongation of DNA ends in rolling-circle replication processes (Nabetani and Ishikawa, 2011).

T-circles, which can be found in human immortalized and cancer cells, rely on ALT pathways or high TA (Tokutake et al., 1998). In fact, in cells showing high TA, extrachromosomal telomeric DNA circles can be excised from the overextended chromosomal ends and released outside the nucleus (Pickett et al., 2009). T-circles have been detected in blood from patients with ALT + tumors; their analysis may have clinical value for their diagnosis and management (Henson et al., 2009). Importantly, t-circles are also found in healthy tissue (Tomaska et al., 2009).

Recent evidence indicates that telomeric sequences are also found in cell-free (cf)-DNA, including biofluids such as plasma and serum (Zinkova et al., 2017). Most of the DNA found in such biofluids is associated to nanovesicles, mainly exosomes, (Fernando et al., 2017; Németh et al., 2017).

TERRA, the long non-coding RNA molecules deriving from transcription of telomeric/subtelomeric regions, is enriched with 5′-(UUAGGG)-3′ repetitions that are complementary to AATCCC, the strand opposite to TTAGGG sequences. TERRA can therefore combine with AATCCC strands, generating three-stranded nucleic acid structures that contain a DNA:RNA hybrid molecule and a displaced DNA strand (Balk et al., 2014). These structures are localized at the level of R-loops and are involved in telomere homeostasis (Toubiana and Selig, 2018). In contrast, UUTCCC molecules, the RNA sequence complementary to TTAGGG, are not related to telomere DNA:RNA hybrids in human cells.

Notably, cf-TERRA – which is shorter (measuring about 200 nt) and more stable than TERRA associated to telomeric DNA in the nucleus – has also been detected in extracellular vesicles. These characteristics suggest that some extrachromosomal telomere-related sequences may be RNA and/or DNA:RNA hybrids (Wang and Lieberman, 2016) (see Fig. 1).

In eukaryotic cells, telomeres and TERRA can fold into G-quadruplexes (G4s), which are characterized by four G combined through non-canonical Hoogsten hydrogen bonding to generate planar structures called tetrads (Martadinata et al., 2011; Wang et al., 2018a) (see Fig. 1). Two or more tetrads can overlap and interact with each other *via* π-stacking, generating stable G4s. Experimental studies and bioinformatics predictions support the view that G4s are involved in several cellular functions such as telomeric DNA elongation, recombination, and transcription, and RNA post-transcriptional mechanisms (Cammas and Millevoi, 2017).

Whereas the location of telomere sequences and TERRA in the nucleus is well known, it is more difficult to describe the cytoplasmic localization of telomere repeats and TERRA.

It has been hypothesized that a cytoplasmic location of TAGGG may exert a suppressive effect on innate immune cells due to their ability to form G4s (Gursel et al., 2003). Colocalization of CpG DNA with toll-like receptor (TLR) 9 in endosomal vesicles is disrupted by TTAGGG telomeric repetitive elements, although cell binding and uptake remain unchanged, suggesting that specific host-derived molecules can downregulate the innate immune response elicited by a TLR ligand.

While cytoplasmic (cy-) or cf-telomeric sequences seem to restrain inflammation, TERRA found in extracellular fractions can stimulate innate immune signaling (Wang et al., 2015). TERRA can be activated

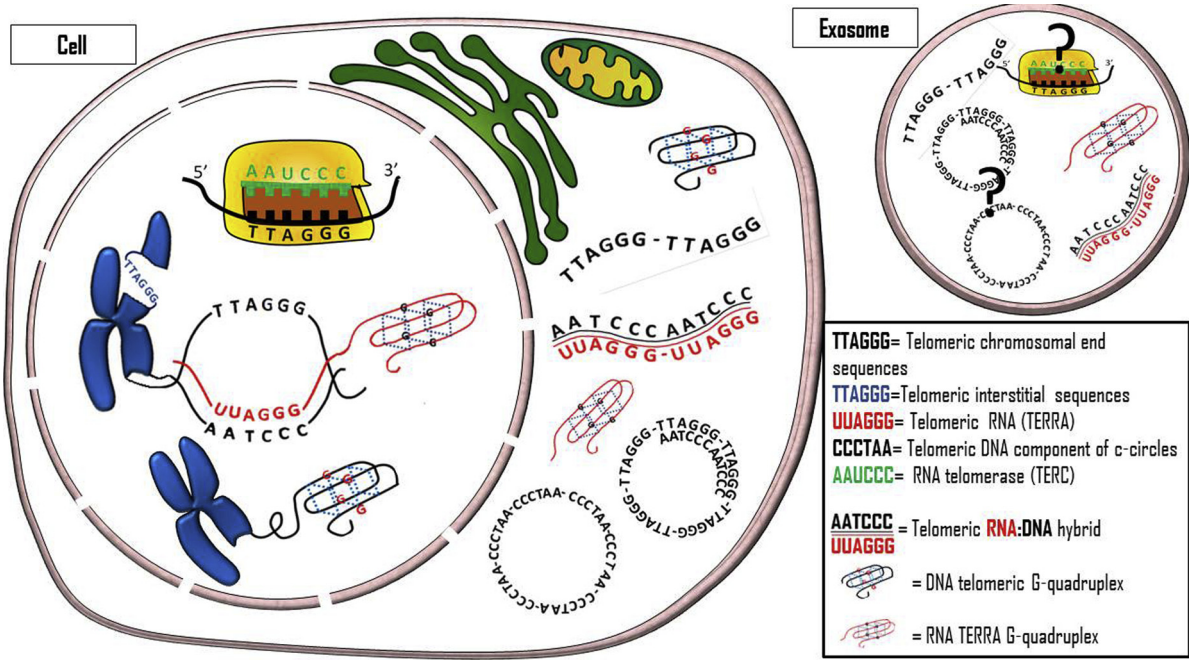

Fig. 1. Telomere-related molecules in cells (nucleus and cytoplasm) and exosomes.
Telomeric sequences can have a number of possible localizations not only in cells (nucleus and cytoplasm) but also in nanovesicles.
DNA, TERRA, DNA:RNA hybrids, and telomerase are not independent molecules: their structures and temporal relationships are closely regulated by key complexes such as shelterin and snoRNPs, besides the transcription machinery. However, these important parameters cannot be depicted here.

through a p53-response element embedded in a retrotransposon-like repeat found in human subtelomeres. TERRA secretion in extracellular vesicles serves as a danger signal, inducing the production of inflammatory cytokines in neighboring cells (Wang et al., 2017c). These findings suggest that TERRA may be part of the innate immune response to viral infection and that exosomes containing cf-TERRA carry a telomere-associated molecular pattern and telomere-specific alarmin from dysfunctional telomeres to the extracellular environment to elicit an inflammatory response (Wang and Lieberman, 2016).

Recently, we have hypothesized that telomeric sequences may be important components of the cy- and cf-DNA payload and that changes in a variety of biochemical features of cy- and cf-DNA (*e.g.* the proportion of DNA hybridized with RNA and the amount of 5-methyl-deoxy-cytosine and 8-oxo-deoxy-guanosine) can affect the ability of these DNA pools to ignite the innate immune system (Storci et al., 2018).

Notably, the localization of telomeric sequences in the cellular and extracellular compartment adds a further layer of complexity to telomere measurement, because different extraction/separation procedures may affect their reciprocal amounts (Komosa et al., 2015).

3. Telomeres and aging

A number of epidemiological studies have found a significant association linking telomere attrition, aging, and several biological outcomes including morbidity and mortality (Gorenjak et al., 2018; Gao et al., 2018). Short leukocyte TL (LTL) has been identified as an independent risk factor for functional decline in elderly European populations (Montiel Rojas et al., 2018) and has been found to be associated to altered fatty acid metabolism and increased oxidative stress in human aging (Zierer et al., 2016). Epidemiological studies provide support for a putative effect of dietary components and exercise on TL and the aging process (Mundstock et al., 2015a; Freitas-Simoes et al., 2018). An association has also been reported between telomere attrition and body mass index gain, suggesting potential pathways linking adiposity and aging outcomes (Müezzinler et al., 2016; Wulaningsih et al., 2018). However, these associations have been described in works performed with different methodological approaches, of which few were prospective; adequately powered randomized controlled trials are therefore warranted. For all these reasons, the causal relationships between important functional parameters and TL remain an open question (Mundstock et al., 2015b; Pérez et al., 2017).

Recent evidence suggests that TL may not be a useful clinical marker of functional aging in older adult populations, whereas it may play a crucial role in longitudinal studies involving young and middle-aged populations (Brown et al., 2018). Since the trajectories of aging begin to diverge as early as in young adulthood (Belsky et al., 2015), it is critical to assess the contribution of telomere-related variations to the diverse aging pathways. Although telomeres shorten during aging, different cell types may have a diverse telomere attrition rate (TAR), *i.e.* a different rate of tissue and organ aging. A yearly TAR of ~25 base pairs during aging has been reported in blood leukocytes, mononuclear cells, and isolated T and B lymphocytes (Müezzinler et al., 2013), whereas high interindividual TAR variation has been described in some longitudinal studies (Chen et al., 2011; Lin et al., 2015).

The marked heterogeneity of health outcomes seen in older individuals suggests that although “chronological” age is a major risk factor for functional impairment, “biological” age may be a more accurate predictor of the rate of aging (Lowsky et al., 2014). TL is currently the most extensively investigated biological age predictor, although new predictors, *e.g.* the epigenetic clock, are emerging (Jylhävä et al., 2017). Mounting evidence suggests that the various biological age predictors may reflect different aspects of the aging process, even though links between the various biomarkers are likely (Vetter et al., 2018). At present, the most stimulating area in telomere research is the study of how progressive TS induces epigenetic alterations and changes in gene expression associated to human healthy and unhealthy aging (Shay, 2018). In this scenario, combinations of the diverse predictors may shed light on the complexity of the aging process and help to improve the estimation of aging trajectories. Composite scores integrating molecular and physiological data have been proposed to measure biological aging in humans (Khan et al., 2017; Belsky et al.,

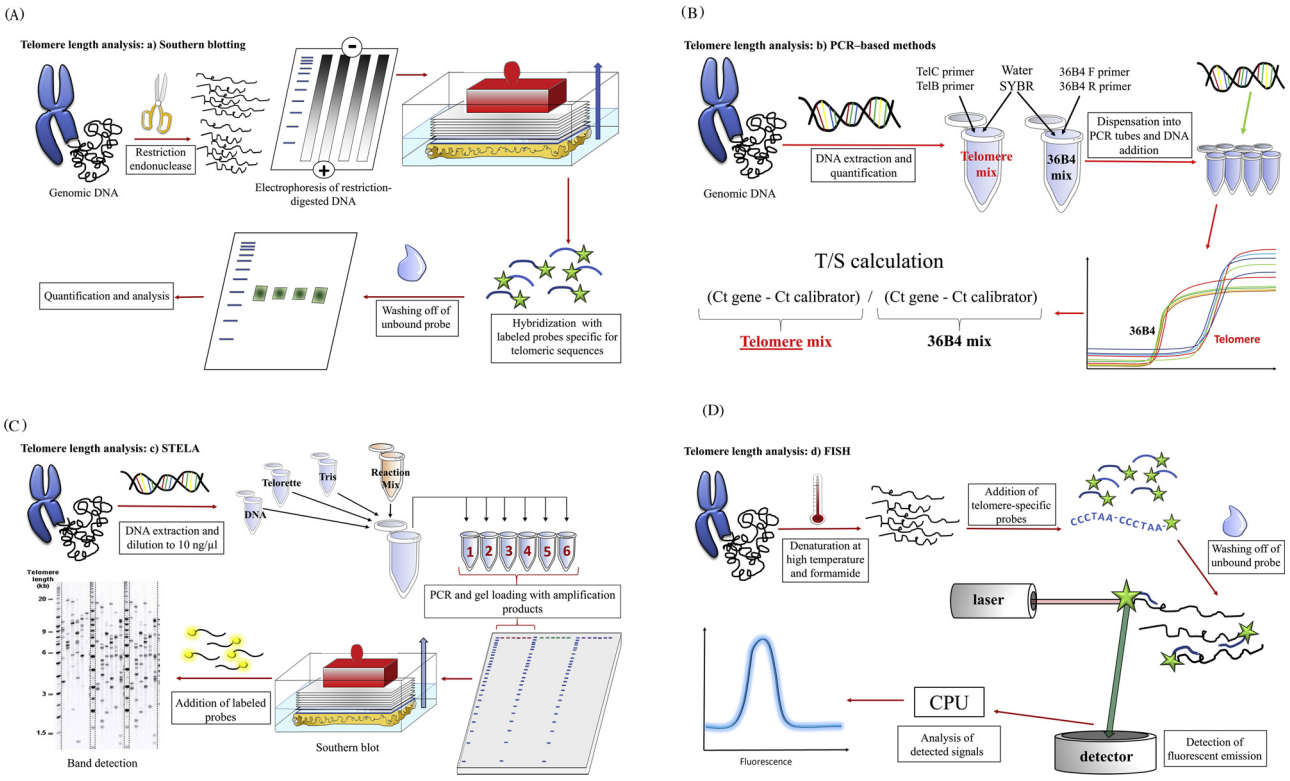

Fig. 2. Most commonly used techniques employed to measure TL.
Schematic representation of the most common techniques employed to measure TL: 2a) Southern blotting-RTF; 2b) PCR-based methods; 2c) STELA; 2d) FISH.

2018).

Growing evidence also suggests that the association of LTL to functional impairment, *i.e.* frailty, may be driven by the unfavorable effect of having short telomeres, rather than reflect a linear dose-response relationship. Short telomeres, rather than TL *per se*, may thus be an informative biomarker of aging and ARDs (Montiel Rojas et al., 2018; Haapanen et al., 2018). Several findings indicate that short telomeres precede the clinical manifestation of the most common ARDs, such as type 2 diabetes and atherosclerotic disease (Toupance et al., 2017).

Overall, even though the factors involved in TAR and the pleiotropic roles of telomeres are not entirely clear, TL is being widely used as a biomarker of the aging process, and intense effort is being devoted to translating TL values into information that can be employed in clinical practice (Hastings et al., 2017). However, translation from the bench to the beside is being hampered by a number of biological factors, including the dependence of TL on genetic, epigenetic, environmental and behavioral factors, as well as by technical aspects such as pre-analytical issues and poor standardization of TL measurement techniques (Danese and Lippi, 2018). With regard to the latter question, since current measurement methods have been developed for use in molecular biology laboratories, reliable and robust TL estimation requires a high level of technical understanding and competence. Since researchers involved in areas other than cell biology, including physiology, psychology, evolution and ecology, have become interested in telomere biology and dynamics (Nussey et al., 2014; Conklin et al., 2018), we feel it is important for all those who work in these varied fields to become familiar with the most common TL measurement techniques and to be aware of their respective advantages and drawbacks.

