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UNIVERSIDADE ESTADUAL PAULISTA
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Instituto de Biociências Unesp Botucatu

# STUDIES ON THE FUNCTION OF THE *LEISHMANIA MAJOR* TELOMERASE TERT COMPONENT IN TELOMERES MAINTENANCE, CELL PROLIFERATION, AND INFECTIVITY

**MARK EWUSI SHIBURAH**

BOTUCATU, SP

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UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquista Filho"
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

# ESTUDOS SOBRE A FUNÇÃO DO COMPONENTE TERT NA MANUTENÇÃO DOS TELÔMEROS E NA PROLIFERAÇÃO CELULAR DE *L. MAJOR*

**CANDIDATO: MARK EWUSI SHIBURAH**
**ORIENTADORA: PROFA. DRA. MARIA ISABEL NOGUEIRA CANO**

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Palavras-chave: DNA damage; Leishmania; TERT knockout; Cell cycle; Loss of infectivity.

# Minutes from the defense/ Ata da defesa

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Câmpus de Botucatu

**ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE MARK EWUSI SHIBURAH, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (GENÉTICA), DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DE BOTUCATU.**

Aos 18 dias do mês de janeiro do ano de 2024, às 09:00 horas, no(a) Sala de Pós-Graduação do Instituto de Biociências, realizou-se a defesa de TESE DE DOUTORADO de MARK EWUSI SHIBURAH, intitulada **ESTUDOS SOBRE A FUNÇÃO DO COMPONENTE TERT NA MANUTENÇÃO DOS TELÔMEROS E NA PROLIFERAÇÃO CELULAR DE L. MAJOR**. A Comissão Examinadora foi constituida pelos seguintes membros: Profa. Dra. MARIA ISABEL NOGUEIRA CANO (Orientador(a) - Participação Presencial) do(a) Departamento de Ciências Químicas e Biológicas / Instituto de Biociências UNESP Câmpus de Botucatu, Prof. Dr. ROBSON FRANCISCO CARVALHO (Participação Presencial) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu - UNESP, PhD MAGALI CASANOVA (Participação Virtual) do(a) Faculty of Pharmacy and Adhesion and Inflammation Laboratory, PhD MIGUEL ANGEL CHIURILLO SIERVO (Participação por Parecer Circunstanciado) do(a) Department of Biological Sciences / University of Cincinnati, PhD MIGUEL GODINHO FERREIRA (Participação Virtual) do(a) Institute for Research on Cancer and Aging of Nice / Nice Université Côte d'Azur. Após a exposição pelo doutorando e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, o discente recebeu o conceito final: APROVADO . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. MARIA ISABEL NOGUEIRA CANO

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# Dedication

This work is dedicated to the God of my fathers and the memory of my beloved grandmother, Aba Ewusiwaa.

# Acknowledgments

Sincerest gratitude to everyone who has been a part of my journey and the people of Sao Paulo whose taxes support FAPESP to push the frontiers of science!

# Resumo

Mais de 98 países e territórios notificam casos de leishmaniose, uma doença que afeta quase um milhão de pessoas anualmente, e contra a qual não há tratamento e controle eficazes. Nosso objetivo foi estudar a função do componente transcriptase reversa da telomerase (TERT) em *Leishmania major* (LmTERT) e verificar se a LmTERT poderá ser considerada alvo para o desenvolvimento de novos medicamentos para tratar a leishmaniose. O componente TERT contém o núcleo catalítico da telomerase, a enzima responsável pelo alongamento dos telômeros e pela manutenção da estabilidade do genoma. Neste estudo utilizamos um sistema CRISPR-Cas9 já padronizado, para induzir o nocaute total e por substituição do gene TERT e inibição da telomerase em *L. major.* Southern blot usando sondas específicas, PCR e sequenciamento Sanger do tipo "primer-walking" confirmaram que as duas metodologias induziram a deleção eficiente dos dois alelos do gene TERT em *L. major.* O nocaute do gene *TERT* teve efeitos no crescimento do parasito, induzindo parada do ciclo celular e problemas na proliferação. O encurtamento progressivo dos telômeros, a marca registrada da ausência de TERT, foi observado tanto de forma qualitativa por Southern TRF como quantitativa por Flow-FISH. Além disso, as linhagens mutantes apresentavam aumento de danos ao DNA e problemas de replicação. As estruturas subcelulares das células nocaute LmTERT comparadas ao tipo selvagem por microscopia eletrônica e de varredura, evidenciaram modificações incomuns no citoplasma e uma abundância de autofagossomos, sugerindo um mecanismo autofágico pró-sobrevivência. Um mecanismo altruísta utilizado pelos parasitas foi abolido, e a análise proteômica demonstrou a existência de alterações na constituição do lipofosfoglicano (LPG) de superfície com expressões significativamente alteradas de leishmanolisina Gp63. Alterações nas proteínas do domínio META (expressas principalmente nas formas metacíclicas) e um número superior ao normal de parasitos de fase estacionária que aglutinam lectina de amendoim, foram igualmente observadas nas linhagens nocaute. Os resultados cumulativos que sugerem um comprometimento do potencial infeccioso do parasita foram confirmados pelo estudo in vivo de infecção em camundongos BALB/c e infecção in vitro usando macrófagos derivados da medula óssea de camundongos BALB/c. Foi observado desenvolvimento significativo de lesões nos camundongos infectados com parasitos controle contra aqueles infectados com as linhagens nocaute. Um índice de infectividade consistentemente mais alto foi observado para linhagens controle versus nocaute 48 horas pós-inoculação. Testes preliminares usando um inibidor não-nucleosídico da telomerase, o BIBR1532, resultou em telômeros mais curtos e diminuição do crescimento do parasito. Juntos, esses efeitos pleiotrópicos causados pela ausência ou inibição da LmTERT sugerem fortemente que ela apresenta grande potencial para ser explorada como alvo para o desenvolvimento de medicamentos contra a leishmaniose.

