Dissertation

The impact of Physical Activity and Nutrition on Telomere Biology

submitted by

Dott.ssa mag.

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

Graz, 26 July 2022 Maria Donatella Semeraro

Disclosures

Animal Experiment Registration

The animal study was approved by the responsible national ethics committee with the code GZ: 66.010/0070-V/3b/2018 and performed in accordance with the guidelines of the Animal Care and Use Committee of the Ministry of Science and Research, Vienna, Austria. The animals were housed the Core Facility Experimental Biomodels, Division of Biomedical Research of the Medical University of Graz, 8036 Graz (Austria) until the end of the study protocol. Ex-vivo analyses and in-silico work presented in this thesis were executed between the Clinical Institute of Medical and Chemical Laboratory Diagnostics (CIMCL), and the Centre for Medical Research (ZMF), with the Medical University of Graz.

Contributions

The following persons have contributed to this thesis:

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List of Publications

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These publications and manuscripts are appended at the end of the thesis, including affiliations for all authors. The contributions of M. Donatella Semeraro for each publication and manuscript:

I. Manuscript conception, writing and editing.
II. Performed the experiments, data analysis and assessment, wrote the original draft of the paper.
III. Conceptualization, investigation, writing— review and editing, visualization.
IV. Methodology, software, formal analysis, investigation, resources, data curation, writing—original draft preparation, visualization.
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Ad Maiora,
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"If you assume that there is no hope, you guarantee that there will be no hope. If you assume that there is an instinct for freedom, that there are opportunities to change things, then there is a possibility that you can contribute to making a better world."

-Noam Chomsky-

"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth."

-Jules Verne-

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List of Acronyms

CVD	Cardiovascular Disease
DALYs	Disability-Adjusted Life Years
YLL	Years of Life Lost
YLD	Years Lost due to Disability
PA	Physical Activity
BMI	Body Mass Index
TL	Telomere Length
LTL	Leucocytes TL
TRF1 or TERF-1	Telomeric Repeat-binding Factor 1
TRF2 or TERF-2	Telomeric Repeat-binding Factor 2
TIN2	TRF1 Interacting Nuclear protein 2
POT1	Telomeric overhang binding protein 1
TPP1	TIN2 and POT1 interacting protein 1

RAP1	Repressor-Activator Protein 1
DDR	DNA Damage Repair
TPE	Telomere Position Effect
ITS	Interstitial Telomeric Sequences
ITL	Interstitial Telomeric Loops
TPE-OLD	TPE-Over Long Distances
TERT	Telomerase Reverse Transcriptase
TERC or hTR	(human) Telomerase RNA Component
scaRNA	small cajal body RNAs
snoRNA	small nucleolar RNAs
TA	Telomerase Activity
PI3K	PhosphatidylInositol 3-Kinase
TRBD	Telomerase RNA Binding Domain

CTE	C-TErminal domain
ROS	Reactive Oxygen Species
mTORC1	mammalian Target Of Rapamycin Complex 1
HR	Hazard Ratio
CI	Confidence Intervall
WHR	Waist-to-Hip Ratio
WC	Waist Circumference
CHD	Coronary Heart Disease
PUFAs	Poly Unsaturated Fatty Acids
PBMCs	Peripheral Blood Mononuclear Cells
MET	Metabolic Equivalents
BDNF	Brain-Derived Neurotrophic Factor
IGF-1	Insulin-like Growth Factor 1

NO	Nitric Oxide
eNOS	endothelial NO Synthase
SD	Sprague Dawley
ND	Normal Diet
HFD	High-Fat Diet
co-	control (ND/HFD)
exe-	exercise (ND/HFD)
BL	Baseline controls
GAPDH	GlycerAldehyde-3-Phosphate DeHydrogenase
oxLDL	oxidized Low-Density Lipoprotein
h-arg	homoarginine
ADMA	Asymmetric DiMethylArginine
HPLC	High-Performance Liquid Chromatography

RANTES	Regulated And Normal T-cell Expressed and Secreted
MIP-1 α	Macrophage Inflammatory Protein 1α
MCP-1 and 3	Monocyte Chemoattractant Proteins 1 and 3
TNF- α	Tumor Necrosis Factor α
IFN- γ	INterFeron γ
IP-10	IFN-γ-Inducible Protein
IL-	InterLeukins-
TC	Total Cholesterol
TG	TriGlycerides
NEFA	Non-Esterified Fatty Acids
HDL	High-Density Lipoproteins
FC	Free Cholesterol
PL	PhosphoLipids

Abstract in German (Zusammenfassung)

(1) Telomere sind schützende Nukleoproteinstrukturen an den Enden aller Chromosomen, die mit zunehmendem Alter kürzer werden. Es gibt Hinweise, dass Fettleibigkeit und Bewegung die altersbedingte Telomerverkürzung mittels Regulation der Expression von Telomerase und Shelterin-Proteinen beeinflussen. Die Ergebnisse bisheriger Studien zu diesem Thema sind allerdings widersprüchlich und auf die Analyse der Telomerlänge (TL) in Leukozyten des peripheren Blutes und einzelnen soliden Geweben beschränkt. Ziel der vorliegenden In-vivo-Studie war deshalb eine systematische Analyse der Auswirkungen von Alter, Ernährung und körperlicher Betätigung auf die TL sowie die Expression Telomer-assoziierter Proteine in peripheren mononukleären Blutzellen (PBMCs) und einem breiten Spektrum solider Gewebe. (2) Dazu wurden 96 weibliche Sprague-Dawley-Ratten über einen Zeitraum von 10 Monaten entweder mit einer Standarddiät (normal Diät, ND) oder fettangereicherten Diät (hoch fetthaltigen Diät, HFD) gefüttert. Die Hälfte der Tiere beider Diätgruppen absolvierte während der gesamten Studiendauer an jeweils fünf aufeinanderfolgenden Tagen pro Woche eine 30-minütige Laufeinheit auf einem Laufband mit einer Geschwindigkeit von 30 cm/s. Die restlichen Tiere dienten als inaktive Kontrollen. Zur Untersuchung von Alterseffekten wurde zusätzlich eine weitere Gruppe von 24 jungen Ratten (4 Monate alt) nach einer Woche Eingewöhnung sakrifiziert und die verschiedenen Gewebe analysiert. Relative Telomerlänge (RTL) und mRNA-Expression von Telomerase (TERT) und den Shelterinen TERF-1 und TERF-2 wurden in PBMCs und den folgenden neun soliden Geweben kartiert: Leber, Skelettmuskel, Brustschlagader, Dickdarm, Milz, Nieren, Gehirn, Lungen und viszerales Fett. (3) Die Korrelationsanalyse ergab keine systematische Korrelation zwischen RTL in PBMCs und soliden Geweben. Während die RTL in PBMCs positiv mit jener in Niere, Skelettmuskel und Leber korrelierte, zeigte sich eine inverse Korrelation für Dickdarm und Aorta. Während der zehnmonatigen Studiendauer kam es zu keiner systematischen Verkürzung der Telomere in PBMCs und soliden Geweben. Im Lungengewebe und in viszeralem Fett älterer Tiere war RTL sogar höher als bei jungen Tieren, während das Gegenteil in der Aorta gefunden wurde. Erwartungsgemäß induzierte die HFD eine ausgeprägte Adipositas mit einem signifikant höheren Körpergewicht (coND = 413,41 ± 6,17 vs. coHFD = 523,75 ± 14,33, $p < 0{,}0001$). Die HFD-bedingte Adipositas führte zu keiner systematischen Veränderung der RTL. Ebenso war die mRNA-Expression der Telomer-assoziierten Gene TERT, TERF-1 und TERF-2 vergleichbar zwischen adipösen Tieren und nicht-adipösen Kontrollen. Lediglich im Nierengewebe und in viszeralem Fett unterschieden sich RTL- und die mRNA-Expression von TERT, TERF-1 und TERF-2 signifikant zwischen adipösen und nicht-adipösen Tieren. Regelmäßiges Laufen hatte heterogene Auswirkungen auf RTL und die mRNA-Expression Telomer-assoziierte Gene. Nur in einzelnen Geweben, wie Aorta und Dickdarm, fand sich eine signifikant höhere

mRNA Expression von einem oder mehrerer Telomer-regulierender Gene. Eine Wechselwirkung zwischen HFD und körperlicher Betätigung wurde nur in den Nieren beobachtet, wo sportlich aktive HFD Ratten eine vergleichbare RTL aufwiesen wie inaktive ND Tiere. Unabhängig von der Ernährung führte langfristiges Training zu erhöhtem oxidativ-nitrosativem Stress, der einen Teil der RTL-Variation im Blut erklärte. Umgekehrt reduzierte regelmäßige Bewegung die Serumkonzentration inflammatorischer Zytokine bei adipösen HFD Tieren. (4) Die vorliegenden Ergebnisse legen nahe, dass RTL und die Expression von Telomer-regulierenden Genen wie Telomerase und Shelterinen gewebespezifisch reguliert sind. Darüber hinaus ließ sich keine Verkürzung der Telomere in PBMCs und soliden Geweben während der zehnmonatigen Alterungsperiode nachweisen. Ebenso wenig wie das Alter hatten auch die beeinflussbaren Lebensstilfaktoren Bewegung und Ernährung keinen systematischen Einfluss auf RTL und die Expression von Telomer-regulierenden Genen. Auf Grundlage dieser Ergebnisse ist die Rolle der RTL in PBMCs als Marker des biologischen Alterungsprozesses in Frage zu stellen.

(1) Hintergrund; (2) Materialien und Methoden; (3) Ergebnisse; (4) Schlussfolgerung

Schlüsselwörter: Telomere; Telomerase; Unterschlupf; moderate Bewegung; Vielfett-Diät; oxidativer und nitrosativer Stress; Sprague-Dawley-Ratten

Abstract in English

(1) Telomeres are protective nucleoprotein structures at the end of chromosomes that shorten with age. Obesity and exercise are believed to modify age-related telomere shortening by regulating telomerase and shelterin expression. Previous studies that have addressed this topic are inconsistent and limited to the analysis of telomere length (TL) in blood and selected solid tissues. The present in-vivo study aimed to analyze systematically the effects of age, diet, and exercise in peripheral blood mononuclear cells (PBMCs) and a broad range of solid tissues. (2) For a period of 10 months, ninety-six female Sprague Dawley rats received either standard (ND) or high-fat diet (HFD). Half of the animals from both diet groups performed 30 min running sessions at 30 cm/s on five consecutive days per week for the entire duration of the study. The remaining animals served as sedentary controls. For the investigation of age effects, an additional group of 24 young animals (four months of age) was sacrificed after one week of acclimatization. Relative telomere length (RTL) and mRNA expression of telomerase (TERT), TERF-1, and TERF-2 were mapped in PBMCs and the following nine solid tissues: liver, skeletal muscle, thoracic aorta, large intestine, spleen, kidneys, brain, lungs, and visceral fat. (3) There was no systematic correlation between RTL in PBMCs and solid tissues. RTL in kidney, skeletal muscle, and liver was positively correlated with that in PBMCs, whereas large intestine and aorta showed an inverse correlation. Ten months of aging did not result in a systematic shortening of telomeres in PBMCs and solid tissues. In lung and visceral fat of aged animals RTL was higher than in young animals, the opposite was found in aorta. HFD induced obesity (coND = 413.41± 6.17 vs. coHFD = 523.75± 14.33, $p < 0.0001$), but RTL was not systematically altered in obese animals when compared to their lean counterparts. In addition, the mRNA expression of the telomere-associated genes TERT, TERF-1, and TERF-2 was comparable in obese and lean animals. Only in kidney and visceral fat of obese animals, RTL, and mRNA expression of TERT, TERF-1, and TERF-2 were significantly different from lean animals. Exercise had heterogeneous effects on RTL and the expression of telomere-associated genes. Again, only in selected tissues, such as aorta and large intestine, an increase of one or more telomere-regulating genes was associated with an increase in RTL of exercising animals compared to lean sedentary controls. An interaction between HFD and exercise was only observed in kidneys, where exercising obese rats exhibited a similar RTL to sedentary lean controls. Regardless of the diet, long-term exercise-induced a state of oxidative-nitrosative stress that explained part of RTL variation in blood. Conversely, exercise significantly reduced inflammatory cytokines in obese rats. (4) The present results suggest that RTL and the expression of telomere-regulating genes, such as telomerase and shelterins, is tissue-specific. Furthermore, ten months of aging do not systematically shorten telomeres in PBMCs and solid tissues. Modifiable lifestyle factors, such as exercise and diet have no systematic

effects on RTL and the expression of telomere-regulating genes. Therefore, the present results challenge the role of RTL in PBMCs as a surrogate marker of RTL in solid tissues. Furthermore, average RTL, as assessed with the PCR-based method used in this study, is not systematically affected by regular exercise and the consumption of HFD.

(1) Background; (2) Materials and methods; (3) Results; (4) Conclusions

Keywords: telomeres; telomerase; shelterin; moderate exercise; high-fat diet; oxidative and nitrosative stress; Sprague Dawley rats

Introduction

Over the last 200 years, average life expectancy in developed countries has more than doubled and is now above 80 years (3). In numerous studies, this linear increase is suggested to rise to an average life span of one hundred years or more (4-6). This dramatic increase in life expectancy was largely driven by changes in lifestyle, sanitation, and a continuous improvement in health care (7). As a result, the major causes of death have shifted from infectious disease to chronic age-related conditions (8, 9). Today, cardiovascular disease (CVD), cancer, and respiratory disease are the most common causes of death worldwide (10, 11). Other lifestyle and age-related conditions such as musculoskeletal disease, diabetes, and dementia are also increasing rapidly and thus impact the number of disability-adjusted life years (DALYs), calculated in a population as the sum of the Years of Life Lost (YLL) due to premature mortality and the Years Lost due to Disability (YLD) (12, 13). Therefore, strategies to promote healthy aging have gained great interest in developed societies. However, the process of aging remains incompletely understood. A better understanding of the complex and interrelated biological mechanisms of aging would help to develop interventions that delay the aging process.

Environmental and lifestyle factors, such as physical activity (PA), nutrition, stress, and smoking, are major determinants of the aging process (14). In particular, regular exercise is a safe and cost-effective way to reduce morbidity and premature mortality (15). Today, the molecular mechanisms that mediate the beneficial effects of exercise are incompletely understood and remain an area of active research. In numerous observational and intervention studies the preservation of telomeres, the protective end-caps of all chromosomes, has been proposed as an appealing putative mechanism that contributes partially to the beneficial health effects of PA (16). Moreover, nutritional factors may amplify or counteract PA-induced health effects. For example, a healthy lifestyle that combines physical exercise, a high intake of vitamins and antioxidants, a low body mass index (BMI), and non-smoking is associated with longer telomeres (17-21). Increased oxidative stress and chronic systemic inflammation are putative mechanisms through which adipose tissue exerts deleterious effects on telomeres (22, 23). Therefore, the amelioration of inflammatory processes through the maintenance of a healthy lifestyle may protect against premature telomere attrition. While several studies have investigated the telomeric effects of PA and nutrition individually, little is known about the interaction between nutritional factors and PA. This thesis aims to provide new and compelling evidence on the effects of PA under a normal or a western-type diet, describing the changes in telomere length and telomere modulatory genes expression, and in the modulation of oxidative stress markers, serum lipids, and systemic inflammation markers;

ultimately, attempting to raise novel ideas for future research in humans, the results of which can be translated into the possibility of developing effective strategies for primary and secondary prevention.

1) Telomeres: Structure and Function

Telomeres are the protective non-coding DNA regions at the end of all chromosomes that are of critical importance for genomic integrity and stability. In humans, they are composed of several hundred repeats of the hexanucleotide 5'-TTAGGGn-3' (24) and associated proteins that form the Shelterin complex. Telomeric DNA is double-stranded for most part of its length and expanded by a short, G-rich single-stranded overhang at the 3′-OH end. This single-stranded overhang is the result of an incomplete lagging strand DNA replication, which leads to telomere shortening with every cell division. Supported by the Shelterin complex, telomeric DNA folds into a three-dimensional structure which is important for telomere function. In 1961, Hayflick and Moorhead discovered the finite lifetime of cultured human cells and interpreted this finding as a manifestation of human aging at the cellular level (25, 26). In the late 1980s, it was made clear that the length of telomeres was not the same in all tissues and that critically shortened telomeres activate the DNA damage response and induce replicative senescence (27-29). Because telomeres shorten throughout life as a result of several modulating factors, telomere length (TL) has been proposed as a biomarker for biological age (30-35).

Telomere function and maintenance are tightly linked to the shelterin protein complex, which consists of six individual proteins: telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TRF1-interacting nuclear protein 2 (TIN2), telomeric overhang binding protein 1 (POT1), TIN2 and POT1 interacting protein 1 (TPP1), and repressor-activator protein 1 (RAP1). TRF1 and TRF2 interact with the double-stranded telomeric DNA, whereas POT1 associates with single-stranded telomeric DNA (36, 37). This nucleoprotein complex is attached to telomeres and forces double-stranded telomeric DNA to fold back forming a looped structure (T-loop). Furthermore, the shelterin proteins aid in displacing a short section of double-stranded telomeric DNA so that the single-stranded G-rich overhang at the 3'-end can be interposed. This structure is referred to as the "D-loop" and protects the free end of the DNA strand from recognition as a strand break, which would induce inappropriate activation of the DNA damage repair (DDR) system at telomeric sites resulting in non-homologous end-joining, alternative non-homologous end-joining or homologous recombination (38). When telomeres become critically short, they can no longer fold into a protective T-loop, which would result in exposing the single-stranded overhang to the DDR system. To prevent the adverse consequences of unwanted DDR activation, affected cells arrest their proliferation cycle and go into senescence.

In addition, the compact DNA structure of telomeres represses the expression of nearby genes through spatial hindering. This transcriptional silencing is known as the telomere position effect (TPE) (39, 40). Telomeric motifs are also interspersed between gene sequences of the coding DNA. These interstitial telomeric sequences (ITS) can interact with telomere-associated shelterin proteins, especially telomeric repeat binding factor 2 (TERF2), resulting in the formation of interstitial telomeric loops (ITL) (41). These ITLs contribute to the complex 3-dimensional chromatin structure and permit telomeres to modify the expression of sub-telomeric and distal genes (Fig. 1). The latter is referred to as the telomere position effect over long distances (TPE-OLD) (42). Critically short telomeres cannot loop back to form genomic ITL, consequently, the expression of sub-telomeric or distal genes is increased (43-45). Interestingly, one of the genes that is regulated via TPE-OLD and ITL is TERT, which encodes the telomere elongating enzyme telomerase (46). The activation of TERT expression in the context of short telomeres is considered a protective mechanism that prevents rapid telomere shortening.

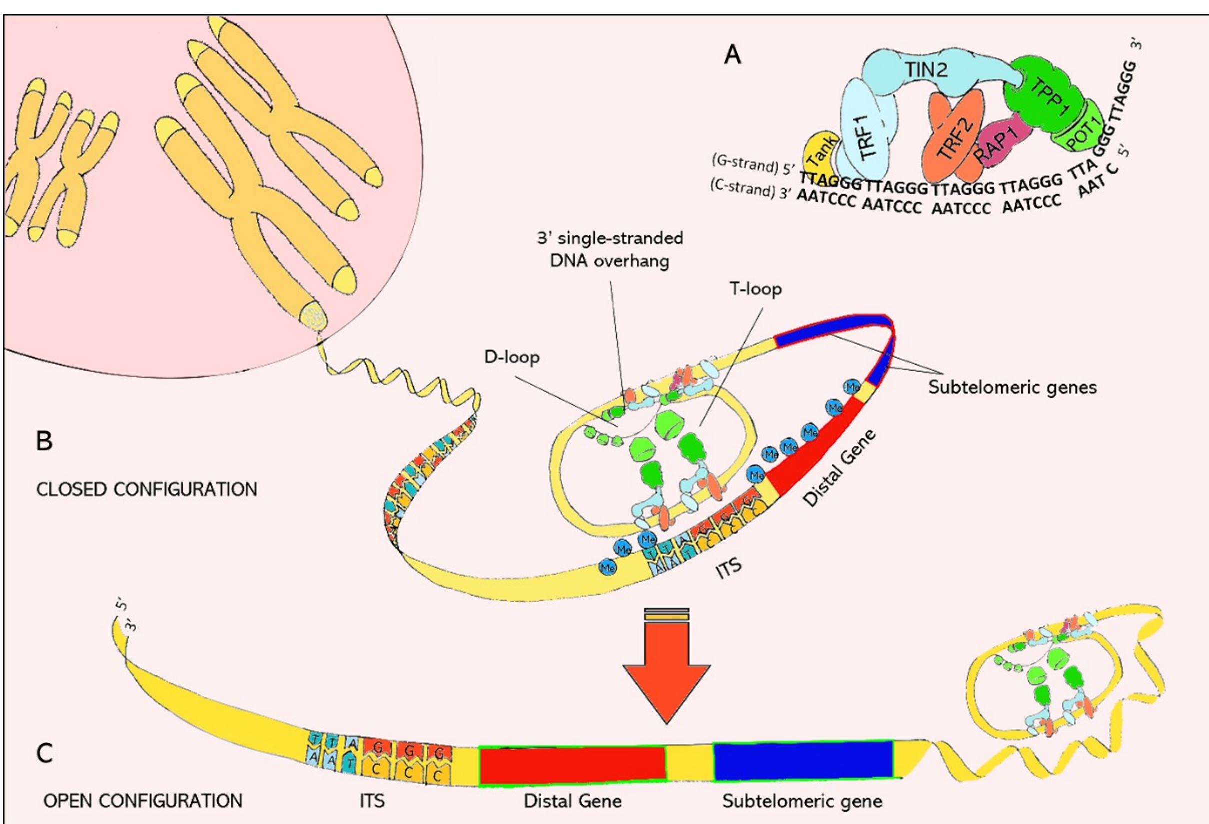


Fig. 1 (A): the Shelterin nucleoprotein complex, which protects coding DNA and telomeric ends from being recognized as free DNA endings or DNA strand breaks. **(B):** closed chromatin configuration at telomeric ends. Due to interactions between proteins and the specific binding of shelterins to telomeres, double-stranded telomeric DNA is forced to fold back in a loop structure (T-loop) while the 3' single-stranded DNA overhang is hidden in a D-loop. The binding of shelterins to interstitial telomeric sequences (ITS) drives the formation of interstitial telomeric loops (ITL) and promotes a closed chromatin structure which impedes the expression of subtelomeric and distal genes. This is also known as the telomere position effect (TPE). **(C):** open chromatin configuration at telomeric ends. Critically short telomeres cannot maintain a compact chromatin structure, and this facilitates the access of the translational machinery to genes that were formerly silenced by TPEs. Freely taken from (47), Copyright: © 2021 by the authors.