4. Measurement of telomere length: analytical caveats

A variety of techniques are employed to measure TL; they include: i) Southern blot analysis of terminal restriction fragments (TRFs), which measures the length distribution and average TL in biological samples; ii) fluorescence *in situ* hybridization (FISH), including Q-FISH and flow-FISH; iii) polymerase chain reaction (PCR)-based methods like quantitative (q)PCR, Single TElomere Length Analysis (STELA), and Telomere Shortest Length Assay (TeSLA); and iv) whole genome sequencing (WGS)-based techniques.

Although Southern blotting is the gold standard technique for TL measurement, the methods used most frequently in clinical and epidemiological studies are qPCR and FISH. Since the correlations among Southern blotting, FISH, and qPCR data from different laboratories can show different strength, laboratory-specific internal validation is clearly essential (Khincha et al., 2017).

The most commonly used matrix for TL measurement is still venous blood, although less invasive DNA collection methods are increasingly used. However, too little is known about how TL values relate in different samples. A recent study of three matrices – whole venous blood, finger-prick dried blood spot (DBS) and saliva – has shown that DBS and saliva are viable alternatives to invasive venous blood draws (Stout et al., 2017). Notably, TL values were higher in saliva than in whole blood or DBS.

Notably, all TL measurement techniques have been developed at a time when telomeric sequences were believed to be measureable only in the nucleus of eukaryotic cells. Now that TL has been found to provide much more complex biological information, it would be useful to distinguish between TL measured in the nucleus and in the other intra- and extracellular compartments. The difficulty of applying some of the techniques devised for the nucleus to sequences found in extra-chromosomal compartments suggests the need for novel technical approaches.

Since all TL measurement methods suffer from considerable weaknesses, several analytical caveats must be resolved before they can be put to routine use (Tarik et al., 2018; Lai et al., 2017, 2018).

The most commonly used methods and their technical drawbacks

are briefly reviewed below and depicted in Fig. 2 (**2a,** Southern blotting- TRF**; 2b,** PCR-based methods; **2c,** STELA; **2d** FISH).

4.1. Terminal restriction fragment (TRF) technique

This is a modified Southern blotting approach that measures the TL range in a cell population using the TRF length distribution expressed in kilobases (Fig. 2a) (Harley et al., 1990; Ouellette et al., 2000). It is one of the earliest TL assessment tools and has become the gold standard in telomere biology. DNA is extracted, digested, resolved by gel electrophoresis, transferred to a membrane, hybridized with labeled probes, and quantified. Though precise and highly accurate, it requires a considerable amount of intact genomic DNA and provides only an average TL for a population of cells (Kimura et al., 2010). The use of this time-consuming, expensive, and labor-intensive technique, which also requires high DNA concentrations, is therefore generally limited to studies involving limited number of samples. Southern blot analysis has recently been proposed to monitor the addition of telomeric sequences to single, newly generated telomeres *in vivo* (Bonetti and Longhese, 2018).

4.2. qPCR technique

qPCR is widely used in epidemiological studies, because it quickly provides mean relative TL (RTL) and requires small amounts of DNA (about 20 ng/reaction) (Fig. 2b). Introduced by Cawthon (2009), it achieves telomeric DNA detection through fluorescent signals (T) using partially mismatched primers. Telomeric DNA is normalized to a single-copy housekeeping gene (S) amplified in the same sample, and the T/S ratio is computed as a measure of RTL. A successive, improved version, Monochrome Multiplex Real-Time PCR (MMQPCR), requires a smaller amount of DNA. Amplification of both telomeric DNA and the single-copy gene in the same well of a plate reduce variability compared with monoplex PCR. Since the use of single-copy genes does not ensure optimal data normalization in cancer DNA, new primer sets have been developed to obtain a more precise evaluation of RTL (Dahlgren et al., 2018).

The chief drawback of measuring TL by qPCR is primer design. The repetitive nature of telomeres involves that primers can crosslink, giving rise to dimers and non-specific PCR signal. The problem can be overcome using samples with a good initial amount of template, since low template concentrations may generate non-specific signal. However, intra- and intersample variability can be high, and the wide range of the coefficient of variation described by a number of studies suggests limited reproducibility (Martin-Ruiz et al., 2015). Moreover, certain methodological conditions are known to affect TL measurement. For instance, some PCR master mixes can influence result specificity and consistency (Jiménez and Forero, 2018). Given the availability of different PCR mixes, the presence of stabilizers for the newly synthesized double-stranded DNA should carefully be checked (Wang et al., 2004). Another limitation of qPCR is that it provides a relative value (RTL) *per* genome rather than an absolute value in kilobases. This is a problem, given the limited number of comparative studies that have been conducted to validate qPCR with the gold standard Southern blotting. Although significant positive correlations have been reported between TL measured by qPCR and Southern blotting, experimental discrepancies do impact TL analysis, requiring optimization of PCR conditions (Tarik et al., 2018). Notably, the difficulty of comparing RTL values entails that only trends can be compared between studies.

Despite these important caveats, TL measurement by PCR is the most widely used method in epidemiological studies, and blood and saliva are the most common matrices in studies of large numbers of samples. The Genetic Epidemiology Research on Adult Health and Aging (GERA) has analyzed TL in 110,266 DNA specimens extracted from saliva (Lapham et al., 2015) using a novel high-throughput robotic system for TL and informatics analysis. Samples were run in triplicate along with control samples; within-sample variability was limited by employing thresholds to eliminate outlying measurements. Interestingly about 99% of samples passed all quality control measures. Another common matrix used for TL measurement by qPCR is leukocytes. LTL analyzed in 12,199 adults participating in two population-based prospective cohort studies in Europe (ESTHER) and the United States (Nurses' Health Study) was found to be associated to all-cause, cardiovascular disease (CVD), and cancer mortality (Mons et al., 2017).

Notably, DNA extraction methods have a pronounced influence on TL values, which in some circumstances can result in spurious or lost associations in epidemiological studies (Raschenberger et al., 2016). A recent meta-analysis of LTL and all-cause mortality suggests that the accuracy of the TL measurement technique used affects risk assessment (Wang et al., 2018b). Whereas qPCR provides reliable TL analysis in blood and saliva, this is not true of cancer cell DNA, because use of single-copy genes in conditions of genomic instability, which is typical of cancer cells, may affect the accuracy of normalization. Dedicated primer sets have recently been developed to achieve RTL measurement in cancer cells (Dahlgren et al., 2018).

The discovery of cf-DNA/RNA has raised the problem of detecting telomeric sequences in samples, such as microvesicles and exosomes, that contain very diluted template sequences, since in these cases qPCR efficiency may also be reduced. Moreover, no single-copy genes have yet been validated for cf-telomere estimation.

A more detailed description is required for STELA (Fig. 2c), which allows assessing the length of a chromosome subset, and TeSLA, which enables TL measurement in the subset of the shortest telomeres, *i.e.* those induced by sudden TS due not to cell replication but to breaks resulting from the action of stressors (Lai et al., 2017). TeSLA has been developed to overcome the problems posed by telomere repeat sequences (TRSs) in detecting abrupt TS in a single chromosome. STELA is labor-intensive and unsuitable for testing large numbers of samples. However, STELA data may have prognostic implications in the clinical assessment of disease cells, *e.g.* to predict clinical outcome in patients with chronic lymphocytic leukemia (Lin et al., 2014) or myelodysplastic syndrome (Williams et al., 2017).

4.3. Probe-based non-PCR methods

Probe-based TL assays, which involve the use of probes for telomeres (T) and a reference gene (R) for a given DNA sample, may provide a cost-effective approach to measure TL in extracted DNA samples (Kibriya et al., 2014). A novel, accurate, high-throughput, pooled-sample multiplex Luminex assay suitable for large-scale studies has recently been described (Jasmine et al., 2018).

4.4. Fluorescence in-situ hybridization (FISH) techniques: Q-FISH and flow-FISH

Q-FISH is a molecular cytogenetic method that can be employed to obtain information from metaphase or interphase cells, depending on the sequence of the fluorochrome-conjugated probe used. FISH methods can be used to measure TL in subsets of cells separated from fresh blood or from tissues (Fig. 2d) (Perner et al., 2003). Whereas the telomeres of each chromosome arm can provide heterogeneous staining results due to a different number of telomeric repeats, those found on the two sister chromatids generally display nearly identical intensities. The use of telomeric probes allows probe binding to its target to be identified by a distinct fluorescent signal within the cell nucleus. Synthetic DNA/RNA analogs capable of binding to DNA/RNA in a sequence-specific manner have been proved to yield more sensitive and specific TL measurements than DNA probes (Marchesini et al., 2016).

Since correct interpretation of the fluorescence signal requires a fluorescence microscope, FISH techniques require expensive equipment that may not be available at all laboratories.

Flow-FISH, first described in 1998 (Rufer et al., 1998), utilizes the

quantitative properties of telomere-specific probe retention to quantify median fluorescence in a population of cells *via* use of a flow cytometer instead of a fluorescence microscope. TL measurement by flow-FISH can have clinical relevance in specific clinical indications and selected settings, *e.g.* patients with mutations in some inherited genes (Alder et al., 2018). Flow-FISH has demonstrated excellent diagnostic sensitivity in measuring TL in patients with short telomere syndrome (STS). STSs are accelerated aging syndromes caused by heritable gene mutations which result in TS. For instance, RTL measurement by qPCR is not optimal to diagnose dyskeratosis congenital, an STS characterized by inherited bone marrow failure and cancer susceptibility caused by germline mutations in telomere biology genes (Gadalla et al., 2016); in these patients LTL analysis by flow-FISH is the recommended molecular diagnostic test (Gutierrez-Rodrigues et al., 2014). Flow-FISH can also be applied to measure TL in stored samples.

4.5. Whole genome sequence (WGS)-based technique

WGS-based TL measurement supplies reliable sequencing reads from telomeres **(**Castle et al., 2010; Parker et al., 2012**)**. However, their standard alignment to the reference sequence provides limited information on the region of origin, due to the repetitive nature of telomeric regions and to the fact that in the human reference sequence the ends of most chromosomes are simply stretches of Ns, which stand for unknown nucleotides. Methods have been developed to measure average TL from whole genome or exome shotgun sequence data (Ding et al., 2014) and to provide simultaneous TL measurement and global transcriptome analysis in the same cell (Wang et al., 2017a). The chief challenge for using next-generation sequences (NGSs) to measure TL is the need for bioinformatics expertise and softwares to decipher massive datasets. Several bioinformatics tools that adopt different approaches to identify, quantify, and normalize telomeric reads – like Motif_counter, TelSeq, Computel, qMotif, and Telomerecat – have been designed to determine telomere content and TL from NGS data (Lee et al., 2017; Farmery et al., 2018). Direct comparison of these WGS-based telomere measurement tools has shown that their best motifs all yielded similar performances when used to measure telomere content (Lee et al., 2017). In contrast, calculations using the best motif for TelSeq and Computel, the two tools that produce a TL estimate, were significantly lower and did not correlate well with TL measurements by TRF analysis (Lee et al., 2017).

Other important factors that should be considered when comparing WGS-based telomere measurement techniques are tool accessibility, ease of tool use, the time required to analyze one sample, and multithreading ability (Lee et al., 2017).

Telomere Dysfunction-Induced Foci (TIF) (Takai et al., 2003; de Lange, 2002; Karlseder et al., 1999) and Extrachromosomal DNA (Tokutake et al., 1998; Cohen and Méchali, 2002; Cesare and Griffith, 2004; Henson et al., 2009; Schvartzman et al., 2013; Komosa et al., 2015; Moye et al., 2015; Henson et al., 2017) analysis are not quantitative techniques and are not optimized for epidemiological studies. Separation of extrachromosomal DNA depends on sample preparation, DNA/RNA extraction procedures, and DNAse or RNase treatment, according to the compartment being assessed.

4.6. TL heterogeneity

TL heterogeneity has been detected in various cell types, including stem and cancer cells and can directly influence the frequency with which chromosomes undergo telomeric fusion and subsequent breakage-fusion-bridge cycles (Londoño-Vallejo, 2004). Single-cell analysis technology has recently been developed and effectively employed to investigate TL heterogeneity. Single-cell analysis of TL, *i.e.* STELA, where each amplicon is derived from a single telomeric molecule from a single cell, can disclose the full detail of TL distribution. Notably, STELA measures TL within the length ranges observed in senescent cells **(**Garcia-Martin et al., 2017), providing a new tool to explore the relationship between TL and the aging process.

5. TERRA and aging

The role of TERRA has been an outstanding issue in telomere biology for the past decade, mainly due to lack of knowledge of its loci. New insights suggest that TERRA molecules can be transcribed from nearly all telomeres in mammalian cells, always in centromere to telomere direction; they also suggest the involvement of subtelomeric regions – transcriptionally active genomic regions that give rise to long non-coding RNAs whose size ranges from 100 to 9 kb according to the position of the transcription starting site (Diman and Decottignies, 2018). These transcripts often display a cap structure added at the 5′ end, and only 7% of these transcripts are polyadenylated (Oliva-Rico and Herrera, 2017).

Cf-TERRA sequences have also been detected in human blood and serum, especially as exosome-associated molecules: the secretory phenotype characterized by release of cf-TERRA has been called TASP (TERRA-associated secretory phenotype) (Wang et al., 2015). Since high TERRA levels may be detected in human tumor tissue, they may prove to be innovative cancer biomarkers (Arora et al., 2014).

Notably, TERRA forms RNA:DNA hybrids at the chromosome ends and can fold into G-quadruplexes (Balk et al., 2014). Experimental evidence has shown that TERRA can also form higher-order structures based on parallel G4 units (Martadinata et al., 2011).

A mounting number of human diseases are being associated to abnormal G4 RNA regulation, suggesting the potential relevance of G4s to human health. Recent work supports the notion that G4s in the promoter regions of oncogene and telomere DNA could be therapeutic targets in cancer patients (Kaulage et al., 2018).

5.1. Measurement of TERRA: analytical caveats

The major problem in TERRA measurement is that transcription can start from the subtelomeric regions of all chromosomes. A number of primers specific for different subtelomeric regions have been used, including primers matching the 1q, 2q, 3p, 7p, 10p, 10q, 12q 13q, 14q, 15q, 17p,17q, 18p, XqYq, and XpYp chromosome, (Feretzaki and Lingner, 2017; Wang et al., 2015). However, some loci seem to be more involved in TERRA transcription than others. Chromosomes 20q and Xp have been suggested to be the main TERRA loci in human cells (Montero et al., 2016).

The techniques used most commonly to quantify TERRA sequences are briefly described below and are depicted in Fig. 3 **(3a,** RT-PCR; **3b,** Northern blotting; **3c,** RNA dot blotting; and **3d,** G-quadruplex detection with specific antibodies).

5.1.1. RT-qPCR

RT-qPCR is currently the fastest and most practical quantitative analysis technique for TERRA measurement.

The initial reverse transcription that yields cDNA can require a different amount of starting RNA, depending on the sample (Feretzaki and Lingner, 2017). DNAse treatment is required after RNA extraction (Iglesias et al., 2011), to ensure the quality and concentration of the starting sample, which can greatly affect results. Contamination with salt, ethanol, or proteins can strongly influence the performance of the downstream application.

