Comparing different methods of measuring cell senescence

Naam: Anastassia Blanter
Studentnummer: 5802075
Adres: Roland Holstlaan 947, Delft
Telefoonnummer: 0648172259
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Intern begeleider: Michel Haring
Extern begeleider: Alexandra Zhernakova
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Summary

Aging is an essential part of the human life cycle and can be described in terms of “chronological age” as determined by the date of birth and the “biological age” as determined by the changes in body physiology. An understanding of relation between chronological and biological age can be important for forensic sciences, disaster victim identification and understanding of pathways or processes of biological age-associated autoimmune diseases.

This study performs a comparative analysis of three different methods of measuring biological age – the Cawthon’s and O’Callaghan & Fenech’s telomere length measurement and the quantifying of T cell development byproducts.

Introduction

Aging process is an essential part of the human life cycle, affecting many cells and being an important factor in phenotype establishment. In this study we distinguish the chronological age and the biological age. The chronological age is determined by the date of birth and established as a number of years passed after the birth. The biological age on contrary is determined by changes in the body physiology and established as e.g. immunosenescence – a decrease in immune competence (Ostan et al., 2008).

Chronological age prediction based on biological material is a powerful tool in forensic context because chronological age is an important factor in civil registrations. Thus an adequate age prediction would facilitate the criminal or victim identification. In the same way prediction of human phenotype through age-cell senescence relation is useful for disaster victim identification. Among other applications of accurate age prediction from biological material are anthropological studies and potentially ecological studies on wild animals. (Zubakov et al., 2010)

Biological age seems also to be associated with some autoimmune diseases, such as rhematoid arthritis (Goronzy & Weyand, 2003) and systemic lupus erythematosus (Beier et al., 2007). An adequate measurement of biological age is therefore a key to understanding of pathways related to development of diseases and mechanisms of immunosenescence.

Different techniques have been developed in order to measure cellular senescence. Among most significant approaches are the measuring of telomere length (Cawthon, 2002) and the exploring of T cell DNA rearrangements (Zubakov et al., 2010).

Telomeres are repetitive DNA sequences at distal ends of the chromosomes and are known to shorten with age. (Aubert & Lansdorp, 2008) A common way to determine telomere length is to measure the mean terminal restriction fragment (TRF) length by a Southern blot analysis. This method, however, requires large amounts of DNA and needs a significant amount of time. Telomere length measurement by a quantitative polymerase chain reaction (qPCR), suggested by R. Cawthon (Cawthon, 2002), can be performed much faster and with relatively small amounts of DNA. According to Cawthon's research, this method gives a 68% correlation with TRF measurements and returns relative telomere length values, allowing comparisons between individuals but not making any predictions about the actual telomere length.

Quantitative PCR can, however, also be used for measuring absolute telomere length, as stated by O'Callaghan and Fenech in 2011 (O'Callaghan&Fenech, 2011). This method is based on Cawthon's qPCR but contains some major modifications, such as different primers and use of oligomer standards in order to calculate the actual telomere length.

Telomere length can also be measured by other methods, such as the FlowFISH (Baerlocher et al., 2006) or the Single Telomere Length Analysis (STELA) method (Hills et al., 2009). Those methods, however, fall beyond the reach of this research.

An approach different from measuring telomere length, is the measuring of T cell DNA rearrangement byproducts. In order to create a broad range of T cell receptors (TCR), immature T cells undergo multiple DNA rearrangements during intra-thymal development. One of the consequences of such rearrangements is the forming of circular episomal DNA molecules, called signal joint TCR excision circles (sjTRECs), and characterized by the presence of small DNA sequences, the so-called signal-joints. As thymus function declines with age, the amount of sjTRECs in blood also goes down. Quantifying the presence of sjTRECs could therefore be a suitable way to predict human age. (Zubakov et al., 2010)

In this article we are comparing Cawthon method, O'Callaghan & Fenech method and sjTREC method in order to determine the most accurate way to predict chronological age and shed some light on the relation between chronological age and biological senescence.

Materials and Methods

In order to compare different methods of measuring cell senescence, we isolated DNA from our testgroup and performed quantitative PCR as described below.

DNA isolation

We isolated genomic DNA from 10 mL whole blood according to the protocol as described in Appendix A. The blood was donated by 20 healthy individuals of various age. Blood was collected at different timepoints (*Fig.1*) and every individual donated blood at least at two timepoints.

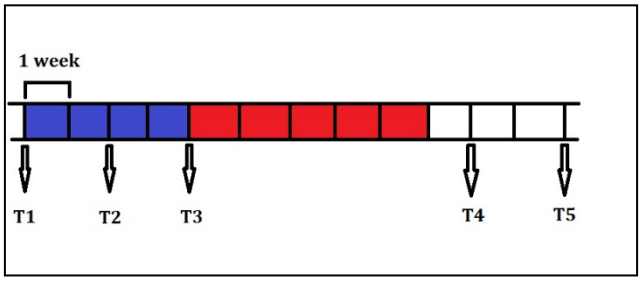

Fig.1 Timepoints of blood collection
Blood for DNA isolation was collected at timepoints indicated with T1-T5. White color marks the periods, during which the blood donors were following their habitual diet, blue color – the gluten free diet. Red color indicates the wash-out period of 5 weeks.

DNA samples

In order to increase the statistical power, we used DNA from two timepoints for every individual, taking the first two timepoints available. For 14 individuals we used T1 and T2, for 4 individuals we used T1 and T3, for 1 individual we used T2 and T4 and for 1 individual we used T3 and T4. This way we had two DNA samples per individual.