2) The Role of The Reverse-Transcriptase Telomerase

TL varies greatly between species (48). At birth, every person has a specific TL that ranges between 5 to 15 kb (35). Telomere attrition is most pronounced during the first two years of life, which are characterized by rapid somatic growth (49-52). Throughout life, telomeres shorten continuously with a rate between 20-50 bp due to the end-replication phenomenon, oxidative stress, and other modulating factors (28, 35). However, telomere shortening rates and consequently also average TL vary amongst different tissue types, which is at least partly explained by tissue-specific proliferation rates (51, 52). In dividing cells, the end replication problem is an important driver of telomere shortening that can be modified by other factors, such as oxidative stress or inflammation (35). In postmitotic cells instead, oxidative stress can directly damage telomeric DNA and drive cells into senescence (53, 54). Mean peripheral blood leucocytes TL (LTL) in adults is approximately 11 kb and declines with an annual rate of 30-35 bp. A recent study comparing the TL of more than 6000 tissue types coming from 952 deceased donors of the Genotype-Tissue Expression (GTEx) project estimated that the rates of telomere shortening in leukocytes and somatic cells are similar and therefore LTL is currently accepted to be a surrogate marker for TL in somatic cells (55).

The shortening of telomeres is not a unidirectional process since the reverse-transcriptase telomerase is capable of adding new hexanucleotides to telomeric ends (56, 57). In contrast to most somatic cells, detectable levels of the active enzyme can typically be found in the germ line and embryonic stem cells, where it ensures appropriate telomere elongation. However, within 18 weeks of gestation, the enzyme is inactivated. On the contrary, single-cell eukaryotes require constantly active telomerase to enable continuous cell division. Telomerase silencing during embryonic life is believed to be mediated either by alternative splicing or epigenetic modifications that alter the three-dimensional chromatin structure (58, 59). Human telomerase consists of the protein component telomerase reverse transcriptase (TERT), which harbors the enzyme activity, and the telomerase RNA component (TERC), also referred to as human telomerase RNA (hTR), endowed with a complementary sequence of telomeric DNA (3'-AUCCC-5') that serves as a template for telomere elongation (60). Telomerase activity is primarily regulated through the expression of TERT (61), while the regulation of TERC transcription is largely unknown. TERC belongs to the family of non-coding small Cajal body RNAs (scaRNA) and small nucleolar RNAs (snoRNA) and has its promotor, unlike most scaRNAs and snoRNAs (62-64). It is worth noting that telomerase expression does not necessarily parallel enzyme activity (60). In the brain, for example, TERT is expressed without detectable telomerase activity (TA) (65). In contrast, the

phosphatidylinositol 3-kinase (PI3K)-Akt kinase pathway and other factors can modulate TA independently from TERT expression (66).

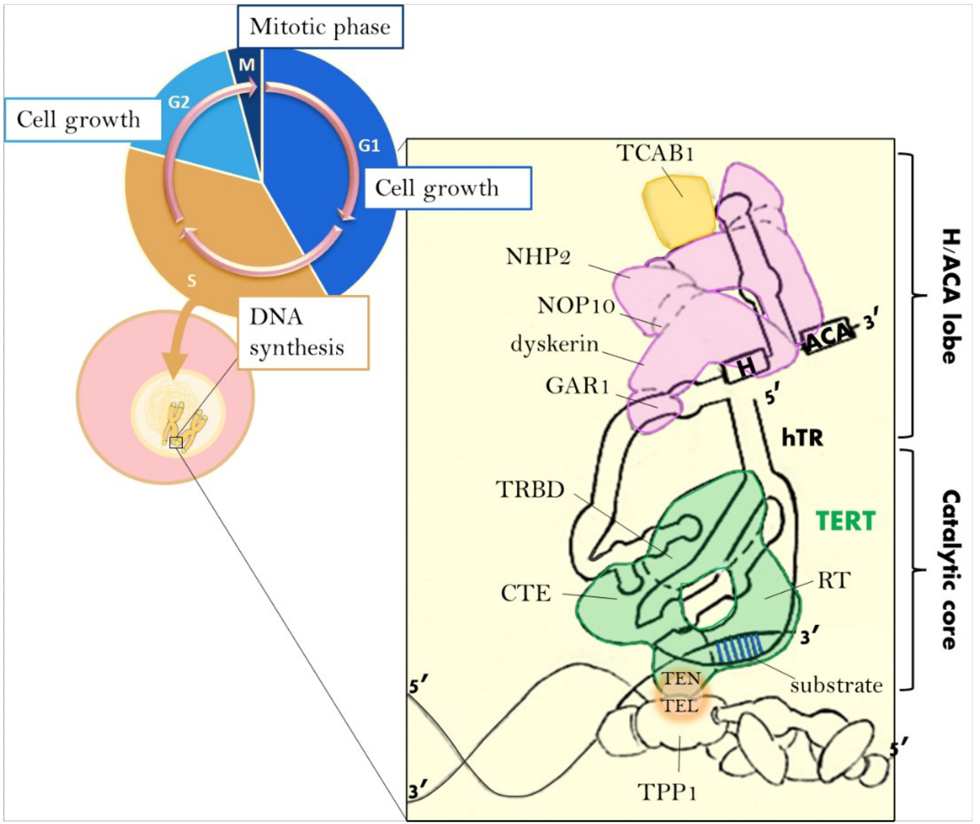


Fig. 2: the active telomerase is recruited to telomeric ends during the S phase of cell cycle. The interaction is mediated by two protein domains named 'TEN' and 'TEL', located respectively on TERT and TPP1. As illustrated, telomerase is characterized by a bilobal structure consisting of a catalytic core, with TERT and DNA bound TERC (hTR), and a H/ACA lobe. The H/ACA lobe contains two protein complexes composed of dyskerin, NOP10, GAR1, and NHP2. The binding of the two dyskerin complexes to the hTR represents the first step of the holoenzyme assembling process. To complete and activate the holoenzyme, also the telomerase Cajal body protein 1 (TCAB1, in yellow) is recruited.

In humans, the assembling of functional telomerase depends on the hTR structure, which can allow or prevent TERT binding (67-69). In particular, TERT is characterized by 3 domains, the N-terminal domain, which is the telomerase RNA binding domain (TRBD), the C-terminal domain (CTE), and the reverse transcriptase domain (RT). These three domains constitute a ring structure allowing RNA-DNA hybridization and DNA synthesis (Fig. 2). Through the action of additional proteins, the holoenzyme is recruited to telomeres and becomes fully activated (70-72).

To add new TTAGGG hexanucleotides the enzyme needs access to the telomere ends, which are hidden in the complex three-dimensional telomere structure (73). Therefore, telomeres can change their conformational status between an 'open' state, where the enzyme has substrate access and a 'closed' state that prevents telomerase action (74). Shelterin proteins play a key role in recruiting the holoenzyme and regulating the conformational state of telomeres, thus modulating TA (75, 76). The recruitment is cell cycle-dependent and occurs in the S phase through the binding of TERT to the shelterin protein TPP1 (77-81). TEN and TEL are the specific protein domains that mediated the binding of TERT to

TPP-1 (82). As regards the conformational status regulation, the low number of TRF1 and POT1 binding sites on short telomeres drives the formation of an open state. Whereas longer telomeres, with more TRF1 and POT1 binding sites, typically assume a closed configuration (83). In this way, telomerase can be efficiently directed to the shortest telomeres within a cell, and sufficiently long telomeres will not undergo any inappropriate lengthening (84, 85). Telomerase is discussed to be involved also in cardiovascular diseases due to its noncanonical and non-nuclear functions. Telomerase is present in mitochondria, thereby improving membrane potential, and reducing mitochondrial reactive oxygen species (ROS) production. These effects counteract the induction of apoptosis by protecting mitochondrial DNA (86-88). In preclinical models, telomerase has also been shown to be involved in autophagy through inhibition of the mammalian target of rapamycin complex 1 (mTORC1), especially under calorie restriction conditions, thereby improving diastolic dysfunction (89, 90).

3) Telomere Biology and Age-Related Diseases

Aging is a gradual and progressive process that changes the individual attitude toward facing environmental challenges, eventually increasing the susceptibility to diseases, frailty, or disability. Advancing aging is indeed the main risk factor for several chronic diseases in humans (91). The aging process is characterized by progressive telomere shortening due to cell division and telomere erosion. Individuals of the same age with the shortest telomeres have compared to those with the longest telomeres a higher hazard ratio for all-cause mortality (92, 93). For example, in the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, adult patients of German ancestry that fell in quartiles 2-4 of RTL had a lower hazard ratio (HR) for all-cause mortality (HR:0.822; 95%CI 0.712–0.915; $p = 0.008$) and CVD-mortality (HR:0.836; 95%CI 0.722–0.969; $p = 0.017$) when compared to those in the 1st quartile (94). TL is also related to the incidence, progression, and disease-specific mortality of individual age-related diseases, such as CVD, type 2 diabetes, cancer, and Alzheimer's disease. In a well-characterized longitudinal cohort of American Indians enrolled in the Strong Heart Family Study, those in the 1st quartile of LTL had an almost 2-fold increased risk of incident diabetes (HR:1.83; 95%CI 1.26-2.66; $p < 0.0001$) compared with subjects in the 4th quartile (95). A prospective, population-based survey conducted on 1000 individuals from South Tyrol (Italy) aged 40 to 79 years yielded similar results. Participants in the lowest quartile of RTL had an HR of 3.22 (95%CI 1.27-8.14; $p = 0.014$) compared with the highest quartile, and 2.86 (95%CI 1.45-5.65; $p = 0.002$) compared with all other quartiles (96). Moreover, a multi-ethnic community-based aging study showed that individuals who developed dementia had significantly shorter mean TL (6131±798 bp for prevalent cases; 6315±817 bp for incident cases) compared with the ones remaining dementia-free (6431±864 bp). Thus, shorter TL

represented a risk for earlier onset of dementia ($p = 0.05$) (97). These associations are believed to be the result of the age-related telomere shortening, which contributes to genomic instability and modulates gene expression through TPE and TPE-OLD (98). It is believed that the physiologic stimulation of TA through physical activity, healthy nutrition, and other modifiable lifestyle factors can reduce the risk for age-related diseases and promote healthy aging (99). This concept is supported by experimental studies in mice showing that constitutive TERT expression delays aging and extends life span (100-102). Moreover, telomerase reactivation reverses tissue degeneration in telomerase deficient mice (103). However, it is also well established that the constitutive expression of TERT is strongly related to carcinogenesis with 85 to 90% of all human cancers having detectable TA (104). In contrast, TERT inhibition in cancer cells reduces tumor growth due to the induction of cell death (105-109). This suggests that a constant unregulated TA activation of oncogenes, and/or silencing of tumor suppressor genes may drive tumor incidence and growth (110), whereas a physiologically regulated telomerase activation appears to have beneficial health effects (100-102).

In light of the extensive body of literature sustaining a fundamental role of telomere biology in the aging process and age-related diseases, in particular, the age-dependent telomere shortening, and genetic instability were associated with shortened life span and a reduced regenerative potential (111). Substantial effort has been invested in the past years in searching for lifestyle factors, such as nutrition or psychological stress, that can modulate TA and preserve telomere length. Not only age but also many other factors contribute to the shortening of telomeres including genetic background (33, 112-114), gender (115), socioeconomic status and consequent stress perceived (116-118), dietary behavior (i.e. antioxidant intake, alcohol consumption, etc.) (19, 119-122), body mass index (BMI) (20, 115), smoking (21, 115) and physical inactivity (123). The influence of specific factors on telomeres will be addressed in the next sections, with a particular focus on the role of PA and nutrition in the modulation of TL and telomere-associated genes.

4) Influencing Factors on Telomere Length

In humans, the mean leukocyte telomere length (LTL) at birth is 11-kilo base pairs (kbp) and declines to less than 4 kbp in elderly individuals (124). However, telomere attrition is not a linear process, where a constant number of base pairs is lost with every successive cell division. TA and telomere trimming events can modulate telomere length in both directions. TL and TA show huge interindividual variability and as a result, most human cohort studies found only a weak correlation between LTL and age. Considering the many factors that affect TL, this observation is not surprising.

Numerous studies have investigated non-modifiable and modifiable factors that influence TL. Gender, for example, is a non-modifiable factor that determines telomere length with longer telomeres being observed in females than in males (125). This effect is mainly driven by estrogens, which mediate antioxidative effects and induces moderate TA. Psychological stress is another well-documented factor that impacts telomere homeostasis by reducing telomerase activity and increasing reactive oxidative species (126, 127). Also, nutritional factors can modulate TL (128-130). In particular, a sufficient supply of micronutrients like vitamin A, D, C, E, B12, folate, and nicotinamide is positively associated with TL (19, 120, 131-133). Minerals like magnesium, zinc, iron, and other micronutrients, such as omega-3 fatty acids, polyphenols, and curcumin, are additional modulators of TL. The effect of vitamins on telomere homeostasis seems to be mediated by their antioxidative properties and consequent prevention of DNA damage. In addition to a healthy diet, regular physical activity also contributes to the preservation of TL via reducing sustained oxidative stress and inflammatory mechanisms. Furthermore, exercise has been shown to increase TA (99, 134, 135). Other lifestyle-related factors that potentially influence TL include smoking and alcohol consumption. However, to date the evidence for a significant association between alcohol consumption and TL is insufficient (136). Regarding smoking, a recent meta-analysis of 84 studies showed significantly shorter telomeres in ever smokers compared to those who never smoked (137). Furthermore, oxidative stress and chronic inflammation are key factors that impact TL (132). Besides many lifestyle and environmental factors, TL and TA are strongly determined by the inherited genetic background, with heritability estimates ranging from 34% to 82% (138).

Taken together, there is good evidence that a healthy and active lifestyle with sufficient sleep and a low psychological stress level contributes to the preservation of telomeres. Physical inactivity, nutritional deficits, overweight, stress, and smoking can exacerbate telomere attrition and thus promote age-related diseases. In the following sections of this introduction, a more detailed overview of the existing evidence regarding the effects of nutritional habits and exercise on telomere physiology will be reported.

a. Body composition, obesity, and telomeres

Obesity is a major risk factor that increases the prevalence of age-related diseases and mortality (139). However, it is unclear whether or not obesity modifies the aging process through accelerated telomere attrition and impaired genomic integrity as existing studies on relative telomere length (RTL) and obesity-related phenotypes are incongruent. A recent study suggests that it may not simply be a high body weight to predict short telomeres, but a specific pattern of central obesity with subcutaneous adipose tissue accumulation at the neck and hip (140). Other studies reported an inverse correlation between TL and body mass index (BMI), waist-to-hip ratio (WHR), total fat, waist circumference (WC), and abdominal adipose tissue is directly associated with TL attrition and promotes aging (141-148). A meta-analysis of 39 studies showed a weak to moderate correlation between obesity and RTL (149),

while others report no correlation neither in middle-aged nor in elderly individuals (150-152). These inconsistencies may be due to major methodological, clinical, anthropological, and statistical differences.

Metabolic diseases associated with obesity appear to be correlated with the amount of adipose tissue and TL attrition (144, 148, 153). While weight gain and obesity are reported to promote telomere attrition independently of age (154), weight loss has been correlated with TL elongation, with the lengthening rate increasing with the degree of weight loss (155). 521 adults aged 55 to 80 years who underwent the Mediterranean diet intervention for 5 years, showed increased TL and an inverse correlation between TL and body weight, BMI, WC, and WHR (156). It appears that weight loss prevented telomere shortening and DNA damage (157).

It is known that obesity might induce a state of chronic inflammation and oxidative stress, which in turn exacerbates telomere attrition (158). Telomeric DNA is especially sensitive to ROS-mediated DNA damage, with the telomeric DNA GGG triplet being most vulnerable, due to the low electron potential of the G nucleotide (159, 160). Oxidative stress-induced cellular senescence represents an important response to DNA damage because it stops the growth of cells with a high risk of mutation (161, 162). Some authors demonstrated that decreased TL correlates with increased oxidative stress in patients with type 1 and type 2 diabetes (22, 23). Furthermore, oxidative stress has been linked to TL shortening in a study comparing elderly Greek men, with lower indices of oxidative stress and higher antioxidant levels, to a corresponding population of elderly Dutch males (18). It might be plausible to hypothesize that TL correlates with weight loss and decreased inflammation and oxidative stress status. However, TL of severely obese subjects measured immediately after bariatric surgery and at 3-month intervals from surgery was significantly shortened, most likely due to the catabolic state caused by the intervention (163). Yet, Latifovic et al. reported no correlation between weight loss and changes in TL after a 12-month dietary and exercise treatment (164). Therefore, prospective, or long-term studies are needed to investigate potential changes in TL and telomere-associated genes.

The few animal studies that have explored the relationship between obesity and telomere biology are also inconclusive. Burchfield et al. showed how C57BL/6J mice fed a high-fat/high-sucrose diet, compared to control animals developed metabolic alterations, obesity, physical inactivity, and cognitive impairment (165). However, LTL was comparable between the two groups. In the myocardium of DahlS.Z-*Lepr*^{fa}/*Lepr*^{fa} (DS/obese) rats, an animal model of metabolic syndrome, RTL was comparable to DahlS.Z-*Lepr*$^{+}$/*Lepr*$^{+}$ (DS/lean) control animals whereas TA and TERT gene expression were increased (166). The expression of senescence-associated genes and markers of cell cycle arrest including *Chk2*, *p53*, and *p21*, were also upregulated in the myocardium of DS/obese rats. These observations support the concept of a direct damaging effect of adipose tissue on telomeres through

obesity-induced conditions such as oxidative stress and inflammation. As already mentioned, both factors have been shown to accelerate telomere attrition (158). Recent evidence suggests that the interaction between adipose tissue and telomeres is not a one-way road. RAP1, a component of the shelterin complex, appears to upregulate energy metabolism. Specifically, RAP1 deficient mice are characterized by an early onset of obesity and multiple metabolic abnormalities (167, 168). Also, in a genome-wide gain-of-function screen RAP1 was identified as a critical modulator of the NF-κB signaling pathway (169). NF-κB is a downstream intermediate of PI3k-α/Akt, a pathway involved in several oxidative stress and inflammatory-related conditions. RAP1 is in turn regulated by NF-κB, establishing a feedback regulatory mechanism. Méndez-Pertuz et al. demonstrated that the inhibition of PI3k-α and Akt by small molecules, as well as the genetic depletion of the PI3kCA gene, resulted in increased telomeric fragility and an increase in telomere aberrations (170). However, despite the first promising results, our knowledge of the relationship between obesity and telomere dysfunction is still limited.

b. Nutrients, dietary patterns, and their influence on telomeres

Several nutritional factors including vitamins, minerals, and other bioactive dietary compounds such as omega-3 fatty acids, polyphenols, and curcumin, can influence LTL directly or indirectly through several mechanisms (119, 130, 171, 172). Numerous studies have reported a correlation between the availability of B and D vitamins with LTL (120, 130, 173-175). Vitamins B, C, D, E, β-carotene, and minerals, including zinc and magnesium, appear to have a protective effect against oxidative stress and inflammation, which aids the preservation of telomeres (119). Also, polyphenols like theaflavins, found in green and black tea, were found to possess antioxidant properties and a negative association with inflammatory biomarkers (176). Resveratrol, another naturally occurring polyphenol found in the skin of red grapes, has been reported to have antioxidant and anti-inflammatory properties, reducing the risk of CVD and diabetes (177). In addition, Resveratrol activates SIRT1, an intracellular regulatory protein that regulates important metabolic and physiological processes including cellular senescence, TL maintenance, and TA in rodents (178, 179).

Of particular importance for the cardiovascular system are omega-3 fatty acids. The blood concentrations of docosahexaenoic and eicosapentaenoic acids were inversely correlated with the rate of telomere attrition over 5 years in patients with coronary heart disease (CHD) (180). A more recent study has shown that it is rather the ratio between omega-3 (n-3): omega-6 (n-6) fatty acids that is inversely correlated with telomere attrition over time (181). During the study period, TA remained unchanged, but TL inversely correlated with markers of oxidative stress and inflammation.

While it is important to know the effects of individual foods, it is even more crucial to assess the cumulative role of dietary patterns on TL, which better reflects reality. For example, in 2015 Lee et al. compared the influence of two major dietary patterns on TL in 1958 middle-aged Korean adults. With a multiple linear regression model adjusted for age, sex, BMI, and other confounders, they found that a healthy diet characterized by a high intake of whole grains, fish and seafood, legumes, vegetables, and seaweed was found positively associated with LTL. In contrast, a Western-type diet characterized by a high intake of refined grain, red or processed meat, and sweetened carbonated beverages was inversely associated with LTL. In this study, dietary patterns were assessed by a semi-quantitative food frequency questionnaire at baseline, and LTL was determined by qPCR 10 years later (182). Tsoukalas et al. administered a mix of nutraceuticals including antioxidant vitamins, PUFAs, and probiotics to a healthy Greek cohort for 6-12 months (122). At the end of the study, the experimental group showed an increment of 953 bp in LTL compared to the control group not taking any nutritional supplements. Moreover, Ornish et al. demonstrated that a 3-month intervention with a low-fat diet based on unrefined plant-based food and supplementation with omega-3 fatty acids (from fish oil), soy, and vitamins C and E increased the TA of PBMCs in men. However, the intervention time was not long enough to detect any change in TL (127). The increase in TA was correlated with a decrease in plasma concentration of low-density lipoprotein (LDL) and psychological distress (127).