Reverse transcription primers can be five repetitions of the oligonucleotide CCCTAA, random hexamers, or different set of primers. Poly-T, which is frequently used for reverse transcription of mRNA, is unsuitable due to the lack of TERRA transcript polyadenylation. GAPDH, U6snRNA, 36B4, GUSB, actin, and 18S have been described as suitable reference genes.

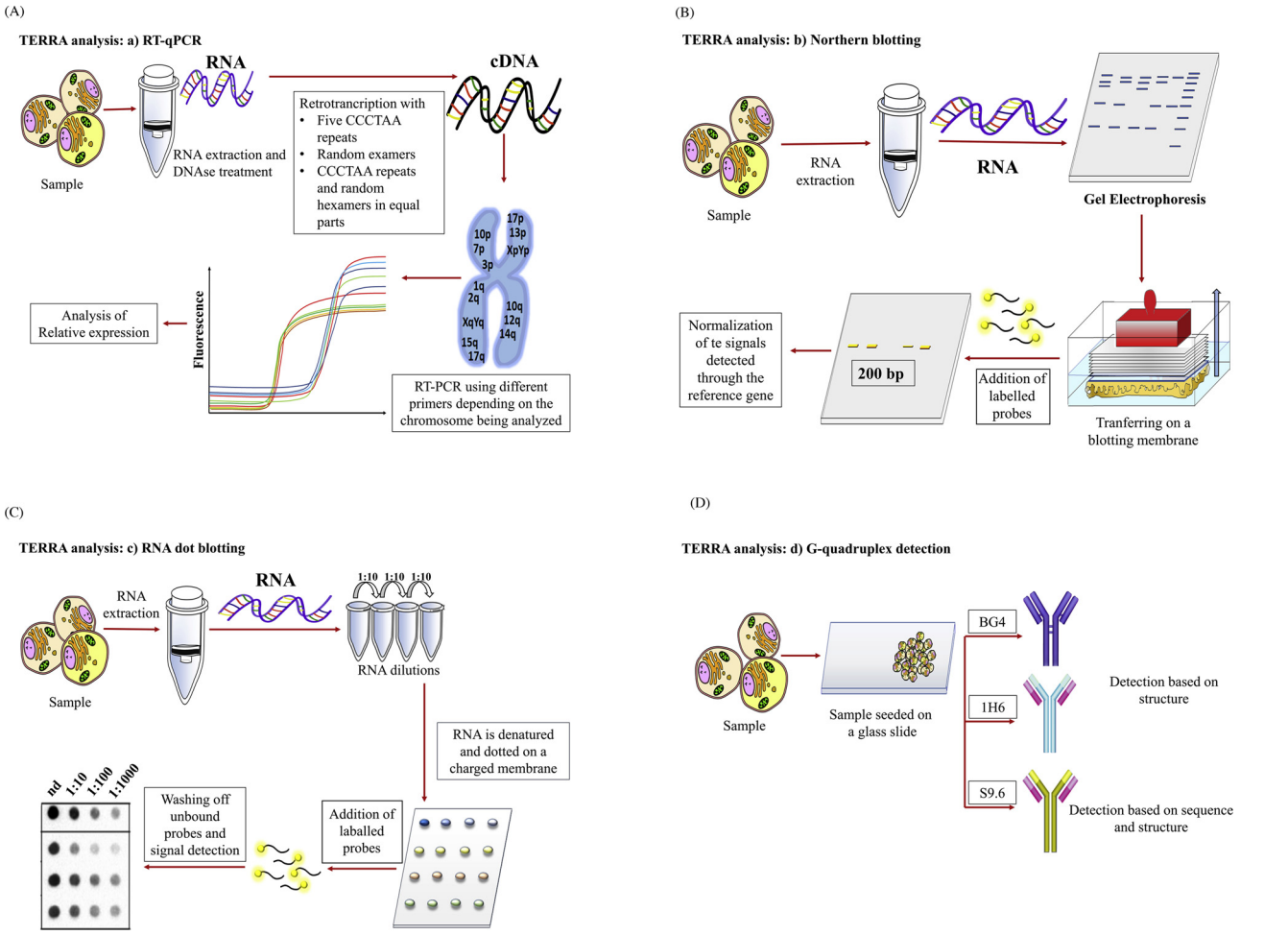

Fig. 3. Most commonly used techniques employed to quantify TERRA sequences.
Schematic representation of the three most common techniques employed to measure TERRA: 3a) RT-PCR; 3b) Northern blotting; 3c) RNA dot blotting (a simplification of Northern blotting that does not require gel electrophoresis); 3d) G-quadruplex detection with specific antibodies. G-quadruplex can be detected using engineered antibodies, such as clones 1H6 and BG4, which have specificity for the structure, or S9.6, which has specificity for structure and sequence.

5.1.2. Northern blotting

Northern blotting can detect the overall amount of TERRA transcripts, but unlike RT-qPCR it cannot measure minor changes in transcription or quantify TERRA levels in single chromosomes (Feretzaki and Lingner, 2017).

RNA extracted from samples is denatured and electrophoresed on agarose formamide gel, since the size of cf-TERRA is about 200 bp. Next, RNA can be transferred onto a blotting membrane and cross-linked to it using UV radiation. For RNA detection, the blot is hybridized using labeled probes specific for TERRA sense and antisense strands; 18S and U6 RNA have been used for normalization (Wang et al., 2015).

5.1.3. RNA dot blotting

RNA dot blotting is a simplification of Northern blotting. It does not require gel electrophoresis and it makes it possible to analyze different samples with different concentrations in a single test.

Extracted RNA is denatured using high temperatures or denaturing solutions, dotted on a charged nylon transfer membrane, and cross-linked to it by UV radiation. Subsequent steps involve a radiolabeled probe, washes in buffer to remove excess probe, and RNAse A treatment to eliminate single-strand RNA. The most common probes for TERRA detection are CCCTAA repeats labeled at their 5′ end with ^{32}P-γ-ATP by T4 polynucleotide kinase (Koskas et al., 2017).

5.1.4. RNA-FISH

RNA-FISH is used to analyze the cellular localization of TERRA by hybridizing detergent-extracted nuclei with a fluorescently labeled probe. It can even be used after immunofluorescence. Different probes, including C-rich peptidonucleic acid (PNA) coupled with a cyanine 3 fluorochrome, a fluorescently labeled probe containing CCCTAA repeats, or a light switching pyrene probe, can be used for hybridization to identify TERRA G4s in living cells (Koskas et al., 2017; Xu et al., 2010).

5.1.5. Detection of G-quadruplexes

G4s can be detected using engineered antibodies, such as clones 1H6 and BG4, which have specificity for the structure, not the sequence. These antibodies are used to detect the cellular localization of G4s by immunofluorescence. Since detection is based on structure, different types of G4s, *e.g.* DNA:RNA (hybrids), RNA-RNA, and DNA-DNA, can be identified.

Other critical aspects concern the labeling obtained with BG4 using different fixatives. Cytoplasmic TERRA is preferentially detected by fixation with PFA-Triton X-100, whereas nuclear quadruplexes (TERRA or DNA-DNA) can be detected by MeOH fixation (Laguerre et al., 2016). S9.6 is another monoclonal antibody with high affinity for DNA-RNA G4s formed at R-loops, because it is both structure- and sequence-specific. Maximum affinity is obtained with 52% of GC in the target sequence, whereas weak or no binding is obtained with amounts lower

than 25% or higher than 75% (König et al., 2017). Unfortunately, commercial kits including these antibodies are currently not available.

6. TA activity and aging

Telomerase is the only enzyme that can elongate a telomeric DNA chain to compensate for TS. It is different from other polymerases, because it involves a distinctive, reiterative reuse of an internal single-stranded telomeric template (Wu et al., 2017). Notably, the human TERT gene may be capable of autoregulation, because it is located very close to the telomere end of the chromosome, ˜ 1.2 Mb from the 5p end.

Most somatic cells lack TA; exceptions include embryonic tissue, stem cells, reproductive organs, and some rapidly regenerating tissues. Since TA is seen in 80–90% of tumor cells, its value as a diagnostic/prognostic tumor marker has prompted the development of reliable and standardized assays. As noted above, in somatic cells TA usually declines after birth; the successive cell divisions gradually lead to TS and trigger cellular senescence (Kim and Shay, 2018).

Most studies have investigated cells that express high levels of TERT, such as immortalized and cancer cells. However, TA regulation is a dynamic process also in normal human cells, suggesting that active telomere maintenance is required for the proliferation of normal cells (Masutomi et al., 2003). However, the role of telomerase in normal senescent cells has not been analyzed in depth. Reduced TERT protein levels and reduced TA have been described in senescent primary cultures of adult human dermal fibroblasts (HDFs) (Yehuda et al., 2017).

Telomerase activation/replacement has potential in treating patients with telomere maintenance deficiency syndrome as well as in tissue engineering approaches designed to treat degenerative conditions that are associated to normal aging. Telomerase-deficient mice have been proposed as a model to study the adverse cellular and organismal consequences of widespread DNA damage signaling activation *in vivo*. Telomerase reactivation in such mice extends TL, reduces DNA damage signaling, and eliminates degenerative phenotypes in multiple organs (Jaskelioff et al., 2011). Conversely, clinical research work is focusing on telomerase inhibition therapies to treat tumors, which induce telomerase overexpression by overcoming the short telomere barrier to unrestricted proliferation (Fleisig and Wong, 2007). Since telomerase-complex components are upregulated in most tumor cells, TERT restriction is a potential therapeutic option (Kim et al., 1994). Given that most somatic cells show low TA, selective inactivation of telomerase expression in cancer cells fails to influence most normal cells, suggesting that telomerase is a good target for cancer therapy (Sprouse et al., 2012).

TA is also considered as a biomarker for cardiovascular aging and CVD. Recent studies suggest a link between statins and telomere biology that may be explained by the anti-inflammatory actions of statins (Strazhesko et al., 2016).

Overall, TL and TA regulation is a complex and dynamic process that is impaired in patients with ARDs.

A greater understanding of the mechanisms underpinning telomerase regulation and the development of reliable TA assessment technologies is essential to help research in therapeutic strategies for telomerase modulation. A number of TA determination techniques, including those devised most recently, are described below. However, not all of them are suitable for epidemiological and clinical studies.

6.1. TA measurement: analytical caveats

TA detection assays can be divided into two main groups: those based on direct detection of telomerase products and those based on different systems of product amplification (Skvortsov et al., 2011). New bioanalytical methods based on direct detection of telomerase products, such as PCR-free assays, isothermal amplification, and Single Molecule Stochastic Binding assay, have recently been applied to TA detection not only in cells and tissues, but also in body fluids (Zhang et al., 2017a, 2017b; Su et al., 2017).

Multiple approaches have also been proposed to assess telomerase gene activity, including engagement of TERT alternative splicing, TERT gene amplification, and epigenetic changes (reviewed in Shay, 2016). Interestingly, some human TERT mutations/deletions have been associated to premature aging diseases, and patients carrying mutations in genes crucial for telomere maintenance show accelerated aging phenotypes (Chu et al., 2016). Recently, alterations in the gene encoding human TERT have been identified as points of interest for elucidating the oncogenic mechanism of several different cancer types (Bollam et al., 2018).

6.2. TA determination assays based on direct detection of telomerase products

6.2.1. TA detection using direct incorporation of a radioactively labeled substrate

The earliest TA detection method was based on the use of radioactive labeling without additional amplification (Blackburn et al., 1989). The method is still employed for qualitative determination of TA in cell line extracts. Since radioactive substances are complex and dangerous to use, new methods that do not use radioactive substances have been developed. Some of these approaches are briefly described in Table 1a.

6.3. TA detection assays based on telomerase product amplification

6.3.1. Telomere repeat amplification protocol (TRAP)

The Telomere Repeat Amplification Protocol (TRAP) is a widely used assay to determine TA in mammalian cells, tissues, and other biological samples (Kim et al., 1994). It involves three steps: extension, amplification, and detection of telomerase products. In the extension step, telomeric repeats are added to the telomerase substrate. In the amplification step, the extension products are amplified by PCR using specific primers (Wege et al., 2003). In the detection step, TA is estimated by electrophoretic analysis of the extension products. However, since amplification is often associated to poor reproducibility and high background, it is unsuitable for diagnostic applications. The main analytical challenges include throughput, the replacement of radioactive labels with non-labeled compounds, the reduction of the amount of side product, the elimination of primer-dimer artifacts and of false-positive amplification products, and the reduction of intra- and inter-sample variation (Wu et al., 2000; Skvortsov et al., 2011). Techniques employed to quantify telomerase activity (TA) were depicted in Fig. 4.

The main changes made to the basic TRAP protocol are summarized in Table 1b.

7. Biological variables related to TL, TERRA, and TA

Although TL is the area characterized by the largest number of applications, it is likely that similar biological, disease-mediated processes will be found to be relevant to TERRA and TA, which are interconnected.

Age, gender, and ethnicity have been found to be significantly associated to TL; for instance, the female gender and an African or Hispanic origin have been associated to a predisposition to have longer telomeres compared to the male gender and a European origin (Gardner et al., 2014; Hansen et al., 2016). Significant but weak correlations have also been described between LTL and hematological parameters (Meyer et al., 2016). A number of biomarkers related to a variety of physiological processes are also strongly associated to accelerated TS; however, the complex relationships between TL and the other chemical and clinical biomarkers are only beginning to be understood (Barrett et al., 2015).

The significant associations between inflammaging and telomere attrition, reported by several studies, suggest that TS is related to an

Table 1a
Some of the most relevant modification of TA detection assays based on direct detection of telomerase products.