Real-time PCR

With real-time PCR or quantitative PCR (qPCR) a DNA sequence can be amplified and quantified simultaneously. A fluorescent dye binds to PCR products and after each PCR cycle the fluorescence is measured by a detector, returning an amplification curve. For each sample a C_t value can be determined: the cycle number at which the cumulative fluorescence crosses a set fluorescence threshold. The more target sequences are produced by the PCR, the sooner the fluorescence crosses the threshold and the lower the C_t value. (Higuchi et al., 1993)

In this study the qPCR was performed in 384-wells optical plates on Applied Biosystems ViiA 7 Real-Time PCR System.

Telomere primer design

Telomeres consist of hexamer repeats, which make telomere primers also consistent of repetitive sequences and thus likely to anneal to each other, forming primer dimers. Such dimers can affect the results of real-time PCR. In order to reduce the amount of primer dimers, primers for telomere length measurement were specifically designed (*Fig. 2)*. During the DNA hybridization telomere primers anneal to genomic DNA in such a way that every sixth base pair is mismatched. When two complementary primers anneal to each other, only four out of six base pairs are hybridized, leaving two bases mismatched and making it less likely for DNA polymerase to extend from the 3'-end of the primer.

Telomere primers in Cawthon method were designed according to the above scheme. Telomere primers design in O'Callaghan & Fenech method is not reported.

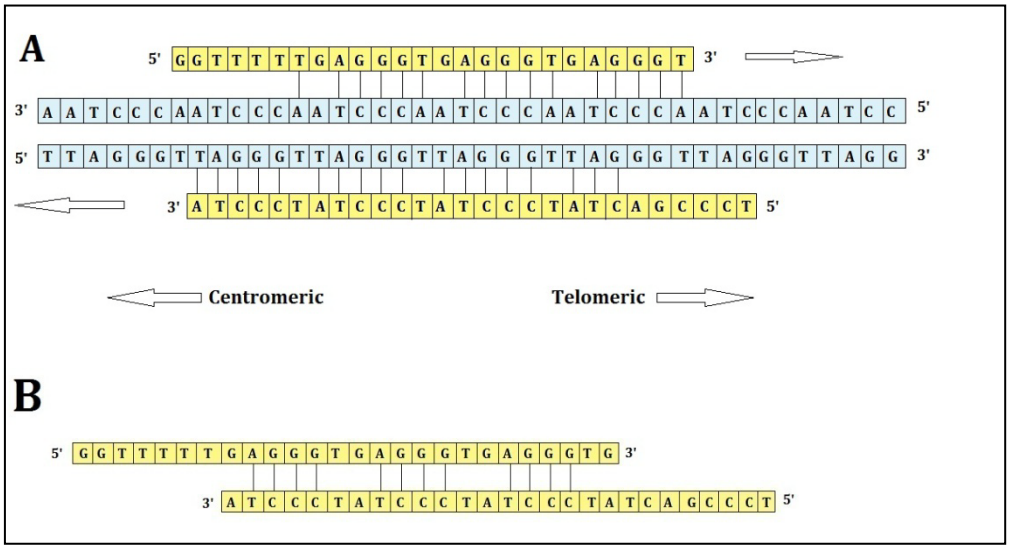

Fig.2 Telomere primer design

A. In blue a part of the telomeric region of a chromosome is indicated, in yellow – the telomere primers. When the primers anneal to the DNA, every sixth base is mismatched. B. During primer dimer forming, two primers anneal to each other and two out of six bases are mismatched.

Figure based on Cawthon, 2002

Cawthon method

This method determines the relative telomere length by comparing the results of qPCR for telomeres and a reference gene, a so-called single copy gene, which has two copies in a diploid genome. In this study beta-globin gene was used as the reference gene.

Two master mixes were prepared: the T-mix (telomere master mix) and the B-mix (beta-globin master mix). The T-mix consisted of telomere primers and the Applied Biosystems mix. The B-mix consisted of beta-globin primers and the Applied Biosystems mix. For primer sequences see *Table 1*, for final concentration of the reagents see *Table 2*.

For each individual 20 ng of isolated genomic DNA was added to the PCR plate and dried overnight. Every DNA sample was present in triplo for the telomere master mix and in triplo for the beta-globin master mix. Subsequently to every well 20 µL of corresponding master mix was added. The plate was covered with optical transparent adhesive cover and centrifuged for 2 min at 4700 rpm.

The qPCR was performed immediately after centrifuging. An incubation step of 95°C for 10 min in order to activate the DNA polymerase was followed by 40 cycles of 95°C for 15 sec, 58°C for 1 min.

	Forward primer sequence (written 5' → 3')	**Reverse primer sequence (written 5' → 3')**
Cawthon method	GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAG GGT	TCCCGACTATCCCTATCCCTATCCCTATCCCTAT CCCTA
O'Callaghan & Fenech method	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTG GGTT	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTT ACCCT
SjTREC measurement method	CCATGCTGACACCTCTGGTT	TCGTGAGAACGGTGAATGAAG
Beta-globin reference gene	GCTTCTGACACAACTGTGTTCACTAGC	CACCAACTTCATCCACGTTCACC
Albumin reference gene	TGAACAGGCGACCATGCTT	CTCTCCTTCTCAGAAAGTGTGCATAT

Table 1. Primer sequences for quantitative PCR

	Final concentration of the reagents
Applied Biosystems mix	150 nM 6-ROX and 0.2xSybr Green I 15 mM Tris-HCl (pH=8.0) 50 mM KCl 2 mM $MgCl_2$ 0.2 mM dNTP (each) 5 mM DTT, 1% DMSO 1.25 U AmpliTaq Gold DNA Polymerase
Cawthon telomere primers	Forward: 54 nM, Reverse: 180 nM
O' Callaghan & Fenech telomere primers	Forward: 80 nM, Reverse: 80 nM
SjTREC primers and probe	*Concentrations in probe-primermix:* Forward primer: 18μM Reverse primer: 18μM FAM/TAMRA probe: 5μM
Beta-globin primers	Forward: 80 nM, Reverse: 80 nM
Albumin primers and probe	*Concentrations in probe-primer mix:* Forward primer: 18μM Reverse primer: 18μM FAM/TAMRA probe: 5μM

Table 2. Final concentrations of the PCR reagents

O' Callaghan & Fenech method

This method allows calculating the absolute telomere length, based on oligomer standards. Hereby both a telomere standard and a single-copy gene standard are required (for sequences see *Table 3*, for calculations see section *Results*). Both oligomer standards were PAGE purified. Just as in Cawthon method, beta-globin gene was used as a reference single-copy gene.