The Mediterranean diet is considered one of the healthiest diets worldwide. It contains all the beneficial nutrients described above and includes a moderate to high intake of fish, a high intake of unsaturated lipids, particularly olive oil, and a regular but moderate intake of alcohol, specifically red wine. This dietary pattern has been linked to low morbidity and lower occurrence of CVD (128, 183). In the Nurses' Health Study, one of the largest prospective cohort studies into the risk factors for major chronic diseases in women, adherence to this diet resulted in the preservation of TL equivalent to 4.5 years of aging (184). A Mediterranean diet also appears to modulate TA in PBMCs and, when combined with moderate exercise, improves endothelial, microvascular, and cardiorespiratory fitness (128, 185).

Taken together, nutritional habits considered 'healthy', i.e., high intake of vitamins, antioxidants, PUFAs, and unrefined grains, have been associated with longevity at least partly due to the preservation of telomeres. However, nutrition is not the only factor affecting telomeres. In the next sections, an excursus on the influence of physical activity on telomere length and the mechanisms of telomere maintenance will be given.

c. The beneficial health effects of regular exercise in cohort studies

Regular exercise is a well-established approach to reduce the risk of morbidity and premature mortality (15, 186). Prospective cohort studies demonstrate that men and women who regularly exercise, have a 30% lower all-cause mortality risk than sedentary individuals (15, 186). In older persons, the beneficial

effects of regular physical activity (above 20 minutes a day) are even more pronounced reaching up to >40% mortality risk reduction (186-188). Some studies have calculated that the gain of life years ranges between 2 to 4 years depending on the individual level of activity (189-193). Despite strong evidence that supports beneficial health effects through regular exercise, comparability between individual studies is limited because of differences in the composition of study cohorts, exercise protocols, and the duration of follow-up (189-193). However, the pooled analysis of six major cohort studies including 632,091 participants of diverse ethnicity and an average age of 61 years showed that the effect of regular exercise on mortality is dose-dependent, and already mild physical activity is associated with a significant reduction of mortality risk and a 1.8-year gain in life expectancy (194). Metabolic equivalents (MET) are used to compare energy consumption between different activities by dividing the actual energy expenditure of a given activity by the energy expenditure at rest (195). Of note, even intermittent exercise sessions with a limited duration offer considerable health benefits, also in obese individuals and those with major risk factors (196). The health effects of exercise are not only determined by the frequency and duration of training sessions, but also by the intensity. Vigorous exercise is more effective than mild or moderate exercise in improving cardiorespiratory fitness (197-199). When adjusted to their specific needs and abilities, even in older individuals, regular physical activity attenuates the age-dependent decline in cardiorespiratory fitness (200), improves mobility and physical functioning (201), and reduces the risk of falls (202).

Besides a substantial decrease in mortality, regular exercise also reduces the incidence and progression of CHD, hypertension, stroke, diabetes, metabolic syndrome, colon cancer, breast cancer, and depression (186). When compared to inactive individuals, physically active adults exhibit better cardiorespiratory fitness and muscular strength, a healthier body mass and composition, and a favorable metabolic profile (186). Furthermore, they report better quality of sleep and health-related quality of life (186). In a 1-year randomized controlled study regular aerobic exercise (moderate-intensity aerobic exercise 3 days/week at 50–60% of the maximum heart rate reserve for week 1 to 7 and at 60–75% for the remainder of the program of 1 year) was shown to attenuate age-related brain atrophy and improve cognitive function in older individuals (203). The authors reported that in 120 older persons aged 55–80 years, regular exercise improved memory function and age-related brain atrophy was reversed by approximately 1-2 years (203). A key mechanism that mediates the neuronal effects of aerobic exercise is the secretion of neurotrophins and in particular brain-derived neurotrophic factor (BDNF) (204-206). Despite the existence of robust evidence for multiple health benefits of regular exercise, the underlying mechanisms are yet insufficiently understood.

d. Exercise and telomere biology in human studies

The first study to explore the relationship between exercise and TL in humans was conducted by Cherkas et al. In a cross-sectional survey of 2401 white men and women they showed that LTL was positively associated with higher physical activity levels (142). Similar results were reported by Du et al. analyzing 7,813 adult women from the Nurses' Health Study, where even moderate amounts of activity were associated with longer telomeres (207). In 5823 adult men and women of the National Health and Nutrition Examination Survey (NHANES 1999-2002) Tucker et al. showed that average LTL decreases by 15.6 bp per year of chronological age (208). Individuals with higher levels of physical activity had substantially longer telomeres in peripheral blood leucocytes, corresponding to a gain of biological age of approximately 9 years (208). In their study, Werner et al. demonstrated that the LTL of middle-aged athletes was preserved at the level of young controls. In contrast, the LTL of middle-aged controls was approximately 30-40% lower than in young controls and thus, suggesting an age-related attenuation (209). The preservation of TL was confirmed by two independent methods, qPCR, and flow-FISH. Furthermore, when compared with untrained individuals, athletes showed increased TA and expression of telomere-stabilizing shelterin proteins, such as TRF2. The effects on telomere biology were accompanied by a pronounced inhibition of *Chk2*, *p16*, and *p53*, which are involved in DNA damage response, cell-cycle progression, and survival (209). In line with these results, Denham et al. analyzed LTL and the expression of telomere-regulating genes in 61 Australian endurance athletes and 61 healthy controls (84). LTL in athletes was 7.1% (208-416 nucleotides (nt)) higher than in sedentary controls. In addition, athletes showed a higher expression of TERT and TPP1 mRNA expression. Interestingly, resting heart rate emerged as an independent predictor of LTL, TERT, and TPP1 mRNA expression in this study. Denham et al. also showed that training volume determines the effect of exercise on telomere biology with the greatest effects seen in the most active athletes. On the molecular level telomere-associated genes, including *tert*, *terf2ip* (which encodes RAP1), sirtuin-6 (*sirt6*), and TATA-box binding protein (*tbp*) and miRNAs that target these genes are upregulated after a single running session of 30 minutes at 80% of peak oxygen uptake (VO2Peak). The analysis of white blood cells from 22 healthy male volunteers, immediately after and 60 min after exercise, showed that 56 miRNAs were differentially regulated post-exercise (FDR <0.05) and that 4 of these (miR-186, miR-181, miR-15a, and miR-96) potentially target telomere-associated mRNA species (210). Although cross-sectional observational studies suggest that regular exercise preserves TL through activation of telomerase, experimental and prospective studies are necessary to prove causality.

A recent study on 124 healthy, previously inactive individuals explored the effects of regular endurance training, intensive interval training, and resistance training over 6 months (211). Participants trained 3 times per week for 45 min. Compared to non-exercising controls, TA in PBMCs was up-regulated 2 to 3-fold in the endurance- and interval-training groups, but not in the resistance-training group. The activation of telomerase was accompanied by longer telomeres in lymphocytes, granulocytes, and

leucocytes. In addition to this training study, Werner et al. also explored the effects of a single bout of exhaustive exercise using a stepwise ramp protocol on a treadmill. When compared to baseline, CD14+ and CD34+ leucocytes collected after exercise, exhibited increased TA, which was still measurable 24h post-exercise. Insulin-like growth factor-1 (IGF-1), a potential mediator of the exercise-induced activation of telomerase (209), showed a biphasic response. However, after the 6-month training program, IGF-1 was comparable to baseline levels. Furthermore, blood collection was performed from 48 hours to 7 days after the last exercise session. This suggests that whilst the exercise-induced effects on telomere biology are of short duration, any health benefit is the result of a cumulative effect achieved by regular training. Besides the secretion of IGF-1, another putative hypothesis to explain the exercise-induced activation of telomerase with subsequent telomere elongation is the release of nitric oxide (NO) as a result of increased vascular shear stress (211). Endothelial NO synthase (eNOS) and TA appear to be linked in a signaling pathway that mediates vascular protection (209).

Despite robust evidence from cross-sectional and prospective intervention studies, not all previously published analyses support a relationship between exercise and telomere biology (212-217). In a cross-sectional and longitudinal analysis of 582 older adults, Soares-Miranda et al. found no consistent relationship between physical activity and LTL (215). Only some general functional measures, such as walking distance and 'chair test performance', were cross-sectionally related to LTL. Results from the 'Berlin Aging Study' suggest that, in adult men aged over 61 years, long periods of physical activity are necessary for the prevention of telomere shortening (at least 10 years), with intensive sports activities having the greatest effect (218). This concept is confirmed in a study by Laine et al. where former elite athletes were found to have comparable LTL to age-matched, sedentary individuals (219).
Some researchers suggest that the relationship between LTL and exercise is U-shaped (84, 220, 221). For example, Savela et al. analyzed physical activity levels, LTL, and the proportion of short telomeres in 204 randomly selected survivors of the 'Helsinki Businessman Study'. Moderate physical activity was associated with the longest mean LTL. A cross-sectional comparison of endurance athletes and healthy controls provides additional support that moderate amounts of exercise training protect against biological aging, while higher amounts may not elicit additional benefits (84).
In summary, the evidence implies that the protective effects of exercise require a rather long-time span and continuity to become evident. In addition, there is insufficient data to judge if different training modalities exert differential effects on telomeres, telomerase, and shelterin expression. However, existing studies suggest that aerobic endurance exercise, but not resistance training, is helpful to preserve TL, at least in leucocytes.

e. Exercise and telomere biology in animal studies

Although human studies suggest that regular exercise preserves telomeres, they are unable to unveil the underlying mechanisms. Animal models can help to close this gap as they allow the investigation of the mechanistic pathways. At present, only a few animal studies have been performed (209, 222-224). It appears that telomeres of murine blood leucocytes and other cell types (e.g., myocardium, liver, aorta) also become shorter with advancing age (209, 222, 223). However, this process is rather slow and may take between 12 to 18 months. For example, TL of blood leucocytes and cardiomyocytes was comparable in 3-week-old and 6-month-old C57/Bl6 mice but was significantly reduced after 18 months (209, 222). Interestingly, the myocardium of these exercising mice also showed increased telomerase and shelterin expression and a reduction of apoptosis and cell-cycle arrest (209, 222, 225). Regular running exercise has been shown to attenuate the age-related erosion of TL in hepatocytes and cardiomyocytes of CAST/Ei J mice, a wild-derived inbred strain of mice, over 1 year (223). Moreover, in skeletal muscles and cardiomyocytes, the age-related shortening of telomeres is accompanied by a decreased gene expression of the shelterin components TRF1 and TRF2 (223). Chronic exercise can counteract the reduced expression of shelterins and thus aid to stabilize telomeres (223). TRF1 and TRF2 protein content showed similar trends. In line with these results, Werner et al. reported a persistent up-regulation of telomere-stabilizing proteins TRF2 and TERT in cardiomyocytes after 6 months of daily running exercise (222). In parallel, the senescence-related proteins Chk2, p53, and p16 were down-regulated. Together, these effects lead to a substantial reduction of apoptotic cardiomyocytes in the heart of exercising mice. Regular running exercise also ameliorated the cardiotoxic effects of doxorubicin (222). Additional data from these experiments suggest that the beneficial cardiac effects of regular exercise are primarily mediated by TERT, eNOS, and IGF-1.

Exercise-mediated telomere preservation and other beneficial health outcomes are most likely the results of a cumulative effect over an extended period. However, even a single bout of exercise has been shown to increase the protein levels of TRF1 and TRF2 as well as *pot1a*, but not *pot1b* gene expression (224). These changes are accompanied by a greater expression of DNA-repair and -response genes (Chk2 and Ku80) and greater protein content of phosphorylated p38 MAPK (224). Ludlow et al. have speculated that the rapid increase in shelterin gene expression represents a direct adaptive reaction to the exercise stimulus, which depends on the duration, intensity, and type of exercise (224). Conversely, the fast increase in protein content is probably the result of improved proteostasis rather than increased mRNA translation. The rapid increase of shelterin expression in response to a single exercise session does not necessarily lead to a prompt increase in TA (224). However, after three weeks of regular training, a persistent upregulation of myocardial *tert* expression has been shown by Werner et al. (209, 222). This activation of *tert* appears to be essential for the cardioprotective effects of physical activity.

Although existing evidence is rather limited, available data suggest that exercise induces an immediate short-lived regulatory response in shelterin mRNA expression, but only a continuous stimulation over

an extended period results in a preservation of telomeres and delays cellular aging. Furthermore, regular exercise is directly involved in the establishment of an anti-apoptotic and anti-senescent cellular environment through the up-regulation of genes implicated in the DNA damage response and repair, including *ku70/ku80*, and down-regulation of *p16*, *p53*, and *Chk2* (222, 223). Therefore, it is not surprising, that the myocardium of exercising mice displays increased telomerase and shelterin expression and a reduction in apoptosis and cell-cycle arrest (209, 222, 225).

5) Aim of the Study

Telomere shortening and telomeric dysfunction are key aspects of aging that can be modified by numerous lifestyle factors including physical activity, nutrition, stress, sleep, and smoking. Most age-related diseases have been found to be associated with telomere length. Therefore, effective strategies for the preservation of telomeres may be of critical importance to promote healthy aging. Based on existing evidence, physical activity and nutrition appear as key factors that may help to protect telomere length and function. However, mechanistic studies that proof causality are limited and mainly confined to animal experiments. Furthermore, the majority of studies measured average telomere length by PCR in PBMCs assuming that this measure would reflect the situation in the entire body. However, evidence that supports this assumption is largely lacking. Systematic studies that map telomere length and the expression of shelterin proteins in PBMCs and solid organs of the same individual at juvenile and advanced adult age are also lacking. Furthermore, systematic investigations into the dynamic changes of TL upon different stimuli, such as different dietary regimens, have not been performed. While several studies have investigated the telomeric effects of physical activity and nutrition individually, and most of them are only observational, little is known about the interaction and the causal relationships between nutritional factors, physical activity, and telomere length regulation.

Thus, an in-vivo study was performed in rats where TL was assessed in young and old animals in PBMCs and nine solid organ tissues including liver, skeletal muscle, aorta, large intestine, spleen, kidneys, brain, lung, and visceral fat. The mapping of TL in different tissues of young and old animals aimed to provide new insights into telomeric aging across all organs. In addition, this study explored the telomeric effects of an unhealthy high fat diet and regular running exercise alone or in combination. Therefore, half of the animals were fed a high fat diet whereas the other half received a standard chow-based diet. From both diet groups, half of the animals performed regular moderate running exercise on a treadmill whereas the other half of the animals remained inactive. RTL and the mRNA expression of TERT, TERF-1 and TERF-2 were analysed in peripheral blood leucocytes and several solid organs. In

an exploratory approach lipid metabolism, NO signalling, pro- and anti-inflammatory pathways were profiled by measuring a series of relevant biomarkers.

Materials And Methods

f. Study design

To expand the existing knowledge about the impact of lifestyle factors, such as exercise and nutrition, on age-related telomere shortening in PBMCS and different solid tissues, an aging study has been performed in rats. For 10 months, forty-eight healthy young-adult female Sprague Dawley (SD) rats performed regular running exercise sessions on a treadmill. In a 1:1 ratio, they were either fed a standard (ND) or a high-fat (HFD) diet. For control purposes, a similar number of animals were kept inactive and received the same two diets. All animal experiments were approved and received ethical permission from the competent local and national authorities.

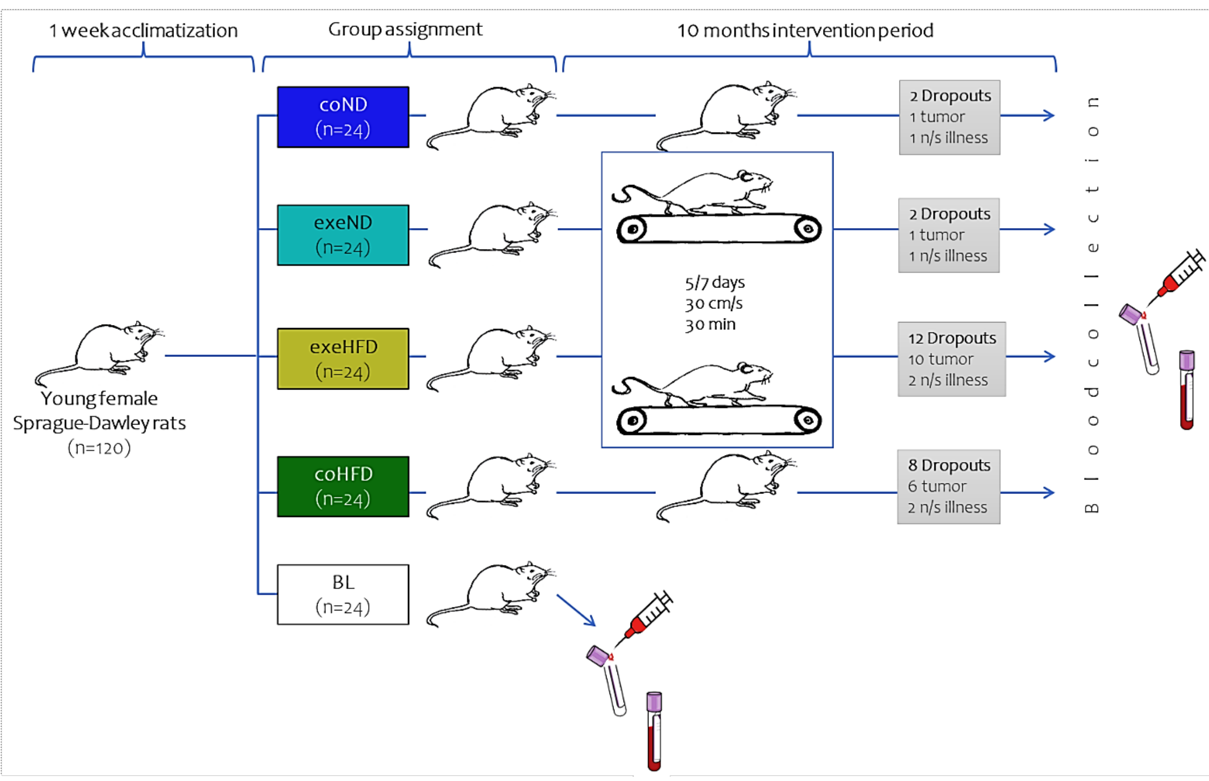


Fig. 3: Illustration of the study design. The study groups have been abbreviated as follows: baseline group (BL, white box), control normal diet (coND, blue box), exercise normal diet (exeND, cyan box), control high fat diet (coHFD, green box) and exercise high fat diet (exeHFD, olive green box). Modified from (226), Copyright is by © The Author(s) 2021.

120 four-month-old SD rats with a body weight between 335 - 375 g were purchased from Janvier Labs (France). After one week of acclimatization, the animals were divided into 5 groups (Fig. 3):

1. baseline control (BL, n = 24):

animals were sacrificed at the beginning of the study without any intervention.

2. control - standard diet (coND, n = 24):

animals did not exercise and had free access to a standard diet with 3235 kcal/kg, 11% fat (Altromin, Germany), and water.

3. control - high-fat diet (coHFD, n = 24):

animals did not exercise and had free access to a custom-designed beef-tallow HFD, rich in saturated fatty acids (SFA), C16:0, and C18:0, with 5150 kcal/kg and 60% fat (Ssniff, Germany) and water.

4. exercise - standard diet (exeND, n = 24):

animals ran on 5 consecutive days for 30 min on a treadmill at a speed of 30 cm/s followed by 2 days of rest. They had free access to a ND and water.

5. exercise - high fat diet (exeHFD, n = 24):

animals ran on 5 consecutive days for 30 min on a treadmill at a speed of 30 cm/s followed by 2 days of rest. They had free access to the same 60% fat diet as their sedentary controls and water.

For the entire study period, the animals were kept on a 12 h / 12 h light/dark cycle at 22 to 25 °C and 55 to 58 % of relative humidity. Animals were weighed at baseline and every 2 months thereafter.

At the end of the 10-months study period, blood was drawn by heart puncture under deep isoflurane anesthesia (Forane, Abbott, Austria). Blood and plasma were collected using S-Monovette Serum-Gel tubes and S-Monovette Plasma-EDTA tubes (Sarstedt, Nümbrecht, Germany), respectively.

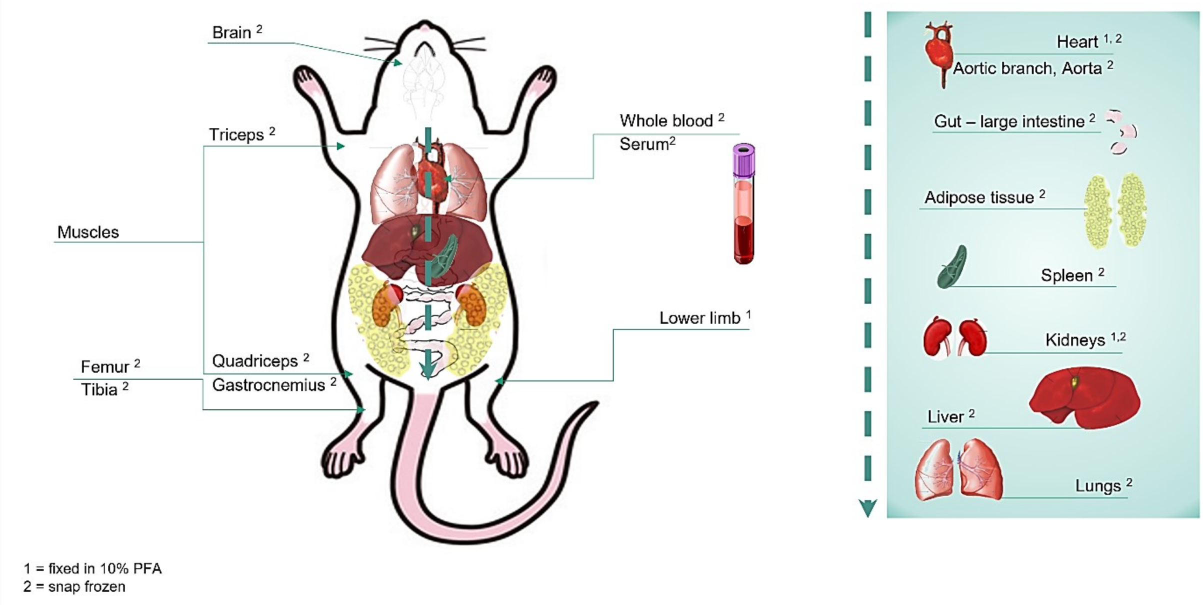


Fig. 4: Illustration of the specimen collection procedure at the end of the study period.