Modifications of TA detection assays based on direct detection of telomerase products	Brief description of the protocol	References
Surface Plasmon Resonance ***(SPR)***	Surface Plasmon Resonance (SPR) can be applied to measure small local changes in refractive index on metal layers, linked directly to alterations in concentration on the surface. Biotin-conjugated oligonucleotides containing telomeric repeats can be immobilized on the surface of a sensor pretreated with streptavidin (). The oligomers associated with the telomerase extracts can be elongated and TA can be calculated by measuring the SPR signals. The main limiting factors are reaction time and sample concentration.	Maesawa et al., 2003
Detection of telomerase activity using oligo-modified magnetic particles and nuclear magnetic resonance ***(NMR)***	Another physical phenomenon employed for TA detection is nuclear magnetic resonance (NMR). Through a complementary interaction, the magnetic particles bind the telomeric sequences synthesized by telomerase. The spin-spin relaxation time is measured by a relaxometer. Since the formation of an organized nanoparticle ensemble involves a change in the magnetic relaxation time of surrounding water molecules, the magnetic field increases in presence of nanoparticle ensembles and decreases with non-ordered ones. Since the change reaches half of the maximum after 30 seconds and plateaus after 40-60 minutes, the analysis is fast, sensitive, and high-throughput.	Grimm et al., 2004
Quartz crystal microbalance technique (QCM)	This highly sensitive method involves microgravitometric analysis of TA based on the piezoelectric effect of quartz. The application of an alternating current promotes the oscillation of a quartz crystal, whose resonance frequency is then determined. Ligands on the crystal surface in a liquid environment can induce a reduction in resonance frequency. Telomerase induces oligonucleotide binding to the sensor's surface.	Pavlov et al., 2004.
Biobarcode assay	This approach uses magnetic particles, *i.e.* gold spheres, and two types of oligonucleotides, complementary and non complementary to telomerase-synthesized DNA. Binding of the electroactive complex $[Ru(NH_3)_6]^{3+}$ to the non-complementary telomerase-synthesized DNA chain permits quantitative detection. The method can measure TA in cell line extracts but is not used in tissues.	Li et al., 2010; Skvortsov et al., 2011
Optical biosensor assay	The assay is based on the change in the refraction index induced by the amount of telomerase products detected on the surface of the optical biosensor in real time. A three-oligonucleotide system, *i.e.* a "cassette system" including a sequence complementary to the RNA template of telomerase modifies the sensor surface to avoid steric impediments and to ensure process reversibility.	Buckle et al., 1996; Schmidt et al., 2002; Kulla and Katz, 2008
Quantum dots	Nanoparticles are conductor or semiconductor particles that represent quantum dots (QDs) in quantitative TA detection. Similar to the way a photon can be emitted during the transition of an atom between energy levels, a photon can be emitted during a transition in a quantum blot. In this system, photon absorption and emission occur at specific wavelengths that are related to QD size (smaller nanoparticles correspond to higher wavelengths). A modification involving a thio group at the end of the telomere oligonucleotide bound to the QD makes it capable of fluorescence when it absorbs a quantum with a wavelength of 400 nm and emits a quantum with wavelength of 560 nm. When a modified fluorescent oligonucleotide, TR-dUTP, is incorporated into telomerase DNA products bound to the QD, a fluorescence energy transfer occurs with a higher wavelength (610 nm). This method is not recommended for clinical materials.	Patolsky et al., 2003; Zavari-Nematabad et al., 2017; Li et al., 2018a, 2018b
Graphene oxide (GO)-based fluorescent nanosensor	A fluorescent DNA is adsorbed on a GO surface that can bind dye-labeled single-stranded DNA (ssDNA) complementary to the telomeric repeated sequence and efficiently quench the fluorescence of the dye *via* fluorescence resonance energy transfer (FRET). It is a rapid, sensitive and specific approach to detect telomerase activity.	Zhang et al., 2018

Brief description of NMR, SPR, QCM, Biobarcode assay, Optical biosensor assay, Quantum dots, Graphene oxide (GO)-based fluorescent nanosensor protocols.

enhanced susceptibility to the development and progression of ARDs, such as type 2 diabetes, CVD, and cancer (Bonfigli et al., 2016; Testa et al., 2011; Wang et al., 2016; Jose et al., 2017). With regard to cancer, long telomeres have been associated to an increased risk of several cancers; however, emerging data suggest that short telomeres might predict poor survival in cancer patients (Zhang et al., 2015; Rachakonda et al., 2018). Association studies of TL and cancer have identified significant, but not clinically relevant, differences in TL between cases and controls (Savage, 2018). A recent meta-analysis has highlighted that the inconsistent effect of TL on cancer outcomes may be due to different measurement methods (Adam et al., 2017; Wang et al., 2017b). Therefore, standardization of TL measurement and reporting has the potential to enhance the prognostic value of TL in human diseases.

A significant relationship, reported among exposure to factors promoting inflammation or oxidative stress (smoking) and shorter telomeres (Astuti et al., 2017), has been confirmed in the offspring of smoking mothers (Oerther and Lorenz, 2018). A recently described association between accelerated telomere attrition and marked weight gain in middle life suggests the importance of lifetime weight management to preserve functional telomeres (Hang et al., 2018). Nonetheless, the inconsistent effects of weight loss on TL and DNA repair indicate that interventions and assays should be reassessed (Himbert et al., 2017).

Different age-related telomere attrition trajectories and circulating inflammatory cytokine levels have been described in aging individuals (Lustig et al., 2017). This is not unexpected, since a number of stressors, including infectious agents, microbiota composition, cellular senescence, and misplaced nucleic acids (DNA/RNA), can promote TS in immune cells as well as in other somatic cells, fueling inflammation. These stressors are so closely interconnected that the respective effects are difficult to unravel. This could be one of the weakness of the studies exploring the relationship linking stressors, inflammaging, and TL: only the integrated analysis of a number of components in the same

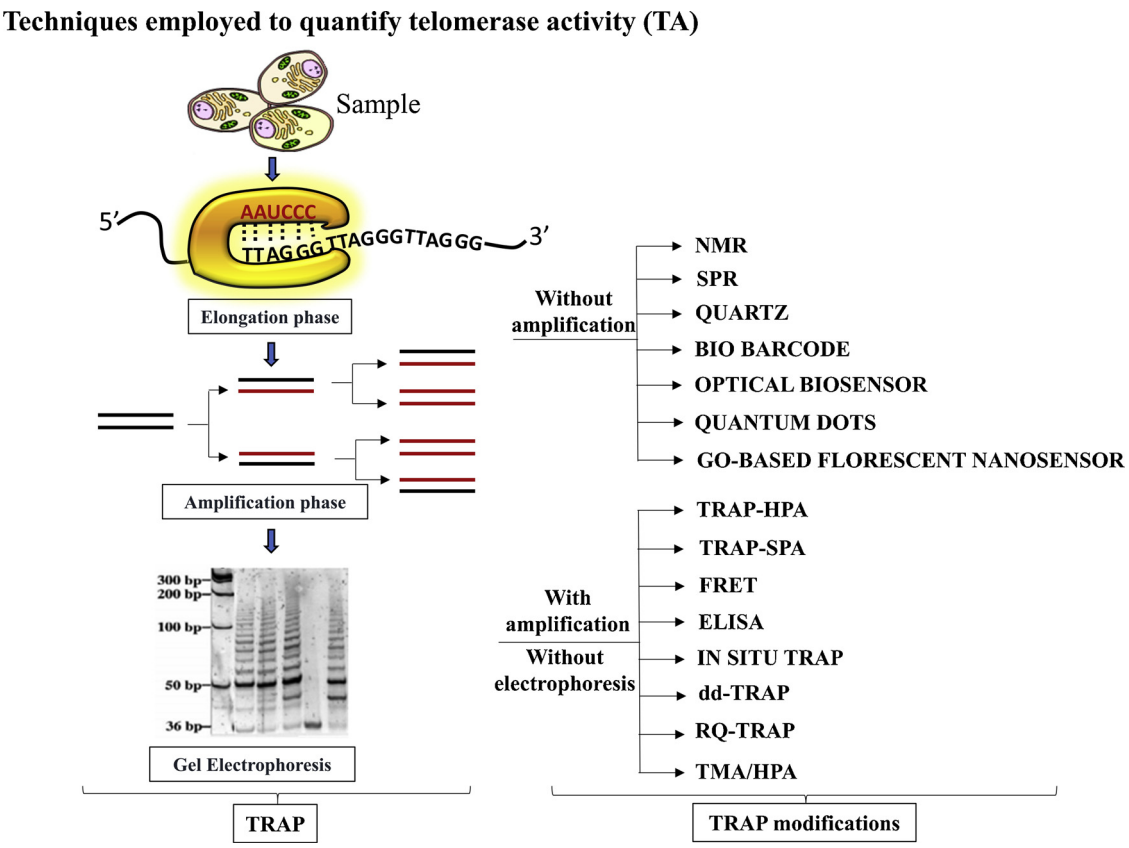

Fig. 4. Techniques employed to quantify telomerase activity (TA).
Schematic representation of the most common techniques employed to measure TA. The methods are grouped into two major categories: those based on direct detection of telomerase products and those based on different systems of amplification of these products (TRAP-based methods).

subjects/patients could clarify the issue. Moreover, the complex signaling affecting telomere balance during life requires multivariate analysis approaches to test for associations between TL and multiple biological variables; bias should be minimized especially in association studies involving TL and ARDs and TL and psychological and bio-behavioral variables.

TL measurement is also a useful approach to evaluate the pathogenicity of the genetic variants associated to telomere-related disorders. STSs are often caused by heritable gene mutations that result in decreased TL (Norberg et al., 2018). Their diverse clinical presentation – like bone marrow failure and idiopathic pulmonary fibrosis associated to gene mutations involving TERT, TERC, and other genes – make STSs a diagnostic challenge (Mangaonkar and Patnaik, 2018). Large-scale molecular epidemiological studies have uncovered novel associations between ARDs such as CVD, cancer, and impaired mental health, and both TL and common genetic variants in telomere biology genes (Savage, 2018).

Finally, it is conceivable that RNA-dependent factors will constitute additional biological variables for TERRA and TA measurement, both of which are RNA-dependent.

Advantages and disadvantages (sensitivity, specificity, number of samples that can be managed, ease to use and suitability for clinical studies) are reported in Table 2 for telomere length (TL), telomeric repeat-containing RNA (TERRA) and telomerase activity (TA) measurement techniques.

8. Conclusion

The outstanding challenge in telomere research is the development of accurate and reliable measurement methods ensuring simple and sensitive TL, TERRA, and TA detection. Reaching these goals requires guidelines for the development of standardized and automated measurement procedures. Studies of the reproducibility, accuracy, reliability, and sensitivity of the different TL, TERRA, and TA measurement methods at independent laboratories should be encouraged. Bench to bedside translation of biological metrics related to the telomere world requires the establishment of normal range values as well as critical values for interpretation. This is essential not only in studies of individual molecules, but also to shed light on the associations of TL, TERRA, and TA with physiological, psychological, and bio-behavioral phenomena and human pathological conditions. These tasks are complex and the data provided by the latest studies are not always clear and conclusive. For these reasons TL, TERRA, and TA are still not included in everyday clinical practice. Nevertheless, considerable progress has been made, and researchers are increasingly close to finding the solutions for better and faster measurement. Importantly, researchers undertaking the study of telomeres should be aware of the methodological problems involved in TL, TERRA, and TA analysis. Careful consideration is warranted when selecting measurement methods for research or clinical studies.

Notably, most of the sample material used for epidemiological studies is extracted banked DNA and/or RNA. Since the results of TL and TERRA measurement are affected by extraction methods and degradation, sample quality checks are critical for correct measurement and data interpretation.

Furthermore, RNA species detection in cells depends on metabolic activity, and although it may not be detectable in resting blood, it may be detected in tumor samples.

What is essential for future studies of the “telomere world” is that researchers publish full details of their methods and the quality control thresholds they employ. Notably, it is critical to be familiar with the relationships between telomeres and a number of other variables if

Table 1b
Some of the most relevant modifications made to the basic TRAP protocol.

Modifications of TRAP protocol	Brief description of the protocol	References
TRAP with a post-PCR hybridization protection protocol ***(TRAP-HPA)***	TRAP-HPA uses a probe labeled with covalently bound chemiluminescent acridine to detect DNA after amplification and provides high-throughput semi-quantitative determination of TA.	Hirose et al., 1997; Fajkus, 2006
Scintillation proximity assay ***(TRAP-SPA)***	TRAP-SPA enables detection of amplification products thought the conjugation of telomerase products with biotin and amplification in presence of [3 H] TTP. Semi-quantitative determination can be obtained in large series of tissue and cell line extracts. The main drawbacks are the use of tritium and PCR artifacts.	Savoysky et al., 1996; Fajkus,2006
TRAP with Fluorescence Resonance Energy Transfer ***(FRET)***	FRET uses two primers (amplifluors) whose hairpin structure contains a donor (fluorescein) and an acceptor (4'-dimethylaminophenyl-azobenzoic acid. Its main advantages include the absence of radioisotopes, the limited volume of the elongation products, the elimination of post-PCR processing, the reduction of carryover contamination risk, and fast analysis.	Uehara et al., 1999; Ding et al., 2010; Kawamura et al., 2014; Fajkus,2006
TRAP combined with Enzyme-Linked Immunosorbent Assay ***(ELISA)***	TRAP-ELISA is a colorimetric qualitative and semi-quantitative assay, where biotin conjugation of telomere-imitating oligonucleotide (TS) primers permits binding of amplified DNA to streptavidin-coated microplates. This step is followed by DNA denaturation and hybridization with digoxigenin (DIG)-labeled probes specific for telomeric sequences. The addition of polyclonal DIG antibodies conjugated to horseradish peroxidase prompts the colorimetric reaction and enables TA detection. Some commercial kits include positive and negative internal standards. Since TRAP-ELISA is fast and allows analyzing multiple samples in a single run, it is the most commonly used assay for TA determination in screening studies.	Fajkus, 2006; Wu et al., 2000; Sue et al., 2014
In situ TRAP	In *in situ* TRAP the cell suspensions are immobilized on silane-coated slides. PCR commonly uses FITC-labeled primers, and the final analysis is made under a fluorescence microscope. Fluorescence intensity and localization are employed to determine TA in individual cells (cancer cells usually show bright fluorescence). *In situ* TRAP can also be used to obtain a semi-quantitative determination of TA in tissue sections.	Skvortsov et al., 2011; Fajkus, 2006
Droplet Digital Telomere Repeat Amplification Protocol ***(ddTRAP)***	This is a two-step assay where cell lysates are analyzed by droplet digital PCR (ddPCR). It involves the same steps as a conventional TRAP assay, except that the PCR products are detected by ddPCR. This system has improved the throughput, sensitivity, and reproducibility of the TRAP assay. As a consequence, it can test a variety of cell types, including cell lines and primary adult human cells, without radioactive compounds but with comparable sensitivity.	Ludlow et al., 2018; Fajkus, 2006
Real-time Quantitative TRAP ***(RQ-TRAP)***	Real-time Quantitative TRAP (RQ-TRAP) uses fluorescent dyes (*e.g.* PicoGreen or SYBR Green) to quantify double-stranded DNA products in the PCR elongation and amplification steps. Its main advantage is that TA quantification is achieved without any additional time-consuming steps besides sample extraction and real-time cycling. The assay provides accurate TA measurement, hence highly effective TA monitoring in cultured cells, and can be used to analyze multiple samples. Its main disadvantages are the inhibitory effects of PCR contaminants, the possibility that PCR reaction saturation in the final step may level small TA differences compared to the results of conventional TRAP, and the risk of false-positive signals.	Wege et al., 2003; Gelmini et al., 1998; Hou et al., 2001; Saldanha et al., 2003; Hou et al., 2001; Skvortsov et al., 2011; Fajkus, 2006
TRAP with isothermic transcription-mediated amplification (TMA/HPA)	This approach provides a semi-quantitative determination of TA based on the presence of a polymerase that uses synthesized DNA as a matrix for RNA synthesis. Telomerase-synthesized DNA contains an additional sequence acting as a substrate for reverse primer hybridization. This tecnique also avoids the necessity of performing and evaluating polyacrylamide gel electrophoresis of reaction products and it is characterized by high sensitivity (1-1000 cells). Its main advantage is that it does not require sample heating. The assay is sensitive to the presence of RNase.	Saldanha et al., 2003; Skvortsov et al., 2011

Brief description of TRAP HPA, TRAP-SPA, FRET, ELISA, *in situ* TRAP, ddTRAP, RQ-TRAP and TMA/HPA protocols.

errors generated by confounding variables that are not controlled for are to be avoided.