**Palavras-chave:** TERT, leishmaniose, encurtamento de telômeros, CRISPR-Cas9, autofagia, parada do ciclo celular, alterações de crescimento e ultraestruturais, proliferação celular comprometida, danos ao DNA, perda de infectividade, inibição da telomerase por BIBR1532

# Abstract

Over 98 countries and territories have reported cases of leishmaniasis, a disease affecting nearly a million individuals annually but with ineffective remedies. Our goal was to study the function of the telomerase reverse transcriptase (TERT) in *Leishmania major* (LmTERT) and leverage this knowledge in developing new drugs. The TERT component contains the catalytic core of telomerase, the enzyme responsible for elongating telomeres and maintaining genome stability. A loss of function study using CRISPR-Cas9 and inhibition of the telomerase in *L. major* was conducted. Probe-specific Southern blot, PCR, and primer walk Sanger sequencing confirmed the efficient deletion of the *TERT* gene in *L. major*. The knockout of the *TERT* gene resulted in parasite growth defects, DNA fragmentation, cell cycle arrest, and problematic replication measured by flow cytometry. Progressive telomere shortening, the hallmark of TERT absence, was observed by Southern TRF and Flow-FISH assessments. We also assessed the subcellular structures of the LmTERT knockout cells against a wild type using scanning and electron microscopy and found unusual modifications in the cytoplasm, and an abundance of autophagosomes, suggesting a pro-survival autophagic mechanism. Changes in the metacyclic domain proteins were seen equally in the knockout lines. An altruistic mechanism used by the parasites was abolished. The cumulative results suggesting a compromise on parasite infective potential was confirmed by *in vivo* BALB/c mice infection study and *in vitro* bone-marrow derived macrophage infection. Significant lesion development was observed in the mice infected with control parasites against those infected with the knockout lineage. A consistently higher infectivity index was observed for control versus knockout lineages at 48 h post-inoculation. Consistent with the growth challenges and telomere shortening, preliminary tests of the inhibition of the telomerase using BIBR1532, a non-nucleoside small molecule, resulted in shorter telomeres and poor parasite growth. These results together suggest the usefulness of LmTERT to the parasite, putting the protein in a space to be explored for drug development against leishmaniasis.

**Keywords:** TERT, Leishmaniasis, telomere shortening, CRISPR-Cas9, Autophagy, Cell cycle arrest, growth, and ultrastructural changes, compromised cell proliferation, DNA damage, loss of infectivity, BIBR1532 inhibition.

**THESIS ARRANGEMENT:**

This thesis begins with a general introduction, followed by a section on the objectives, followed by a section entitled Introduction to the Chapters where the materials, methods, and results for the project are presented as two separate Chapters. Each chapter is presented as an article and begins with an introduction followed by a conclusion and references. A brief general conclusion section ends the chapters followed by a reference section referring specifically to citations from the general introduction.

To aid clarity, the lists of figures and tables are divided into various sections: e.g., a list of figures for the general introduction, a list of figures for Chapter 1, and a list of figures for Chapter 2.

# Table of Contents


Catalog page/ Ficha catalográfica.....i

Minutes from the defense/ Ata da defesa.....i

Dedication..... ii

Acknowledgments ..... iii

Resumo .....iv

Abstract.....v

Table of Contents.....vi

List of Figures..... viii

List of tables.....x

List of Abbreviations .....xi

1. Introduction .....1

1.1 Leishmaniasis, a Neglected Tropical Disease and a quest for new remedies .....4

1.2 Biology of *Leishmania* parasites .....5

1.3 Telomeres, End replication problem and Hayflick limit .....9

1.4 The telomerase enzyme .....16

1.5 Alternative mechanisms in telomere maintenance: ALT pathways .....19

1.6 Justification: Telomerase as a drug target in *Leishmania* .....20

2. Objectives .....22

2.1 Main objective .....22

2.2 Specific Objectives .....22

3. Materials, Methods, Results and Discussion: Introduction to the chapters .....23

3.1 Chapter 1 .....24

3.2 Chapter 2 .....93

4. General conclusion and perspectives .....107

5. References .....108

6. Appendix .....116

# List of Figures in Introduction

Figure 1. Distribution of leishmaniasis co-infection with HIV across the globe.. .....2

Figure 2. Visceral leishmaniasis distribution. .....3

Figure 3. Cutaneous leishmaniasis global assessment.....4

Figure 4. Life cycle of the *Leishmania* parasite. .....7

Figure 5. A cartoon of the end replication problem. .....11

Figure 6. Schematic representation of the 3'-G overhang structural conformations. .....12

Figure 7. The association of telomeres with the shelterin complex. .....14

Figure 8. *L. major* and *L. amazonensis* chromosome termini. .....15

Figure 9. Graphic representation of TERT domains and motifs. .....18

Figure 10. Secondary Structure of Telomerase RNA (TER) in different organisms.. .....18

# List of figures in Chapter 1 (Page 24 - 92)

Fig 1. Defective growth, DNA synthesis, and accumulation of DNA damage in TERT-depleted parasites. .....33

Fig 2. Absence of cell death and increased PNA-negative parasites among the TERT-depleted *L. major* promastigotes .....36

Fig 3. TERT-depleted parasites show a senescent-like phenotype. .....39

Fig 4. TERT depletion induces telomere attrition in *L. major* promastigotes. The deletion of LmTERT leads to a significant reduction in telomeres.....41

Fig 5. TERT-depleted parasites showed reduced or no capacity to infect BALB/c mouse models and bone marrow macrophages .....47

S1 Fig. Expression of pTB007 in *L. major* (Lm007) does not affect the growth and cell cycle of the parasite. .....83

S2 Fig. Strategies for the deletion of LmTERT and the methods of confirmation.. .....84

S3 Fig. Representative gating paths used in analyzing flow cytometry data.. .....85

S4 Fig. Fluorescent antibody test of *Leishmania major* infection in experimentally infected mice. .....86

S5 Fig. SCG gene amplifications. .....87

S6 Fig. Plasmid engineering for LmTERT reintroduction in knockout lineages (complementation). .....88

S7 Fig. Differential protein expression.....89

## List of figures in Chapter 2 (Page 92 – 106)

FIG 1. Growth comparisons of BIBR1532 treated and non-treated parasites. (A) Growth comparisons between untreated parasites and parasites treated with three different concentrations of BIBR1532. (B), (C), (D) comparative proliferation in percentages of parasites at 24, 48, and 72 h respectively. .....97

FIG 2. Telomere length attrition due to BIBR1532 treatment. (A) Southern blot TRF assessment of the sizes of telomeres in the parasites 72 h after treatment of BIBR1532. (B) WALTER intensity profiles of the TRF sizes obtained from the southern blot. .....99

# List of tables

## Tables in Chapter 1 (Pages 24-92)

Table 1 Comparative changes in telomere size .....42

S1 Table. List of oligonucleotides used in this study .....91

# List of Abbreviations

NTDs- Neglected Tropical Diseases

*L. major- Leishmania major*

LmTERT- *Leishmania major* TERT

ORF- Open Reading Frame

TERT- Telomerase reverse transcriptase

TEN- telomerase essential N-terminal

TRBD- telomerase RNA binding domain

RT- reverse transcriptase

CTE- C-terminal extension

TER- Telomerase RNA

TBE- template boundary element

STE- stem terminus element

CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats

Cas9- CRISPR-Associated protein 9

T7RNAP- T7 RNA Polymerase

pTB007- Expression plasmid carrying Cas9, T7RNAP and Hygromycin genes used in transfection

Lm007- *Leishmania major* parasite carrying plasmid pTB007

sgRNA- single guide RNA

tracrRNA- trans-activating CRISPR RNA

DT- donor template

EdU- 5-ethynyl-2'-deoxyuridine

LPG- Lipophosphoglycan

PBS- phosphate-buffered saline

BIBR1532- 2-[[(E)-3-naphthalen-2-ylbut-2-enoyl]amino]benzoic acid

SCG- scβ-Galactosyltransferases

# 1. Introduction

The search for an alternative therapeutic strategy in treating leishmaniasis caused by protozoan parasites from the *Leishmania* genus is a matter of global public health interest [1]. *Leishmania* parasites have a digenetic lifecycle that involves living inside a mammalian host and a phlebotomine insect vector. This lifecycle imposes on the parasite a need to develop strategies to survive two different extreme environments using two major developmental phases: intracellular amastigotes and extracellular promastigotes, with observable differences but shared basic molecular and cellular characteristics [1–4].

