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# Age-specific telomere length percentiles in the Argentine population: a diagnostic tool for telomere biology disorders

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## ABSTRACT

***Introduction.*** Telomere length (TL) is a biomarker of cellular aging. Its excessive shortening is associated with telomere biology disorders (TBD), which can manifest from childhood with bone marrow failure and a predisposition to cancer.

The clinical interpretation of TL requires age-adjusted reference curves specific to each population. In Argentina, there is no curve of its own. The objective of the study was to construct an age-adjusted telomere length reference curve representative of the Argentine population.

***Methods.*** Using the Monochrome Multiplex Quantitative PCR (MMQPCR) technique, TL was estimated in 159 samples from healthy individuals (0-50 years old). The percentile curve was adjusted using a generalized linear model with a gamma distribution. Validation was performed using 19 controls (normal and pathological) that had previously been evaluated by MMQPCR or Southern blot in international laboratories.

***Results.*** The curve allowed us to estimate age-adjusted TL percentiles (P1-P95). All samples with a clinical or molecular diagnosis of TBD were below P10, and cases with severe phenotypes were below P1. Normal controls were above P10. The technique demonstrated good reproducibility and adequate model adjustment.

***Conclusions.*** The first age-adjusted TL reference curve was generated in the Argentine population. It is a valuable tool for local laboratories that use the same methodology, guiding the diagnosis of TBD. Its integration with next-generation sequencing techniques increases diagnostic sensitivity and allows for a more accurate approach to these syndromes.

***Keywords:*** *telomere shortening; multiplex polymerase chain reaction; telomere, pathology.*

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## INTRODUCTION

Telomeres are repeats of the hexanucleotide TTAGGG associated with proteins located at the ends of chromosomes to protect their integrity. In human cells, the average length of the 92 telomeres is a heritable trait influenced by sequence variants in genes that maintain telomeres.[1,2] Telomere length (TL) is a biological marker of cellular age, as telomeres progressively physiologically shorten with age.[3-5]

In recent years, accelerated telomere shortening has been reported in certain inherited bone marrow failure syndromes (IBMFS) and in specific pathologies generally associated with aging (cardiovascular disease, diabetes mellitus, Alzheimer's disease, schizophrenia, osteoporosis), thereby increasing the predisposition to certain oncological diseases.[6-9] The severity of clinical expression is usually correlated with telomere length.

Telomere biology disorders (TBD) result from excessive telomere shortening. In the pediatric population, they can present with great clinical heterogeneity and varying severity, ranging from isolated cytopenias or progressive bone marrow failure to mucocutaneous alterations, skeletal abnormalities, growth retardation, neurological alterations, dental and ophthalmological abnormalities, and multiorgan complications. The presence of a TL below the first percentile relative to healthy individuals of the same age is highly sensitive (97%) and specific (91%) for the diagnosis of TBD, thereby guiding molecular studies of associated genes and supporting a different clinical approach. Dyskeratosis congenita (DC), Hoyeraal-Hreidarsson syndrome (HH), and Revesz syndrome (RS) are severe clinical forms of TBD, characterized by extremely short telomere lengths, defined as below the first percentile for age, in all cell types.[3,4,10-12]

In TBD, as in many IBMFS, hematopoietic stem cell transplantation (HSCT) is the only curative treatment for bone marrow failure and the first therapeutic option if a histocompatible donor is available. Excessive TL shortening suggests possible constitutional bone marrow failure, which requires different conditioning before transplantation and evaluation of potential related donors.[13,14]

Various methodologies have been developed to determine LT, including Southern blot, Flow-Fish, Q-Fish, HT-Stela, and MMQPCR.[15-17] MMQPCR is a technique that enables semi-quantitative measurement of TL. This measurement is based on the amplification signal obtained from the telomeric region (T), which is normalized with the amplification signal of a single-copy gene (S). Telomere length values obtained using this methodology are expressed as the T/S ratio.[18-20]

TL values can be affected by methodological factors,[21] as well as by ethnic and socio-environmental factors that influence TL in reference populations.[22-24] Therefore, it is not advisable to use percentile curves derived from different populations. Generating an age-adjusted TL percentile curve for the Argentine population is essential to obtain a TL value that can guide TBD. The development of a reference population curve representing percentiles (P) from 1 to 100 would require the analysis of many samples from healthy individuals, including newborns and children.