Starting from the most recent evidence in telomere biology, we hypothesize that combined TL, TERRA, and TA analysis may provide highly reliable information to estimate the rate of cell, tissue and organismal aging and the risk of ARD development (Kim and Shay, 2018). TL, TERRA, and TA have been associated singly to aging and a number of common ARDs, yet no studies have evaluated them in an integrated way, either using cellular or organismal models.

Although red blood cells and platelets are a significant source of exosomes from blood, different cell types can release exosomes into human plasma/serum, including endothelial cells, monocytes and astrocytes (Goetzl et al., 2017; Goetzl et al., 2016; Halim et al., 2016). Plasma exosomes released by endothelial cells and monocytes should be extensively investigated for telomeres and TERRA content, especially in patients with ARDs.

9. Future perspective

A new geriatric medicine branch, geroscience, postulates that it should be possible to delay the aging process and the onset of the most common chronic ARDs (Vaiserman and Lushchak, 2017), and mounting evidence suggests that the human healthspan could be increased by nutraceutical or pharmaceuticals approaches (Gurău et al., 2018; Klimova et al., 2018). The hypothesis has prompted the organization of geroscience-guided therapeutic trials, *e.g.* TAME (Targeting Aging with

Table 2
Advantages and disadvantages of the most common TL, TERRA, and TA measurement techniques.

	Tecnique	Time efficient	Sensitivity	Specificity	Reproducibility	Easy to use	Suited for clinical studies	References
TL	**STELA/TeSLA**	No	High	High	High	No	No	Lai et al., 2018; Martin-Ruiz et al., 2015.
	(q)PCR	Yes	Low	Intermediate	Intermediate	Yes	Yes	Gutierrez-Rodrigues et al., 2014; Martin-Ruiz et al., 2015.
	Southern blot	No	Intermediate	High	High	Yes	No	Tarik et al., 2018; Martin-Ruiz et al., 2015.
	FISH-qFISH-Flow	No	Intermediate	Intermediate	Intermediate	Yes	No	Baerlocher et al., 2002
	FISH	Yes	High	High	High	Yes	Yes	Gutierrez-Rodrigues et al., 2014
	WGS	No	High	High	High	No	Yes	Lee et al., 2017
TERRA	**RT-qPCR**	Yes	High	Intermediate	–	Yes	Yes	Feretzaki and Lingner, 2017
	Northern Blot	No	Low	High	–	Yes	No	Feretzaki and Lingner, 2017
	RNA dot blotting	Yes	Low	High	–	Yes	No	Koskas et al., 2017
	RNA-FISH	Yes	High	Intermediate	–	Yes	No	Koskas et al., 2017; Xu et al., 2010
TA	**Classic**	No	Intermediate	Intermediate	Low	Yes	No	Ohuchida et al., 2005; Fajkus, 2006
	HPA	No	Intermediate	Intermediate	Low	Yes	No	Hirose et al., 1997; Fajkus, 2006
	SPA	No	Intermediate	Intermediate	Low	No	No	Savoysky et al., 1996; Fajkus,2006
		Yes	Intermediate	Intermediate	Intermediate	Yes	Yes	Uehara et al., 1999; Ding et al., 2010; Kawamura et al., 2014; Fajkus,2006
		Yes	High	High	Intermediate	Yes	Yes	Wu et al., 2000; Sue et al., 2014; Fajkus, 2006
		Yes	High	High	Intermediate	Yes	No	Skvortsov et al., 2011; Fajkus, 2006
		Yes	High	High	High	Yes	Yes	Ludlow et al., 2018; Fajkus, 2006
		Yes	High	Intermediate	High	Yes	Yes	Wege et al., 2003; Gelmini et al., 1998; Hou et al., 2001; Saldanha et al., 2003; Hou et al., 2001; Skvortsov et al., 2011; Fajkus, 2006
		Yes	High	High	–	Yes	No	Saldanha et al., 2003; Skvortsov et al., 2011
	SPR	Yes	Intermediate	Intermediate	High	Yes	No	Maesawa et al., 2003
	NMR	Yes	High	Intermediate	High	No	No	Grimm et al., 2004.
	QCM	Yes	High	Intermediate	High	No	No	Pavlov et al., 2004.
	Biobarcode	No	High	High	Intermediate	Yes	No	Li et al., 2010; Skvortsov et al., 2011
	Optical biosensor	Yes	High	High	High	Yes	No	Buckle et al., 1996; Schmidt et al., 2002; Kulla and Katz, 2008
	Quantum dots	No	High	High	–	Yes	No	Patolsky et al., 2003; Zavari-Nematabad et al., 2017; Li et al., 2018a, 2018b
	GO-based fluorescent nanosensor	Yes	High	High	–	Yes	Yes	Zhang et al., 2018

Advantages and disadvantages (sensitivity, specificity, number of samples that can be managed and ease to use) are reported for telomere length (TL), telomeric repeat-containing RNA (TERRA), and telomerase activity (TA) measurement techniques.
-Not yet addressed.

MEtformin) (Justice et al., 2018). However, ongoing studies highlight the scarcity of well-vetted "rate of aging" biomarkers for human studies and the need for such biomarkers to move from the assessment of chronological age to the evaluation of physiological age (Kohanski et al., 2016; Sierra and Kohanski, 2017).

Cutting-edge technologies for TL, TERRA, and TA measurement are still in their infancy, but they promise great future discoveries through method improvements and the multiplication of laboratories offering them. Technological advances are expected to provide fast, automated, and standardized measurement of TL, TERRA, and TA in the same sample not only in cells and tissues, but in all biological fluids and circulating vesicles. These new technologies are expected to provide more concordant data in association studies both with regard to aging *per se* and to a number of ARDs.

Conflicts of interest

None.

Authors' contributions

Emanuela Mensà, Silvia Latini, Deborah Ramini and Gianluca Storci wrote the paper, which was revised by Massimiliano Bonafè and Fabiola Olivieri.

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References

Adam, R., Díez-González, L., Ocaña, A., Šeruga, B., Amir, E., Templeton, A.J., 2017. Prognostic role of telomere length in malignancies: a meta-analysis and meta-regression. Exp. Mol. Pathol. 102, 455–474. https://doi.org/10.1016/j.yexmp.2017.05.010.

Alder, J.K., Hanumanthu, V.S., Strong, M.A., DeZern, A.E., Stanley, S.E., Takemoto, C.M., Danilova, L., Applegate, C.D., Bolton, S.G., Mohr, D.W., Brodsky, R.A., Casella, J.F., Greider, C.W., Jackson, J.B., Armanios, M., 2018. Diagnostic utility of telomere length testing in a hospital-based setting. Proc. Natl. Acad. Sci. U. S. A. 115, E2358–E2365. https://doi.org/10.1073/pnas.1720427115.

Arora, R., Lee, Y., Wischnewski, H., Brun, C.M., Schwarz, T., Azzalin, C.M., 2014. RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells. Nat. Commun. 21, 5–5220.

Astuti, Y., Wardhana, A., Watkins, J., Wulaningsih, W., PILAR Research Network, 2017. Cigarette smoking and telomere length: a systematic review of 84 studies and meta-analysis. Environ. Res. 158, 480–489.

Baerlocher, G.M., Mak, J., Tien, T., Lansdorp, P.M., 2002. Telomere length measurement by fluorescence in situ hybridization and flow cytometry: tips and pitfalls. Cytometry 47, 89–99.

Balk, B., Dees, M., Bender, K., Luke, B., 2014. The differential processing of telomeres in response to increased telomeric transcription and RNA-DNA hybrid accumulation. RNA Biol. 11, 95–100.

Barrett, J.H., Iles, M.M., Dunning, A.M., Pooley, K.A., 2015. Telomere length and common disease: study design and analytical challenges. Hum. Genet. 134, 679–689.

Belsky, D.W., Caspi, A., Houts, R., Cohen, H.J., Corcoran, D.L., Danese, A., Harrington, H., Israel, S., Levine, M.E., Schaefer, J.D., Sugden, K., Williams, B., Yashin, A.I., Poulton, R., Moffitt, T.E., 2015. Quantification of biological aging in young adults. Proc. Natl. Acad. Sci. U. S. A. 112, E4104–10. https://doi.org/10.1073/pnas.1506264112.

Belsky, D.W., Moffitt, T.E., Cohen, A.A., Corcoran, D.L., Levine, M.E., Prinz, J.A., Schaefer, J., Sugden, K., Williams, B., Poulton, R., Caspi, A., 2018. Eleven telomere, epigenetic clock, and biomarker-composite quantifications of biological aging: do they measure the same thing? Am. J. Epidemiol. 187, 1220–1230.