Over 20 species of *Leishmania* can cause the disease and may manifest in different clinical forms depending on the parasite species and host immune system. The three main clinical forms of leishmaniasis are Cutaneous, Mucocutaneous, and Visceral [5,6]. Most of the cutaneous forms are self-healed in contrast to the dilacerating and mutilating mucocutaneous and the lethal visceral forms [6–9].

Over a million new leishmaniasis cases are reported annually [10]. According to the World Health Organization, 94% of leishmaniasis cases reported in 2017 were recorded in 7 countries, including Brazil (www.who.int/leishmaniasis/burden/en). In the last two decades, a great number of HIV/leishmaniasis coinfections have also been reported (Figure 1) [11,12]. Due to the challenges associated with existing treatment and control methods against the disease, an alternative therapeutic target has become necessary [13].

The WHO outlines a road map for tackling leishmaniasis by looking at the data from different perspectives, including mortalities from vector-disease transmission from 2021 to 2030. Efforts are still being made to combat the spread of leishmaniasis through several health interventions,

including the Elimination Programme of Kala-azar in Southeast Asia (https://www.who.int/publications-detail-redirect/who-wer9635-401-419). The 200 territories and countries that reported cases of leishmaniasis to the WHO show nearly 50% of these regions still have endemic cases of leishmaniasis (Figure 2 and Figure 3) [11].

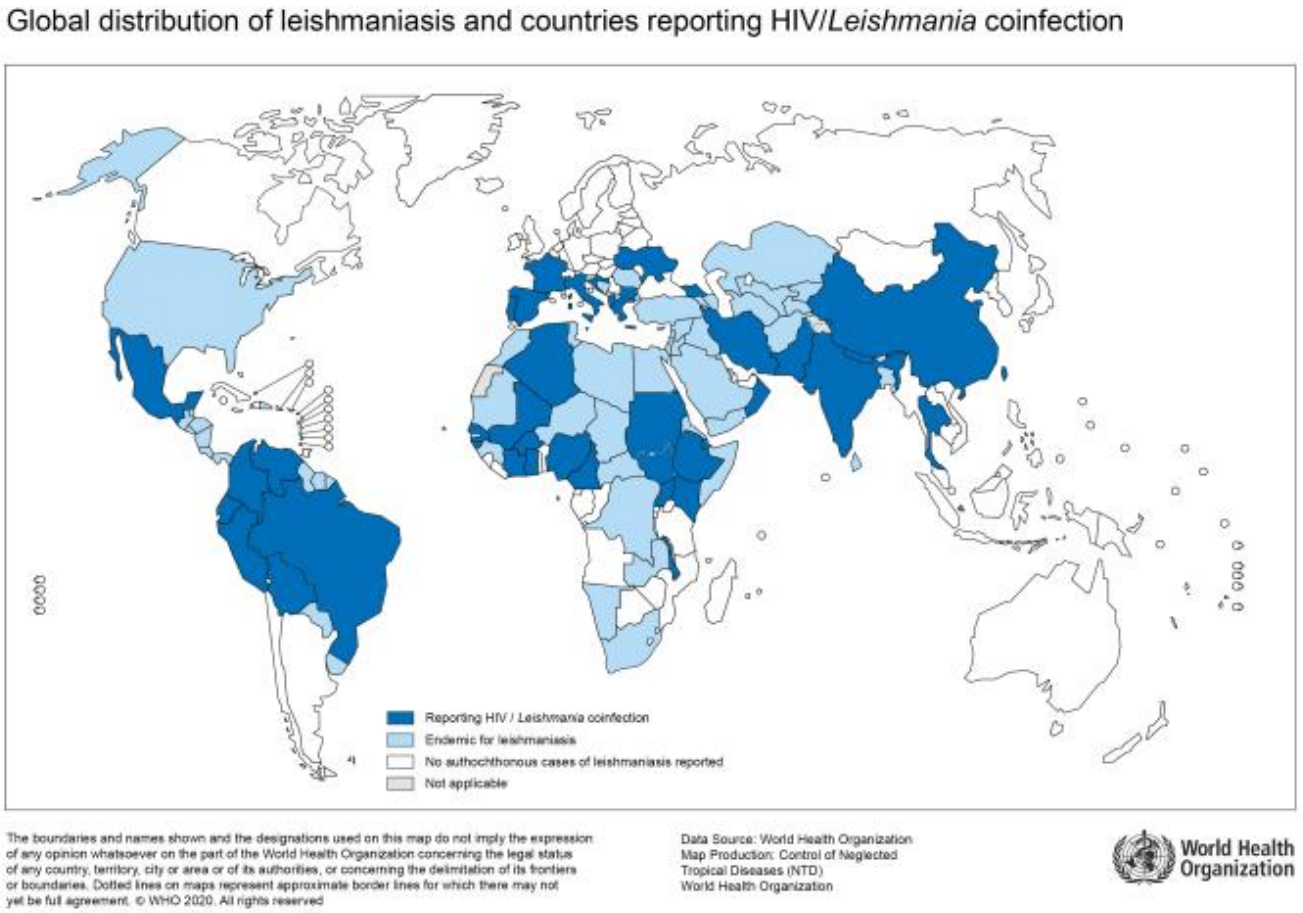


**Figure 1. Distribution of leishmaniasis co-infection with HIV across the globe.** The 2020 report on the global distribution of countries reporting cases of leishmaniasis co-infection with HIV. WHO classification of countries within the boundaries of Middle East and the Americas seems to have an increased incidence of co-infection cases. Source of image: WHO website.