The telomere master mix (T-mix) and the beta-globin master mix (B-mix) were prepared in the same way as in the Cawthon method. However, this time different telomere primers were used. (for sequence see *Table 1,* for final concentration see *Table 2*).

Serial dilutions (10^{-1} through to 10^{-6} dilution) were performed on the oligomer standards in order to generate a standard curve. The highest concentration of telomere standard was 60 pg per reaction, the highest concentration of beta-globin oligomer standard was 200 pg per reaction. The oligomers were added to the PCR plate in triplo for every dilution. Also every oligomer standard well contained 20 ng of plasmid DNA in order to maintain the overall DNA mass in every well. DNA samples from individuals were added to the plate in the same way as in the Cawthon method – 20 ng per well, two timepoints, in triplo for every sample. The DNA on PCR plate was dried overnight and subsequently to every well 20 μL of corresponding master mix was added. The plate was covered with optical transparent adhesive cover and centrifuged for 2 min at 4700 rpm.

The qPCR was performed immediately after centrifuging. An incubation step of 95°C for 10 min in order to activate the DNA polymerase was followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min.

	Sequence (written 5'→ 3')	Amplicon size
Telomere standard	TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG GGTTAGGGTTAGGGTTAGGG	84 bp
Beta-globin standard	GCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCATCTGACTCCTGAG GAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTG	120 bp

Table 3. Oligomer standard sequences for O' Callaghan & Fenech method

sjTREC measurement method

During intra-thymal development, T cells undergo several rearrangements in TCR loci. One of these rearrangements is the deletion of TCRD locus (*Fig. 3)*, during which a circularized episomal DNA molecule (signal joint TREC) is formed, containing a δRecψJα signal joint. When applying the qPCR to the signal joint with corresponding primers, the amount of signal joint TRECs (sjTRECs) can be quantified.

The sjTREC method is performed on the same genomic DNA as the Cawthon and O'Callaghan & Fenech method. A single copy gene is required in order to correct for the amount of input DNA. In this study albumin gene was used as a reference gene.

First, two primer-probe mixes were prepared – the sjTREC mix (S mix) and the albumin mix (A mix). The S mix consisted of sjTREC primers and sjTREC hydrolysis probe. The A mix consisted of albumin primers and albumin hydrolysis probe. For primer sequences see *Table 1*, for probe sequences see *Table 4* and for final concentration of the reagents, see *Table 2*.

	Sequence (written 5' → 3')	**Fluorophore and quencher**
sjTREC probe	CACGGTGATGCATAGGCACCTGC	FAM/TAMRA
Albumin probe	TGCTGAAACATTCACCTTCCATGCAGA	FAM/TAMRA

Table 4. Hydrolysis probe sequences for sjTREC method

DNA was added to the plate in the same way as in Cawthon method – 20 ng DNA per well, two timepoints per individual – and dried overnight. Every sample was present in triplo per primer-probemix. Subsequently to each well 0.485 µL of corresponding primer-probemix, 6.25 µL of AB-gene mastermix was added. Each well was filled out with Milli-Q water to a total volume of 12 µL per well. The plate was covered with optical transparent adhesive cover and centrifuged for 2 min at 4700 rpm.

The qPCR was performed immediately after centrifuging. One cycle of 50°C for 2 min was followed by one cycle of 95°C for 15 min and then by 45 cycli of 95°C for 15 sec, 60°C for 30 sec.