Blackburn, E.H., 1990. Telomeres: structure and synthesis. J. Biol. Chem. 265,

5919–5921.
Blackburn, E.H., 2005. Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. FEBS Lett. 579, 859–862.
Blackburn, E.H., Greider, C.W., Henderson, E., Lee, M.S., Shampay, J., Shippen-Lentz, D., 1989. Recognition and elongation of telomeres by telomerase. Genome 31, 553–560.
Blackburn, E.H., Epel, E.S., Lin, J., 2015. Human telomere biology: a contributory and interactive factor in aging, disease risks, and protection. Science 350, 1193–1198.
Blasco, M.A., Gasser, S.M., Lingner, J., 1999. Telomeres and telomerase. Genes Dev. 13, 2353–2359.
Bollam, S.R., Berens, M.E., Dhruv, H.D., 2018. When the ends are really the beginnings: targeting telomerase for treatment of GBM. Curr. Neurol. Neurosci. Rep. 18, 15.
Bonetti, D., Longhese, M.P., 2018. Analysis of de novo telomere addition by southern blot. Methods Mol. Biol. 1672, 363–373.
Bonfigli, A.R., Spazzafumo, L., Prattichizzo, F., Bonafè, M., Mensà, E., Micolucci, L., Giuliani, A., Fabbietti, P., Testa, R., Boemi, M., Lattanzio, F., Olivieri, F., 2016. Leukocyte telomere length and mortality risk in patients with type 2 diabetes. Oncotarget 7, 50835–50844. https://doi.org/10.18632/oncotarget.10615.
Brown, L., Zhang, Y., Mitchel, C., Ailshire, J., 2018. Does telomere length indicate biological, physical and cognitive health among older adults? Evidence from the health and retirement study. J. Gerontol. A Biol. Sci. Med. Sci. https://doi.org/10.1093/gerona/gly001.
Buckle, M., Williams, R.M., Negroni, M., Buc, H., 1996. Real time measurements of elongation by a reverse transcriptase using surface plasmon resonance. Proc. Natl. Acad. Sci. U. S. A. 93, 889–894.
Byrd, A.K., Zybailov, B.L., Maddukuri, L., Gao, J., Marecki, J.C., Jaiswal, M., Bell, M.R., Griffin, W.C., Reed, M.R., Chib, S., Mackintosh, S.G., MacNicol, A.M., Baldini, G., Eoff, R.L., Raney, K.D., 2016. Evidence that G-quadruplex DNA accumulates in the cytoplasm and participates in stress granule assembly in response to oxidative stress. J. Biol. Chem. 291, 18041–18057.
Cammas, A., Millevoi, S., 2017. RNA G-quadruplexes: emerging mechanisms in disease. Nucleic Acids Res. 45, 1584–1595.
Castle, J.C., Biery, M., Bouzek, H., Xie, T., Chen, R., Misura, K., Jackson, S., Armour, C.D., Johnson, J.M., Rohl, C.A., Raymond, C.K., 2010. DNA copy number, including telomeres and mitochondria, assayed using next-generation sequencing. BMC Genomics 11, 244.
Cawthon, R.M., 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. 37, e21.
Cesare, A.J., Griffith, J.D., 2004. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. Mol. Cell. Biol. 24, 9948–9957.
Chen, W., Kimura, M., Kim, S., Cao, X., Srinivasan, S.R., Berenson, G.S., 2011. Longitudinal versus cross-sectional evaluations of leukocyte telomere length dynamics: age-dependent telomere shortening is the rule. J. Gerontol. A Biol. Sci. Med. Sci. 66, 312–319.
Chiang, Y.J., Calado, R.T., Hathcock, K.S., Lansdorp, P.M., Young, N.S., Hodes, R.J., 2010. Telomere length is inherited with resetting of the telomere set-point. Proc. Natl. Acad. Sci. U. S. A. 107, 10148–10153.
Chu, T.W., MacNeil, D.E., Autexier, C., 2016. Multiple mechanisms contribute to the cell growth defects imparted by human telomerase insertion in fingers domain mutations associated with premature aging diseases. J. Biol. Chem. 291, 8374–8386.
Cohen, S., Méchali, M., 2002. Formation of extrachromosomal circles from telomeric DNA in Xenopus laevis. EMBO Rep. 3, 1168–1174.
Cohen, S., Agmon, N., Sobol, O., Segal, D., 2010. Extrachromosomal circles of satellite repeats and 5S ribosomal DNA in human cells. Mob. DNA 1, 11.
Conklin, Q.A., King, B.G., Zanesco, A.P., Lin, J., Hamidi, A.B., Pokorny, J.J., Álvarez-López, M.J., Cosín-Tomás, M., Huang, C., Kaliman, P., Epel, E.S., Saron, C.D., 2018. Insight meditation and telomere biology: the effects of intensive retreat and the moderating role of personality. Brain Behav. Immun. 70, 233–245. https://doi.org/10.1016/j.bbi.2018.03.003.
Cusanelli, E., Romero, C.A., Chartrand, P., 2013. Telomeric noncoding RNA TERRA is induced by telomere shortening to nucleate telomerase molecules at short telomeres. Mol. Cell 51, 780–791.
d'Adda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., Jackson, S.P., 2003. A DNA damage checkpoint response in telomere-initiated senescence. Nature 426, 194–198.
Dahlgren, P.N., Bishop, K., Dey, S., Herbert, B.S., Tanaka, H., 2018. Development of a new monochrome multiplex qPCR method for relative telomere length measurement in Cancer. Neoplasia 20, 425–431.
Danese, E., Lippi, G., 2018. Telomere length: is the future in our "ends"? Ann. Transl. Med. 6, 280. https://doi.org/10.21037/atm.2018.06.24.
de Lange, T., 2002. Protection of mammalian telomeres. Oncogene 21, 532–540.
Diman, A., Decottignies, A., 2018. Genomic origin and nuclear localization of TERRA telomeric repeat-containing RNA: from Darkness to Dawn. FEBS J. 285, 1389–1398.
Ding, C., Li, X., Ge, Y., Zhang, S., 2010. Fluorescence detection of telomerase activity in cancer cells based on isothermal circular strand-displacement polymerization reaction. Anal. Chem. 82, 2850–2855.
Ding, Z., Mangino, M., Aviv, A., Spector, T., Durbin, R., UK10K Consortium, 2014. Estimating telomere length from whole genome sequence data. Nucleic Acids Res. 42, e75.
Fajkus, J., 2006. Detection of telomerase activity by the TRAP assay and its variants and alternatives. Clin. Chim. Acta 371, 25–31.
Farmery, J.H.R., Smith, M.L., NIHR BioResource - Rare Diseases, Lynch, A.G., 2018. Telomerecat: a ploidy-agnostic method for estimating telomere length from whole genome sequencing data. Sci. Rep. 8, 1300.
Feretzaki, M., Lingner, J., 2017. A practical qPCR approach to detect TERRA, the elusive telomeric repeat-containing RNA. Methods 114, 39–45.
Fernando, M.R., Jiang, C., Krzyzanowski, G.D., Ryan, W.L., 2017. New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. PLoS One 12, e0183915.
Fleisig, H.B., Wong, J.M., 2007. Telomerase as a clinical target: current strategies and potential applications. Exp. Gerontol. 42, 102–112.
Freitas-Simoes, T.M., Ros, E., Sala-Vila, A., 2018. Telomere length as a biomarker of accelerated aging: is it influenced by dietary intake? Curr. Opin. Clin. Nutr. Metab. Care 21, 430–436.
Gadalla, S.M., Khincha, P.P., Katki, H.A., Giri, N., Wong, J.Y., Spellman, S., Yanovski, J.A., Han, J.C., De Vivo, I., Alter, B.P., Savage, S.A., 2016. The limitations of qPCR telomere length measurement in diagnosing dyskeratosis congenital. Mol. Genet. Genomic Med. 4, 475–479.
Gao, X., Zhang, Y., Mons, U., Brenner, H., 2018. Leukocyte telomere length and epigenetic-based mortality risk score: associations with all-cause mortality among older adults. Epigenetics 13, 846–857. https://doi.org/10.1080/15592294.2018.1514853.
Garcia-Martin, I., Janssen, A.B., Jones, R.E., Grimstead, J.W., Penketh, R.J.A., Baird, D.M., John, R.M., 2017. Telomere length heterogeneity in placenta revealed with high-resolution telomere length analysis. Placenta 59, 61–68. https://doi.org/10.1016/j.placenta.2017.09.007.
Gardner, M., Bann, D., Wiley, L., Cooper, R., Hardy, R., Nitsch, D., Martin-Ruiz, C., Shiels, P., Sayer, A.A., Barbieri, M., Bekaert, S., Bischoff, C., Brooks-Wilson, A., Chen, W., Cooper, C., Christensen, K., De Meyer, T., Deary, I., Der, G., Diez Roux, A., Fitzpatrick, A., Hajat, A., Halaschek-Wiener, J., Harris, S., Hunt, S.C., Jagger, C., Jeon, H.S., Kaplan, R., Kimura, M., Lansdorp, P., Li, C., Maeda, T., Mangino, M., Nawrot, T.S., Nilsson, P., Nordfjall, K., Paolisso, G., Ren, F., Riabowol, K., Robertson, T., Roos, G., Staessen, J.A., Spector, T., Tang, N., Unryn, B., van der Harst, P., Woo, J., Xing, C., Yadegarfar, M.E., Park, J.Y., Young, N., Kuh, D., von Zglinicki, T., Ben-Shlomo, Y., Halcyon study team, 2014. Gender and telomere length: systematic review and meta-analysis. Exp. Gerontol. 51, 15–27.
Gelmini, S., Caldini, A., Becherini, L., Capaccioli, S., Pazzagli, M., Orlando, C., 1998. Rapid, quantitative nonisotopic assay for telomerase activity in human tumors. Clin. Chem. 44, 2133–2138.
Goetzl, E.J., Mustapic, M., Kapogiannis, D., Eitan, E., Lobach, I.V., Goetzl, L., Schwartz, J.B., Miller, B.L., 2016. Cargo proteins of plasma astrocyte-derived exosomes in Alzheimer's disease. FASEB J. 30, 3853–3859.
Goetzl, E.J., Schwartz, J.B., Mustapic, M., Lobach, I.V., Daneman, R., Abner, E.L., Jicha, G.A., 2017. Altered cargo proteins of human plasma endothelial cell-derived exosomes in atherosclerotic cerebrovascular disease. FASEB J. 31, 3689–3694.
Gomez, D.E., Armando, R.G., Farina, H.G., Menna, P.L., Cerrudo, C.S., Ghiringhelli, P.D., Alonso, D.F., 2012. Telomere structure and telomerase in health and disease (review). Int. J. Oncol. 41, 1561–1569. https://doi.org/10.3892/ijo.2012.1611.
Gorenjak, V., Akbar, S., Stathopoulou, M.G., Visvikis-Siest, S., 2018. The future of telomere length in personalized medicine. Front. Biosci. (Landmark Ed) 23, 1628–1654.
Graf, M., Bonetti, D., Lockhart, A., Serhal, K., Kellner, V., Maicher, A., Jolivet, P., Teixeira, M.T., Luke, B., 2017. Telomere length determines TERRA and R-Loop regulation through the cell cycle. Cell 170, 72–85.
Grimm, J., Perez, J.M., Josephson, L., Weissleder, R., 2004. Novel nanosensors for rapid analysis of telomerase activity. Cancer Res. 64, 639–643.
Gurău, F., Baldoni, S., Prattichizzo, F., Espinosa, E., Amenta, F., Procopio, A.D., Albertini, M.C., Bonafè, M., Olivieri, F., 2018. Anti-senescence compounds: a potential nutraceutical approach to healthy aging. Ageing Res. Rev. 46, 14–31. https://doi.org/10.1016/j.arr.2018.05.001.
Gursel, I., Gursel, M., Yamada, H., Ishii, K.J., Takeshita, F., Klinman, D.M., 2003. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. J. Immunol. 171, 1393–1400.
Gutierrez-Rodrigues, F., Santana-Lemos, B.A., Scheucher, P.S., Alves-Paiva, R.M., Calado, R.T., 2014. Direct comparison of Flow-FISH and qPCR as diagnostic tests for telomere length measurement in humans. PLoS One 9, e113747.
Haapanen, M.J., Perälä, M.M., Salonen, M.K., Guzzardi, M.A., Iozzo, P., Kajantie, E., Rantanen, T., Simonen, M., Pohjolainen, P., Eriksson, J.G., von Bonsdorff, M.B., 2018. Telomere length and frailty: the helsinki birth cohort study. J. Am. Med. Dir. Assoc. 19, 658–662. https://doi.org/10.1016/j.jamda.2018.05.011.
Halim, A.T., Ariffin, N.A., Azlan, M., 2016. Review: the multiple roles of monocytic microparticles. Inflammation 39, 1277–1284.
Hang, D., Nan, H., Kværner, A.S., De Vivo, I., Chan, A.T., Hu, Z., Shen, H., Giovannucci, E., Song, M., 2018. Longitudinal associations of lifetime adiposity with leukocyte telomere length and mitochondrial DNA copy number. Eur. J. Epidemiol. 33, 485–495.
Hansen, M.E., Hunt, S.C., Stone, R.C., Horvath, K., Herbig, U., Ranciaro, A., Hirbo, J., Beggs, W., Reiner, A.P., Wilson, J.G., Kimura, M., De Vivo, I., Chen, M.M., Kark, J.D., Levy, D., Nyambo, T., Tishkoff, S.A., Aviv, A., 2016. Shorter telomere length in Europeans than in Africans due to polygenetic adaptation. Hum. Mol. Genet. 25, 2324–2330.
Harley, C.B., Futcher, A.B., Greider, C.W., 1990. Telomeres shorten during ageing of human fibroblasts. Nature 345, 458–460.
Hastings, W.J., Shalev, I., Belsky, D.W., 2017. Translating Measures of Biological Aging to Test Effectiveness of Geroprotective Interventions: What Can We Learn from Research on Telomeres? Front. Genet. 8, 164.
Henson, J.D., Cao, Y., Huschtscha, L.I., Chang, A.C., Au, A.Y., Pickett, H.A., Reddel, R.R., 2009. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. Nat. Biotechnol. 27, 1181–1185.
Henson, J.D., Lau, L.M., Koch, S., Martin La Rotta, N., Dagg, R.A., Reddel, R.R., 2017. The C-Circle assay for alternative-lengthening-of-telomeres activity. Methods 114, 74–84.
Himbert, C., Thompson, H., Ulrich, C.M., 2017. Effects of intentional weight loss on markers of oxidative stress, DNA repair and telomere length - a systematic review. Obes. Facts 10, 648–665.
Hirose, M., Abe-Hashimoto, J., Ogura, K., Tahara, H., Ide, T., Yoshimura, T., 1997. A