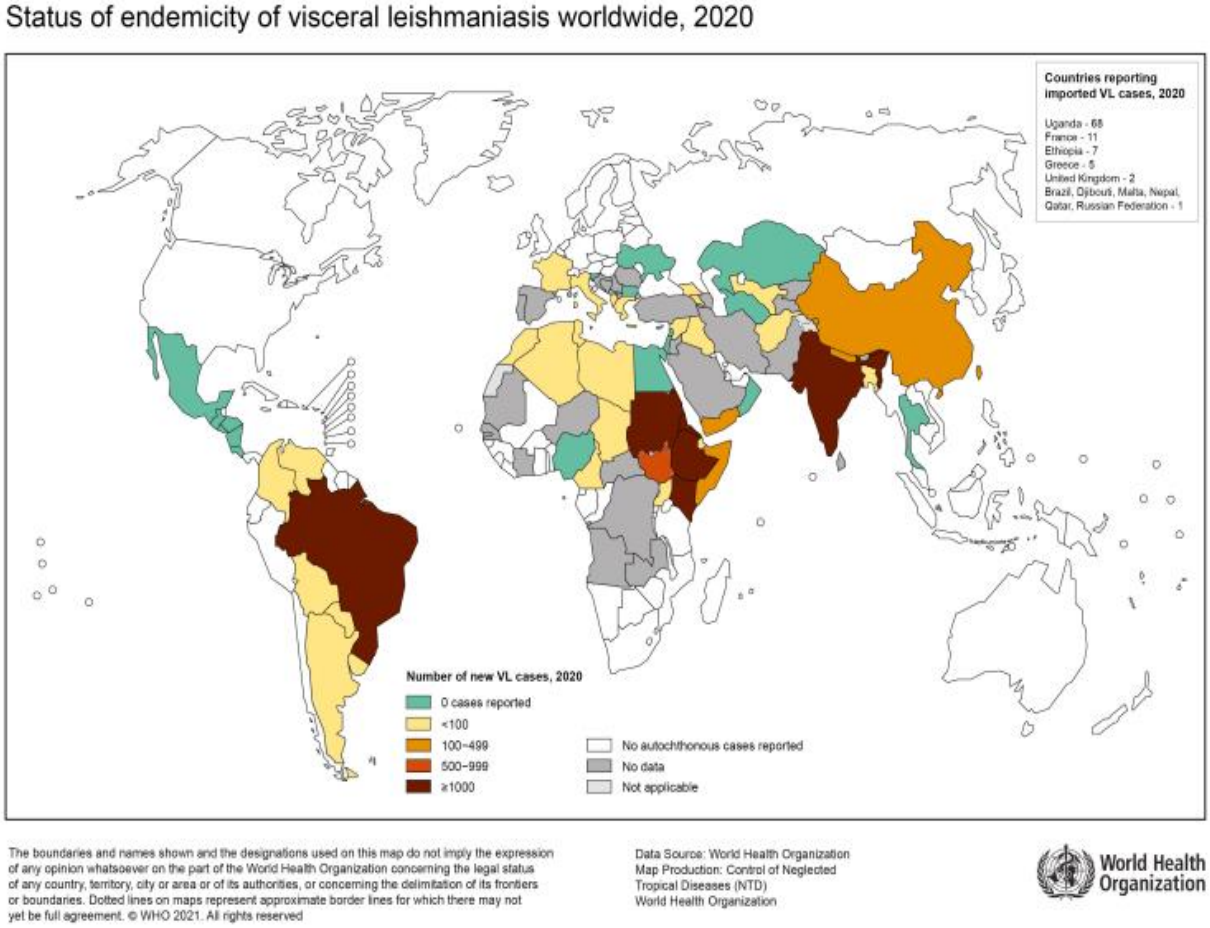


**Figure 2. Visceral leishmaniasis distribution.** The burden of visceral leishmaniasis across the globe. Some regions in Africa including Ghana, report no cases of Visceral leishmaniasis. Source: World Health Organization.

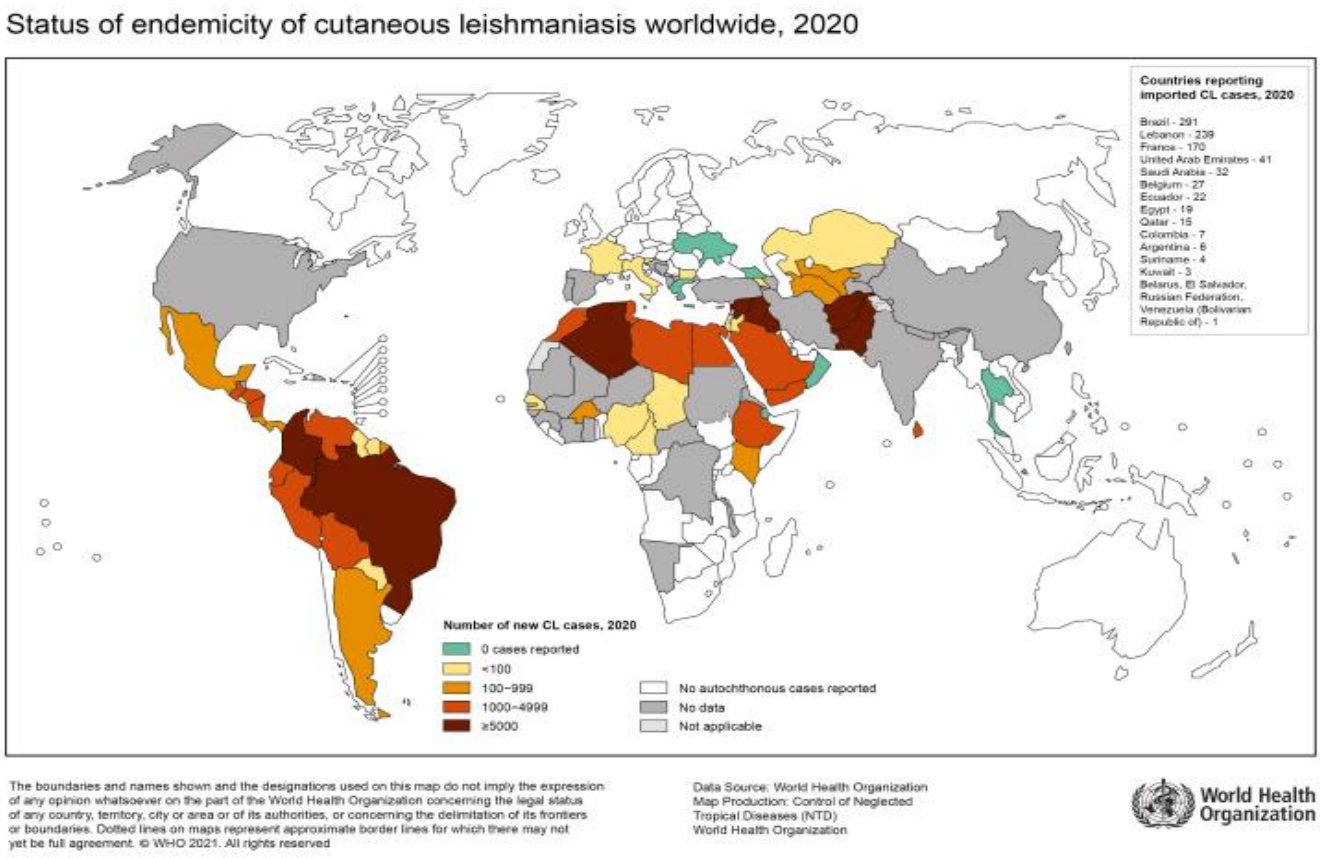


**Figure 3. Cutaneous leishmaniasis global assessment.** The global burden of cutaneous leishmaniasis in different countries described according to their impact in the various regions. Source: World Health Organization.