The objective of this study was to construct an age-adjusted telomere length reference curve representative of the Argentine population.

## METHODS

### Samples

To develop the TL reference curve based on age, 159 samples from healthy individuals with no personal or family history of TBD were analyzed. These samples were collected from children undergoing pre-surgical studies for trauma surgery or surgery unrelated to hematological pathologies, and from adult donors at the hospital's blood therapy center during the period 2015-2020 (*Table 1*). The Research Review Committee of the Hospital de Pediatría Prof. Dr. Juan P. Garrahan approved the study. Patients and/or their parents signed informed consent forms for genetic testing and for the use of the results for scientific and academic purposes.

Genomic DNA was isolated from total leukocytes using commercial columns from whole blood anticoagulated with EDTA (QIAamp® DNA Mini Kit Qiagen). The concentration of each DNA sample was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The integrity of each sample was evaluated by loading 100 ng of DNA onto a 1% agarose gel.

A reference sample was used for inter-assay normalization, and a pooled sample was used to construct the standard curve, with a dynamic range of DNA concentration from 2 to 0.0625 ng/µl.

To validate the TL percentile curve vs. age, 11 control samples from healthy individuals and 8 samples from individuals with a clinical and/or molecular diagnosis of TBD were evaluated.[12]

**Table 1. Characteristics of the samples used to construct the TL vs. age reference population curve and its distribution according to sex and age**

| Reference population | | Total = 159 |
|---|---|---|
| **Healthy individuals** | Pediatric | 125 (58% M) |
| | Adults | 34 (59% M) |
| **Gender** | Male (M) | 92 (58%) |
| | Female (F) | 67 (42%) |
| **Age range (years)** | 0-3 | 23 |
| | 4-6 | 26 |
| | 7-10 | 32 |
| | 11-15 | 27 |
| | 16-20 | 24 |
| | 21-25 | 10 |
| | 26-30 | 6 |
| | 31-50 | 11 |

*M: male.*

Eleven healthy controls analyzed by MMQPCR at the Hospital das Clinicas da Faculdade de Medicina de Ribeirão Preto (Brazil) and eight with a diagnosis of TBD evaluated by Southern blot and provided by the Centre for Genomics and Child Health, Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, were included.

## Real-time PCR

TL was evaluated semi-quantitatively using the MMQPCR technique described by Cawthon et al.[18], with modifications. The Telg and Telc primers were used for the amplification of telomeric sequences, while Hbgu and Hbgd were used for the amplification of the single-copy gene (SCG) β-globin. A reaction volume of 25 µl was used with 6 ng of DNA, 850 nM of Telg and Telc, 400 nM of Hbgu and Hbgd, and 1X IQTM SYBR Green Supermix (Bio-Rad) containing 0.625 U of Taq DNA polymerase (iTaqTM polymerase), 3 mM $MgCl_2$, 0.2 mM dNTPs, SYBR Green I, and fluorescein. The following cycling protocol was used: 15 minutes at 95 °C (1 cycle); 15 seconds at 94 °C (2 cycles); 15 seconds at 94 °C, 10 seconds at 62 °C, 15 seconds at 74 °C (32 cycles with signal reading at 74 °C); 10 seconds at 84 °C and 15 seconds at 88 °C with signal reading. The telomere and SCG sequence data were analyzed using BioRad's CFX Manager 3.0 software. For the standard curves, the baseline was established between cycles 2-6 (telomere) and 2-15 (SCG). The fluorescence threshold was experimentally defined at 100 units for both curves.

Only assays with $R^2 \geq 0.980$, efficiency between 95% and 105%, and a difference in efficiency between the two standard curves of less than 5% were accepted.