The organs were collected following an established standard operating procedure (SOP, Fig. 4). Blood samples were centrifuged at 2000g for 12 min at room temperature, aliquoted, and stored together with tissue samples at −80°C until analysis. Animals that showed evidence of solid tumors at the time of sacrification were excluded from further analysis.

g. Measurement of RTL in PBMCs and solid tissues

After diluting 100µl of whole blood with 100µl of dH_2O, DNA was isolated with the MagNA Pure LC instrument (Roche, Austria) using the Total Nucleic Isolation Kit (Roche, Austria). Subsequently, RTL of peripheral blood leukocytes was measured by a quantitative real-time PCR (qPCR) method that is based on a protocol developed by Cawthon (227). This method uses glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) as a single copy reference gene. All primers have been purchased from Eurofins Genomics (Austria) and their sequence is described below.

Telomere Forward	5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
Telomere Reverse	3'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-5'
GAPDH Forward	5'-CACCTAGACAAGGATGCAGAG-3'
GAPDH Reverse	3'-GCATGACTGGAGGAATCACA-5'

The assay quantifies the ratio of average telomere length (T) to a single copy reference gene (S). The single copy gene GAPDH is used as an amplification control for each sample and to determine genome copies per sample. All qPCR analyses were performed on the Thermocycler CFX384 Touch™ (Biorad, Germany). Each run included a standard curve made by dilutions of isolated and pooled DNA from 21 different rat blood samples, to determine the quantity of the targeted templates. The RTL is calculated as the ratio of telomere quantity to single-copy reference gene quantity (T/S ratio).
For the analysis of RTL in solid tissues, approximately 10 mg of tissues were homogenized using a MagnaLyser (Roche, Austria) instrument. From these lysates, DNA was extracted, and RTL was analyzed using the same protocols as for blood (Fig. 5).

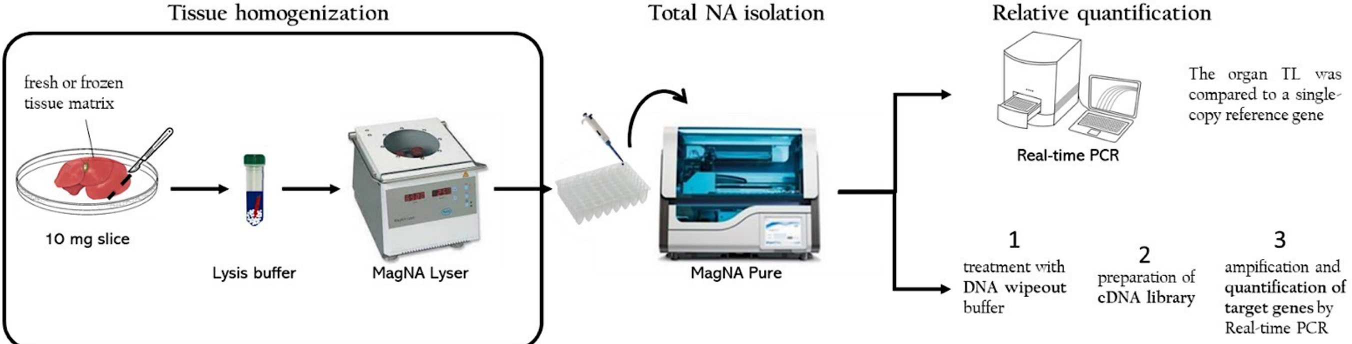


Fig. 5: General workflow for preparation of tissue homogenates and nucleic acids (NA) isolation. The total NA extracts from each solid organ were aliquoted and used for the quantification of relative telomere length as ratio of telomere quantity to a single copy reference gene quantity (T/S ratio). A second aliquot of NA solution was treated with DNA wipeout buffer. The RNA samples were then retro transcribed into cDNA. The cDNA was amplified and quantified by Real-time PCR with TaqMan probes for the gene expression analysis of *tert*, *terf-1* and *terf-2*.

h. Measurement of mRNA gene expression of *tert*, *terf-1*, and *terf-2* in tissues

From all solid tissues, the mRNA expression of TERT, TERF-1, and TERF-2 was analyzed. For this purpose, 10 mg of tissue were homogenized with the MagnaLyser (Roche, Austria), and nucleic acids were extracted from the lysate with the Total Nucleic Isolation Kit (Roche, Austria) on a MagNA Pure LC instrument (Roche, Austria). Subsequently, samples were prepared for analysis using RNA preparation kits from Qiagen as recommended by the manufacturer (Fig. 5). After reverse transcription of mRNA into cDNA using the cDNA library Kit (Qiagen, Germany), quantitative real-time PCR with TaqMan probes (Life Technologies, California) was performed. Each target gene expression was calculated with the ΔΔCT method using β-actin as a reference gene. The probes sequence is the following:

β-actin	5'-CTTCCTTCCTGGGTATGGAATCCTG-3'
Tert	5'-ATCGAGCAGAGCATCTCCATGAATG-3'
Terf-1	5'-AAAACAGACATGGCTTTGGGAAGAA-3'
Terf-2	5'-GAGAAAATTTAGACTGTTCCTTTGA-3'

i. Assessment of oxidative-nitrosative stress and inflammation

One hundred µl of serum was used to measure circulating levels of Oxidized Low-Density Lipoprotein (oxLDL) with a commercial Sandwich ELISA (USCN Life Sciences, USA). The concentration of oxLDL in the samples is calculated from a standard curve, as recommended by the manufacturer. NO

was estimated in serum (100 µl) by measuring the degradation products nitrate (NO_3^-) and nitrite (NO_2^-) using a commercial photometric method (NO quantification kit, Active Motif, California) on a FlexStation3 (Molecular devices, California). In addition, plasma concentrations of homoarginine (h-arg), and ADMA were quantified by a reverse-phase high-performance liquid chromatography (HPLC) method as described previously (228, 229). Table 1 summarizes the analytical performance characteristic for all analytes with the assays used.

Table 1: Description of performance characteristics for the commercial assays used in the study.

Analyte	Test Method	Detection Range	Sensitivity	Intra-assay Precision	Inter-assay Precision
			Stress markers		
oxLDL	Double-antibody Sandwich	31.2-2,000pg/mL	<13.9pg/mL	[1]CV<10%	<12%
nitrite/nitrate	Photometric assay	2-55 µM	< 1 µM		
			Cytokines		
TNF- α	Multiplex immunoassay	4-920,000pg/mL	0-4pg/mL	<15%	<15%
IFN-γ	Multiplex immunoassay	9-840,000pg/mL	0-6pg/mL	<15%	<15%
IL-1β	Multiplex immunoassay	15-965,000pg/mL	1-1pg/mL	<15%	<15%
IL-2	Multiplex immunoassay	4-920,000pg/mL	1-9pg/mL	<15%	<15%
IL-5	Multiplex immunoassay	2-410,000pg/mL	0-3pg/mL	<15%	<15%
IL-6	Multiplex immunoassay	4-920,000pg/mL	1-9pg/mL	<15%	<15%
IL-10	Multiplex immunoassay	9-840,000pg/mL	1-6pg/mL	<15%	<15%
IL-12	Multiplex immunoassay	9-840,000pg/mL	5-9pg/mL	<15%	<15%
IL-17	Multiplex immunoassay	3-715,000pg/mL	0-2pg/mL	<15%	<15%
			Chemokines		
RANTES	Multiplex immunoassay	85-5350,000pg/mL	6-1pg/mL	<15%	<15%
IP-10	Multiplex immunoassay	4-920,100pg/mL	0-9pg/mL	<15%	<15%

MIP-1 α	Multiplex immunoassay	2-39,500pg/mL	1-0pg/mL	<15%	<15%
MCP-1	Multiplex immunoassay	18-375,000pg/mL	10-9pg/mL	<15%	<15%
MCP-3	Multiplex immunoassay	3-514,200pg/mL	1-1pg/mL	<15%	<15%

[1]CV (%) = SD/meanX100

Matrix: serum

A comprehensive panel of inflammatory markers and chemokines was analyzed in 25 µl of serum with a preconfigured multiplex immunoassay (ThermoFisher Scientific, Austria) using the BioPlexTM 200 detection system (Bio-Rad, Austria). This assay included the following analytes: regulated and normal T-cell expressed and secreted (RANTES), eotaxin, macrophage inflammatory protein 1α (MIP-1 α), monocyte chemoattractant proteins 1 and 3 (MCP-1 and 3), tumor necrosis factor α (TNF- α), interferon γ (IFN-γ), IFN-γ-inducible protein (IP-10), and interleukins (IL-1β, IL-2, IL-5, IL-6, IL-10, IL-12, IL-17). The BioPlexTM 200 detection system is equipped with two lasers, one to distinguish the spectral signature of each bead and the second to quantify the amount of streptavidin-R-phycoerythrin (RPE) fluorescence, which is proportional to the amount of protein present in the sample (Table 1, Fig. 6).

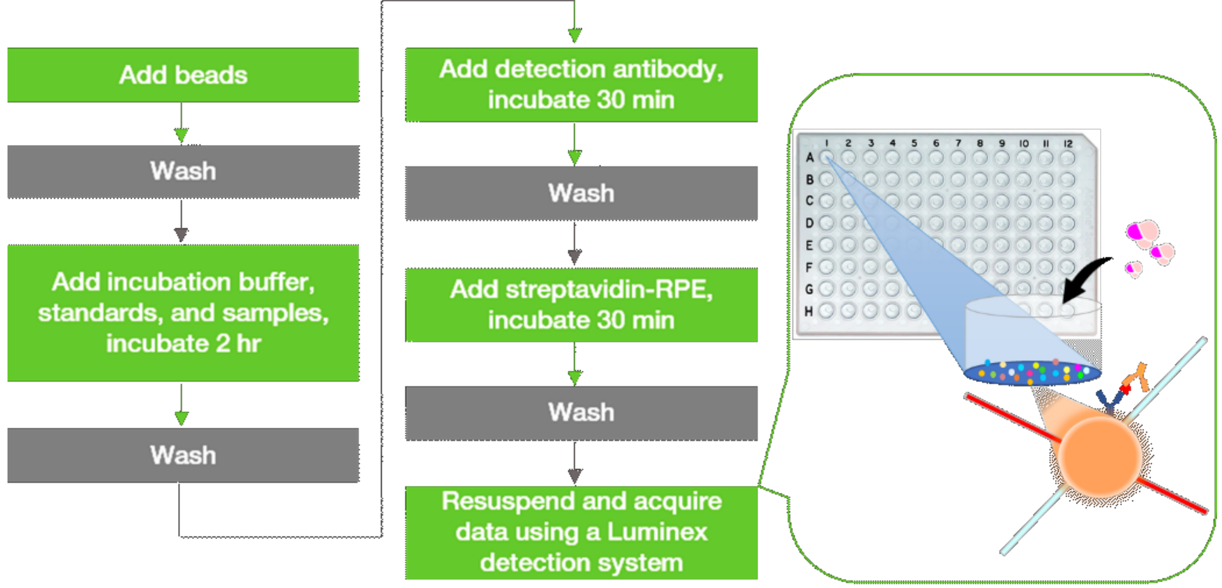


Fig. 6: General workflow for the Invitrogen ProcartaPlex Multiplex Immunoassay.

j. Assessment of lipid metabolism and adipocytokines

The serum lipid profile was determined on a fully automated Olympus AU640 analyzer (Olympus, Hamburg, Germany) using established commercial enzymatic assays from Diasys (Holzheim,

Germany). Briefly, total cholesterol (TC), triglycerides (TG), non-esterified fatty acids (NEFA), and HDL cholesterol (HDL-C; homogeneous assay) were measured using enzymatic methods and reagents from Diasys (Holzheim, Germany). The instrument was calibrated using secondary standards from Roche Diagnostics (Mannheim, Germany; for TC, TG) and Dyasis (Holzheim, Germany; for FC, PL). The coefficients of variation (between days) were < 5%. IGF-1, leptin, and adiponectin were determined in serum by commercial sandwich ELISAs (Demeditec Diagnostics GmbH, Germany) according to the manufacturer's instructions.

k. Statistical analyses

Statistical data analysis was performed using the IBM SPSS v. 26 for Windows software package. Results are shown as mean ± standard deviations (SD). Qualitative variables such as tumor abundance and type were assessed with the Fisher's exact test or the Chi-squared test. Group differences were assessed using the two-tailed Student's *t*-test for dependent or independent samples or the Mann-Whitney *U*-test depending on the distribution of the data. Group comparisons with three or more groups were analyzed using the two-way ANOVA or the Kruskall-Wallis test for independent samples. Correlations between variables were determined by linear regression analysis according to Pearson (r, Pearson Correlation coefficient; p, univariate ANOVA). Data were plotted using Python programming language with Jupyter Notebook within the data science package Anaconda3 for Windows. The level of acceptance of the null hypothesis was set at $p = 0.05$.

Results

6) Telomere Biology (Paper II-III)

I. Descriptive results and body composition

From the 120 rats included, 6 animals had to be sacrificed before the end of the study because of illness. Additional 18 animals developed tumors and, thus, were excluded from the final analysis. Tumors were more frequent in animals on high-fat diet rather than on standard diet (16 vs. 2 rats, p = 1,289e-4). The tumors in the high-fat diet group were heterogeneous compared to the normal diet group ($p < 0.001$), as masses were found in the breasts, ovaries, and abdomen of obese animals. Regular exercise did not significantly change tumor incidence in both diet groups (coND vs. exeND, p = 0.975; coHFD vs. exeHFD, p = 0.347) nor tumor diversity in the HFD group (p = 0.197). After the exclusion of dropouts, 96 eligible animals were included in the final statistical analyses.

At the end of the 10-month study period, the median body weight of all four experimental groups was significantly higher than in the juvenile baseline group (Fig. 7). Weight gain was higher in the two HFD groups than in ND animals. In non-exercising animals, body weight differed by 104 g. In line with this finding, also the weight of individual organs and tissues, such as the heart, spleen, liver, and visceral fat, were significantly higher in HFD animals (Table 2).

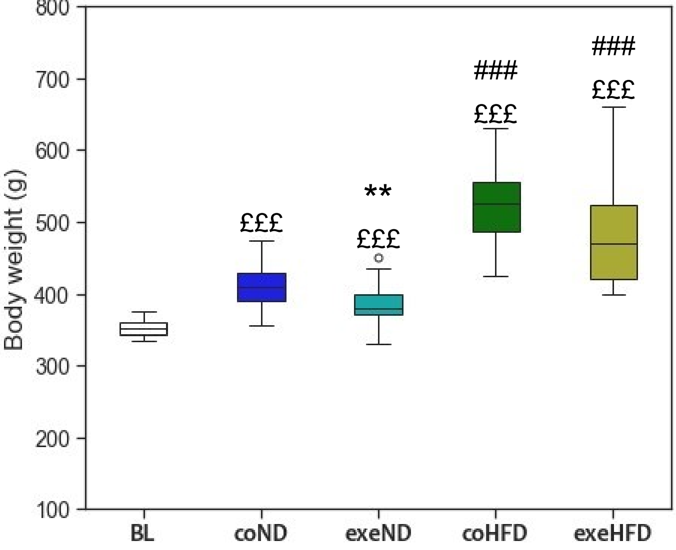


Fig. 7: Box and Whisker Blot of the body weight at the end of the 10 months study period. Outliers are shown as white circles above the box plots. The two-tailed Student's t-test was used for group comparison of independent samples. ***p < 0.01* compared to appropriate sedentary control group; *### p < 0.001* compared to appropriate normal diet control group; *£££ p < 0.001* compared to baseline control group. Modified from (226), Copyright is by © The Author(s) 2021.

The exercise protocol was well tolerated by the animals in both diet groups. Weight gain was significantly lower in exercising ND animals than in their sedentary counterparts *($p < 0.01$)*, whereas the two HFD groups showed no significant difference in final body weight. In the factorial ANOVA, the main effects of diet and exercise on body weight were significant with $F(1, 67) = 80.92$, $p = 3.89e\text{-}13$, and $F(1, 67) = 8.29$, $p = 0.005$, respectively. There was no significant interaction between diet and exercise, $F(1, 67) = 0.138$, $p = 0.712$. Regular moderate exercise induced a higher organ weight of the heart and liver in HFD animals, but not in ND animals (Table 2).

Table 2: Organ weight in female Sprague Dawley rats after 10 months of treadmill exercise.

		BL *n = 24*	**coND** *n* = 22	**exeND** *n* = 22	**coHFD** *n* = 16	**exeHFD** *n* = 12
heart	*average weight*	1.18 ± 0.12	1.31 ± 0.21 £	1.24 ± 0.11	1.40 ± 0.14 £££	1.46 ± 0.19 ###£££
	normalized weight	0.26 ± 0.03	0.28 ± 0.04 £	0.27 ± 0.03	0.30 ± 0.03 £££	0.31 ± 0.03 ###£££
spleen	*average weight*	0.93 ± 0.11	0.98 ± 0.16	0.97 ± 0.15	1.20 ± 0.16###£££	1.18 ± 0.23##£££
	normalized weight	0.20 ± 0.02	0.21 ± 0.03	0.21 ± 0.03	0.24 ± 0.07	0.25 ± 0.04###£££
liver	*average weight*	2.39 ± 0.25	12.53 ± 1.72 £££	12.50 ± 1.80 ££	14.03 ± 2.36 #£££	15.16 ± 4.40 #£££
	normalized weight	2.39 ± 0.25	2.67 ± 0.31 ££	2.56 ± 0.68	2.98 ± 0.52 #£££	3.28 ± 0.89 #£££
visceral fat	*average weight*	5.81 ± 2.07	13.20 ± 5.26 £££	10.46 ± 4.48 £££	40.13 ± 12.81 ###£££	39.46 ± 23.20 ###£££
	normalized weight	0.01 ± 0.006	0.03 ± 0.01 £££	0.03 ± 0.01 £££	0.08 ± 0.018 ###£££	0.07 ± 0.03 ###£££

The organ weight is given in grams. Heart and liver's weight were normalized to total tibia length (cm), while visceral fat's weight was normalized to body weight (g).

Data are presented as mean ± standard deviation * *$p < 0.05$*, ** *$p < 0.01$*, *** *$p < 0.001$* compared to appropriate sedentary control group; # *$p < 0.05$*, ## *$p < 0.01$*, ### *$p < 0.001$* compared to the appropriate normal diet control group; £ *$p < 0.05$*, ££ *$p < 0.01$*, £££ *$p < 0.001$* compared to the baseline control group. Freely taken from (230), Copyright: © 2021 by the authors.

m. Distribution of RTL in blood leucocytes and solid organ tissues in young and adult SD rats

The analysis of RTL showed pronounced interindividual variability across all tissues with the greatest scatter in the large intestine, spleen, and brain (Fig. 8). The juvenile animals in the BL group had a mean RTL in PBMCs of 0.88 ± 0.15. Mean RTLs of the solid tissues ranged from 0.64 ± 0.26 in the large intestine to 1.07 ± 0.22 in skeletal muscle. Liver, skeletal muscle, aorta, and kidney had significantly higher mean RTLs than PBMCs. In contrast, RTL in the large intestine and lung was lower than in PBMCs. In aged animals, the distribution of RTL across organs was comparable to juvenile animals. Similar to young animals, mean RTL in the large intestine and lung was significantly lower than in PBMCs of aged animals. Also, in the aortic tissue of aged animals, RTL was significantly lower than in PBMCs (Fig. 8).

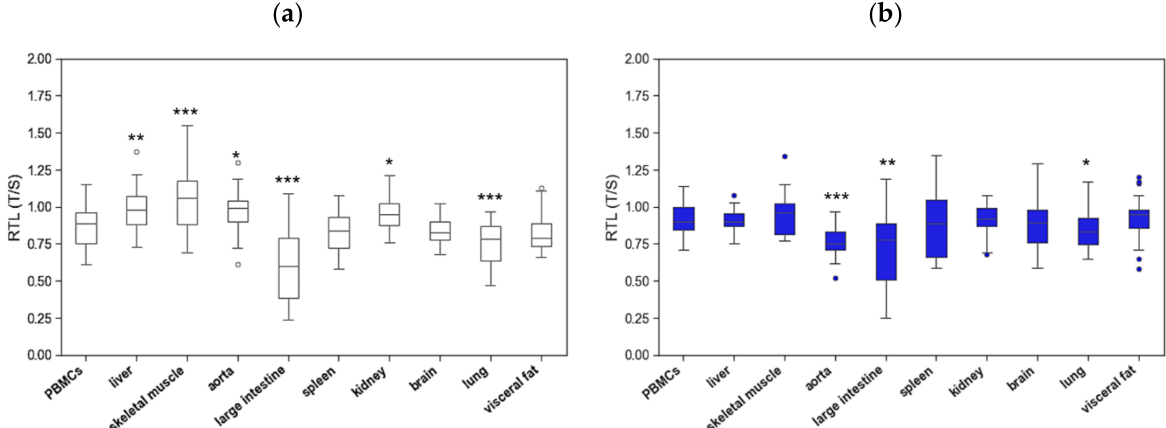


Fig. 8: Relative telomere length (RTL) of peripheral blood mononuclear cells (PBMCs) and nine different solid organs including liver, skeletal muscle, aorta, large intestine, spleen, kidney, brain, lung, and visceral fat. (a) young rats, (b) aged adult rats. Outliers are shown as white circles above the box plots. RTL is expressed as ratio of average telomere length to the reference gene GAPDH. The paired samples *t*-test was used for tissue-type comparison. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. PBMCs RTL. Modified from (1), Copyright: © 2021 by the authors.

To test the hypothesis that RTL in PBMCs represents the situation in other organs, RTL from PBMCs and solid organs were correlated in young and aged animals. As shown in Fig. 9, there was no consistent correlation between RTL in PBMCs and solid organs. Positive correlations were found between RTL in PBMCs and liver, skeletal muscle, and kidney. Inverse correlations were found between RTL in PBMCs and RTL in the large intestine and aorta. RTL of all other tissues was not significantly correlated to that of PBMCs.