## 1.1 Leishmaniasis, a Neglected Tropical Disease and a quest for new remedies

The WHO classifies 20 conditions mainly identified in tropical regions under the umbrella name 'Neglected Tropical Diseases' (NTDs). Leishmaniasis is one such condition. A disease under the NTD classification like leishmaniasis affects largely impoverished communities where women and children are unduly affected (https://www.who.int/health-topics/neglected-tropical-diseases#tab=tab_1).

Even though there has been some progress in getting countries to fight these diseases, there is still little attention being paid to the development of new and effective remedies. Also, there is little

# References


[1] Greider CW, Blackburn EH. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 1989;337:331–7. https://doi.org/10.1038/337331a0.

[2] Cano MIN, Dungan JM, Agabian N, Blackburn EH. Telomerase in kinetoplastid parasitic protozoa. Proc Natl Acad Sci U S A 1999;96:3616–21. https://doi.org/10.1073/pnas.96.7.3616.

[3] Ségal-Bendirdjian E, Geli V. Non-canonical Roles of Telomerase: Unraveling the Imbroglio. Front Cell Dev Biol 2019;7:1–12. https://doi.org/10.3389/fcell.2019.00332.

[4] Ale-Agha N, Dyballa-Rukes N, Jakob S, Altschmied J, Haendeler J. Cellular functions of the dual-targeted catalytic subunit of telomerase, telomerase reverse transcriptase - Potential role in senescence and aging. Exp Gerontol 2014;56:189–93. https://doi.org/10.1016/j.exger.2014.02.011.

[5] Mozdy AD, Cech TR. Low abundance of telomerase in yeast: Implications for telomerase haploinsufficiency. Rna 2006;12:1721–37. https://doi.org/10.1261/rna.134706.

[6] Giardini MA, Segatto M, Da Silva MS, Nunes VS, Cano MIN. Telomere and telomerase biology. vol. 125. 2014. https://doi.org/10.1016/B978-0-12-397898-1.00001-3.

[7] Zhang A, Zheng C, Hou M, Lindvall C, Li KJ, Erlandsson F, et al. Deletion of the telomerase reverse transcriptase gene and haploinsufficiency of telomere maintenance in cri du chat syndrome. Am J Hum Genet 2003;72:940–8. https://doi.org/10.1086/374565.

[8] Shiburah ME, Cristina Dias de Oliveira B, Bisetegn H, Silva A, Henrique de Castro Assis L, Menna Barreto R, et al. Ablation of telomerase reverse transcriptase in *Leishmania major* results in a senescent-like 2 phenotype and loss of infectivity 3 n.d. https://doi.org/10.1101/2023.11.10.566596.

[9] Haendeler J, Hoffmann J örg, Rahman S, Zeiher AM, Dimmeler S. Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation. FEBS Lett 2003;536:180–6. https://doi.org/10.1016/S0014-5793(03)00058-9.

[10] Xi L, Schmidt JC, Zaug AJ, Ascarrunz DR, Cech TR. A novel two-step genome editing strategy with CRISPR-Cas9 provides new insights into telomerase action and TERT gene expression. Genome Biol 2015;16:1–17. https://doi.org/10.1186/s13059-015-0791-1.

[11] Zhu J, Liu W, Chen C, Zhang H, Yue D, Li C, et al. TPP1 OB - fold domain protein suppresses cell proliferation and induces cell apoptosis by inhibiting telomerase recruitment to telomeres in human lung cancer cells. J Cancer Res Clin Oncol 2019;145:1509–19. https://doi.org/10.1007/s00432-019-02921-3.

[12] Lee J, Sung YH, Cheong C, Choi YS, Jeon HK, Sun W, et al. TERT promotes cellular and organismal survival independently of telomerase activity. Oncogene 2008;27:3754–60. https://doi.org/10.1038/sj.onc.1211037.

[13] Zhou J, Ding D, Wang M, Cong YS. Telomerase reverse transcriptase in the regulation of gene expression. BMB Rep 2014;47:8–14. https://doi.org/10.5483/BMBRep.2014.47.1.284.

[14] Lai AG, Pouchkina-Stantcheva N, Di Donfrancesco A, Kildisiute G, Sahu S, Aziz Aboobaker A. The protein subunit of telomerase displays patterns of dynamic evolution and conservation across different metazoan taxa. BMC Evol Biol 2017;17. https://doi.org/10.1186/s12862-017-0949-4.

[15] Zvereva MI, Shcherbakova DM, Dontsova OA. Telomerase: Structure, functions, and activity regulation. Biochemistry (Moscow) 2010;75:1563–83. https://doi.org/10.1134/S0006297910130055.

[16] Nguyen THD, Tam J, Wu RA, Greber BJ, Toso D, Nogales E, et al. Cryo-EM structure of substrate-bound human telomerase holoenzyme. Nature 2018;557:190–5. https://doi.org/10.1038/s41586-018-0062-x.

[17] Wang Y, Feigon J. Structural biology of telomerase and its interaction at telomeres. Curr Opin Struct Biol 2017;47:77–87. https://doi.org/10.1016/j.sbi.2017.06.010.

[18] Davis JA, Chakrabarti K. Telomerase ribonucleoprotein and genome integrity—An emerging connection in protozoan parasites. WIREs RNA 2021. https://doi.org/10.1002/wrna.1710.

[19] Elton JRV, Vinícius SN, Marcelo SDS, Marcela S, Peter JM, Maria INC. The putative *Leishmania* telomerase rna (LeishTer) undergoes trans-splicing and contains a conserved template sequence. PLoS One 2014;9:17–20. https://doi.org/10.1371/journal.pone.0112061.

[20] Bryan C, Rice C, Hoffman H, Harkisheimer M, Sweeney M, Skordalakes E. Structural Basis of Telomerase Inhibition by the Highly Specific BIBR1532. Structure 2015;23:1934–42. https://doi.org/10.1016/j.str.2015.08.006.

[21] Berardinelli F, Coluzzi E, Sgura A, Antoccia A. Targeting telomerase and telomeres to enhance ionizing radiation effects in in vitro and in vivo cancer models. Mutat Res Rev Mutat Res 2017;773:204–19. https://doi.org/10.1016/j.mrrev.2017.02.004.

[22] Herbert B, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW, et al. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. 1999.