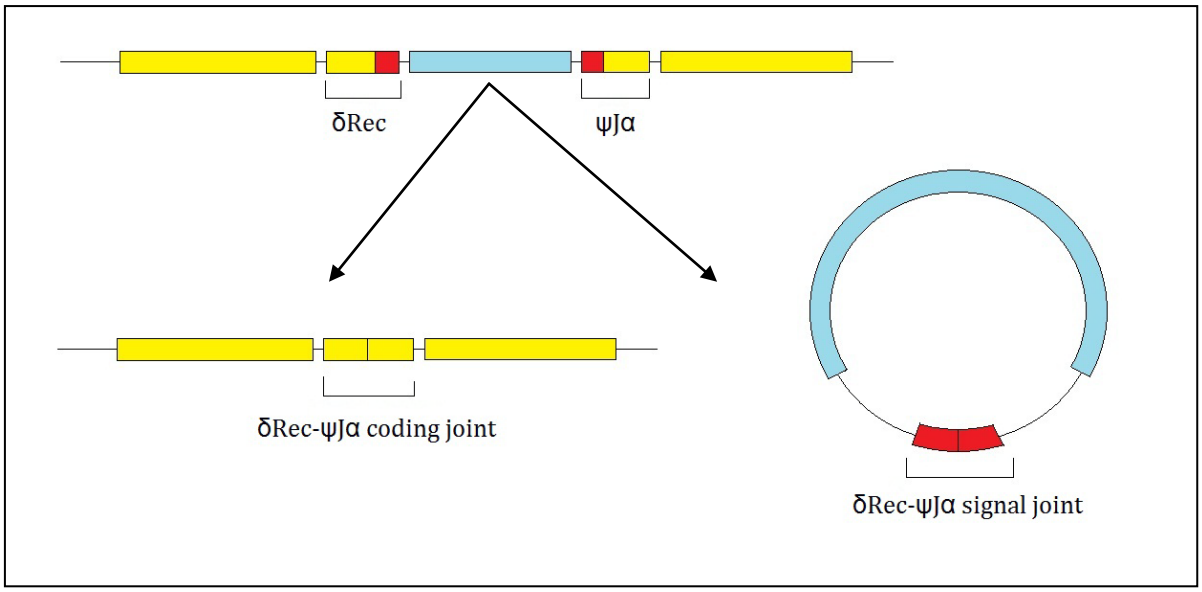

Fig.3 Deletion of TCRD locus
During the δRec-ψJα rearrangement in $\alpha\beta^+$ T cells, the TCRD locus is deleted. The δRec and ψJα sequences flanking the TCRD locus, when excised, form the δRecψJα signal joint sequence in the signal joint TCR excision circle. The remaining δRec and ψJα sequences form the δRecψJα coding joint and are excised in most $\alpha\beta^+$ T cells during later development.
The blue color indicates the TCRD locus, the red color indicates the signal joint forming δRec and ψJα sequences.
Figure adapted from Zubakov et al., 2010

Results

Cawthon method

After performing the qPCR, for every sample mean C_t values were calculated by taking an average of triplo's and timepoints. A single individual, who had his triplo C_t values out of standard deviation range, was excluded from further analysis. Subsequently, for each sample a mean T/S ratio (T stands for telomere, S stands for single-copy gene) was calculated according to the following formula:

$$\frac{T}{S} Ratio = \left(\frac{2^{telomere\ C_t}}{2^{beta-globin\ C_t}}\right)^{-1} = 2^{-\Delta C_t}$$

The PCR efficiency was equal for beta-globin and telomere primers (data not shown). The correlation between age and mean T/S ratio is shown graphically in *Figure 4.* The R^2 coefficient is 0.39 and the figure shows a descending line.

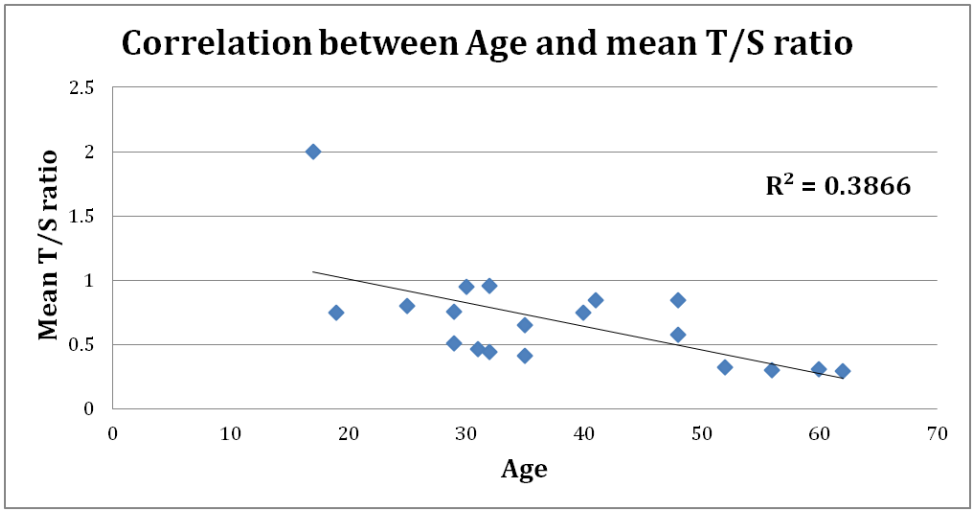

Fig. 4 Correlation between Age and mean T/S ratio (N=19)
On the x-axis we can see the age in years, on the y-axis the relative telomere length represented as the T/S ratio

For this method an additional experiment was performed in order to determine the reproducibility of results for every DNA sample. Two DNA samples were measured six times each with telomere primers and the variation between measurements is shown graphically in *Figure 5.* The maximum variation for DNA 1 was 0.528 cycles, for DNA 2 it was 0.966 cycles.

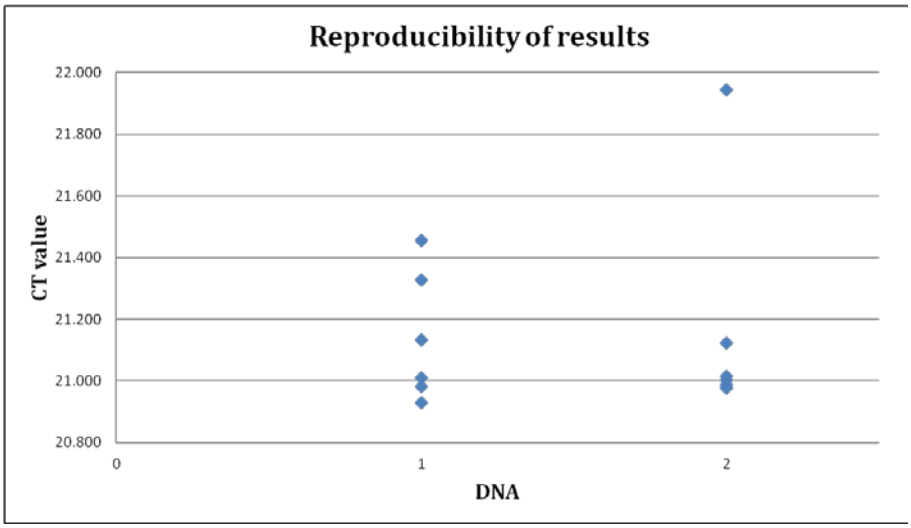

Fig. 5 Reproducibility of results (N=6)
This figure shows the variation of measurements performed on a single DNA. DNA 1: max. variation – 0.528 cycles, mean – 21.139, st. deviation – 0.21. DNA 2: max. variation – 0.966 cycles, mean – 21.174, st.deviation – 0.38

O'Callaghan & Fenech method

For every sample mean C_t values were calculated by taking an average of triplos and timepoints. Individuals who had their triplo C_t values out of standard deviation range, were excluded from further analysis. For every remaining sample the absolute telomere length was determined by first calculating the overall telomere length in base pairs and subsequently correcting for genome copy number, as described below.