DNA samples from the healthy population, controls, standard curve, and reference sample were evaluated under the reaction conditions described above. Only samples that met the assay's integrity and concentration requirements were accepted. Each sample was assessed in triplicate across three independent assays, with an interassay coefficient of variation (CV%) of less than 15% accepted.

## Construction of the percentile curve

As described by Cawthon in 2002, the ratio (T/S) sample/(T/S) reference sample was calculated, providing a semi-quantitative measure of TL.[25] Based on these values and the age of healthy donors, an age-adjusted percentile curve was developed for the Argentine population.

The analysis of the results was conducted using a parametric approach based on a generalized linear model, which yields highly accurate percentiles. These models assume a parametric distribution for the response variable (TL); in our case, the gamma distribution was chosen. Both parameters of this distribution, µ (position) and σ (scale), are modeled independently as linear functions of age, with a logarithmic link.

The versatility of these models makes them appropriate tools for situations in which the distributional context is neither normal nor symmetric, or when error variability is not constant across values of the predictor variable, as is the case with TL-age values.[26-28]

The choice of the gamma distribution was based on its flexibility and the suitability of its distributional form for these data.

## RESULTS

The average CV% of the MMQPCR technique was 10.3%, calculated from 25 samples analyzed in three independent assays. Only assays that met the established quality criteria were accepted: coefficient of determination ($R^2$) ≥ 0.980, amplification efficiency between 95% and 105%, and efficiency difference between the standard curves (telomeric and SCG) less than 5%. Only samples with adequate integrity and concentration and an inter-assay CV% of less than 15% were included in the curve.

From the 159 samples from healthy individuals, a TL (T/S)-age curve was obtained between the 95th and 1st percentiles (*Figure 1*). This figure shows the adjusted TL distribution as a function of age. The percentiles of this curve were calculated from a smaller sample size with high precision using the statistical model with a gamma distribution, as detailed in *Figure 2*. *Table 2* details the median TL and 95% confidence intervals for each age group. This table was compiled by drawing 1,000 Bootstrap samples from the original sample and applying the GAMLSS (generalized additive models for location, scale, and shape) gamma methodology to each sample. The Bootstrap sampling technique allows multiple simulated samples to be generated from a single original sample. In this way, it is possible to obtain a more accurate estimate of percentile variability and greater confidence in the results.

The statistical model allowed us to estimate specific percentiles by age, from P1 to P95.

Age proved to be a significant factor in estimating the TL distribution parameters, μ and σ, with the influence most pronounced on the position parameter μ. *Figure 3* shows the relationship between age and the estimation of both model parameters.

*Figure 1* shows the degree of fit of the TL (T/S) curves versus age constructed from the generalized linear model. TL values for the 11 healthy control samples and the 8 samples with a clinical/molecular diagnosis of DBT were interpolated from the curve and used for validation. The pathological control samples were all below P10, although the samples associated with more severe phenotypes (DC and HH) were below P1. The healthy controls were all above P10.