[23] Ding X, Cheng J, Pang Q, Wei X, Zhang X, Wang P, et al. BIBR1532, a Selective Telomerase Inhibitor, Enhances Radiosensitivity of Non-Small Cell Lung Cancer Through Increasing Telomere Dysfunction and ATM/CHK1 Inhibition. Int J Radiat Oncol Biol Phys, vol. 105, Elsevier Inc.; 2019, p. 861–74. https://doi.org/10.1016/j.ijrobp.2019.08.009.

[24] Amin A, Morello M, Petrara MR, Rizzo B, Argenton F, De Rossi A, et al. Short-Term TERT Inhibition Impairs Cellular Proliferation via a Telomere Length-Independent Mechanism and Can Be Exploited as a Potential Anticancer Approach. Cancers (Basel) 2023;15. https://doi.org/10.3390/cancers15102673.

[25] Pascolo E, Wenz C, Lingner J, Hauel N, Priepke H, Kauffmann I, et al. Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. Journal of Biological Chemistry 2002;277:15566–72. https://doi.org/10.1074/jbc.M201266200.

[26] Zhang Y, Yang X, Zhou H, Yao G, Zhou L, Qian C. BIBR1532 inhibits proliferation and enhances apoptosis in multiple myeloma cells by reducing telomerase activity. PeerJ 2023;11:e16404. https://doi.org/10.7717/peerj.16404.

[27] Turkmen E, Sogutlu F, Erdogan M, Biray Avci C. Evaluation of the anticancer effect of telomerase inhibitor BIBR1532 in anaplastic thyroid cancer in terms of apoptosis, migration and cell cycle. Medical Oncology 2023;40. https://doi.org/10.1007/s12032-023-02063-0.

[28] Altamura G, degli Uberti B, Galiero G, De Luca G, Power K, Licenziato L, et al. The Small Molecule BIBR1532 Exerts Potential Anti-cancer Activities in Preclinical Models of Feline Oral Squamous Cell

Carcinoma Through Inhibition of Telomerase Activity and Down-Regulation of TERT. Front Vet Sci 2021;7. https://doi.org/10.3389/fvets.2020.620776.

[29] Ding X, Cheng J, Pang Q, Wei X, Zhang X, Wang P, et al. BIBR1532, a Selective Telomerase Inhibitor, Enhances Radiosensitivity of Non-Small Cell Lung Cancer Through Increasing Telomere Dysfunction and ATM/CHK1 Inhibition. Int J Radiat Oncol Biol Phys, vol. 105, Elsevier Inc.; 2019, p. 861–74. https://doi.org/10.1016/j.ijrobp.2019.08.009.

[30] Pandya VA, Crerar H, Mitchell JS, Patani R. A non-toxic concentration of telomerase inhibitor BIBR1532 fails to reduce tert expression in a feeder-free induced pluripotent stem cell model of human motor neurogenesis. Int J Mol Sci 2021;22. https://doi.org/10.3390/ijms22063256.

# 4. General conclusion and perspectives

Our data on the study of the TERT as a potential drug target against leishmaniasis has provided useful insights on the possible effects on the biology of the parasite. Particularly, the druggability of the target was successfully confirmed by the use of BIBR1532. However, there are still lingering questions on the mechanism of action and whether the BIBR1532 acts in similar ways as in other tested models. Additionally, the proposed model of action after TERT ablation will need to be experimentally proven to aid in therapeutic modulation of the target. Future studies may look at the processivity of the telomerase during the inhibition at different concentrations. Toxicity levels of the various concentrations can also be tested to avert potential threats to normal cells with telomerase activity.

Lastly, studying further the infectivity *in vitro* and *in vivo* will be an additional benefit to developing potential therapeutics.

# 5. References

References in this section refer to the citations made before the beginning of the objectives section.

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[1] Wheeler RJ, Gluenz E, Gull K. The cell cycle of Leishmania: Morphogenetic events and their implications for parasite biology. Mol Microbiol 2011;79:647–62. https://doi.org/10.1111/j.1365-2958.2010.07479.x.

[2] Da Silva R, Sacks DL. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. Infect Immun 1987;55:2802–6. https://doi.org/10.1128/iai.55.11.2802-2806.1987.

[3] Calderano SG, Moretti NS, Araujo C, Silva MS, Cristina T, Jesus L de, et al. CHAPTER 1 The Cellular Organization of Trypanosomatids During Life Cycle. Frontiers in Parasitology 2016;1:3–60.

[4] Sunter J, Gull K. Shape, form, function and *Leishmania* pathogenicity: from textbook descriptions to biological understanding. Open Biol 2017;7. https://doi.org/10.1098/rsob.170165.

[5] Ramos CS, Franco FAL, Smith DF, Uliana SRB. Characterisation of a new *Leishmania* META gene and genomic analysis of the META cluster. FEMS Microbiol Lett 2004;238:213–9. https://doi.org/10.1016/j.femsle.2004.07.037.

[6] Kweku MA, Odoom S, Puplampu N, Desewu K, Nuako GK, Gyan B, et al. An outbreak of suspected cutaneous leishmaniasis in Ghana: lessons learnt and preparation for future outbreaks. Glob Health Action 2011;4. https://doi.org/10.3402/gha.v4i0.5527.

[7] Faulde M, Schrader J, Heyl G, Amirih M, Hoerauf A. Zoonotic cutaneous leishmaniasis outbreak in Mazar-e Sharif, northern Afghanistan: An epidemiological evaluation. International Journal of Medical Microbiology 2008;298:543–50. https://doi.org/10.1016/j.ijmm.2007.07.015.

[8] Kwakye-Nuako G, Mosore MT, Duplessis C, Bates MD, Puplampu N, Mensah-Attipoe I, et al. First isolation of a new species of *Leishmania* responsible for human cutaneous leishmaniasis in Ghana and classification in the *Leishmania* enriettii complex. Int J Parasitol 2015;45:679–84. https://doi.org/10.1016/j.ijpara.2015.05.001.

[9] Kanyina EW. Characterization of visceral leishmaniasis outbreak, Marsabit County, Kenya, 2014. BMC Public Health 2020;20. https://doi.org/10.1186/s12889-020-08532-9.

[10] Handman E, Kedzierski L, Uboldi AD, Goding JW. Fishing for anti-*Leishmania* drugs: Principles and problems. Adv Exp Med Biol 2008;625:48–60. https://doi.org/10.1007/978-0-387-77570-8_5.

[11] Weekly epidemiological record Relevé épidémiologique hebdomadaire. n.d.

[12] Lindoso JAL, Lindoso AABP. Neglected tropical diseases in Brazil. Rev Inst Med Trop Sao Paulo 2009;51:247–53. https://doi.org/10.1590/S0036-46652009000500003.