Calculating overall telomere length in base pairs

Every telomere oligomer standard has a molecular weight of 26667.2 g/mol. Given that

$$Weight\ of\ 1\ molecule = \frac{Molecular\ weight}{Avogadro's\ number}$$

we can calculate that the weight of 1 molecule telomere standard is $0.44*10^{-19}$ g. For each dilution of the standard we know the concentration per reaction. The highest concentration was 60 pg per reaction which corresponds to $1.36*10^{9}$ molecules. The length of each telomere oligomer standard is 84 bp. The overall length of telomere standard in the first dilution (60 pg) was then $1.36 * 10^{9} * 84 = 1.15 * 10^{11}$bp.

In a similar way the overall length was calculated for every dilution of the telomere standard. The relation between the overall length and the C_t is shown graphically in *Figure 6*. The correlation between the overall length and C_t was 99%, which is higher than the minimal confidence value of 95%, indicated in the literature (O' Callaghan & Fenech, 2011).

For every sample the average C_t is known, so the corresponding overall length in base pairs could be measured from the standard curve. This was done, using the value for telomere primers.

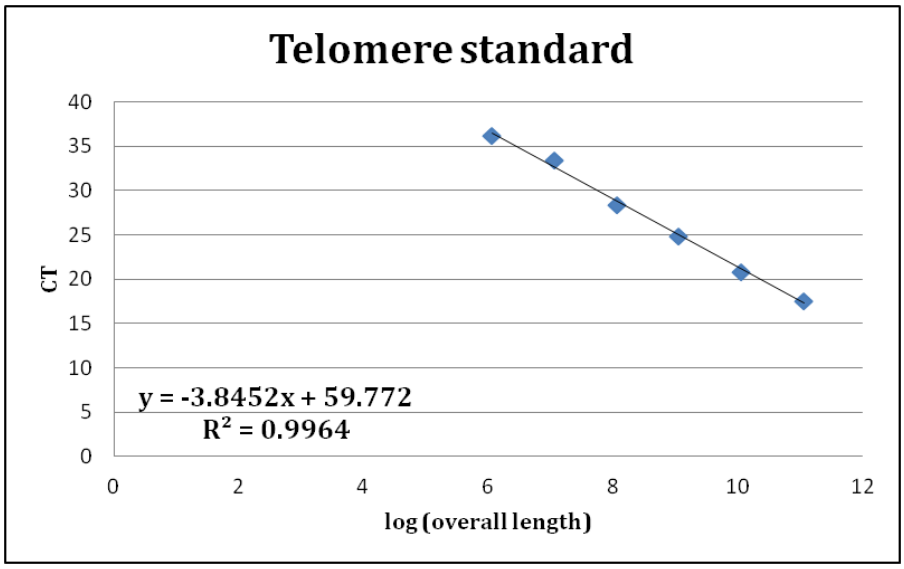

Fig. 6 Telomere oligomer standard curve
On the x-axis we can see the logarithm of overall telomere length in bp. On the y-axis the corresponding C_t values are displayed. The trend line equation is used for calculating overall telomere length for telomere C_t values.

Correcting for genome copy number

Beta-globin gene is a single copy gene and thus has two copies (molecules) in a diploid genome. Beta-globin oligomer standard has a molecular weight of 37118.1 g/mol and the highest concentration of 200 pg per reaction. In the same way as described above, we can calculate that the first dilution (200 pg) consists of $3.24*10^9$ copies in a haploid genome, which corresponds to $1.62*10^9$ copies in a diploid genome.

In a similar way the number of copies was calculated for every dilution of the beta-globin standard. The relation between the number of copies and the C_t is shown graphically in *Figure 7*. The correlation is 96%, which is higher than the minimal confidence value.

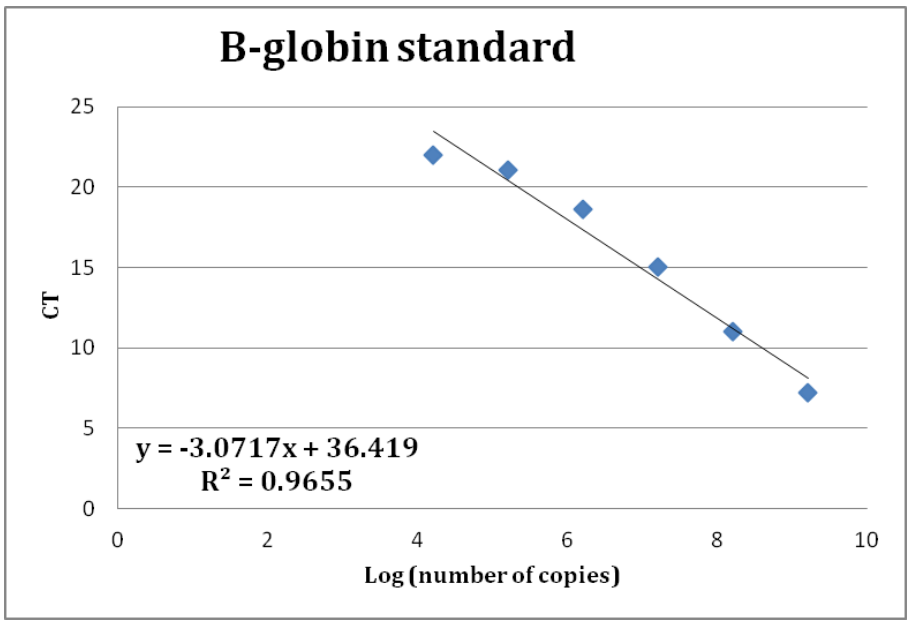

Fig. 7 Beta-globin oligomer standard
On the x-axis the logarithm of the genome copy number is displayed, on the y-axis the corresponding C_t values. The trend line equation is used for calculating beta-globin genome copy number for telomere C_t values.