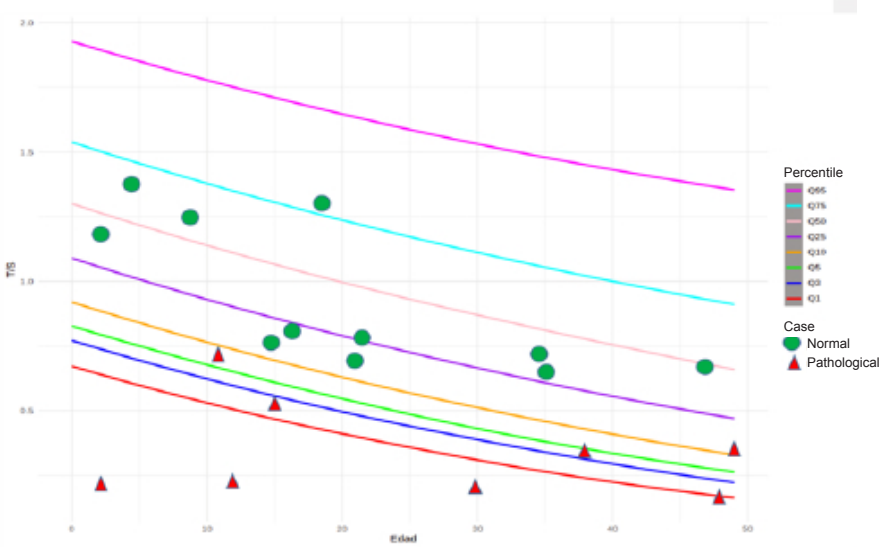
## DISCUSSION

Telomeres play a crucial role in population health.[29-32] Accumulated evidence shows that patients with TBD are at increased risk of developing bone marrow failure, pulmonary fibrosis, liver disease, hematological tumors, and solid tumors.[2]

TL measurement complements molecular gene studies by revealing the functional importance of sequence variants in genes involved in telomere maintenance.

Determining TL is also helpful for pre-HSCT evaluation of related donors of patients with a

**Figure 1. Percentile curve obtained from 159 samples from healthy donors**




Telomere length of 11 normal and 8 pathological controls.

**Figure 2. Adjusted gamma distribution for each age**

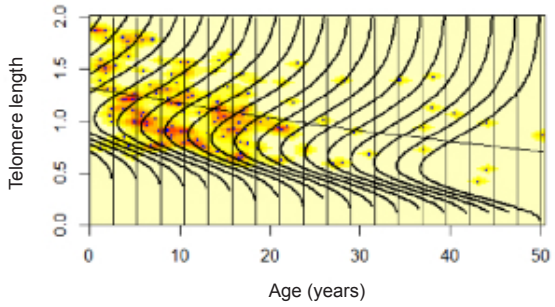
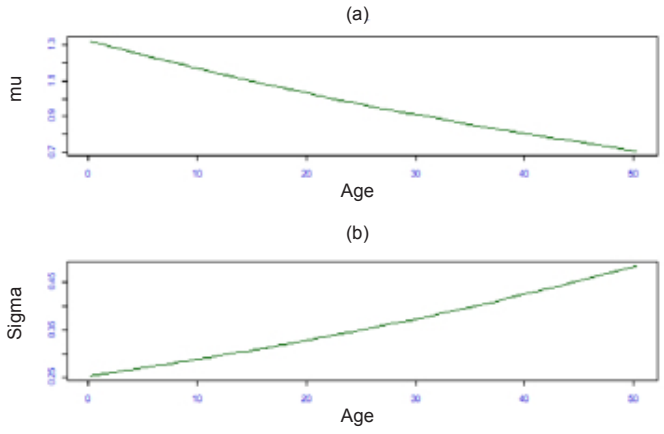



**Figure 3. Estimation of parameters based on age: (a) estimation for μ (b) estimation for σ**




clinical diagnosis of TBD who lack an identified genetic alteration. Because of the anticipation phenomenon that characterizes these disorders, a potential related donor may be asymptomatic at the time of selection but share the same genetic alteration and telomere shortening as the recipient. This classifies them as an unsuitable donor for HSCT.[10]

Although a certain degree of telomere shortening can be observed in other IBMFS, values rarely fall below P1. For this reason, TL represents a valuable biomarker in differential diagnosis and therapeutic decision-making.[13,14]

The MMQPCR technique was selected for its simplicity, low cost, and ability to process many samples with minimal DNA input.[18,33] This is particularly useful in patients with cytopenias associated with bone marrow failure. Among the limitations of MMQPCR, we observed greater inter-assay variability and dependence of the TL value on the extraction method and DNA integrity.[19,20,34]

One of the main strengths of this study is the development of an age-adjusted TL percentile reference curve for the Argentine population, with broad pediatric representation, which allows its use in place of international curves and reduces variability attributable to ethnic and socio-environmental factors.

A statistical approach based on the gamma distribution enabled precise estimation of percentiles from a small sample, representing a methodological improvement over classical models such as quantile regression. One limitation of this study is the underrepresentation of the healthy adult population in TL relative to the age reference curve. However, using a GAMLSS model with a four-parameter generalized gamma distribution yielded highly accurate percentiles across all age groups, with estimates based on the entire sample, thereby reducing the impact of the smaller sample sizes in the older age groups.