rapid, useful and quantitative method to measure telomerase activity by hybridization protection assay connected with a telomeric repeat amplification protocol. J. Cancer Res. Clin. Oncol. 123, 337–344.
Honig, L.S., Kang, M.S., Cheng, R., Eckfeldt, J.H., Thyagarajan, B., Leiendecker-Foster, C., Province, M.A., Sanders, J.L., Perls, T., Christensen, K., Lee, J.H., Mayeux, R., Schupf, N., 2015. Heritability of telomere length in a study of long-lived families. Neurobiol. Aging 36, 2785–2790.
Hou, M., Xu, D., Björkholm, M., Gruber, A., 2001. Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. Clin. Chem. 47, 519–524.
Hu, Y., Shi, G., Zhang, L., Li, F., Jiang, Y., Jiang, S., Ma, W., Zhao, Y., Songyang, Z., Huang, J., 2016. Switch telomerase to ALT mechanism by inducing telomeric DNA damages and dysfunction of ATRX and DAXX. Sci. Rep. 6, 32280.
Iglesias, N., Redon, S., Pfeiffer, V., Dees, M., Lingner, J., Luke, B., 2011. Subtelomeric repetitive elements determine TERRA regulation by Rap1/Rif and Rap1/Sir complexes in yeast. EMBO Rep. 12, 587–593.
Jaskelioff, M., Muller, F.L., Paik, J.H., Thomas, E., Jiang, S., Adams, A.C., Sahin, E., Kost-Alimova, M., Protopopov, A., Cadiñanos, J., Horner, J.W., Maratos-Flier, E., Depinho, R.A., 2011. Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. Nature 469, 102–106.
Jasmine, F., Shinkle, J., Sabarinathan, M., Ahsan, H., Pierce, B.L., Kibriya, M.G., 2018. A novel pooled-sample multiplex luminex assay for high-throughput measurement of relative telomere length. Am. J. Hum. Biol. 30, e23118. https://doi.org/10.1002/ajhb.23118.
Jiang, J., Wang, Y., Sušac, L., Chan, H., Basu, R., Zhou, Z.H., Feigon, J., 2018. Structure of telomerase with telomeric DNA. Cell 173, 1179–1190.
Jiménez, K.M., Forero, D.A., 2018. Effect of master mixes on the measurement of telomere length by qPCR. Mol. Biol. Rep. 45, 633–638.
Jose, S.S., Bendickova, K., Kepak, T., Krenova, Z., Fric, J., 2017. Chronic inflammation in immune aging: role of pattern recognition receptor crosstalk with the telomere complex? Front. Immunol. 8, 1078.
Justice, J.N., Ferrucci, L., Newman, A.B., Aroda, V.R., Bahnson, J.L., Divers, J., Espeland, M.A., Marcovina, S., Pollak, M.N., Kritchevsky, S.B., Barzilai, N., Kuchel, G.A., 2018. A framework for selection of blood-based biomarkers for geroscience-guided clinical trials: report from the TAME Biomarkers Workgroup. Geroscience. https://doi.org/10.1007/s11357-018-0042-y.
Jylhävä, J., Pedersen, N.L., Hägg, S., 2017. Biological age predictors. EBioMedicine 21, 29–36. https://doi.org/10.1016/j.ebiom.2017.03.046.
Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., de Lange, T., 1999. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science 283, 1321–1325.
Kaulage, M.H., Maji, B., Pasadi, S., Ali, A., Bhattacharya, S., Muniyappa, K., 2018. Targeting G-quadruplex DNA structures in the telomere and oncogene promoter regions by benzimidazole–carbazole ligands. Eur. J. Med. Chem. 148, 178–194.
Kawamura, K., Yaku, H., Miyoshi, D., Murashima, T., 2014. A simple "add and measure" FRET-based telomeric tandem repeat sequence detection and telomerase assay method. Org. Biomol. Chem. 12, 936–941.
Khan, S.S., Singer, B.D., Vaughan, D.E., 2017. Molecular and physiological manifestations and measurement of aging in humans. Aging Cell 16, 624–633. https://doi.org/10.1111/acel.12601.
Khincha, P.P., Dagnall, C.L., Hicks, B., Jones, K., Aviv, A., Kimura, M., Katki, H., Aubert, G., Giri, N., Alter, B.P., Savage, S.A., Gadalla, S.M., 2017. Correlation of leukocyte telomere length measurement methods in patients with dyskeratosis congenita and in their unaffected relatives. Int. J. Mol. Sci. 18 pii: E1765.
Kibriya, M.G., Jasmine, F., Roy, S., Ahsan, H., Pierce, B., 2014. Measurement of telomere length: a new assay using QuantiGene chemistry on a Luminex platform. Cancer Epidemiol. Biomarkers Prev. 23, 2667–2672.
Kim, W., Shay, J.W., 2018. Long-range telomere regulation of gene expression: telomere looping and telomere position effect over long distances (TPE-OLD). Differentiation 99, 1–9.
Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., Shay, J.W., 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266, 2011–2015.
Kimura, M., Stone, R.C., Hunt, S.C., Skurnick, J., Lu, X., Cao, X., Harley, C.B., Aviv, A., 2010. Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths. Nat. Protoc. 5, 1596–1607. https://doi.org/10.1038/nprot.2010.124.
Klimova, B., Novotny, M., Kuca, K., 2018. Anti-aging drugs - prospect of longer life? Curr. Med. Chem. 25, 1946–1953. https://doi.org/10.2174/0929867325666171129215251.
Kohanski, R.A., Deeks, S.G., Gravekamp, C., Halter, J.B., High, K., Hurria, A., Fuldner, R., Green, P., Huebner, R., Macchiarini, F., Sierra, F., 2016. Reverse geroscience: how does exposure to early diseases accelerate the age-related decline in health? Ann. N. Y. Acad. Sci. 1386, 30–44. https://doi.org/10.1111/nyas.13297.
Komosa, M., Root, H., Meyn, M.S., 2015. Visualization and quantitative analysis of extrachromosomal telomere-repeat DNA in individual human cells by Halo-FISH. Nucleic Acids Res. 43, 2152–2163.
König, F., Schubert, T., Längst, G., 2017. The monoclonal S9.6 antibody exhibits highly variable binding affinities towards different R-loop sequences. PLoS One 12, e0178875.
Koskas, S., Decottignies, A., Dufour, S., Pezet, M., Verdel, A., Vourc'h, C., Faure, V., 2017. Heat shock factor 1 promotes TERRA transcription and telomere protection upon heat stress. Nucleic Acids Res. 45, 6321–6333.
Kulla, E., Katz, E., 2008. Biosensor techniques used for determination of telomerase activity in Cancer cells. Sensors (Basel) 8, 347–369.
Kuttler, F., Mai, S., 2007. Formation of non-random extrachromosomal elements during development, differentiation and oncogenesis. Semin. Cancer Biol. 17, 56–64.
Laguerre, A., Wong, J.M., Monchaud, D., 2016. Direct visualization of both DNA and RNA quadruplexes in human cells via an uncommon spectroscopic method. Sci. Rep. 6, 32141.
Lai, T.P., Zhang, N., Noh, J., Mender, I., Tedone, E., Huang, E., Wright, W.E., Danuser, G., Shay, J.W., 2017. A method for measuring the distribution of the shortest telomeres in cells and tissues. Nat. Commun. 8, 1356.
Lai, T.P., Wright, W.E., Shay, J.W., 2018. Comparison of telomere length measurement methods. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 373, 20160451.
Lapham, K., Kvale, M.N., Lin, J., Connell, S., Croen, L.A., Dispensa, B.P., Fang, L., Hesselson, S., Hoffmann, T.J., Iribarren, C., Jorgenson, E., Kushi, L.H., Ludwig, D., Matsuguchi, T., McGuire, W.B., Miles, S., Quesenberry, C.P.Jr., Rowell, S., Sadler, M., Sakoda, L.C., Smethurst, D., Somkin, C.P., Van Den Eeden, S.K., Walter, L., Whitmer, R.A., Kwok, P.Y., Risch, N., Schaefer, C., Blackburn, E.H., 2015. Automated assay of telomere length measurement and informatics for 100,000 subjects in the genetic epidemiology research on adult health and aging (GERA) cohort. Genetics 200, 1061–1072. https://doi.org/10.1534/genetics.115.178624.
Lee, M., Napier, C.E., Yang, S.F., Arthur, J.W., Reddel, R.R., Pickett, H.A., 2017. Comparative analysis of whole genome sequencing-based telomere length measurement techniques. Methods 114, 4–15.
Li, B., Jog, S.P., Reddy, S., Comai, L., 2008. WRN controls formation of extrachromosomal telomeric circles and is required for TRF2DeltaB-mediated telomere shortening. Mol. Cell. Biol. 28, 1892–1904.
Li, Y., Liu, B., Li, X., Wei, Q., 2010. Highly sensitive electrochemical detection of human telomerase activity based on bio-barcode method. Biosens. Bioelectron. 25, 2543–2547.
Li, C.C., Hu, J., Lu, M., Zhang, C.Y., 2018a. Quantum dot-based electrochemical biosensor for stripping voltammetric detection of telomerase at the single-cell level. Biosens. Bioelectron. 122, 51–57. https://doi.org/10.1016/j.bios.2018.09.049.
Li, Y., Pan, G., Chen, Y., Yang, Q., Hao, T., Zhao, L., Zhao, L., Cong, Y., Diao, A., Yu, P., 2018b. Inhibitor of the human telomerase reverse trancriptase (hTERT) gene promoter induces cell apoptosis via a mitochondrial-dependent pathway. Eur. J. Med. Chem. 145, 370–378.
Lin, T.T., Norris, K., Heppel, N.H., Pratt, G., Allan, J.M., Allsup, D.J., Bailey, J., Cawkwell, L., Hills, R., Grimstead, J.W., Jones, R.E., Britt-Compton, B., Fegan, C., Baird, D.M., Pepper, C., 2014. Telomere dysfunction accurately predicts clinical outcome in chronic lymphocytic leukaemia, even in patients with early stage disease. Br. J. Haematol. 167, 214–223. https://doi.org/10.1111/bjh.13023.
Lin, Y., Damjanovic, A., Metter, E.J., Nguyen, H., Truong, T., Najarro, K., 2015. Age-associated telomere attrition of lymphocytes in vivo is co-ordinated with changes in telomerase activity, composition of lymphocyte subsets and health conditions. Clin. Sci. (Lond.) 128, 367–377.
Londoño-Vallejo, J.A., 2004. Telomere length heterogeneity and chromosome instability. Cancer Lett. 212, 135–144.
Lowsky, D.J., Olshansky, S.J., Bhattacharya, J., Goldman, D.P., 2014. Heterogeneity in healthy aging. J. Gerontol. A Biol. Sci. Med. Sci. 69, 640–649.
Ludlow, A.T., Shelton, D., Wright, W.E., Shay, J.W., 2018. ddTRAP: a method for sensitive and precise quantification of telomerase activity. Methods Mol. Biol. 1768, 513–529.
Lustig, A., Liu, H.B., Metter, E.J., An, Y., Swaby, M.A., Elango, P., Ferrucci, L., Hodes, R.J., Weng, N.P., 2017. Telomere shortening, inflammatory cytokines, and anti-cytomegalovirus antibody follow distinct age-associated trajectories in humans. Front. Immunol. 8, 1027.
Maesawa, C., Inaba, T., Sato, H., Iijima, S., Ishida, K., Terashima, M., Sato, R., Suzuki, M., Yashima, A., Ogasawara, S., Oikawa, H., Sato, N., Saito, K., Masuda, T., 2003. A rapid biosensor chip assay for measuring of telomerase activity using surface plasmon resonance. Nucleic Acids Res. 31 E4-4.
Mangaonkar, A.A., Patnaik, M.M., 2018. Short telomere syndromes in clinical practice: bridging bench and bedside. Mayo Clin. Proc. 93, 904–916.
Marchesini, M., Matocci, R., Tasselli, L., Cambiaghi, V., Orleth, A., Furia, L., Marinelli, C., Lombardi, S., Sammarelli, G., Aversa, F., Minucci, S., Faretta, M., Pelicci, P.G., Grignani, F., 2016. PML is required for telomere stability in non-neoplastic human cells. Oncogene 35, 1811–1821.
Martadinata, H., Heddi, B., Lim, K.W., Phan, A.T., 2011. Structure of long human telomeric RNA (TERRA): G-quadruplexes formed by four and eight UUAGGG repeats are stable building blocks. Biochemistry 50, 6455–6461.
Martin-Ruiz, C.M., Baird, D., Roger, L., Boukamp, P., Krunic, D., Cawthon, R., Dokter, M.M., van der Harst, P., Bekaert, S., de Meyer, T., Roos, G., Svenson, U., Codd, V., Samani, N.J., McGlynn, L., Shiels, P.G., Pooley, K.A., Dunning, A.M., Cooper, R., Wong, A., Kingston, A., von Zglinicki, T., 2015. Reproducibility of telomere length assessment: an international collaborative study. Int. J. Epidemiol. 44, 1673–1683. https://doi.org/10.1093/ije/dyu191.
Masutomi, K., Yu, E.Y., Khurts, S., Ben-Porath, I., Currier, J.L., Metz, G.B., Brooks, M.W., Kaneko, S., Murakami, S., DeCaprio, J.A., Weinberg, R.A., Stewart, S.A., Hahn, W.C., 2003. Telomerase maintains telomere structure in normal human cells. Cell 114, 241–253.
Meyer, A., Salewsky, B., Buchmann, N., Steinhagen-Thiessen, E., Demuth, I., 2016. Relative leukocyte telomere length, hematological parameters and Anemia - data from the Berlin aging study II (BASE-II). Gerontology 62, 330–336. https://doi.org/10.1159/000430950.
Mons, U., Müezzinler, A., Schöttker, B., Dieffenbach, A.K., Butterbach, K., Schick, M., Peasey, A., De Vivo, I., Trichopoulou, A., Boffetta, P., Brenner, H., 2017. Leukocyte telomere length and all-cause, cardiovascular disease, and Cancer mortality: results from individual-participant-Data meta-analysis of 2 large prospective cohort studies. Am. J. Epidemiol. 185, 1317–1326. https://doi.org/10.1093/aje/kww210.
Montero, J.J., López de Silanes, I., Graña, O., Blasco, M.A., 2016. Telomeric RNAs are essential to maintain telomeres. Nat. Commun. 7, 12534.
Montiel Rojas, D., Nilsson, A., Ponsot, E., Brummer, R.J., Fairweather-Tait, S., Jennings,

A., de Groot, L.C.P.G.M., Berendsen, A., Pietruszka, B., Madej, D., Caumon, E., Meunier, N., Malpuech-Brugère, C., Guidarelli, G., Santoro, A., Franceschi, C., Kadi, F., 2018. Short Telomere Length Is Related to Limitations in Physical Function in Elderly European Adults. Front. Physiol. 9, 1110. https://doi.org/10.3389/fphys.2018.01110. eCollection 2018.

Montpetit, A.J., Alhareeri, A.A., Montpetit, M., Starkweather, A.R., Elmore, L.W., Filler, K., Mohanraj, L., Burton, C.W., Menzies, V.S., Lyon, D.E., Jackson-Cook, C.K., 2014. Telomere length: a review of methods for measurement. Nurs. Res. 63, 289–299.

Moye, A.L., Porter, K.C., Cohen, S.B., Phan, T., Zyner, K.G., Sasaki, N., Lovrecz, G.O., Beck, J.L., Bryan, T.M., 2015. Telomeric G-quadruplexes are a substrate and site of localization for human telomerase. Nat. Commun. 6, 7643.

Müezzinler, A., Zaineddin, A.K., Brenner, H., 2013. A systematic review of leukocyte telomere length and age in adults. Ageing Res. Rev. 12, 509–519.

Müezzinler, A., Mons, U., Dieffenbach, A.K., Butterbach, K., Saum, K.U., Schick, M., Stammer, H., Boukamp, P., Holleczek, B., Stegmaier, C., Brenner, H., 2016. Body mass index and leukocyte telomere length dynamics among older adults: results from the ESTHER cohort. Exp. Gerontol. 74, 1–8. https://doi.org/10.1016/j.exger.2015.11.019.

Mundstock, E., Zatti, H., Louzada, F.M., Oliveira, S.G., Guma, F.T., Paris, M.M., Rueda, A.B., Machado, D.G., Stein, R.T., Jones, M.H., Sarria, E.E., Barbé-Tuana, F.M., Mattiello, R., 2015a. Effects of physical activity in telomere length: systematic review and meta-analysis. Ageing Res. Rev. 22, 72–80. https://doi.org/10.1016/j.arr.2015.02.004.

Mundstock, E., Sarria, E.E., Zatti, H., Mattos Louzada, F., Kich Grun, L., Herbert Jones, M., Guma, F.T., Mazzola In Memoriam, J., Epifanio, M., Stein, R.T., Barbé-Tuana, F.M., Mattiello, R., 2015b. Effect of obesity on telomere length: Systematic review and meta-analysis. Obesity 23 (Silver Spring), 2165–2174. https://doi.org/10.1002/oby.21183.

Nabetani, A., Ishikawa, F., 2011. Alternative lengthening of telomeres pathway: recombination-mediated telomere maintenance mechanism in human cells. J. Biochem. 149, 5–14. https://doi.org/10.1093/jb/mvq119.

Németh, A., Orgovan, N., Sódar, B.W., Osteikoetxea, X., Pálóczi, K., Szabó-Taylor, K.É., Vukman, K.V., Kittel, Á., Turiák, L., Wiener, Z., Tóth, S., Drahos, L., Vékey, K., Horvath, R., Buzás, E.I., 2017. Antibiotic-induced release of small extracellular vesicles (exosomes) with surface-associated DNA. Sci. Rep. 7, 8202.

Norberg, A., Rosén, A., Raaschou-Jensen, K., Kjeldsen, L., Moilanen, J.S., Paulsson-Karlsson, Y., Baliakas, P., Lohi, O., Ahmed, A., Kittang, A.O., Larsson, P., Roos, G., Degerman, S., Hultdin, M., 2018. Novel variants in Nordic patients referred for genetic testing of telomere-related disorders. Eur. J. Hum. Genet. 26, 858–867.

Nussey, D.H., Baird, D., Barrett, E., Boner, W., Fairlie, J., Gemmell, N., Hartmann, N., Horn, T., Haussmann, M., Olsson, M., Turbill, C., Verhulst, S., Zahn, S., Monaghan, P., 2014. Measuring telomere length and telomere dynamics in evolutionary biology and ecology. Methods Ecol. Evol. 5, 299–310.

Oerther, S., Lorenz, R., 2018. State of the science: using telomeres as biomarkers during the first 1,000 days of life. West. J. Nurs. Res. https://doi.org/10.1177/0193945918762806.

Ohuchida, K., Mizumoto, K., Ogura, Y., Ishikawa, N., Nagai, E., Yamaguchi, K., Tanaka, M., 2005. Quantitative assessment of telomerase activity and human telomerase reverse transcriptase messenger RNA levels in pancreatic juice samples for the diagnosis of pancreatic cancer. Clin. Cancer Res. 11, 2285–2292.

Oliva-Rico, D., Herrera, L.A., 2017. Regulated expression of the lncRNA TERRA and its impact on telomere biology. Mech. Ageing Dev. 167, 16–23.

Ouellette, M.M., Liao, M., Herbert, B.S., Johnson, M., Holt, S.E., Liss, H.S., Shay, J.W., Wright, W.E., 2000. Subsenescent telomere lengths in fibroblasts immortalized by limiting amounts of telomerase. J. Biol. Chem. 275, 10072–10076.

Parker, M., Chen, X., Bahrami, A., Dalton, J., Rusch, M., Wu, G., Easton, J., Cheung, N.K., Dyer, M., Mardis, E.R., Wilson, R.K., Mullighan, C., Gilbertson, R., Baker, S.J., Zambetti, G., Ellison, D.W., Downing, J.R., Zhang, J., Pediatric Cancer Genome Project, 2012. Assessing telomeric DNA content in pediatric cancers using whole-genome sequencing data. Genome Biol. 13, R113.

Patolsky, F., Gill, R., Weizmann, Y., Mokari, T., Banin, U., Willner, I., 2003. Lighting-up the dynamics of telomerization and DNA replication by CdSe-ZnS quantum dots. J. Am. Chem. Soc. 125, 13918–13919.

Pavlov, V., Xiao, Y., Gill, R., Dishon, A., Kotler, M., Willner, I., 2004. Amplified chemiluminescence surface detection of DNA and telomerase activity using catalytic nucleic acid labels. Anal. Chem. 76, 2152–2156.

Pérez, L.M., Amaral, M.A., Mundstock, E., Barbé-Tuana, F.M., Guma, F.T.C.R., Jones, M.H., Machado, D.C., Sarria, E.E., Marques E Marques, M., Preto, L.T., Epifanio, M., Meinem Garbin, J.G., Mattiello, R., 2017. Effects of diet on telomere length: systematic review and meta-analysis. Public Health Genomics 20, 286–292. https://doi.org/10.1159/000486586.

Perner, S., Brüderlein, S., Hasel, C., Waibel, I., Holdenried, A., Ciloglu, N., 2003. Quantifying telomere lengths of human individual chromosome arms by centromere-calibrated fluorescence in situ hybridization and digital imaging. Am. J. Pathol. 163, 1751–1756.