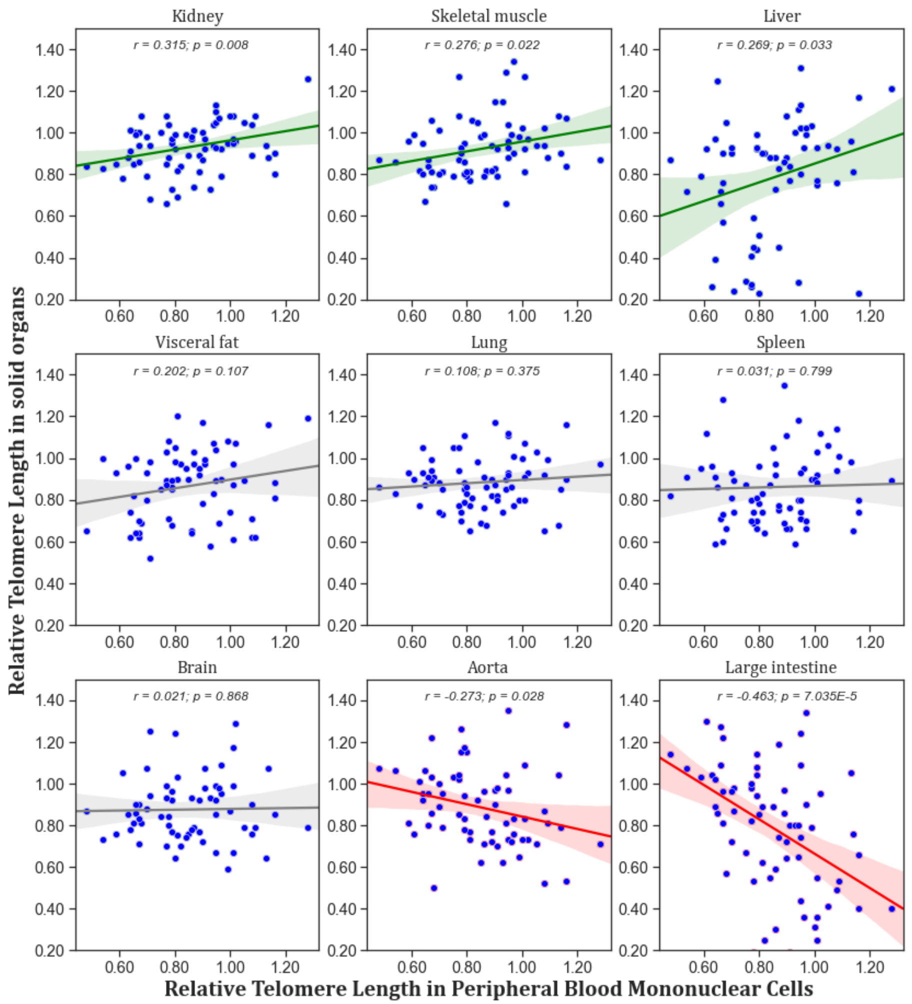


Fig. 9: Correlation between RTL in PBMCs and RTL in different organs isolated from adult rats (n = 72). r – Pearson correlation coefficient (r values above 0.250 are shown in green, r values below -0.250 are shown in red); p – p value. Each panel includes the regression line with the respective 95% confidence interval (shaded area). Modified from (1), Copyright: © 2021 by the authors.

To identify age-related differences in RTL in PBMCs and solid tissues, a comparison between RTL of aged and young animals was performed for each tissue type (Fig. 10). The only organ with lower RTL in aged animals was the aorta. Lung and visceral fat tissue showed higher RTL in aged animals. All other organs had comparable RTL in both age groups.

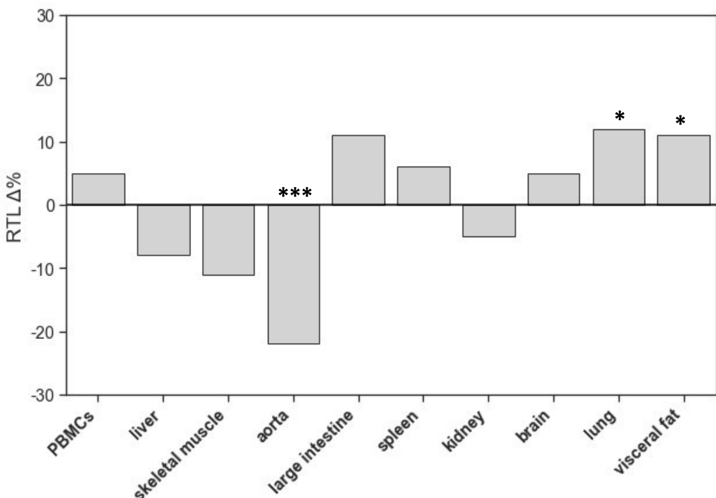


Fig. 10: The impact of age on relative telomere length (RTL) of different tissue types. The boxes represent the delta% variation of RTL in aged adults compared to young rats. * $p < 0.05$; *** $p < 0.001$ vs. young rats. Modified from (1), Copyright: © 2021 by the authors.

n. Telomerase and shelterin mRNA gene expression from different tissue types in young and adult SD rats

The mRNA expression of TERT markedly differed between the tested organs with the highest levels in the liver and kidneys (Fig. 11). In the liver, TERT mRNA expression was 40 times higher than in spleen and lung tissue.

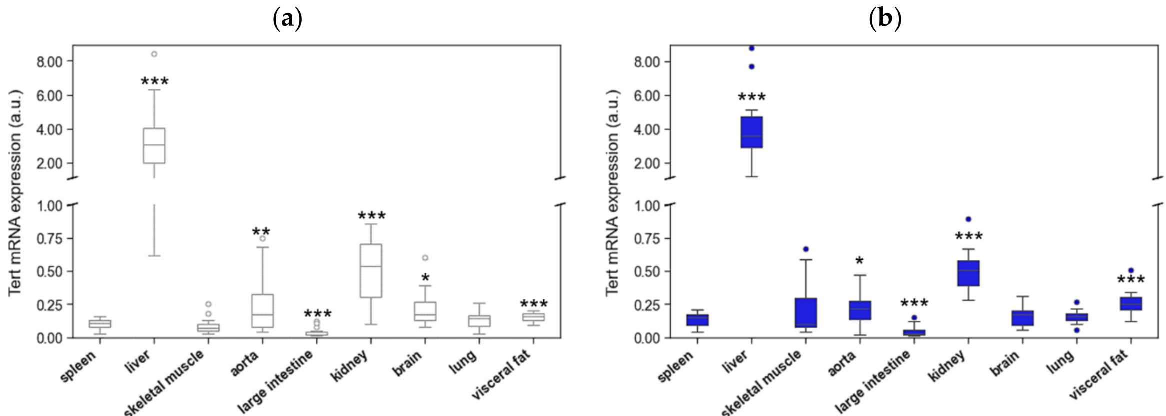


Fig. 11: mRNA expression of TERT in spleen and eight other solid organs including liver, skeletal muscle, aorta, large intestine, kidney, brain, lung, visceral fat. (a) young rats, (b) aged adult rats. Outliers are shown as white circles above the box plots. TERT mRNA expression is shown in arbitrary units. The paired samples *t*-test was used for tissue-type comparison. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. TERT mRNA expression in spleen. Modified from (1), Copyright: © 2021 by the authors.

Age-related differences in TERT mRNA expression were only found in the spleen, skeletal muscle, and visceral fat (Fig. 12).

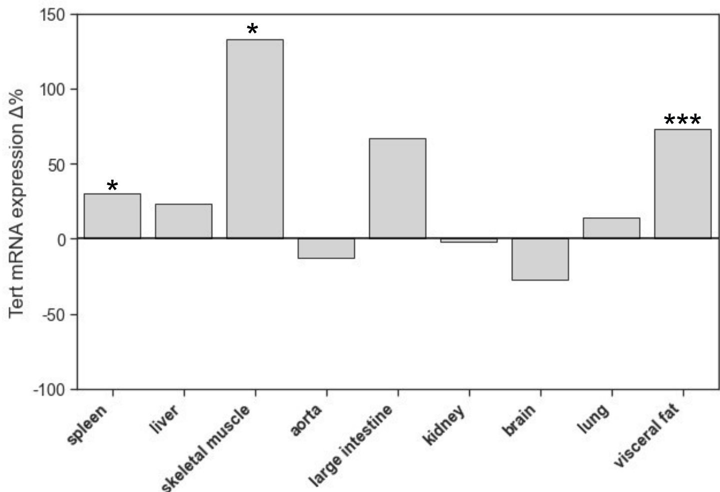
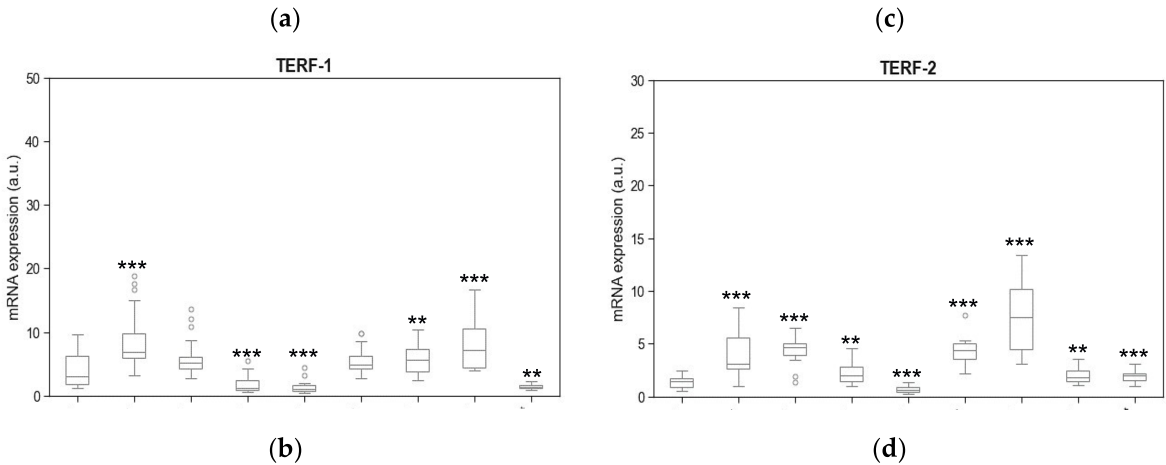


Fig. 12: The impact of age on TERT mRNA expression of different tissue types. The boxes represent the delta% variation of TERT mRNA expression in adults compared to young. * $p < 0.05$; *** $p < 0.001$ vs. young. Modified from (1), Copyright: © 2021 by the authors.

The mRNA expression of TERF-1 and TERF-2 was tissue-specific showing pronounced variation between the different organs and spleen. The highest expression levels of both genes were found in the liver (Fig. 13). In addition, TERF-2 was highly expressed in the brain. In six out of nine tissue types TERF-2 and TERT were positively associated with r = 0.801; p = 4,615e-11 (spleen), r = 0.560; p = 0.00017 (liver), r = 0.707; p = 1,002e-6 (aorta), r = 0.783; p = 1,278e-10 (large intestine), r = 0.748; p = 3,602e-9 (kidney), r = 0.562; p = 0.000106 (lung), but not in skeletal muscle, brain, and visceral fat.



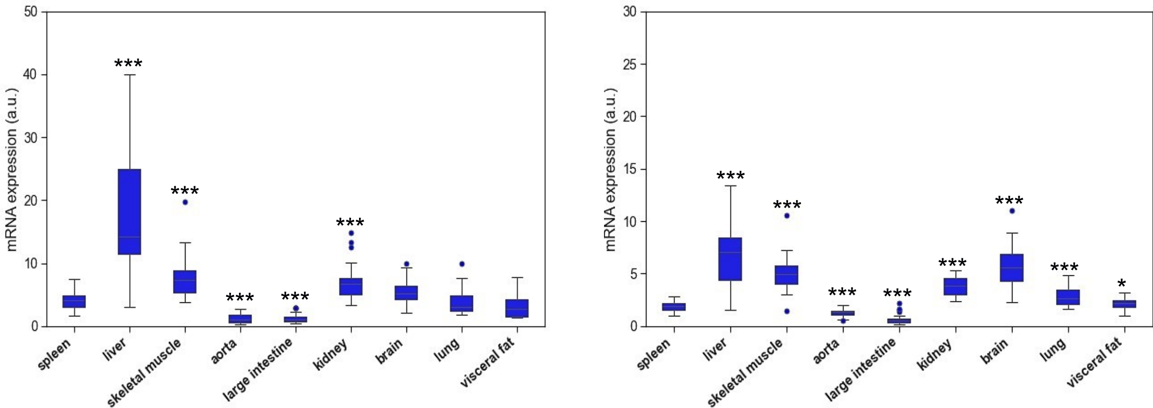


Fig. 13: mRNA expression of TERF-1 and TERF-2 in spleen and eight other solid organs including liver, skeletal muscle, aorta, large intestine, kidney, brain, lung, visceral fat. (a) TERF-1 in young rats, (b) TERF-1 in aged adult rats, (c) TERF-2 in young rats and (d) TERF-2 in aged adult rats. Outliers are shown as white circles above the box plots. The paired samples *t*-test was used for tissue-type comparison. Terf-1 and Terf-2 mRNA expression is shown in arbitrary units. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. TERF-1 or TERF-2 mRNA expression in spleen. Modified from (1), Copyright: © 2021 by the authors.

Age-related differences in mRNA expression of these two shelterins were inconsistent. In aged animals, TERF-1 showed higher mRNA expression levels in the liver, kidneys, and visceral fat but lower levels in the lung. TERF-2 expression was higher in the spleen, liver, and lung of aged animals, whereas the aorta showed a lower expression level (Fig. 14).

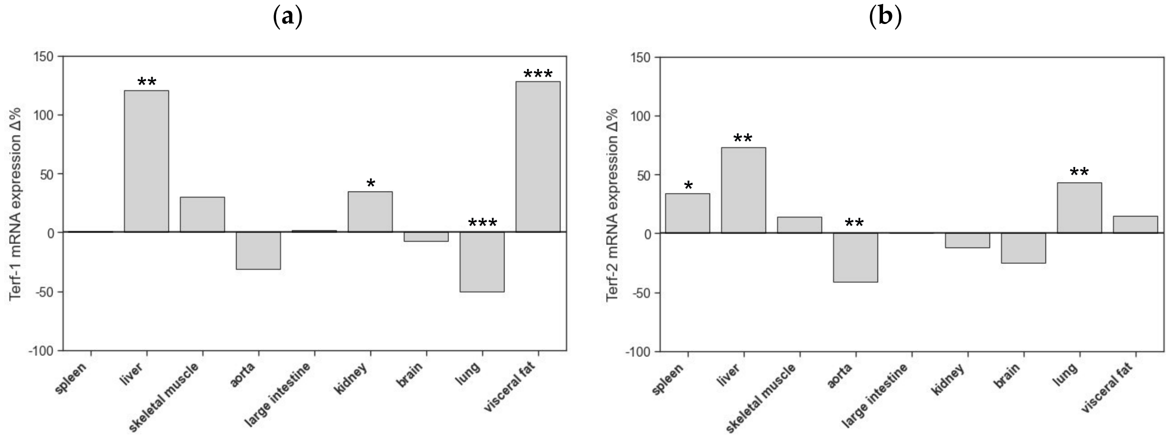


Fig. 14: The impact of age on mRNA expression of (a) TERF-1 and (b) TERF-2 in different solid organs. The boxes represent the delta% variation of mRNA expression of Terf-1 and Terf-2 in aged adult rats compared to juvenile animals. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. juvenile animals. Modified from (1), Copyright: © 2021 by the authors.

o. Impact of high-fat diet on RTL, and mRNA expression of telomere-related genes in different tissue types

Aged animals fed with a 60% fat diet exhibited a comparable organ distribution of RTLs with age-matched animals on a standard diet. Compared to lean rats, obese animals showed an increased RTL in the kidney, while RTL was slightly but significantly reduced in visceral fat (Fig. 15).

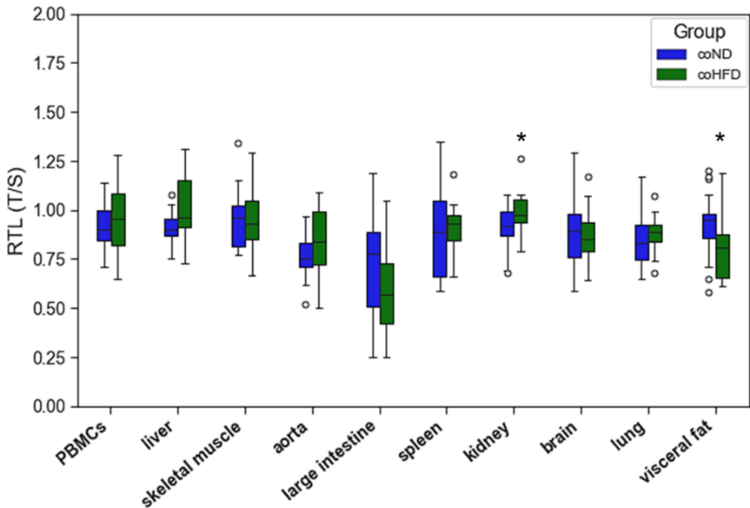
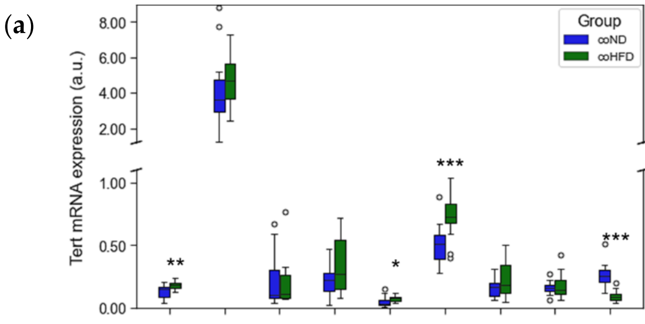


Fig. 15: Distribution of RTL in PBMCs and nine solid organs isolated from lean (coND) and obese rats (coHFD). Outliers are shown as white circles above the box plots. RTL is expressed as ratio of average telomere length to the reference gene GAPDH. The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. * $p < 0.05$ vs. coND. Freely taken from (230), Copyright: © 2021 by the authors.

Significant differences in TERT mRNA expression between obese and lean animals were only found in the spleen, large intestine, kidney, and visceral fat (Fig. 16a). Spleen, large intestine, and kidney showed higher TERT mRNA expression levels in obese compared to lean rats, whereas in visceral fat a lower TERT mRNA expression was observed.

The mRNA expression of both shelterins was significantly reduced in visceral fat of obese rats. Compared to lean animals the mRNA expression of TERF-1 was significantly reduced also in the liver while it was significantly increased in skeletal muscle, aorta, and large intestine (Fig. 16b). The mRNA expression of TERF-2 showed consistently higher levels in five out of nine solid organs of obese rats compared to lean ones (Fig. 16c).



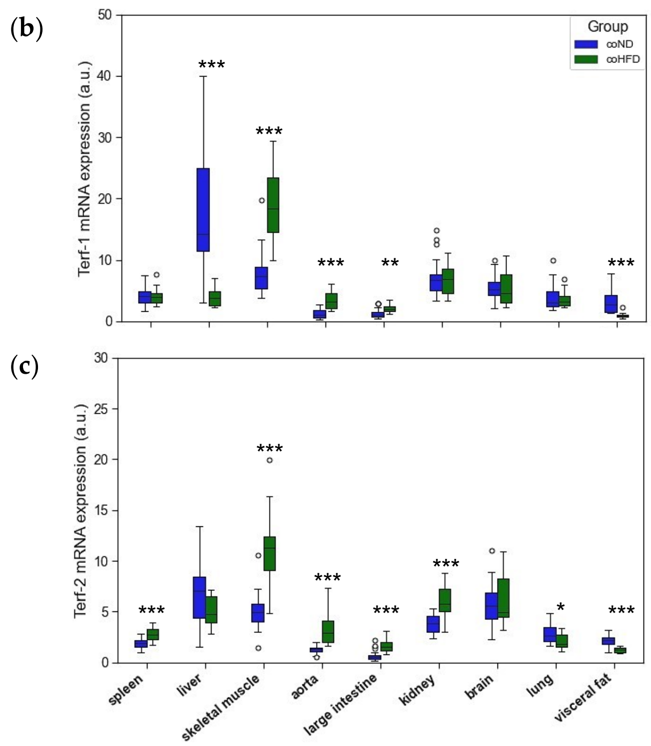


Fig. 16: Differences in the gene expression of (a) TERT, (b) TERF-1, and (c) TERF-2, isolated from different solid organs of lean (coND) and obese rats (coHFD). TERT, TERF-1, and TERF-2, mRNA expression is shown in arbitrary units. Outliers are shown as white circles above the box plots. The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. conD. Freely taken from (230), Copyright: © 2021 by the authors.

p. The impact of exercise on RTL, and mRNA expression of telomere-related genes in different tissue types

Ten months of regular treadmill running had heterogeneous effects on RTL in different tissues with significantly longer telomeres in the aorta and large intestine of trained animals, whereas PBMCs and liver showed lower RTL (Fig. 17). In all other tissues, RTL did not significantly differ between sedentary and exercising animals. The administration of HFD did not substantially change this pattern.