Although there is some overlap in the 95% CI between different TL percentiles, this overlap does not invalidate the clinical usefulness of this curve. From a clinical standpoint, the objective is not to accurately discriminate between adjacent percentiles, but rather to identify individuals with

**Table 2. Distribution of telomere length (T/S) percentiles according to age**

| Age. years | P1_ lower | P1_ mean | P1_ upper | P5_ lower | P5_ middle | P5_ upper | P50_ lower | P50_ medium | P50 uper | P95_ lower | P95_ mean | P95_ upper |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.548 | 0.668 | 0.797 | 0.705 | 0.824 | 0.95 | 1.18 | 1.301 | 1.43 | 1.746 | 1.936 | 2.123 |
| 2 | 0.541 | 0.654 | 0.774 | 0.701 | 0.809 | 0.925 | 1.174 | 1.284 | 1.402 | 1.742 | 1.918 | 2.096 |
| 3 | 0.537 | 0.64 | 0.75 | 0.695 | 0.794 | 0.9 | 1.163 | 1.267 | 1.374 | 1.734 | 1.901 | 2.063 |
| 4 | 0.532 | 0.626 | 0.726 | 0.689 | 0.779 | 0.877 | 1.153 | 1.25 | 1.347 | 1.728 | 1.883 | 2.032 |
| 5 | 0.525 | 0.612 | 0.704 | 0.68 | 0.764 | 0.853 | 1.145 | 1.233 | 1.32 | 1.725 | 1.866 | 2.003 |
| 6 | 0.519 | 0.599 | 0.682 | 0.672 | 0.749 | 0.831 | 1.135 | 1.217 | 1.295 | 1.716 | 1.849 | 1.981 |
| 7 | 0.513 | 0.586 | 0.66 | 0.664 | 0.735 | 0.808 | 1.126 | 1.201 | 1.273 | 1.71 | 1.833 | 1.957 |
| 8 | 0.507 | 0.573 | 0.64 | 0.656 | 0.721 | 0.787 | 1.117 | 1.185 | 1.252 | 1.695 | 1.816 | 1.936 |
| 9 | 0.5 | 0.56 | 0.621 | 0.648 | 0.707 | 0.766 | 1.106 | 1.169 | 1.23 | 1.683 | 1.8 | 1.914 |
| 10 | 0.494 | 0.547 | 0.603 | 0.641 | 0.693 | 0.747 | 1.095 | 1.154 | 1.21 | 1.671 | 1.784 | 1.896 |
| 11 | 0.486 | 0.535 | 0.586 | 0.631 | 0.68 | 0.729 | 1.084 | 1.138 | 1.192 | 1.656 | 1.769 | 1.88 |
| 12 | 0.474 | 0.522 | 0.57 | 0.619 | 0.666 | 0.713 | 1.072 | 1.123 | 1.177 | 1.64 | 1.754 | 1.864 |
| 13 | 0.464 | 0.51 | 0.557 | 0.61 | 0.653 | 0.698 | 1.059 | 1.108 | 1.162 | 1.624 | 1.738 | 1.85 |
| 14 | 0.452 | 0.499 | 0.545 | 0.595 | 0.64 | 0.686 | 1.045 | 1.093 | 1.146 | 1.61 | 1.724 | 1.837 |
| 15 | 0.44 | 0.487 | 0.535 | 0.583 | 0.627 | 0.673 | 1.03 | 1.079 | 1.133 | 1.591 | 1.709 | 1.825 |
| 16 | 0.426 | 0.475 | 0.527 | 0.569 | 0.615 | 0.663 | 1.015 | 1.064 | 1.119 | 1.568 | 1.695 | 1.815 |
| 17 | 0.412 | 0.464 | 0.52 | 0.554 | 0.602 | 0.654 | 0.997 | 1.05 | 1.108 | 1.55 | 1.68 | 1.809 |
| 18 | 0.396 | 0.453 | 0.512 | 0.537 | 0.59 | 0.647 | 0.98 | 1.036 | 1.096 | 1.527 | 1.666 | 1.803 |
| 19 | 0.382 | 0.442 | 0.505 | 0.52 | 0.578 | 0.64 | 0.963 | 1.022 | 1.084 | 1.503 | 1.653 | 1.796 |
| 20 | 0.368 | 0.432 | 0.501 | 0.505 | 0.566 | 0.631 | 0.945 | 1.009 | 1.074 | 1.482 | 1.639 | 1.791 |
| 21 | 0.354 | 0.421 | 0.496 | 0.489 | 0.555 | 0.626 | 0.928 | 0.995 | 1.064 | 1.464 | 1.626 | 1.786 |
| 22 | 0.339 | 0.411 | 0.492 | 0.472 | 0.543 | 0.619 | 0.911 | 0.982 | 1.054 | 1.442 | 1.613 | 1.781 |
| 23 | 0.325 | 0.401 | 0.486 | 0.456 | 0.532 | 0.613 | 0.892 | 0.969 | 1.044 | 1.419 | 1.6 | 1.777 |