For every sample the corresponding number of copies could be measured from the standard curve. This was done, using the values for beta-globin primers. Subsequently the absolute telomere length was calculated according to the following formula:

$$Absolute\ length = \frac{Overall\ length}{Number\ of\ copies}$$

The correlation between age of the individuals and their telomere length is shown in *Figure 8.* The R^2 coefficient is 0.33, and the figure shows a descending line.

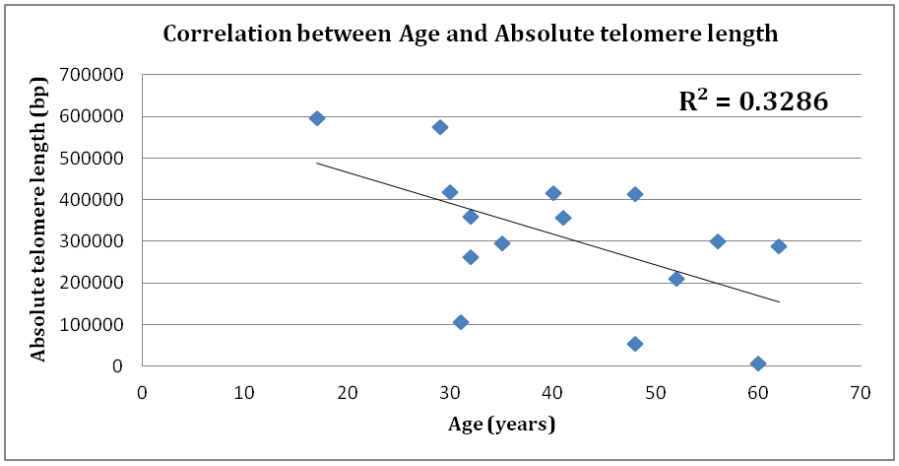

Fig. 8 Correlation between age and absolute telomere length (N=15)
On the x-axis we can see the age in years, on the y-axis the calculated absolute telomere length in base pairs.

sjTREC method

When the qPCR returned the C_t values for each sample, mean C_t was calculated for every individual in the same way as it was done for Cawthon method and O'Callaghan & Fenech method. Individuals who had their triplo C_t values out of standard deviation range, were excluded from further analysis. Subsequently an average ΔC_t was calculated, according to the following formula:

$$Average\ \Delta C_t = mean\ sjTREC\ C_t - mean\ albumin\ C_t$$

The relation between age and average ΔC_t is shown graphically in *Figure 9.* The R^2 coefficient is 0.71 and the figure shows an ascending line.

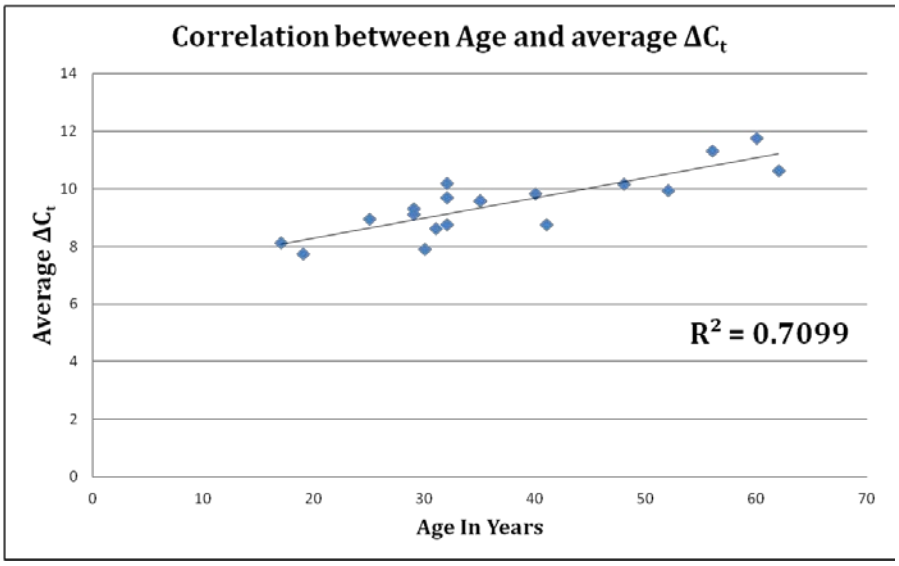

Fig.9 Correlation between age and average ΔC_t (N=16)
On the x-axis we can see the age in years, on the y-axis the corresponding average ΔC_t (telomere – albumin).

Comparison of the methods

In order to compare the three methods, we first made a comparison of the two telomere length measurement methods. An ideal correlation of 100% would indicate a perfect similarity between Cawthon method and O'Callaghan & Fenech method. The results of this comparison are shown graphically in *Figure 10.* The R^2 coefficient is 0.39 and the graph shows an ascending line.