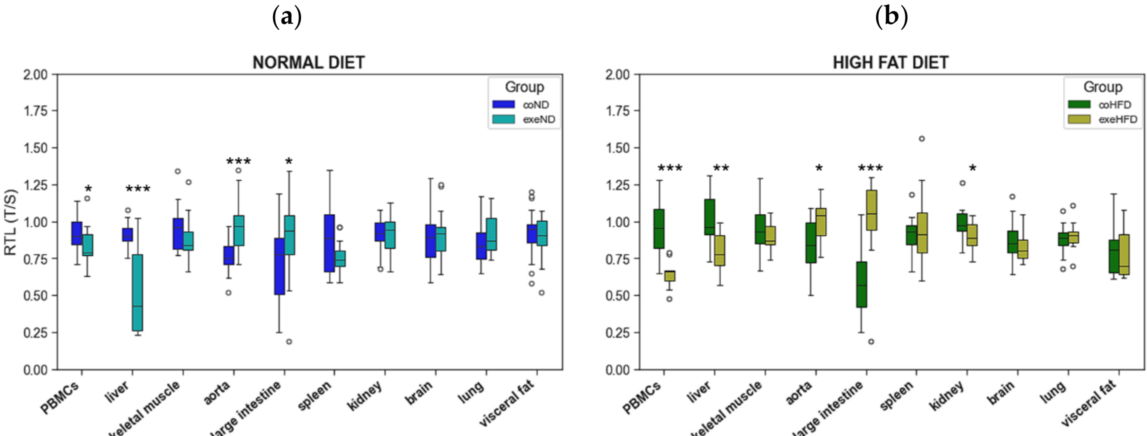


Fig. 17: Comparison of RTL in PBMCs and nine solid organs isolated from exercising and sedentary SD rats that received normal diet or HFD for 10 months. (a) sedentary (coND) vs. exercising (exeND) animals on ND, (b) sedentary (coHFD) vs. exercising (exeHFD) animals on HFD. Outliers are shown as white circles above the box plots. RTL is expressed as ratio of average telomere length to the reference gene GAPDH. The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. respective sedentary controls. Freely taken from (230), Copyright: © 2021 by the authors.

The exercise-induced regulation of mRNA expression of telomerase and shelterin proteins differed profoundly amongst solid organs and also between dietary groups. TERT mRNA expression was increased in the spleen, liver, kidney, and lung of lean exercising animals compared to lean sedentary controls (Fig. 18a). Conversely, in obese exercising animals, TERT expression in the large intestine and kidney was significantly lower than in obese sedentary controls (Fig. 18b).

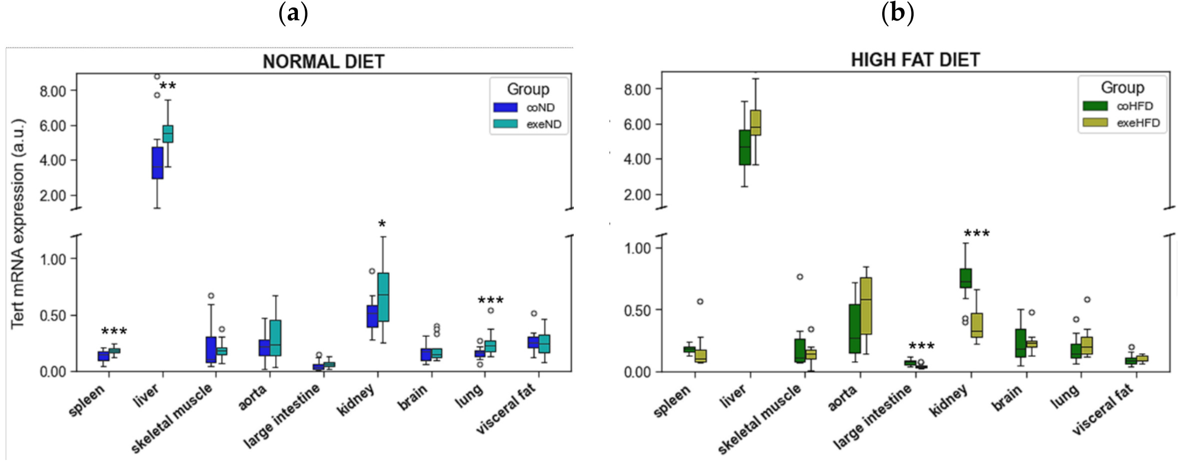


Fig. 18: Comparison of TERT expression in nine solid organs from exercising and sedentary SD rats that received either normal diet or HFD for 10 months. (a) sedentary lean animals (coND) vs. exercising lean animals (exeND), (b) sedentary obese animals (coHFD) vs. exercising obese animals (exeHFD). Outliers are shown as white circles above the box plots. TERT mRNA expression is shown in arbitrary units. The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. respective sedentary controls. Freely taken from (230), Copyright: © 2021 by the authors.

TERF-1 mRNA expression was significantly reduced in the liver, lung, and visceral fat but increased in skeletal muscle, aorta, and large intestine of exercising lean rats compared to their sedentary counterpart (Fig. 19a). In exercising obese animals instead, TERF-1 mRNA expression was increased in spleen,

liver, large intestine, and kidney, but reduced in skeletal muscle compared to sedentary obese animals (Fig. 19c).

TERF-2 mRNA expression was significantly increased in five out of nine solid organs of exercising lean rats compared to their sedentary counterpart (Fig. 19b). In exercising obese animals instead, TERF-2 mRNA expression was increased in liver but reduced in skeletal muscle and kidney when compared to sedentary obese animals (Fig. 19d).

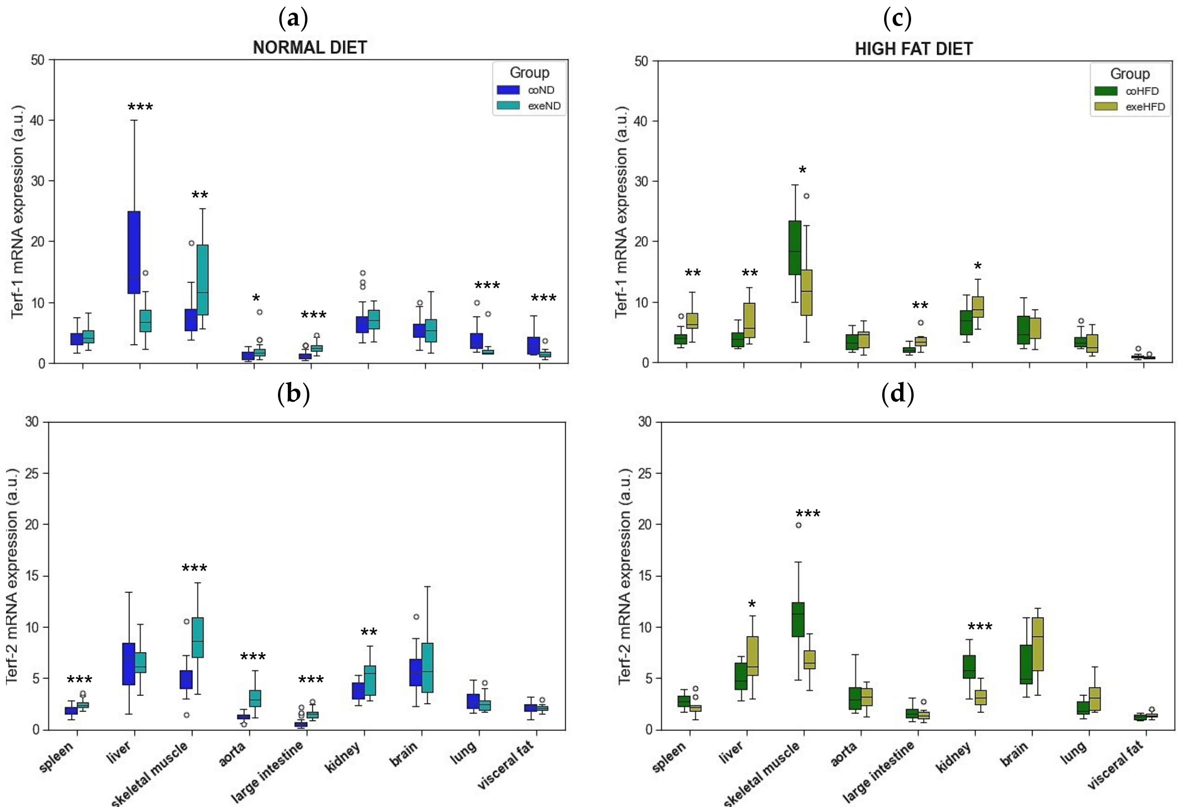


Fig. 19: Comparison of TERF-1 and TERF-2 expression in nine solid organs from exercising and sedentary SD rats that received either normal diet or HFD for 10 months. (a) TERF-1 in sedentary lean animals (coND) vs. exercising lean animals (exeND), (b) TERF-2 in sedentary lean animals (coND) vs. exercising lean animals (exeND) (c) TERF-1 in sedentary obese animals (coHFD) vs. exercising obese animals (exeHFD), (d) TERF-2 in sedentary obese animals (coHFD) vs. exercising obese animals (exeHFD). Outliers are shown as white circles above the box plots. TERF-1 and TERF-2 mRNA expression is shown in arbitrary units. The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. respective sedentary controls. Freely taken from (230), Copyright: © 2021 by the authors.

Summarizing all the results from sedentary and exercising lean animals in the effect matrix (left column), it becomes clear that only in the aorta and large intestine an increase of one or more telomere-regulating genes was associated with an increase in RTL (Fig. 20). All other differences in mRNA expression of telomere-regulating genes were unrelated to RTL. Similar to exercise, also HFD failed to induce systematic effects on RTL and telomere-regulating genes. Only in kidney and visceral fat of obese sedentary animals, did RTL and telomere-regulating genes show changes directed in the same way. However, the changes in both tissues pointed in opposite directions. An interaction between HFD and exercise was only observed in kidneys, where exercising obese rats exhibited a similar RTL to

sedentary lean controls (Fig. 20). Also, both exercising groups show reductions in hepatic RTL, but inconsistent changes in the hepatic expression of telomere-regulating genes.

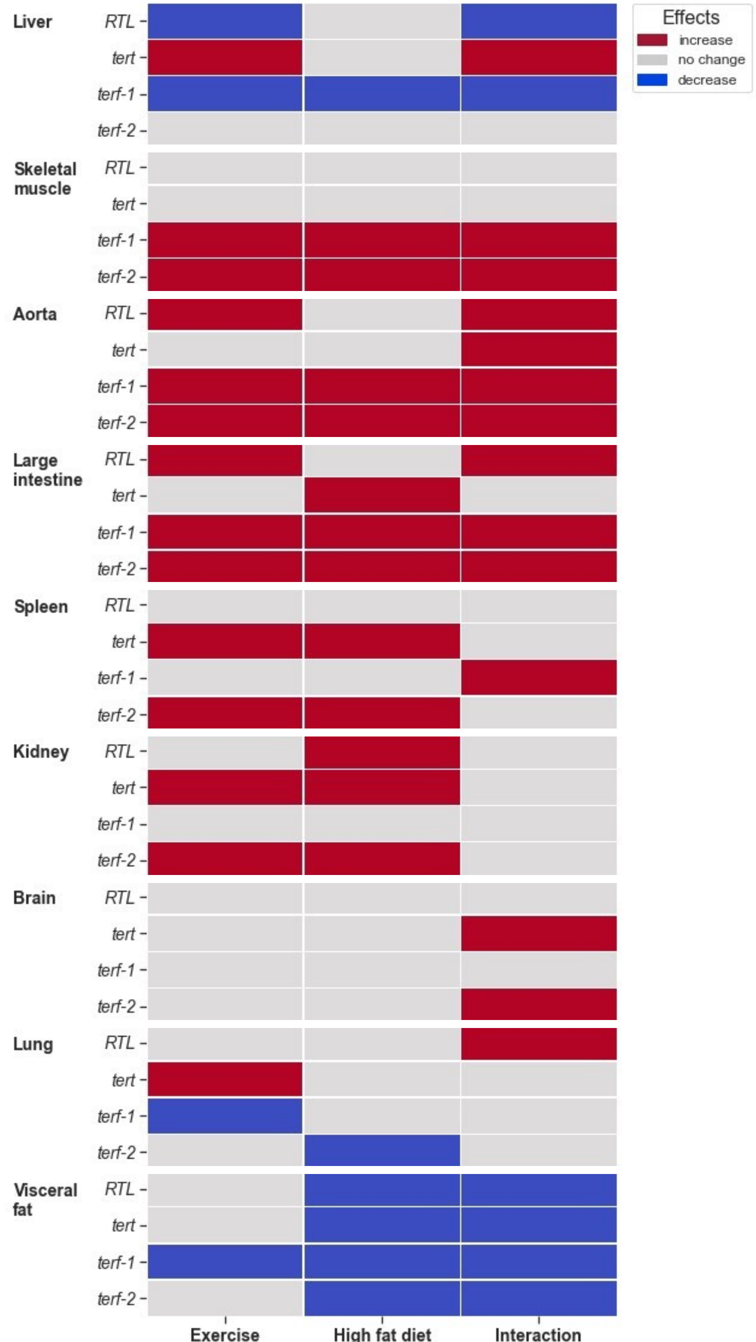


Fig. 20: Effects matrix that visualizes the effects of exercise, HFD, and the interaction of both lifestyle factors on RTL and the mRNA expression of telomere-regulating genes. The two-tailed Student's t-test or the Mann-Whitney U-test were used for group comparison of independent samples. Significant effects are shown in color and refer to the coND group ($p \leq 0.05$). Grey boxes indicate the lack of a significant effect. Freely taken from (230), Copyright: © 2021 by the authors.

q. Correlation analysis

To further explore our hypothesis that lifestyle factors can modify RTL through regulatory effects on the expression of telomere-regulating genes, we performed correlation analyses that included the animals from all 4 intervention groups. Fig. 21 illustrates that RTL was not consistently correlated to any of the telomere-regulating genes. For example, in kidney (R = 0.337; p = 0.004) and visceral fat (R = 0.337; p = 0.004) RTL and TERT mRNA expression were positively correlated, whereas large intestine (R = -0.252; p = 0.036) and spleen (R = -0.263; p = 0.028) showed the opposite. Likewise, RTL and TERF-2 were positively correlated in aorta (R = 0.373; p = 0.002), kidney (R = 0.318; p = 0.007) and visceral fat (R = 0.332; p = 0.007), but negatively correlated in liver (R = -0.247; p = 0.053).

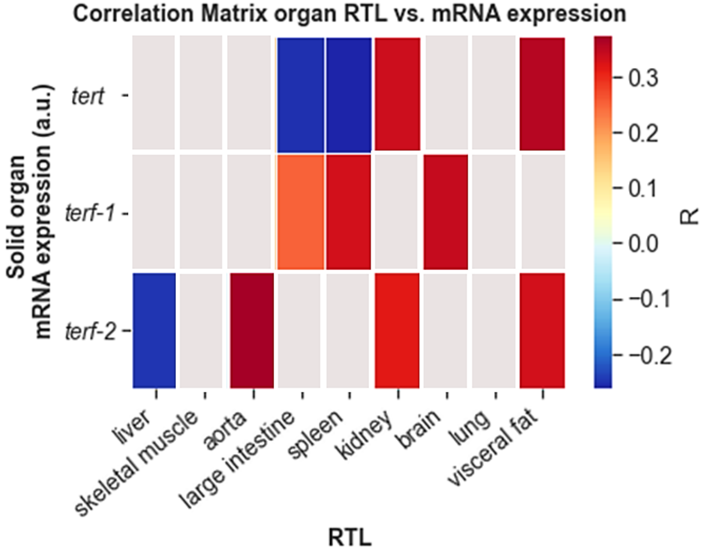


Fig. 21: Correlation matrix for RTL and telomere-regulating genes in 9 solid tissue types. The color bar on the right side of the figure shows the Pearson correlation coefficient as R. Only significant correlations are shown in color (p ≤ 0.05). Grey boxes indicate the lack of significant correlation. Freely taken from (230), Copyright: © 2021 by the authors.

7) Serum Biomarkers (Paper IV)

r. The lipid profile

After 10 months of intervention, average TC and TG concentrations were significantly higher compared to baseline, regardless of diet and exercise (Fig. 22a-b). Obese animals showed additional alterations of the lipid profile beyond simple age-related changes. TG and NEFA were significantly higher than in lean animals, whereas non-HDL-C was lower (Fig. 22b, c, e). Exercise induced a decrease in TC and HDL-C in obese, but not lean animals (Fig. 22a, d). All other lipid parameters were comparable between sedentary and exercising animals in both diet groups. In an attempt to explore potential mechanisms that mediate the changes in plasma lipids, correlation analyses have been performed. At the study end body

weight was positively correlated with TG (r = 0.598; p < 0.001), NEFA (r = 0.238; p = 0.025) and non-HDL-C (r = 0.271; p = 0.010), whereas TC (r = − 0.291; p = 0.006) and HDL-C (r = − 0.365; p < 0.001) were inversely correlated to adiponectin.

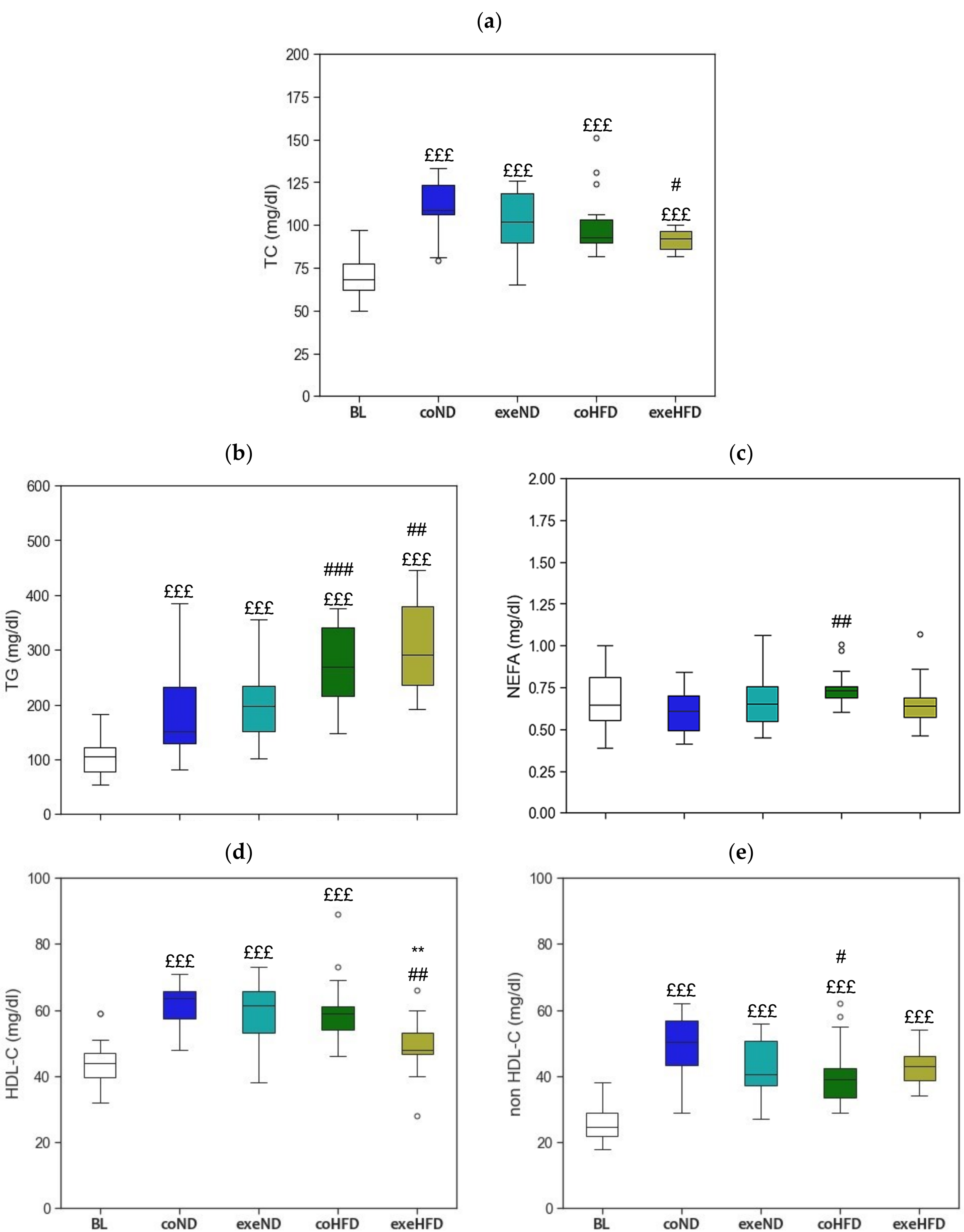


Fig. 22: Box and Whisker plots of the lipid profile (a-e) after the 10 months study period. The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. ***p < 0.01* compared to appropriate sedentary control group; *# p < 0.05, ## p < 0.01, ### p < 0.001* compared to appropriate normal diet control group; *£££ p < 0.001* compared to baseline control group. Modified from (226), Copyright is by © The Author(s) 2021.

s. Nitric oxide metabolism and oxidative-nitrosative stress

Advanced age, obesity, and physical inactivity are known modulators of the equilibrium between oxidant and antioxidant compounds, which may alter lipid peroxidation and NOS function. Therefore, we analyzed the effects of HFD and exercise on oxLDL and NOx. Figure 19 shows that age and diet had a substantial effect neither on oxLDL nor on NOx (Fig. 23a-b). Exercise instead altered both biomarkers significantly. After 10 months of regular training, oxLDL was increased in ND and HFD by 44% and 68%, respectively. In contrast, NOx was markedly lower in exercising animals with the lowest concentrations in the HFD group. To corroborate the NOx results, we also measured the non-proteogenic amino acid h-arg, a substrate of NOS for the production of NO, and ADMA, a competitive inhibitor of NOS. h-arg was significantly higher in HFD animals than in ND and baseline controls (Fig. 23c). Exercise increased h-arg in lean animals, but not in obese animals. The serum concentrations of ADMA were highest at baseline and decreased with age. This decrease was less pronounced in obese than in lean animals. Exercise reduced ADMA in the HFD group, but not in the ND group (Fig. 23d).