| 24 | 0.312 | 0.391 | 0.482 | 0.44 | 0.52 | 0.606 | 0.874 | 0.955 | 1.035 | 1.398 | 1.588 | 1.774 |
| 25 | 0.296 | 0.381 | 0.478 | 0.425 | 0.509 | 0.6 | 0.858 | 0.943 | 1.027 | 1.379 | 1.575 | 1.77 |
| 26 | 0.281 | 0.371 | 0.475 | 0.408 | 0.499 | 0.594 | 0.841 | 0.93 | 1.02 | 1.36 | 1.563 | 1.766 |
| 27 | 0.267 | 0.362 | 0.47 | 0.393 | 0.488 | 0.589 | 0.823 | 0.917 | 1.012 | 1.34 | 1.551 | 1.765 |
| 28 | 0.253 | 0.353 | 0.466 | 0.378 | 0.477 | 0.583 | 0.808 | 0.905 | 1.003 | 1.319 | 1.539 | 1.764 |
| 29 | 0.241 | 0.343 | 0.462 | 0.362 | 0.467 | 0.578 | 0.79 | 0.893 | 0.995 | 1.301 | 1.528 | 1.763 |
| 30 | 0.229 | 0.335 | 0.457 | 0.347 | 0.457 | 0.573 | 0.772 | 0.88 | 0.987 | 1.281 | 1.516 | 1.761 |
| 31 | 0.216 | 0.326 | 0.452 | 0.332 | 0.447 | 0.568 | 0.755 | 0.868 | 0.98 | 1.261 | 1.505 | 1.76 |
| 32 | 0.206 | 0.317 | 0.449 | 0.318 | 0.437 | 0.563 | 0.739 | 0.857 | 0.972 | 1.238 | 1.494 | 1.759 |
| 33 | 0.193 | 0.309 | 0.446 | 0.305 | 0.427 | 0.559 | 0.724 | 0.845 | 0.964 | 1.218 | 1.483 | 1.757 |
| 34 | 0.182 | 0.301 | 0.443 | 0.29 | 0.418 | 0.553 | 0.709 | 0.833 | 0.956 | 1.199 | 1.473 | 1.756 |
| 35 | 0.171 | 0.293 | 0.439 | 0.277 | 0.408 | 0.549 | 0.694 | 0.822 | 0.949 | 1.181 | 1.462 | 1.757 |
| 36 | 0.161 | 0.285 | 0.435 | 0.264 | 0.399 | 0.545 | 0.68 | 0.811 | 0.941 | 1.163 | 1.452 | 1.756 |
| 37 | 0.151 | 0.277 | 0.431 | 0.251 | 0.39 | 0.539 | 0.666 | 0.799 | 0.933 | 1.147 | 1.442 | 1.756 |
| 38 | 0.141 | 0.269 | 0.427 | 0.238 | 0.381 | 0.535 | 0.651 | 0.788 | 0.926 | 1.131 | 1.432 | 1.756 |
| 39 | 0.131 | 0.262 | 0.422 | 0.226 | 0.372 | 0.531 | 0.636 | 0.777 | 0.919 | 1.116 | 1.422 | 1.757 |
| 40 | 0.123 | 0.255 | 0.419 | 0.214 | 0.363 | 0.527 | 0.621 | 0.767 | 0.912 | 1.098 | 1.413 | 1.762 |
| 41 | 0.115 | 0.248 | 0.416 | 0.203 | 0.355 | 0.522 | 0.606 | 0.756 | 0.906 | 1.081 | 1.403 | 1.764 |
| 42 | 0.106 | 0.241 | 0.413 | 0.192 | 0.346 | 0.518 | 0.592 | 0.745 | 0.899 | 1.064 | 1.394 | 1.77 |
| 43 | 0.098 | 0.234 | 0.41 | 0.181 | 0.338 | 0.514 | 0.578 | 0.735 | 0.891 | 1.048 | 1.385 | 1.771 |
| 44 | 0.09 | 0.227 | 0.406 | 0.171 | 0.33 | 0.51 | 0.565 | 0.725 | 0.884 | 1.031 | 1.376 | 1.77 |
| 45 | 0.083 | 0.221 | 0.402 | 0.161 | 0.322 | 0.506 | 0.552 | 0.714 | 0.877 | 1.016 | 1.368 | 1.768 |
| 46 | 0.076 | 0.215 | 0.4 | 0.152 | 0.314 | 0.502 | 0.54 | 0.704 | 0.87 | 1.001 | 1.359 | 1.767 |
| 47 | 0.069 | 0.209 | 0.397 | 0.143 | 0.307 | 0.497 | 0.526 | 0.694 | 0.863 | 0.986 | 1.351 | 1.77 |
| 48 | 0.062 | 0.203 | 0.394 | 0.134 | 0.299 | 0.493 | 0.514 | 0.684 | 0.856 | 0.971 | 1.342 | 1.775 |
| 49 | 0.056 | 0.197 | 0.391 | 0.125 | 0.292 | 0.488 | 0.503 | 0.675 | 0.849 | 0.957 | 1.334 | 1.781 |
| 50 | 0.051 | 0.191 | 0.389 | 0.117 | 0.284 | 0.484 | 0.492 | 0.665 | 0.842 | 0.942 | 1.326 | 1.787 |
| 51 | 0.045 | 0.186 | 0.385 | 0.109 | 0.277 | 0.479 | 0.479 | 0.655 | 0.834 | 0.926 | 1.319 | 1.791 |