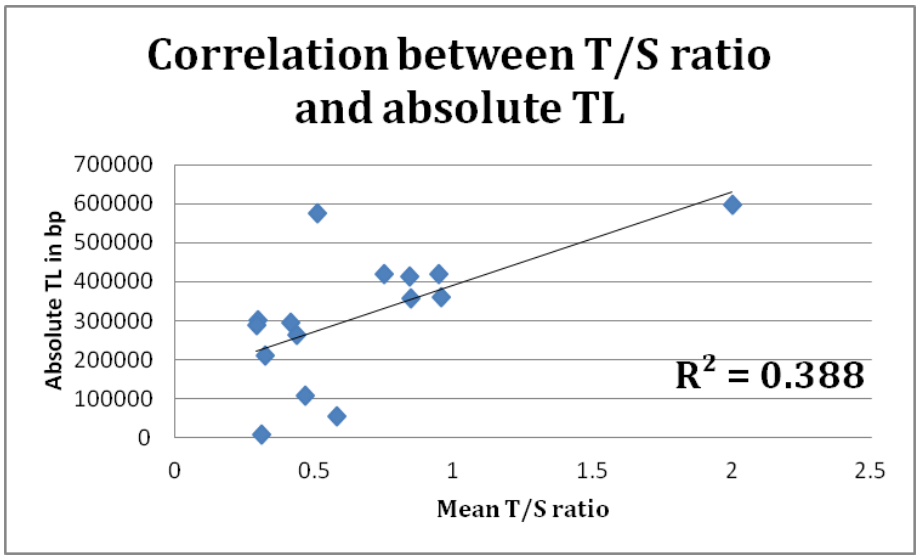

Fig. 10 The correlation between relative and absolute telomere length (N=15)
On the x-axis we see the relative telomere length, represented as the T/S ratio. On the y-axis the absolute telomere length in base pairs is displayed.

Methods, however, are not only characterized by the correlation with chronological age, but also by different practical parameters, displayed in *Table 5.* Comparing the methods, we considered the amount of DNA needed, time for preparing one qPCR plate and the number of samples excluded from further analysis. We can see that O'Callaghan & Fenech method is most time consuming, which is due to oligomer standard dilutions that must be added to the plate. In Cawthon method only 5% of the samples failed, while O'Callaghan & Fenech method and the sjTREC method have a higher failure rate.

	Cawthon method	**O'Callaghan & Fenech method**	**sjTREC method**
Failure rate	5%	25%	20%
Time needed for 1 qPCR plate	Approx. 6h	Approx. 7h	Approx. 6h
DNA needed	20 ng	20 ng	20-50 ng

Table 5. Comparison of three methods of measuring biological age

Discussion

During this research we made several major modifications to original study protocols in order to increase the efficiency of the methods. Regardless of the particular telomere primer design (*Fig. 2*), PCR products were observed in negative controls for both Cawthon method and O'Callaghan & Fenech method. Corresponding amplification curves returned C_t values in the range between 26-28 cycles and the corresponding melt curves were different from melt curves of PCR products, suggesting a primer dimer origin. In order to reduce the primer dimer effect, we tested different primer concentrations. A five times dilution of the concentration given in study protocols turned out to be optimal.

Another modification concerns the use of one qPCR plate instead of two plates recommended in Cawthon method. According to the original protocol samples should be run separately for telomere primers and single-copy gene primers due to different PCR programs. However, telomere primers showed a satisfactory result when run with single copy gene's PCR program during optimization phase. The use of single PCR plate also keeps the conditions for both primer sets as equal as possible, making comparisons and calculations more confident and reducing the costs. It is possible, however, for telomere primers not to work optimally due to changes in PCR program, affecting the results. Since qPCR is a very sensitive technique (Higuchi et al., 1993), even the smallest changes in primer effectiveness could affect the result significantly.

During our study we also used dry DNA instead of solutions as indicated in Cawthon method and O'Callaghan & Fenech method. Adding DNA on the plate in advance and drying it overnight allows preparing several plates simultaneously and storing them for a long time without losses in PCR quality, thus saving time. The biggest risk factor is the dissolving of DNA when back in solution – it is possible that DNA would not dissolve again properly, reducing the overall amount of DNA per reaction with an unknown factor. However, proper centrifuging of the plate and the PCR incubation step of 95°C make proper DNA dissolving very likely.

In contrast to recommended 36B4-gene we used beta-globin gene as the reference single-copy gene for both telomere length measurement methods and therefore a beta-globin oligomer standard instead of 36B4 oligomer standards. The beta-globin standard has a length of 120 bp, which is longer than the 36B4 standard (75 bp). A larger transcript size could explain variation between triplos of the same sample and thus the number of excluded samples (25% for the Cawhon method).

The difference in reference gene can also be an explanation for the calculated absolute telomere lengths in O'Callaghan & Fenech method (*Fig. 8*). The telomere lengths obtained in this study (0 – 700 kb range) differ clearly from the lengths reported in the literature (0 – 350 kb range; O'Callaghan & Fenech, 2011). The factor two difference can be explained by the difference in gene copy number. 36B4 is reported as a single-copy gene, thus having two copies in a diploid genome, while In Silico PCR in the USCS Genome Bioinformatics database returns three copies in a haploid genome – on chromosomes 12, 2 and 5. Previous research mentioned the 36B4 gene to be homologous to several pseudogene sequences (Kumar et al., 2006). This is, however, not reflected in the calculations of the absolute telomere length, as presented by O'Callaghan & Fenech.

Another possible explanation could lie in the high sensitivity of qPCR and the instability of the obtained C_t values, shown in *Figure 5*. We can see that a variation up to an entire cycle is possible, while a difference of one cycle would give a factor two difference in the amount of input DNA and thus of absolute telomere length. Such variation could also explain the presence of samples with absolute telomere lengths below 100 kb per genome.

There are several other possible reasons for high variation of results. One of them is the repetitive nature of telomere hexamers. Since the primers can equally anneal to any hexamer, the PCR product contains transcripts of different length in unknown proportion. Therefore it is unclear how the measured telomere length relates to the actual telomere length. There are also no indications to assume that the proportion of transcripts would be similar between individuals, making relative telomere length measurements as doubtful as absolute telomere length.

Another reason of variation of results lies in blood composition of samples. Since lymphocytes show a faster telomere shortening than granulocytes (Rufer et al., 1999), it is presumable that individuals suffering from infections and thus having a higher lymphocyte fraction in blood, should return lower telomere length values than hypothetical values based on their chronological age only.