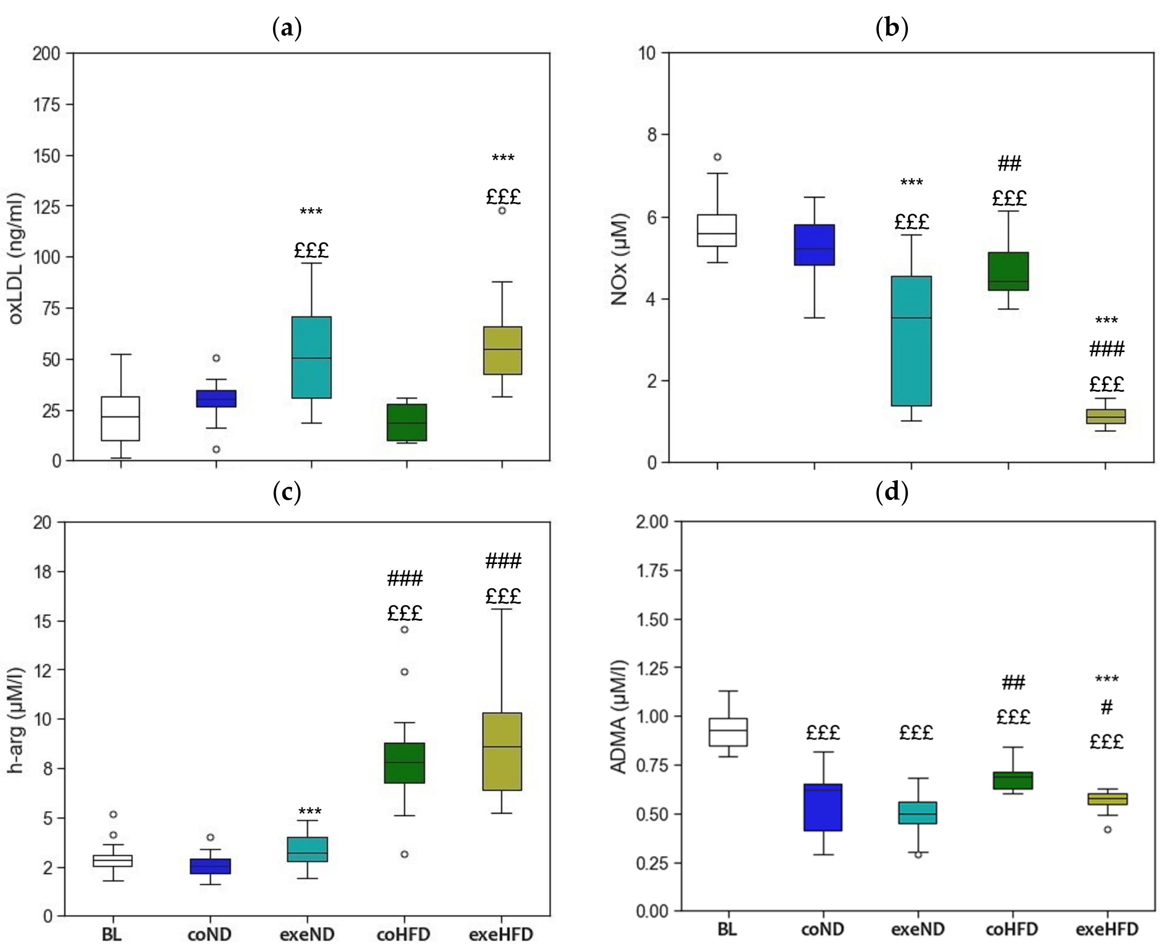


Fig. 23: Box and whisker plots of (a) oxidized LDL, (b) nitric oxide metabolites, (c) homo-arginine, and (d) ADMA. The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. $^{***}p < 0.001$ compared to appropriate sedentary control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to appropriate normal diet control group; £££ $p < 0.001$ compared to baseline control group. Modified from (226), Copyright is by © The Author(s) 2021.

Considering that oxidative stress and NOS function are linked, we performed linear regression analyses that showed an inverse association between oxLDL and NOx (Fig. 24a). An inverse relationship was also found between h-arg and NOx (Fig. 24b). ADMA was positively related to NOx with r = 0.401; p < 0.001 and but inversely associated with oxLDL with r = − 0.338; p < 0.001.

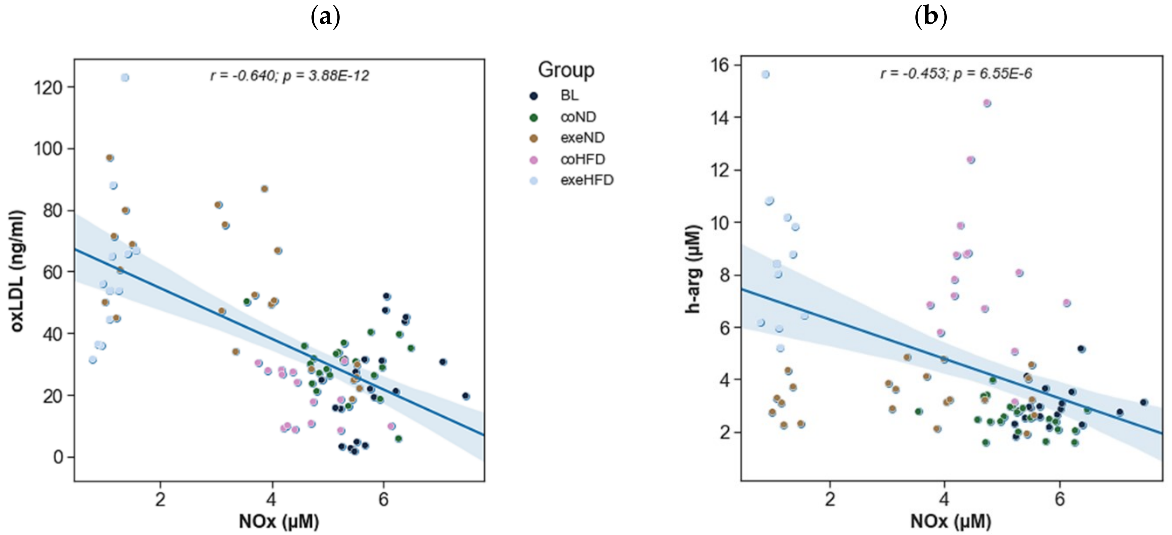


Fig. 24: Simple scatter dot plots of the linear regression analysis between (a) oxidized LDL-C and nitric oxide metabolites with $r = -0.640$ and $p = 3.88e\text{-}12$ and between (b) h-arg and nitric oxide metabolites $r = -0.453$ and $p = 6.55e\text{-}6$. R = Pearson correlation coefficient; p-value = significance level. Modified from (226), Copyright is by © The Author(s) 2021.

Table S1: Additional serum biomarkers in female Sprague Dawley rats after 10 months of treadmill exercise.

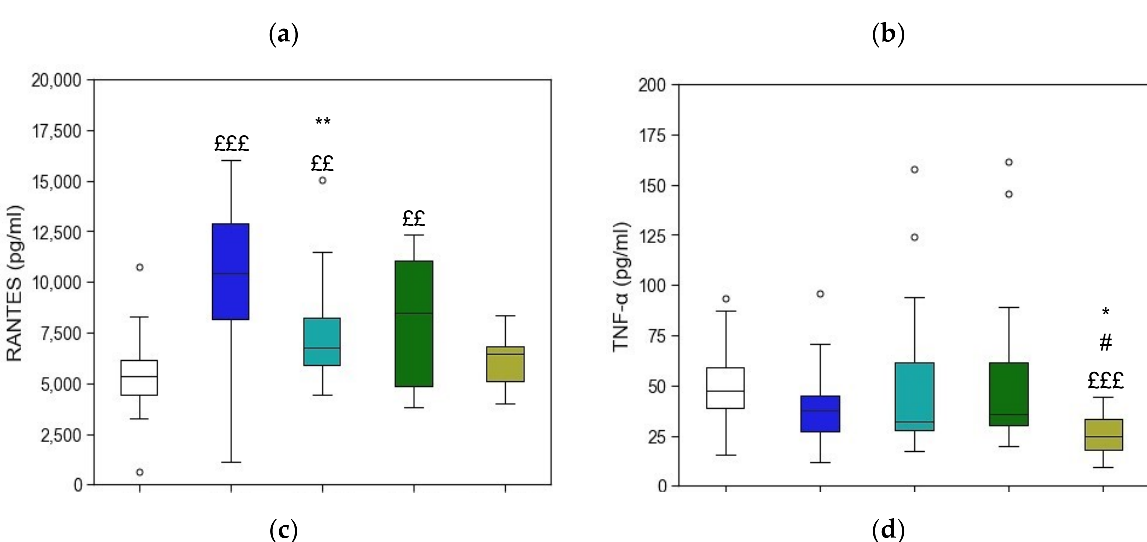
	BL *n = 24*	coND *n* = 22	exeND *n* = 22	coHFD *n* = 16	exeHFD *n* = 12
SDMA	0.43 ± 0.08	0.27 ± 0.11 $^{£££}$	0.28 ± 0.08 $^{£££}$	0.33 ± 0.04 $^{\#£££}$	0.20 ± 0.04 $^{***\#\#£££}$
Resistin	14.97 ± 5.15	8.16 ± 3.45 $^{£££}$	7.72 ± 2.94 $^{£££}$	7.78 ± 3.86 $^{£££}$	7.49 ± 3.99 $^{£££}$
Eotaxin (CCL11)	230.53 ± 46.15	420.79 ± 104.54 $^{£££}$	335.76 ± 94.67 $^{**£££}$	372.79 ± 143.41 $^{£££}$	294.85 ± 150.66
MIP-1α (CCL3)	18.42 ± 5.05	18.96 ± 5.06	22.47 ± 9.27	25.68 ± 16.33	17.66 ± 4.07 *
IFN-γ	38.36 ± 11.58	46.20 ± 31.04	44.22 ± 28.37	42.11 ± 22.80	53.36 ± 39.07
IP-10 (CXCL10)	109.25 ± 33.01	153 ± 35.70 $^{£££}$	163.78 ± 46.04 $^{£££}$	125.05 ± 51.60	118.30 ± 23.12 $^{\#\#}$
IL-5	79.43 ± 29.89	31.59 ± 17.50 $^{£££}$	38.57 ± 18.05 $^{£££}$	43.41 ± 17.29 $^{\#£££}$	37.27 ± 21.91 $^{*£££}$
IL-6	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
IL-10	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
IL-12	58.62 ± 17.45	58.07 ± 27.91	121.53 ± 83.54 $^{**£££}$	52.18 ± 25.04	45.72 ± 21.98
IL-17	21.79 ± 8.02	18.73 ± 11.90	22.45 ± 15.95	11.28 ± 3.91 $^{£££}$	16.37 ± 11.40
MMPs activity	10881 ± 1177	12136 ± 1488 $^{££}$	14092 ± 1832 $^{***£££}$	11876 ± 2026	9966 ± 2943 $^{\#\#\#}$

SDMA is expressed in µM, while resistin in ng/ml. All other cytokines and chemokines are expressed in pg/ml.

Data are presented as mean ± standard deviation * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to appropriate sedentary control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the appropriate normal diet control group; £ $p < 0.05$, ££ $p < 0.01$, £££ $p < 0.001$ compared to the baseline control group. Modified from (226), Copyright is by © The Author(s) 2021.

t. The impact of exercise and high-fat diet on cytokines and chemokines

Dyslipidaemia and oxidative-nitrosative stress are established drivers of chronic systemic inflammation, an important factor in the pathogenesis of atherosclerosis (231, 232). To study the immunological response of HFD and regular exercise, we analyzed a broad panel of pro-and anti-inflammatory cytokines (Fig. 25). Regular treadmill exercise reduced the serum concentrations of the pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-2 in rats consuming HFD, but not those fed ND (Fig. 25b, e, f). All other cytokines were not significantly affected by exercise. Age and diet alone had no significant effects on the serum concentrations of the measured pro-and anti-inflammatory cytokines. Chemokines are secreted signaling proteins that mediate the migration of immune cells in response to pro-inflammatory cytokines (233). In the present study, several pro-inflammatory chemokines, including RANTES, MCP-1, and 3, were measured. In 14-month-old sedentary lean and obese animals, the average serum concentrations of RANTES and MCP-3 were significantly higher than in young baseline controls (Fig. 25a, d). Regular exercise attenuated this age-related increase irrespective of diet. MCP-1 showed similar trends, but due to greater inter-individual variability of this marker, significant effects were present only in obese animals (Fig. 25c).



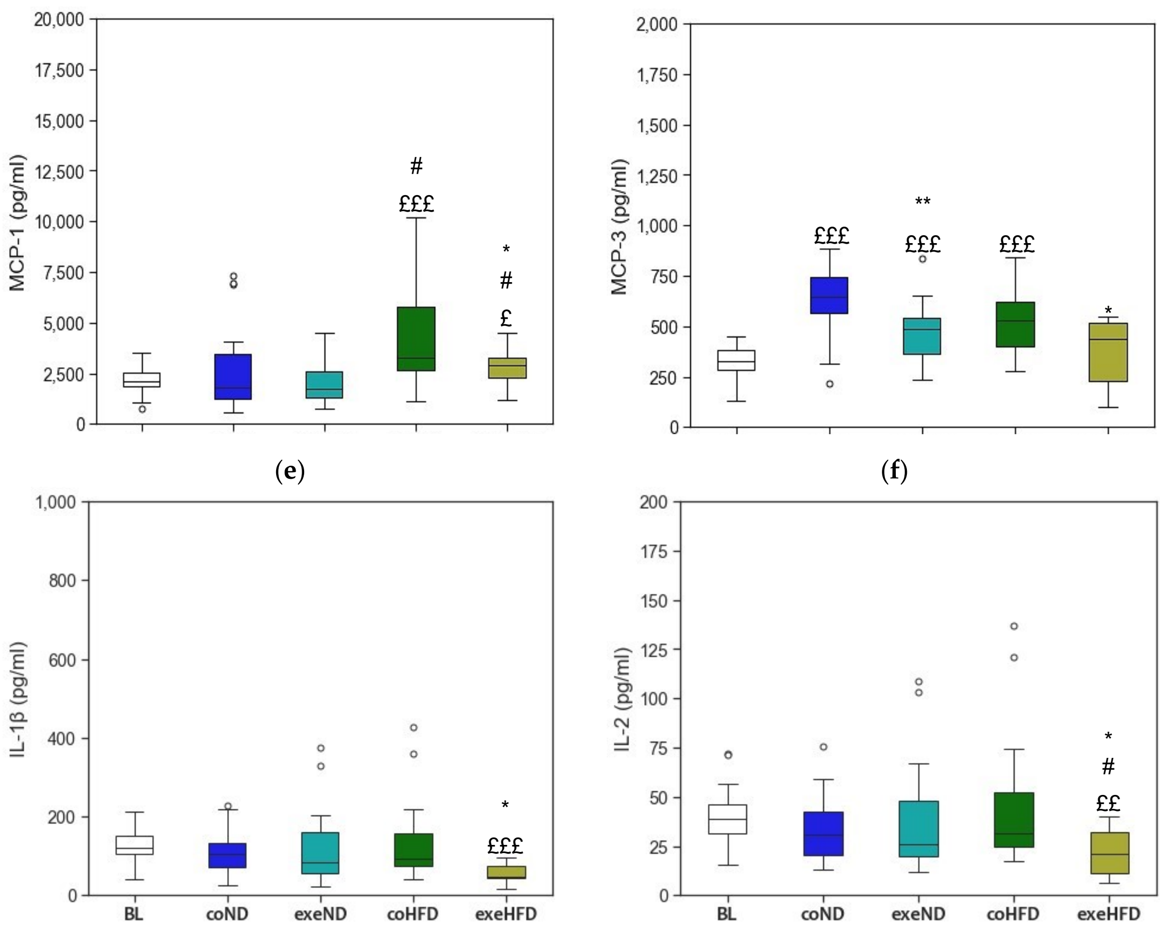


Fig. 25: Box and whisker plots of cytokines and chemokines (a-f). The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. $*p < 0.05$, $**p < 0.01$ compared to appropriate sedentary control group; # $p < 0.05$ compared to appropriate normal diet control group. £ $p < 0.05$, ££ $p < 0.01$, £££ $p < 0.001$ compared to baseline control group. Modified from (226), Copyright is by © The Author(s) 2021.

u. The impact of exercise and high-fat diet on adipocytokines

Adipocytokines, such as adiponectin, leptin, and IGF-1 are key regulators of energy metabolism and fat storage and they possess also immune-modulatory properties (234). In the present study, age, diet, and exercise had profound effects on the serum concentrations of IGF-1, adiponectin, and leptin (Fig. 26). At the end of the 10-month protocol, IGF-1 concentration was approximately 50% lower than in young baseline controls, irrespective of diet (Fig. 26a). Exercising animals fed HFD, had slightly higher IGF-1 concentrations than their respective controls. Adiponectin was not affected by age and exercise in ND animals but increased in HFD animals (Fig. 26b). This increase was greater in exercising HFD animals. Leptin was markedly higher in old than in young animals and this increase was substantially amplified by HFD (Fig. 26c). Exercise reduced serum leptin concentrations slightly in both diet groups. Resistin decreased with age in HFD and ND animals ($p < 0.001$). In both dietary groups, exercising animals had even lower resistin serum concentrations than their sedentary counterparts.

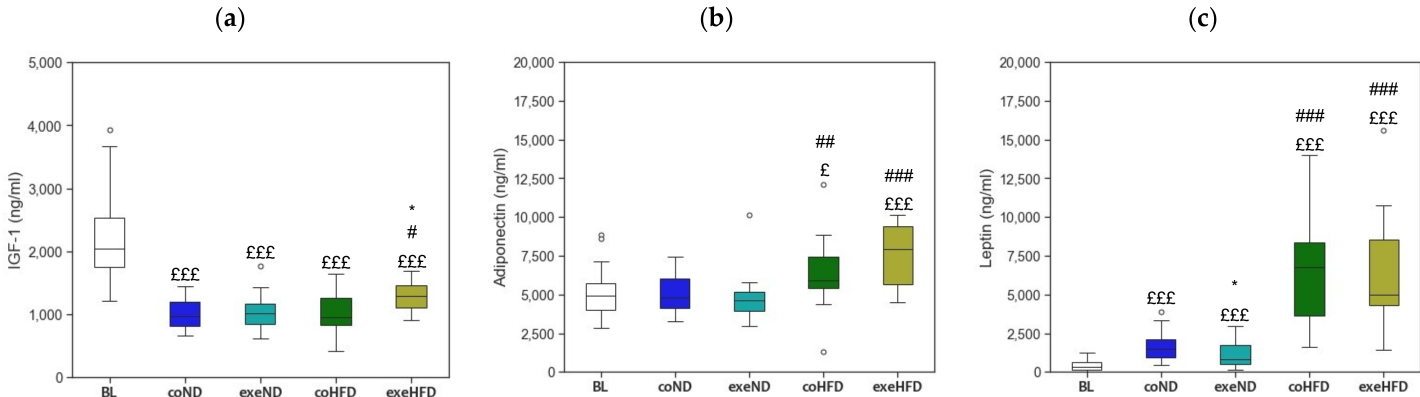


Fig. 26: Box and whisker plots of IGF-1 (a), adiponectin (b) and leptin (c). The two-tailed Student's *t*-test or the Mann-Whitney *U*-test were used for group comparison of independent samples. *$p < 0.05$ compared to appropriate sedentary control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to appropriate normal diet control group. £ $p < 0.05$, £££ $p < 0.001$ compared to baseline control group. Modified from (226), Copyright is by © The Author(s) 2021.

v. Linear Regression Analysis

A linear stepwise multiple testing was performed to predict PBMCs RTL from age, body weight, NOx, TG, MCP-1, RANTES, IGF-1, and leptin. The assumptions of linearity, independence of errors, homoscedasticity, unusual points, and normality of residuals were met. Only NOx and IGF-1 were retained in the regression model as statistically significant predictors of LTL, $F (2, 89) = 9.394$, $p = 0.0001$, adj. $R2 = 0.156$. The two variables added statistically significantly to the prediction, $p < 0.05$. Regression coefficients and standard errors can be found in Table 3. Despite this being the best regression model, it only explains approximately 16% of RTL variability, and the two factors are not independent predictors of TL as they were also correlated with an $R = 0.254$, $p = 0.007$.

Table 3: Coefficients and standard errors table

Variable	B	SEB	Beta	p-value
Intercept	.778	.047		3.522e-29
NOx (µM)	.037	.009	.409	.000087
IGF-1 (ng/ml)	-5.527e-5	.000023	-.236	.020

Note: B = unstandardized regression coefficient; SEB = Standard error of the coefficient; Beta = standardized coefficient; p-value = significance level. Dependent Variable: PBMCs RTL, and Predictors: (Constant), Nitric Oxid metabolites (µM), IGF-1 (ng/ml)

Summary and Discussion of Results

This section summarizes and discusses the results following the same order as the previous chapter. In the first part, the relationships between TL in blood and solid tissues as well as the association with age are addressed. Subsequently, the impact of modifiable lifestyle factors, such as diet and exercise, on telomere biology in different tissues is discussed. The focus of the final part will be on biomarkers of different pathways that may interact with telomere biology. This section also addresses the strengths and limitations of the project and provides future prospective.

8) Telomere length in leucocytes and solid tissues of young and aged rats

To address the existing gap of knowledge in telomere biology, the study presented in this paper attempted to answer the following questions: a. does TL differ between leucocytes and solid organs? b. is LTL correlated with TL in other organs and tissues? c. does aging induce a systematic shortening of TL in PBMCs and solid tissues? and finally, d. are age-related changes in TL associated with systematic changes in the expression of telomere-related genes which are the critical regulator of telomere length, structure, and function?

The study provides robust evidence for tissue-specific RTLs. In SD rats, the longest and the shortest telomeres were found in skeletal muscle and large intestine, respectively, with a 2-fold difference. To date, similar studies have not been performed on rodents. Human studies that compared RTL between organs also found significant differences of up to 2.5-fold (52, 55). The organ distribution of RTL in rats and humans seems to differ substantially, which is probably due to species effects. Despite, rigorous standardization of age, genetic background, and housing conditions, the animals of the present study displayed great interindividual variation in RTL, as previously observed in human studies.

In contrast to current concepts, RTL in PBMCs was positively correlated to kidney, skeletal muscle, and liver but not to any of the other tissue types. In some tissues, such as the large intestine and aorta, we even observed an inverse correlation. However, our results are aligned with those of others, Dlouha et al. also found a weak correlation of RTL in leucocytes with that in the liver and skeletal muscle (52). Similarly, Hiam et al. showed a correlation between RTL in blood leucocytes and skeletal muscle biopsies of 93 healthy men between 18–87 years of age (217). In contrast, in the large-scale GTEx project including 6391 tissue samples from more than 20 tissue types and 952 individuals, Demanelis et al. showed that RTL was positively correlated amongst different tissues and that whole blood RTL was a reasonable surrogate for RTL in most tissues (55). Inverse associations, as observed by Dlouha et al. and us in humans and rats respectively, may thus be chance findings that are primarily due to the marked

interindividual variability of RTL in most tissues. Based on previous studies and the present result it appears that RTL in peripheral blood cells is not a suitable biomarker to judge RTL in other organs of the same individual. However, in large cohorts, there may be a robust association between RTL in peripheral blood cells and most other tissue types, which makes it an interesting marker for epidemiologic aging studies.