*Note: For each percentile, the average value (PXX_median) was calculated, and the P0.025 and P0.975 percentiles of the Bootstrap samples were calculated (designated as PXX_lower and PXX_upper, respectively). The lower and upper limit values correspond to the 95% CI.*

marked telomere shortening, i.e., those below P10, and, in particular, P1. These cut-off points are the most relevant for prognosis and diagnosis in patients with TBD.

Another limitation is the inability to use the gold-standard technique (Flow-FISH) recommended for diagnostic purposes, due to its high cost and technical requirements.[35] However, cross-validation of this curve with samples previously analyzed by Southern blot and MMQPCR in international reference laboratories supports its clinical reliability.

The prospective application of this curve in patients with a confirmed clinical and/or molecular diagnosis of TBD will strengthen evidence of its reliability, evaluate its performance, and inform consideration of whether it should be expanded to include a larger cohort of healthy individuals.

## CONCLUSION

This study enabled the construction of a reference curve for the TL of healthy individuals in the Argentine population, spanning newborns through adults aged 50 years. The generated curve can be applied in other laboratories in the country, provided that the methodological conditions of the present study are maintained, particularly the DNA extraction method and the MMQPCR protocol used.

The results will enable the identification of individuals with significant telomere shortening, contribute to the diagnosis of constitutional bone marrow failure, guide the molecular analysis of genes associated with telomere maintenance, and facilitate genetic counseling in affected families. ■

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