[13] Cano MIN. Telomere biology of Trypanosomatids: More questions than answers. Trends Parasitol 2001;17:425–9. https://doi.org/10.1016/S1471-4922(01)02014-1.

[14] Geneva: World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030. 2020.

[15] Kamhawi S. Phlebotomine sand flies and *Leishmania* parasites: friends or foes? Trends Parasitol 2006;22:439–45. https://doi.org/10.1016/j.pt.2006.06.012.

[16] Reis-cunha JL, Valdivia HO, Castanheira D. CHAPTER 2 Trypanosomatid Genome Organization and Ploidy. Frontiers in Parasitology 2016;1:61–103.

[17] Wheeler RJ. The trypanolytic factor-mechanism, impacts and applications. Trends Parasitol 2010;26:457–64. https://doi.org/10.1016/j.pt.2010.05.005.

[18] Calado R, Young N. Telomeres in disease. F1000 Med Rep 2012;4. https://doi.org/10.3410/M4-8.

[19] Srinivas N, Rachakonda S, Kumar R. Telomeres and telomere length: A general overview. Cancers (Basel) 2020;12. https://doi.org/10.3390/cancers12030558.

[20] blackburn_and_gall_1978 n.d.

[21] Moyzis RK, Buckingham JM, Crams LS, Dani M, Deavent LL, Jones MD, et al. A highly conserved repetitive DNA sequence, (TTAGGG)., present at the telomeres of human chromosomes (human repetitive DNA/in situ hybridization/trypanosome telomeres/BAL-31 nuclease/flow cytometry). vol. 85. 1988.

[22] Blackburn EH, Challoner PB. Identification of a Telomeric DNA Sequence in Trypanosoma brucei. vol. 36. 1984.

[23] Cano MIN, Dungan JM, Agabian N, Blackburn EH. Telomerase in kinetoplastid parasitic protozoa. Proc Natl Acad Sci U S A 1999;96:3616–21. https://doi.org/10.1073/pnas.96.7.3616.

[24] Ohki R, Tsurimoto T, Ishikawa F. In Vitro Reconstitution of the End Replication Problem. Mol Cell Biol 2001;21:5753–66. https://doi.org/10.1128/mcb.21.17.5753-5766.2001.

[25] Barbé-Tuana F, Kich Grun L, Pierdoná V, Dias De Oliveira BC, Paiva SC, Shiburah ME, et al. Human genome structure, function and clinical considerations. 2021. https://doi.org/https://doi.org/10.1007/978-3-030-73151-9_7#DOI.

[26] Dreesen O, Li B, Cross GAM. Telomere structure and shortening in telomerase-deficient Trypanosoma brucei. Nucleic Acids Res 2005;33:4536–43. https://doi.org/10.1093/nar/gki769.

[27] Bryan TM. G-quadruplexes at telomeres: Friend or foe? Molecules 2020;25. https://doi.org/10.3390/molecules25163686.

[28] Martínez P, Blasco MA. Cardiovascular Aging Compendium. Circ Res 2018;123:787–802. https://doi.org/10.1161/CIRCRESAHA.118.312202.

[29] Lange T De. How Shelterin Solves the Telomere End-Protection Problem. Cold Spring Harb Symp Quant Biol 2011;75:167–77. https://doi.org/10.1101/sqb.2010.75.017.

[30] Diotti R, Loayza D. Shelterin complex and associated factors at human telomeres. Nucleus 2011;2:2:119–35. https://doi.org/10.4161/nucl.2.2.15135.

[31] Lange T De. Shelterin : the protein complex that shapes and safeguards human telomeres. Genes Dev 2005;19:2100–10. https://doi.org/10.1101/gad.1346005.quence.

[32] Schmutz I, Lange T De. Quick guide Shelterin. Current Biology 2016;26:R387–407. https://doi.org/10.1016/j.cub.2016.01.056.

[33] Jones M, Bisht K, Savage SA, Nandakumar J, Keegan CE, Maillard I. The shelterin complex and hematopoiesis. J Clin Invest 2016;126:1621–9. https://doi.org/10.1172/JCI84547.unit.

[34] Janovič T, Stojaspal M, Veverka P, Horáková D, Hofr C. Human Telomere Repeat Binding Factor TRF1 Replaces TRF2 Bound to Shelterin Core Hub TIN2 when TPP1 Is Absent. Journal of Environmental Issues and Agriculture in Developing Countries 2019;431:3289–301. https://doi.org/10.1016/j.jmb.2019.05.038.

[35] Lan J, Zhu Y, Xu L, Yu H, Yu J, Liu X, et al. The 68-kDa Telomeric Repeat Binding Factor 1 ( TRF1 ) - associated Protein ( TAP68 ) Interacts with and Recruits TRF1 to the Spindle Pole during Mitosis. Journal of Biological Chemistry 2014;289:14145–56. https://doi.org/10.1074/jbc.M113.526244.

[36] Chen Y. The structural biology of the shelterin complex. Biol Chem 2019;400:457–66. https://doi.org/10.1515/hsz-2018-0368.

[37] Conte FF, Cano MIN. Genomic organization of telomeric and subtelomeric sequences of *Leishmania* (Leishmania) amazonensis. Int J Parasitol 2005;35:1435–43. https://doi.org/10.1016/j.ijpara.2005.05.011.

[38] T Van der Ploeg LH, C Liu AY, Borst P. Structure of the Growing Telomeres of Trypanosomes. vol. 36. 1964.

[39] Dossin F de M, Dufour A, Dusch E, Siqueira-Neto JL, Moraes CB, Yang GS, et al. Automated nuclear analysis of *Leishmania major* telomeric clusters reveals changes in their organization during the parasite's life cycle. PLoS One 2008;3. https://doi.org/10.1371/journal.pone.0002313.

[40] Fu G, Barker DC. Characterisation of *Leishmania* telomeres reveals unusual telomeric repeats and conserved telomere-associated sequence. vol. 26. 1998.

[41] Zvereva MI, Shcherbakova DM, Dontsova OA. Telomerase: Structure, functions, and activity regulation. Biochemistry (Moscow) 2010;75:1563–83. https://doi.org/10.1134/S0006297910130055.

[42] Collins K, Mitchell JR. Telomerase in the human organism. Oncogene 2002;21:564–79. https://doi.org/10.1038/sj/onc/1205083.

[43] Greider CW, Blackburn EH. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 1989;337:331–7. https://doi.org/10.1038/337331a0.

[44] Gilley D, Lee MS, Blackburn EH. Altering specific telomerase RNA template residues affects active site function. Genes Dev 1995;9:2214–26. https://doi.org/10.1101/gad.9.18.2214.

[45] Davis JA, Chakrabarti K. Telomerase ribonucleoprotein and genome integrity—An emerging connection in protozoan parasites. WIREs RNA 2021. https://doi.org/10.1002/wrna.1710.