Pfeiffer, V., Lingner, J., 2012. TERRA promotes telomere shortening through exonuclease 1-mediated resection of chromosome ends. PLoS Genet. 8, e1002747.

Pickett, H.A., Cesare, A.J., Johnston, R.L., Neumann, A.A., Reddel, R.R., 2009. Control of telomere length by a trimming mechanism that involves generation of t-circles. EMBO J. 28, 799–809.

Rachakonda, S., Srinivas, N., Mahmoudpour, S.H., Garcia-Casado, Z., Requena, C., Traves, V., Soriano, V., Cardelli, M., Pajnova, D., Molven, A., Gruis, N., Nagore, E., Kumar, R., 2018. Telomere length and survival in primary cutaneous melanoma patients. Sci. Rep. 8, 10947. https://doi.org/10.1038/s41598-018-29322-9.

Raschenberger, J., Lamina, C., Haun, M., Kollerits, B., Coassin, S., Boes, E., Kedenko, L., Köttgen, A., Kronenberg, F., 2016. Influence of DNA extraction methods on relative telomere length measurements and its impact on epidemiological studies. Sci. Rep. 6, 25398. https://doi.org/10.1038/srep25398.

Rufer, N., Dragowska, W., Thornbury, G., Roosnek, E., Lansdorp, P.M., 1998. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. Nat. Biotechnol. 16, 743–747.

Saldanha, S.N., Andrews, L.G., Tollefsbol, T.O., 2003. Analysis of telomerase activity and detection of its catalytic subunit, hTERT. Anal. Biochem. 315, 1–21.

Savage, S.A., 2018. Beginning at the ends: telomeres and human disease. F1000Res. 7https://doi.org/10.12688/f1000research.14068.1. pii: F1000.

Savoysky, E., Akamatsu, K., Tsuchiya, M., Yamazaki, T., 1996. Detection of telomerase activity by combination of TRAP method and scintillation proximity assay (SPA). Nucleic Acids Res. 24, 1175–1176.

Schmidt, P.M., Matthes, E., Scheller, F.W., Bienert, M., Lehmann, C., Ehrlich, A., Bier, F.F., 2002. Real-time determination of telomerase activity in cell extracts using an optical biosensor. Biol. Chem. 383, 1659–1666.

Schvartzman, J.B., Martínez-Robles, M.L., Hernández, P., Krimer, D.B., 2013. Plasmid DNA topology assayed by two-dimensional agarose gel electrophoresis. Methods Mol. Biol. 1054, 121–132.

Shay, J.W., 2016. Role of telomeres and telomerase in aging and Cancer. Cancer Discov. 6, 584–593.

Shay, J.W., 2018. Telomeres and aging. Curr. Opin. Cell Biol. 52, 1–7. https://doi.org/10.1016/j.ceb.2017.12.001.

Sierra, F., Kohanski, R., 2017. Geroscience and the trans-NIH geroscience interest group, GSIG. Geroscience 39, 1–5. https://doi.org/10.1007/s11357-016-9954-6.

Skvortsov, D.A., Zvereva, M.E., Shpanchenko, O.V., Dontsova, O.A., 2011. Assays for detection of telomerase activity. Acta Naturae 3, 48–68.

Smith, E.D., Garza-Gongora, A.G., MacQuarrie, K.L., Kosak, S.T., 2018. Interstitial telomeric loops and implications of the interaction between TRF2 and lamin A/C. Differentiation 102, 19–26.

Sprouse, A.A., Steding, C.E., Herbert, B.S., 2012. Pharmaceutical regulation of telomerase and its clinical potential. J. Cell. Mol. Med. 16, 1–7. https://doi.org/10.1111/j.1582-4934.2011.01460.x.

Storci, G., De Carolis, S., Olivieri, F., Bonafè, M., 2018. Changes in the biochemical taste of cytoplasmic and cell-free DNA are major fuels for inflamm-aging. Semin. Immunol. https://doi.org/10.1016/j.smim.2018.08.003. pii: S1044-5323(18)30070-30078.

Stout, S.A., Lin, J., Hernandez, N., Davis, E.P., Blackburn, E., Carroll, J.E., Glynn, L.M., 2017. Validation of minimally-invasive sample collection methods for measurement of telomere length. Front. Aging Neurosci. 9, 397. https://doi.org/10.3389/fnagi.2017.00397.

Strazhesko, I.D., Tkacheva, O.N., Akasheva, D.U., Dudinskaya, E.N., Plokhova, E.V., Pykhtina, V.S., Kruglikova, A.S., Kokshagina, N.V., Sharashkina, N.V., Agaltsov, M.V., Kashtanova, D.A., Vygodin, V.A., Ozerova, I.N., Skvortsov, D.A., Vasilkova, D., Boytsov, S.A., 2016. Atorvastatin therapy modulates telomerase activity in patients free of atherosclerotic cardiovascular diseases. Front. Pharmacol. 7, 347.

Su, X., Li, Z., Yan, X., Wang, L., Zhou, X., Wei, L., Xiao, L., Yu, C., 2017. Telomerase activity detection with amplification-free single molecule stochastic binding assay. Anal. Chem. 89, 3576–3582.

Sue, M.J., Yeap, S.K., Omar, A.R., Tan, S.W., 2014. Application of PCR-ELISA in molecular diagnosis. Biomed Res. Int. 2014, 653014.

Takai, H., Smogorzewska, A., de Lange, T., 2003. DNA damage foci at dysfunctional telomeres. Curr. Biol. 13, 1549–1556.

Tarik, M., Ramakrishnan, L., Sachdev, H.S., Tandon, N., Roy, A., Bhargava, S.K., Pandey, R.M., 2018. Validation of quantitative polymerase chain reaction with Southern blot method for telomere length analysis. Future Sci. OA 4, FSO282.

Testa, R., Olivieri, F., Sirolla, C., Spazzafumo, L., Rippo, M.R., Marra, M., Bonfigli, A.R., Ceriello, A., Antonicelli, R., Franceschi, C., Castellucci, C., Testa, I., Procopio, A.D., 2011. Leukocyte telomere length is associated with complications of type 2 diabetes mellitus. Diabet. Med. 28, 1388–1394. https://doi.org/10.1111/j.1464-5491.2011.03370.x.

Tokutake, Y., Matsumoto, T., Watanabe, T., Maeda, S., Tahara, H., Sakamoto, S., Niida, H., Sugimoto, M., Ide, T., Furuichi, Y., 1998. Extra-chromosomal telomere repeat DNA in telomerase-negative immortalized cell lines. Biochem. Biophys. Res. Commun. 247, 765–772.

Tomaska, L., Nosek, J., Kramara, J., Griffith, J.D., 2009. Telomeric circles: universal players in telomere maintenance? Nat. Struct. Mol. Biol. 16, 1010–1015. https://doi.org/10.1038/nsmb.1660.

Toubiana, S., Selig, S., 2018. DNA:RNA hybrids at telomeres - when it is better to be out of the (R) loop. FEBS J. 285, 2552–2566. https://doi.org/10.1111/febs.14464.

Toupance, S., Labat, C., Temmar, M., Rossignol, P., Kimura, M., Aviv, A., Benetos, A., 2017. Short telomeres, but not telomere attrition rates, are associated with carotid atherosclerosis. Hypertension. 70, 420–425.

Uehara, H., Nardone, G., Nazarenko, I., Hohman, R.J., 1999. Detection of telomerase activity utilizing energy transfer primers: comparison with gel- and ELISA-based detection. Biotechniques. 26, 552–558.

Vaiserman, A., Lushchak, O., 2017. Implementation of longevity-promoting supplements and medications in public health practice: achievements, challenges and future perspectives. J. Transl. Med. 15, 160. https://doi.org/10.1186/s12967-017-1259-8.

Vetter, V.M., Antje, M., Karbasiyan, M., Steinhagen-Thiessen, E., Hopfenmüller, W., Demuth, 2018. Epigenetic clock and relative telomere length represent largely different aspects of aging in the Berlin Aging Study II (BASE-II). J. Gerontol. A Biol. Sci. Med. Sci. https://doi.org/10.1093/gerona/gly184.

Wang, Z., Lieberman, P.M., 2016. The crosstalk of telomere dysfunction and inflammation through cell-free TERRA containing exosomes. RNA Biol. 13, 690–695.

Wang, Y., Prosen, D.E., Mei, L., Sullivan, J.C., Finney, M., Vander Horn, P.B., 2004. A novel strategy to engineer DNA polymerases for enhanced processivity and improved performance in vitro. Nucleic Acids Res. 32, 1197–1207.

Wang, Z., Deng, Z., Dahmane, N., Tsai, K., Wang, P., Williams, D.R., Kossenkov, A.V., Showe, L.C., Zhang, R., Huang, Q., Conejo-Garcia, J.R., Lieberman, P.M., 2015. Telomeric repeat-containing RNA (TERRA) constitutes a nucleoprotein component of extracellular inflammatory exosomes. Proc. Natl. Acad. Sci. U. S. A. 112, E6293–300.

Wang, J., Dong, X., Cao, L., Sun, Y., Qiu, Y., Zhang, Y., Cao, R., Covasa, M., Zhong, L., 2016. Association between telomere length and diabetes mellitus: a meta-analysis. J. Int. Med. Res. 44, 1156–1173. https://doi.org/10.1177/0300060516667132.

Wang, H., Zhang, K., Liu, Y., Fu, Y., Gao, S., Gong, P., Wang, H., Zhou, Z., Zeng, M., Wu, Z., Sun, Y., Chen, T., Li, S., Liu, L., 2017a. Telomere heterogeneity linked to metabolism and pluripotency state revealed by simultaneous analysis of telomere length and RNA-seq in the same human embryonic stem cell. BMC Biol. 15, 114.

Wang, W., Zheng, L., Zhou, N., Li, N., Bulibu, G., Xu, C., Zhang, Y., Tang, Y., 2017b. Meta-analysis of associations between telomere length and colorectal cancer survival from observational studies. Oncotarget 8, 62500–62507. https://doi.org/10.18632/oncotarget.20055.

Wang, Z., Deng, Z., Tutton, S., Lieberman, P.M., 2017c. The telomeric response to viral infection. Viruses. 9https://doi.org/10.3390/v9080218. pii: E218.

Wang, Z., Li, J., Liu, J.P., 2018a. Effects of cation charges on the binding of stabilizers with human telomere and TERRA G-quadruplexes. J. Biomol. Struct. Dyn. https://doi.org/10.1080/07391102.2018.1471416.

Wang, Q., Zhan, Y., Pedersen, N.L., Fang, F., Hägg, S., 2018b. Telomere length and all-cause mortality: a meta-analysis. Ageing Res. Rev. 48, 11–20. https://doi.org/10.1016/j.arr.2018.09.002.

Wege, H., Chui, M.S., Le, H.T., Tran, J.M., Zern, M.A., 2003. SYBR Green real-time telomeric repeat amplification protocol for the rapid quantification of telomerase activity. Nucleic Acids Res. 31, E3–3.

Williams, J., Heppel, N.H., Britt-Compton, B., Grimstead, J.W., Jones, R.E., Tauro, S., Bowen, D.T., Knapper, S., Groves, M., Hills, R.K., Pepper, C., Baird, D.M., Fegan, C., 2017. Telomere length is an independent prognostic marker in MDS but not in de novo AML. Br. J. Haematol. 178, 240–249. https://doi.org/10.1111/bjh.14666.

Wu, Y.Y., Hruszkewycz, A.M., Delgado, R.M., Yang, A., Vortmeyer, A.O., Moon, Y.W., Weil, R.J., Zhuang, Z., Remaley, A.T., 2000. Limitations on the quantitative determination of telomerase activity by the electrophoretic and ELISA based TRAP assays. Clin. Chim. Acta 293, 199–212.

Wu, R.A., Upton, H.E., Vogan, J.M., Collins, K., 2017. Telomerase mechanism of telomere synthesis. Annu. Rev. Biochem. 86, 439–460.

Wulaningsih, W., Kuh, D., Wong, A., Hardy, R., 2018. Adiposity, telomere length, and telomere attrition in midlife: the 1946 british birth cohort. J. Gerontol. A Biol. Sci. Med. Sci. 73, 966–972. https://doi.org/10.1093/gerona/glx151.

Xu, Y., Suzuki, Y., Ito, K., Komiyama, M., 2010. Telomeric repeat-containing RNA structure in living cells. Proc. Natl. Acad. Sci. U. S. A. 107, 14579–14584.

Yehuda, S., Yanai, H., Priel, E., Fraifeld, V.E., 2017. Differential decrease in soluble and DNA-bound telomerase in senescent human fibroblasts. Biogerontology. 18, 525–533. https://doi.org/10.1007/s10522-017-9688-6.

Zavari-Nematabad, A., Alizadeh-Ghodsi, M., Hamishehkar, H., Alipour, E., Pilehvar-Soltanahmadi, Y., 2017. Development of quantum-dot-encapsulated liposome-based optical nanobiosensor for detection of telomerase activity without target amplification. Anal. Bioanal. Chem. 409, 1301–1310. https://doi.org/10.1007/s00216-016-0058-z.

Zhang, C., Chen, X., Li, L., Zhou, Y., Wang, C., Hou, S., 2015. The association between telomere length and Cancer prognosis: evidence from a meta-analysis. PLoS One 10, e0133174. https://doi.org/10.1371/journal.pone.0133174.

Zhang, T., Zhang, Z., Li, F., Hu, Q., Liu, H., Tang, M., Ma, W., Huang, J., Songyang, Z., Rong, Y., Zhang, S., Chen, B.P., Zhao, Y., 2017a. Looping-out mechanism for resolution of replicative stress at telomeres. EMBO Rep. 18, 1412–1428. https://doi.org/10.15252/embr.201643866.

Zhang, X., Lou, X., Xia, F., 2017b. Advances in the detection of telomerase activity using isothermal amplification. Theranostics. 7, 1847–1862.

Zhang, L., Peng, J., Hong, M.F., Chen, J.Q., Liang, R.P., Qiu, J.D., 2018. A facile graphene oxide-based fluorescent nanosensor for the in situ "turn-on" detection of telomerase activity. Analyst. 143, 2334–2341.

Zhu, Y., Liu, X., Ding, X., Wang, F., Geng, X., 2018. Telomere and its role in the aging pathways: telomere shortening, cell senescence and mitochondria dysfunction. Biogerontology. https://doi.org/10.1007/s10522-018-9769-1.

Zierer, J., Kastenmüller, G., Suhre, K., Gieger, C., Codd, V., Tsai, P.C., Bell, J., Peters, A., Strauch, K., Schulz, H., Weidinger, S., Mohney, R.P., Samani, N.J., Spector, T., Mangino, M., Menni, C., 2016. Metabolomics profiling reveals novel markers for leukocyte telomere length. Aging (Albany NY) 8, 77–94.

Zinkova, A., Brynychova, I., Svacina, A., Jirkovska, M., Korabecna, M., 2017. Cell-free DNA from human plasma and serum differs in content of telomeric sequences and its ability to promote immune response. Sci. Rep. 7, 2591.