The main course of correlation curves for telomere measurement methods corresponds to previous studies. In O'Callaghan & Fenech curve (*Fig.8)* we can observe a descending line, indicating an age-related decrease in absolute telomere length. For the Cawthon method we can also observe a descending line, indicating the same decrease in relative telomere length. When we compare the telomere measurement assays to each other (*Fig. 5)*, an ascending line can be observed, indicating that when the relative telomere length rises, absolute telomere length

also rises. This corresponds perfectly to previous studies (Aubert & Lansdorp, 2008). However, the two methods do not show an absolute similarity – the correlation between the absolute and relative telomere length is 39%. This correlation is lower than the reported correlation between absolute and relative telomere length, when absolute telomere length is measured with TRF-method – 68% (Cawthon, 2002). A possible explanation could be the high variability of telomere length and previously mentioned high sensitivity of the qPCR method. Another possible reason can be the statistically small test group.

It is remarkable that both telomere length measurement methods showed an R^2 coefficient < 0.50, suggesting that although biological age in common and telomere length in particular depend considerably on the chronological age it is not the only influencing factor. Previous research states several other variables, possibly influencing the telomere length, including gender, Body Mass Index (BMI), psychological stress, presence of markers of oxidative stress and smoking status and history to possibly influence the telomere length. (O'Callaghan & Fenech, 2011)

The sjTREC method, on the contrary, showed an R^2 coefficient > 0.50, thus suggesting that thymic function highly depends on chronological age. This could suggest the sjTREC method to be the most preferable for forensic sciences and fields of study in which age prediction plays a dominant role. Measurements of sjTRECs in a larger cohort of celiac patients (N=69 out of 96), however, returned an R^2 value of 0.48. Two possible explanations are available for this observation. First, a difference in R^2 values can be due to different cohorts. In this study the sjTREC method was performed on 20 individuals only. A larger cohort provides a greater statistical power. Second, the differences can be explained by differences in disease status. Since celiac disease belongs to autoimmune diseases and the fraction of T lymphocytes in blood is different in celiac patients, it is assumable that amounts of sjTREC which are only measured in T cells depend more on the disease state and progression in patients than in healthy individuals.

Results obtained in this research suggest all the three methods to be suitable for measuring biological age. In order to obtain more statistical relevance, the experiments should be repeated in larger cohorts, especially when observing the failure rate. Among possible further research we count the extension of comparative analysis of telomere length measurements with flowFISH and STELA methods. Also a later monochrome multiplex qPCR (MMQPCR) telomere length measurement by R. Cawthon belongs to perspective methods (Cawthon, 2009). In order to minimize the influence of blood composition, the measurements should be performed separately per cell type.

In this study we investigated the influence of chronological age on biological age manifestations, such as telomere length measurements and the amounts of byproducts of T cell development. When combined with studies on other factors influencing biological senescence, the results of this research contribute to a fuller understanding of different aging processes.

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Appendix A – DNA isolation

Reagents:
MQ water (MilliQ)
DNA- and RNA-free water

20%SDS (Sodium dodeculsulphate)
100 mL MQ water was heated to 50°C, then 20 g of SDS was added. The solution was prepared in the fumehood.

0.5 M EDTA pH 8.0 (titriplex II)
186.1 g titriplex III was dissolved in 800 mL MQ water. The pH was set to 8.0 with NaOH. After dissolving, the MQ water was filled out to 1000 mL.

6M NaCl (Sodium chloride)
350 g NaCl was dissolved in 1 L MQ water

1 M Tris-HCl pH 8.0
121.1 g of Tris was dissolved in 800 mL MQ water, the pH was set to 8.0 with HCl. After dissolving, the MQ water was filled out to 1000 mL

TE-4 (Tris-EDTA)
5 mL of 1M Tris-HCl (pH 8.0) was mixed with 0.1 mL 0.5 EDTA (pH 8.0), then MQ water was added to fill the total volume to 500 mL

10xAKE
80.2 g of NH_4Cl , 10.0 g $KHCO_3$ and 0.2 mL 0.5 M EDTA (pH 8.0) were dissolved in 1000 mL MQ water.
In order to obtain 1xAKE, 1000 mL 10xAKE was dissolved with MQ water to 10 L

10xSE
125 mL 6M NaCl and 500 mL 0.5 M EDTA (pH 8.0) were dissolved in 1000 mL MQ water.
In order to obtain 1xSE, 1000 mL 10xSE was dissolved with MQ water to 10 L

Pelleting of the whole blood:
To 10 L whole blood 30 mL 1xAKE buffer was added. The solution was shaked gently, incubated on ice for 30 min and centrifuged (1250 rpm, 15 min, 4°C). The supernatant was removed and the pellet was resuspended in 10 mL 1xAKE buffer, then centrifuged (1250 rpm, 15 min, 4°C). The supernatant was once again removed.
Thus obtained pellet was stored at -20°C.

DNA isolation:
The pellet (previously stored at -20°C) was resuspended by vortexing in 10 mL 1xSE buffer, then 1000 µL 10% SDS and 50 µL proteinase K was added.
The solution was incubated overnight at 55 °C.
To the solution 4.2 mL 6M NaCl and 14.7 mL Chloroform were added, then it was shaken in the top-of-top rotor for 20 min and centrifuged (2500 rpm, 30 min, room temperature). The water phase (upper layer) was transferred into a new tube, and 14 mL isopropanol was added. When gently shaken, the DNA was visible as a white cloud. This white cloud was transferred to 1 mL 70% Ethanol and shaken for 5 min in the top-of-top rotor. Afterwards the Ethanol was removed and the DNA was airdried. When dry and glassy-transparent, DNA was dissolved in TE-4 buffer overnight.
DNA concentration was measured in a nanodrop. Afterwards the DNA was stored on 96-wells masterplate (at 4°C).