[46] Zhang A, Zheng C, Hou M, Lindvall C, Li KJ, Erlandsson F, et al. Deletion of the telomerase reverse transcriptase gene and haploinsufficiency of telomere maintenance in cri du chat syndrome. Am J Hum Genet 2003;72:940–8. https://doi.org/10.1086/374565.

[47] Pestana A, Vinagre J, Sobrinho-Simões M, Soares P. TERT biology and function in cancer: Beyond immortalisation. J Mol Endocrinol 2017;58:R129–46. https://doi.org/10.1530/JME-16-0195.

[48] Giardini MA, Lira CBB, Conte FF, Camillo LR, De Siqueira Neto JL, Ramos CHI, et al. The putative telomerase reverse transcriptase component of *Leishmania* amazonensis: Gene cloning and characterization. Parasitol Res 2006;98:447–54. https://doi.org/10.1007/s00436-005-0036-4.

[49] Giardini MA, Segatto M, Da Silva MS, Nunes VS, Cano MIN. Telomere and telomerase biology. vol. 125. 2014. https://doi.org/10.1016/B978-0-12-397898-1.00001-3.

[50] Giardini MA, Fernández MF, Lira CBB, Cano MIN. *Leishmania* amazonensis: Partial purification and study of the biochemical properties of the telomerase reverse transcriptase activity from promastigote-stage. Exp Parasitol 2011;127:243–8. https://doi.org/10.1016/j.exppara.2010.08.001.

[51] Dey A, Chakrabarti K. Current perspectives of telomerase structure and function in eukaryotes with emerging views on telomerase in human parasites. Int J Mol Sci 2018;19. https://doi.org/10.3390/ijms19020333.

[52] Lai AG, Pouchkina-Stantcheva N, Di Donfrancesco A, Kildisiute G, Sahu S, Aziz Aboobaker A. The protein subunit of telomerase displays patterns of dynamic evolution and conservation across different metazoan taxa. BMC Evol Biol 2017;17. https://doi.org/10.1186/s12862-017-0949-4.

[53] Xia J, Peng Y, Saira Mian I, Lue NF. Identification of Functionally Important Domains in the N-Terminal Region of Telomerase Reverse Transcriptase. vol. 20. 2000.

[54] Bhattacharyya A, Blackburn EH. A functional telomerase RNA swap in vivo reveals the importance of nontemplate RNA domains. vol. 94. 1997.

[55] Theimer CA, Feigon J. Structure and function of telomerase RNA. Curr Opin Struct Biol 2006;16:307–18. https://doi.org/10.1016/j.sbi.2006.05.005.

[56] Elton JRV, Vinícius SN, Marcelo SDS, Marcela S, Peter JM, Maria INC. The putative *Leishmania* telomerase rna (LeishTer) undergoes trans-splicing and contains a conserved template sequence. PLoS One 2014;9:17–20. https://doi.org/10.1371/journal.pone.0112061.

[57] Chen Q, Ijpma A, Greider CW. Two Survivor Pathways That Allow Growth in the Absence of Telomerase Are Generated by Distinct Telomere Recombination Events. Mol Cell Biol 2001;21:1819–27. https://doi.org/10.1128/MCB.21.5.1819-1827.2001.

[58] Dreesen O, Cross GAM. Telomerase-Independent Stabilization of Short Telomeres in Trypanosoma brucei . Mol Cell Biol 2006;26:4911–9. https://doi.org/10.1128/mcb.00212-06.

[59] Bryan TM, Englezou A, Oalla-Pozza2 L, Ounham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. 1997.

[60] Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO Journal 1995;14:4240–8. https://doi.org/10.1002/j.1460-2075.1995.tb00098.x.

[61] Guterres AN, Villanueva J. Targeting telomerase for cancer therapy. Oncogene 2020;39:5811–24. https://doi.org/10.1038/s41388-020-01405-w.

[62] Londoñ O-Vallejo JA, Der-Sarkissian H, Cazes L, Bacchetti S, Reddel RR. Alternative Lengthening of Telomeres Is Characterized by High Rates of Telomeric Exchange. vol. 64. 2004.

[63] Seimiya H. Crossroads of telomere biology and anticancer drug discovery. Cancer Sci 2020;111:3089–99. https://doi.org/10.1111/cas.14540.

[64] Zhang JM, Zou L. Alternative lengthening of telomeres: From molecular mechanisms to therapeutic outlooks. Cell Biosci 2020;10. https://doi.org/10.1186/s13578-020-00391-6.

[65] Ale-Agha N, Dyballa-Rukes N, Jakob S, Altschmied J, Haendeler J. Cellular functions of the dual-targeted catalytic subunit of telomerase, telomerase reverse transcriptase - Potential role in senescence and aging. Exp Gerontol 2014;56:189–93. https://doi.org/10.1016/j.exger.2014.02.011.

[66] Haendeler J, Hoffmann J örg, Rahman S, Zeiher AM, Dimmeler S. Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation. FEBS Lett 2003;536:180–6. https://doi.org/10.1016/S0014-5793(03)00058-9.

[67] de Oliveira BCD, Shiburah ME, Paiva SC, Vieira MR, Morea EGO, da Silva MS, et al. Possible Involvement of Hsp90 in the Regulation of Telomere Length and Telomerase Activity During the *Leishmania amazonensis*Developmental Cycle and Population Proliferation. Front Cell Dev Biol 2021;9. https://doi.org/10.3389/fcell.2021.713415.

[68] Campelo R, Lozano ID, Figarella K, Osuna A, Ramírez JL. *Leishmania major* telomerase TERT protein has a nuclear/mitochondrial eclipsed distribution that is affected by oxidative stress. Infect Immun 2015;83:57–66. https://doi.org/10.1128/IAI.02269-14.

[69] Mozdy AD, Cech TR. Low abundance of telomerase in yeast: Implications for telomerase haploinsufficiency. Rna 2006;12:1721–37. https://doi.org/10.1261/rna.134706.

[70] Lee J, Sung YH, Cheong C, Choi YS, Jeon HK, Sun W, et al. TERT promotes cellular and organismal survival independently of telomerase activity. Oncogene 2008;27:3754–60. https://doi.org/10.1038/sj.onc.1211037.

[71] Lee MK, Hande MP, Sabapathy K. Ectopic mTERT expression in mouse embryonic stem cells does not affect differentiation but confers resistance to differentiation- and stress-induced p53-dependent apoptosis. J Cell Sci 2005;118:819–29. https://doi.org/10.1242/jcs.01673.

[72] Strong MA, Vidal-Cardenas SL, Karim B, Yu H, Guo N, Greider CW. Phenotypes in mTERT+/- and mTERT-/- Mice Are Due to Short Telomeres, Not Telomere-Independent Functions of Telomerase Reverse Transcriptase. Mol Cell Biol 2011;31:2369–79. https://doi.org/10.1128/mcb.05312-11.