In our study, RTL was comparable in different tissue types of young adults and aged animals. This contrasts the concept of telomere shortening with advancing age (55, 235-238). However, existing longitudinal studies measured RTL exclusively in leucocytes (20, 145, 235, 237-241). After a systematic analysis of RTL in multiple organs of young and aged animals, that were kept under standardized conditions, only aortic RTL was significantly shortened by age. In lung and visceral fat RTL increased with advancing age. The absence of telomere shortening in our rats could be related to their age. SD rats have a life expectancy of 2.5 to 3.5 years. In the present study, animals were sacrificed at 14 months of age, which corresponds to mid-adult age. Therefore, it cannot be excluded that a longer aging period would have yielded a different result. Previous work from Werner et al. supports a rather slow shortening of telomeres in leucocytes and cardiomyocytes of C57/Bl6 mice (209, 222). In these animals, a significant reduction of telomere length took up to 18 months. Although it was demonstrated that telomeric sequences are highly conserved among eukaryotic vertebrates, the length of telomeres differs between species (242). While in humans, telomeres reach up to 20 kb in length, rodent telomere length is rather heterogeneous. For example, Mus musculus' telomers reach up to 150 kb in size (243). Rats instead have telomeres ranging from 20 to 100 kb in length, which makes them still the most flexible and suitable animal model to study telomere biology (244). One might need to consider that large-scale human studies have also provided evidence that longitudinal changes of RTL vary substantially between individuals (235, 236, 238). Such heterogeneous results may be due to multiple individual factors that influence telomere dynamics, such as stress, nutrition, physical activity, smoking, and others (16, 18, 93, 116, 119, 130, 134, 174, 181, 224, 245, 246). In the present animal study, most of these factors were rigorously controlled. Moreover, we analyzed 10 different tissues without seeing a systematic effect. Altogether, the present results indicate that neither RTL in PBMCs nor in solid tissues is a useful marker to monitor aging on an individual level. Only in cohorts of substantial size, age-related changes in RTL can be shown reliably.

The organ-specific regulation of telomeric function is further supported by the mRNA expression analyses of TERT, TERF-1, and TERF-2. Liver tissue showed by far the highest TERT mRNA expression. However, this phenomenon was not accompanied by markedly longer telomeres. Considering that hepatocytes divide rapidly, it can be assumed that the high expression of *tert* is needed to prevent excessive telomere shortening in these cells. Also, *terf-1* and *terf-2* are highly expressed in

hepatic tissue supporting tissue-specific maintenance of telomeres in the liver. In contrast, terminally differentiated tissues, such as skeletal muscle and brain, are characterized by a low expression level of *tert* and *terf-1*, but a rather high expression of *terf-2*. TERF-2 is pivotal for t-loop formation and aids the invasion of the single-stranded telomeric DNA overhang into the double-stranded telomeric region upstream. Loss of TERF-2 has been shown to prevent t-loop formation and leads to excessive telomere shortening with premature cell death (247). It can be speculated that the high *terf-2* expression in terminally differentiated tissues reflects the particular need to protect their telomeres from DDR. In the present study, we have not seen systematic age-related differences in the mRNA expression of these proteins. TERT expression was slightly higher in the spleen, skeletal muscle, and visceral fat of the aged animals, but not in any of the other tissues. When considering the interindividual variability of *tert* expression in most tissues, the physiological relevance of these differences is questionable. However, TERF-1 and TERF-2 expression were markedly higher in aged liver tissue, which further supports the concept of organ-specific maintenance of telomere homeostasis in rapidly dividing hepatocytes. In addition, TERF-2 expression was higher in the spleen and lung tissue of aged animals, which also have a great capacity for renewal. Interestingly, aortic tissue showed a substantially lower TERF-2 expression in aged animals, which was paralleled by a reduction in RTL. Considering the rather inconclusive results of *terf-1* and *terf-2* expression, caution is warranted when interpreting them in relation to *tert* and RTL. All of the above-mentioned concepts are speculative and require further research to prove them.

9) Influences of long-term exercise and high-fat diet on age-related telomere shortening in rats

The second objective of our study was to investigate and expands existing knowledge on the influence of modifiable lifestyle factors such as diet (in particular a fat-enriched diet), regular exercise, and the combination of both factors on age-related telomere shortening. Through a comprehensive mapping of RTL and related telomere-regulating genes in exercising and sedentary animals on HFD and control diet, it was possible to demonstrate that neither regular exercise nor the consumption of HFD has a systematic effect on RTL of the tested organs.

The implications of obesity and obesity-related diseases on telomere biology are not yet clarified, and the available results are inconsistent. Although some human studies do not support an inverse relationship between TL and obesity (147, 248, 249), a recent meta-analysis calculated a significantly lower LTL in obese than in normal-weight individuals. LTL was inversely correlated with BMI, and other anthropometric measurements (149). Available results from longitudinal observational studies indicate an improvement in LTL after >2 years from bariatric surgery, probably due to an improvement

in inflammation and oxidative stress (250). However, only a small number of such studies have been published, with rather heterogeneous design and outcomes that impedes any conclusion towards causality. In addition, in-vivo studies were mostly focused on RTL in specific cells or tissue types, such as PBMCs or myocardium (209, 222-224). Moreover, the investigation of TL in mouse models of obesity and metabolic syndrome failed to show accelerated telomere shortening despite upregulation of the senescence-associated genes *Chk2*, *p53*, and *p21* (165, 166). For example, feeding mice for 60 weeks with a high-fat/high-sucrose diet-induced obesity and metabolic dysfunction, but did not accelerate LTL shortening (165). With advancing obesity, the animals were physically less active, which should have amplified the potentially adverse effects of obesity. Also, in genetically modified rats with metabolic syndrome, Takahashi et al. showed comparable myocardial TL than in wild-type controls (166). At the same time, telomerase expression and TA were upregulated together with *Chk2*, *p53*, and *p21*. These results are in line with our present study, showing similar RTL in PBMCs, liver, aorta, and skeletal muscle after 10 months of HFD or ND. Also, the large intestine, spleen, brain, and lung showed comparable RTL in the two groups.

As for the diet, also previous results on the effect of exercise on TL are rather inconsistent. Several observational studies have shown higher LTL in exercising individuals of different age groups and activity levels (142, 207, 209, 211, 251). Additional support from prospective observational and interventional studies is strongly limited. Soares-Miranda L et al. performed serial blood collections over 5 years in 582 older US adults and found no significant association between physical activity, physical performance, and LTL (215). In contrast, Werner et al. reported an increase in LTL, TA, and TERF-2 expression after 6 months of aerobic endurance training or high-intensity training, which was not seen in controls (209, 211).

Ludlow et al. showed in wild-type derived short telomere mice (CAST/Ei) that 1 year of voluntary running preserved TL in the myocardium and liver, but not in skeletal muscle (224). Similarly, in the present study, these effects were not accompanied by consistent alterations of telomere-regulating genes that would explain these effects. Conversely, after 3 weeks of voluntary wheel running, Werner et al. reported an upregulation of TA in the murine aorta and PBMCs and an increased aortic gene expression of *terf-2*. Also, *Chk2*, *p53*, and *p21* were lower expressed in the aorta of exercising animals. However, the increased expression of these telomere-regulating genes did not result in a significant difference in aortic TL after 6 months of exercise when compared to inactive controls (209). The TL results reported by the two studies of Ludlow et al. and Werner et al. are not in line with our findings, where 10 months of regular moderate running exercise reduced RTL in PBMCs and liver, whereas the aorta and large intestine showed a significant increase. These inconsistencies between existing exercise studies in animals might, at least partly, be explained by differences in the animal models used. We worked with SD rats, whereas others used mainly mice models in which telomeres, as explained above, fall in a wider

length range than rats. Also, the duration of exercise varied among existing studies between a few weeks and one year, which further limits comparability. In addition, a greater group size with 22 coND and 22 exeND animals provides robustness to our results. The studies from Ludlow et al. and Werner et al. were performed with no more than 10 animals per group, which limits statistical power and leaves more room for random effects. A major strength of the present study is the strict standardization of the exercise intervention, which consisted of forced treadmill running for 30 min at a fixed speed on 5 consecutive days per week. The efficacy of this intervention is evidenced by significantly lower body weight at the time of scarification. In contrast, most previous studies used voluntary wheel running, which is not standardized.
In our model, neither exercise nor HFD induced a consistent expression pattern of telomere-regulating genes, namely *tert*, *terf-1* and *terf-2*. Only kidney and visceral fat showed significant differences in RTL, but in opposite directions. Similar differences were detected for the expression of *tert* and *terf-2*. However, the relevance of these effects is questionable as *tert*, *terf-1*, and *terf-2* were also altered in several other tissues of HFD animals without affecting the respective RTL. Furthermore, most existing studies reported the effects of exercise and obesity on telomerase and shelterins, but often this was not associated with changes in RTL. In line with existing data, correlation analyses in the present study showed inconsistent correlations between RTL and mRNA expression levels of the three telomere-regulating genes.

A distinctive aspect of the present study is the combination of exercise and HFD. In modern societies, people often try to compensate for adverse nutritional habits with exercise, but the efficacy of this approach is not well documented. Our results show that such an approach does produce a different outcome than exercise alone. Specifically, RTL was lower in PBMCs, liver, and kidney of exercising animals on HFD, but higher in the aorta and large intestine. Despite a comparable pattern of RTL in the different organs of exercising animals on a normal diet and HFD, incongruent results were registered for the mRNA expression of TERT, TERF-1, and TERF-2. For example, TERF-1 and TERF-2 were both increased in skeletal muscle of exercising animals on ND but decreased in exercising animals on HFD. However, both groups showed comparable RTL in this tissue. In line with this argument, also correlation analyses that included all 72 animals did not show consistent correlations between RTL and the expression level of telomerase or shelterins. E.g., an inverse correlation between RTL and TERT was seen in the spleen and large intestine, whereas kidney and visceral fat showed the opposite. In all other tissues, both parameters were not correlated. The absence of systemic effects on RTL in PBMCs and solid tissues and the highly inconsistent mRNA expression pattern of telomerase and shelterins limit the potential scope of these factors as relevant mediators of telomere effects induced by exercise and diet.

10) Effects of exercise and diet on oxidative/nitrosative stress, lipids, and cytokines

Oxidative stress and chronic inflammation are widely known mediators of telomere biology. Physical activity and nutrition strongly impact oxidative and inflammatory processes. To shed further light on the telomeric effects of aging and lifestyle, a broad panel of metabolic biomarkers has been analyzed. The following section discusses the results of these analyses with a particular focus on lipid metabolism, oxidative-nitrosative stress, and systemic inflammation. The discussion will also elude a potential mechanism linking oxidative stress and telomere biology.

In old animals, the lipid profile was substantially different from that of young animals with markedly higher TC and TG concentrations. Moreover, HDL-C, non-HDL-C, and NEFA were higher in old than in young animals. Such age-related changes in the lipid profile are expected and have been described by others before (252). With the HFD used in this study, the concentrations of TG and NEFA increased whereas all other parameters of the lipid profile remained unchanged. Previous studies that treated rodents with HFD reported mixed results. While some studies found increased concentrations of TC, TG, and LDL-C (252-255) others did not (256). However, a direct comparison of these studies is limited due to differences in study design and composition of the diets. For example, in 4-8 weeks old Wistar rats the administration of HFD for 4-8 weeks resulted in increased body weight, adipose tissue weight, TC, TG, and LDL-C concentrations (257, 258). However, both studies did not include baseline measurements, which impedes a longitudinal evaluation of age-related effects. Zelzer et al. treated adult female SD rats for 12 weeks with an HFD comparable to the one used in the present study (256). This intervention did not result in different TC or TG concentrations. Also, HDL-C and non-HDL-C were comparable between controls and HFD-treated animals. The limited comparability of different animal models is not surprising and has already been shown before (252). Similar to animal models, also human studies that compared the lipid profile of obese and non-obese individuals yielded heterogeneous results (259-261). Most existing studies showed higher TC, TG, LDL-C, and small dense LDL-C (sdLDL-C) concentrations in obese individuals when compared to normal weight controls. However, for HDL-C inconsistent results have been reported (259, 261). A controlled dietary intervention study by Egert et al. demonstrated that the substitution of a high-fat diet rich in saturated fatty acids with either a high-fat or a low-fat diet rich in mono-unsaturated fatty acids ameliorated the lipid profile, reducing TC, LDL-C/HDL-C ratio, LDL-C size and its susceptibility to oxidation (262).

The main finding of our study is the exercise-induced increase in oxLDL that is accompanied by a reduction of NO. Elevated oxidative stress is a common condition in sedentary and obese individuals that increases the generation of ROS and leads to the modification of many biochemical targets (263-

265) including LDL-C. OxLDL is supposed to be more aggressive than non-oxidized LDL in driving inflammation, atherogenesis, and ultimately the risk of CVD events (231, 266, 267). Nevertheless, exercise-induced ROS production seems to protect cells against oxidation by maintaining cellular oxidant-antioxidant homeostasis (268, 269). Furthermore, regular physical activity improves blood pressure control through increased production of NO and other vasoactive substances (265). In the present study, however, long-term moderate running exercise was associated with an increase in oxLDL and a reduction in NOx. While the increase in oxLDL was comparable in both dietary groups, the reduction in NOx was more pronounced in HFD animals. These results are in line with previous studies demonstrating an increased susceptibility of LDL-C and other lipoproteins to oxidation after a single intensive exercise session (270, 271). Furthermore, in another study by Zelzer et al., the administration of a similar HFD to rats increased several markers of oxidative stress, such as malondialdehyde (256). When interpreting the modification of oxidative stress biomarkers by physical activity, it should be considered that chronic exercise, as well as high-intensity training can increase oxidative stress through several mechanisms including increased mitochondrial oxygen consumption and activation of oxidase enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major source of ROS (264). This might at least partially explain the exercise-induced increase in oxLDL that we observed in the present study. Additional support for this hypothesis comes from the reduction in NOx by regular exercise. Under normal circumstances, the potent vasodilator NO is released during the conversion of L-arginine to L-citrulline. This reaction is catalyzed by NOS. However, under certain circumstances, NOS can also produce superoxide (O2*-), which reacts avidly with vascular NO· to form peroxynitrite (ONOO−). This metabolite is capable of impairing NOS dimerization and function (263, 272). This phenomenon is known as NOS uncoupling, such an uncoupling of NOS can occur in the absence of either L-arg or BH4 and increased concentrations of ADMA, a competitive inhibitor of NOS. In the present study, HFD and regular exercise increased h-arg, another substrate of NOS for the production of NO. Although h-arg competes with L-arg for NOS binding sites, it seems to be a less efficient substrate for NO synthesis. According to März et al., the role of h-arg in NO metabolism is still insufficiently understood (273). Some studies support the hypothesis that h-arg might increase arginine bioavailability by inhibition of the enzyme arginase, which competes with NOS for the utilization of the key substrate L-arg (273, 274). If this is correct, the substantial increase in h-arg is likely to exert a protective effect under HFD. This concept is supported by a previous study showing that h-arg supplementation ameliorates blood glucose in mice on HFD (275). The reduction of ADMA and SDMA observed in the present study further supports the beneficial effect of h-arg in HFD animals. However, h-arg competes with L-arg in more than one way. They both utilize the same transport system for cell entry, and high extracellular h-arg concentrations will result in reduced L-arg uptake (276). This might at least partially explain the exercise-induced reduction of NOx. The inverse relationship between

oxLDL and NOx strongly supports the concept of NOS uncoupling in exercising animals. Considering that NOx levels were lower in non-exercising obese animals than in non-exercising lean animals, HFD consumption seems to have an independent NO-reducing effect, which is amplified by regular exercise. The very low NOx concentration in exercising obese animals further supports the concept of an additive NO-reducing effect of HFD and exercise.

Obesity is typically associated with increased secretion of adipokines and mild tissue inflammation (234). Therefore, a broad range of adipokines, cytokines, and chemokines was also studied in the present project. As expected, our HFD animals showed markedly higher leptin and adiponectin serum concentrations than ND animals regardless of exercise. Exercise was effective in reducing leptin in ND, but not in HFD animals, suggesting that exercise could not revert the effect of HFD. The mild increase in serum adiponectin in obese exercising animals might simply reflect the weight loss in these animals. Interestingly, the HFD used in this study had only minor pro-inflammatory effects inducing increased serum concentrations only for MCP-1 and IL-5. Other pro-inflammatory cytokines, such as TNF-α, IFN-γ, or IL-6 were comparable between the two dietary groups.

Consistent with previous studies (277, 278), the exercise protocol applied in this project significantly reduced the serum concentrations of TNF-α, IL-1β, IL-2, MCP-1, MCP-3, and RANTES in obese animals. MCP-3 and RANTES were also decreased in lean exercising animals. Both chemokines regulate the migration and infiltration of monocytes and macrophages into solid tissues (233). In the context of obesity, visceral fat-derived resistin, TNF-α, and several other interleukins contribute to insulin resistance (279), whereas weight loss or visceral fat removal decreases serum IL-6 and increases the insulin-sensitizing hormone adiponectin (279, 280). The present results are in line with recent findings from Rocha-Rodrigues et al. showing that regular physical activity reduces inflammation in response to HFD administration (277). The exercise-induced increase of IP-10, IL-12, and MMP-activity in lean exercising animals might reflect a mild activation of cellular immunity and tissue remodeling in response to exercise, which is masked in the obese exercising animals.

In this study, a linear regression analysis was performed to evaluate the influence of age, body weight, and several biochemical parameters on RTL. Interestingly, it was found that only NOx and IGF-1 are related to RTL in PBMCs, and they might predict RTL variability in such tissue.
IGF-1, also called *somatomedin*, is a substance under control and mediates the effects of growth hormone (GH) as well as GH-independent anabolic responses in many cells and tissues (281). The levels of GH and IGF-1 decrease with age in both laboratory animals and humans (282). Also in the present study, aged animals showed markedly lower IGF-1 concentrations than their younger counterparts. The age-associated decreases of GH and IGF-1 are believed to contribute to the development of many signs

of aging, including cardiovascular dysfunction, and are associated with all-cause and cardiovascular mortality (283, 284). Epidemiological studies investigating the relationship between LTL and serum IGF-1 concentrations reported inconsistent results with some authors showing a positive association (285, 286) whereas others reported an inverse correlation between LTL and IGF-1 (287-289). IGF-1 exerts its effects via activation of the IGF-1 receptor (290). This receptor is widely distributed and enables blood-transported IGF-1 to coordinate balanced growth among multiple tissues and organs. Conversely, autocrine-paracrine IGF-1 can stimulate local and unbalanced growth independently of systemic GH. In-vitro studies have shown that IGF-1 reduced TL in fibroblasts (291) but enhanced the effect of phytohemagglutinin to increase TA in cord blood mononuclear cells (292), hinting toward cell-type specific effects of IGF-1. Other studies have reported that IGF-1 induced an increase in TA, mediated via the PI3K-Akt kinase pathway, and increased mRNA expression and protein levels of hTERT (293). The PI3K-Akt kinase pathway regulates various cellular processes, such as proliferation, growth, apoptosis, and cytoskeletal rearrangement and is a fundamental mediator of the effects of exercise at cellular level. For example, long-term voluntary running increased the expression of IGF-1 in the myocardium of C57/Bl6 wild-type mice (222). Treatment with IGF-1 up-regulated myocardial TA and increased the expression of phosphorylated Akt protein kinase and phosphorylated eNOS. The exercise-induced changes were absent in both *mTERT* (-/-) and *eNOS* (-/-) mice (222). In a follow-up study in aortic tissues of mice and mononuclear cells of humans, Werner et al. showed that exercise training increased TA, telomere binding protein content, and mRNA expression, and decreased levels of *Chk2*, *p16*, and *p53* in the murine aorta. These changes in gene expression were again shown to be mediated by TERT and eNOS in the aorta. Based on their results, Werner et al. suggested that TA, eNOS, and IGF-1 are linked in a signaling pathway mediating exercise-induced cardiovascular protection (209, 222). In line with these findings, we observed an increase in circulating concentrations of IGF-1 in obese exercising animals, paralleled with an increase in thoracic aorta RTL, telomerase, and shelterins gene expression. It is known that IGF-1 is also involved in the synthesis of NO in endothelial cells, causing additional vasodilation of the arteries (294). Interestingly, lean exercising animals showed normal IGF-1 serum levels but increased RTL and telomere-associated gene expression in the aorta. The reduction in NO bioavailability and increased expression of MMP activity observed in these animals indicate that long-term exercise promotes arterial remodeling. In obese exercising animals, the increase in IGF-1 and consequent activation of the PI3K-Akt kinase pathway might be a compensatory mechanism to prevent atherogenesis and arterial stiffness. The current dogma of telomere biology suggests that telomeres shorten with every cell division, eventually leading to a sustained DDR and activation of cellular senescence (295). While this may explain age-related degeneration of tissues maintained by a constant contribution of stem cells, it is insufficient to explain how senescence contributes to aging in post-mitotic tissues. Thus, several mechanisms that add to telomerase and

shelterins' function, are in place to control TL. Even though in this study we did not report any mechanistic link between NO, IGF-1, and TL, we have demonstrated by statistical imputation that NO and IGF-1 contribute to TL variability, and, as shown by others before, they possibly do it via activation of a unique signaling pathway.

11) Strengths, Limitations and Future Perspectives

This is the first in-vivo animal study that has systematically analysed the effects of age, exercise, and diet on TL in blood and an extended range of solid tissues in the same individuals. The use of validated and strictly quality controlled methods for the measurement of serum lipids also allowed a proper characterization of the animals' metabolism. The rather large number of animals per group provided robustness to the results. In addition, the intervention period of 10 months was quite long. Thus, this study had the potential of identifying long-term effects on TL and the expression of associated genes. Furthermore, the exercise protocol was rigorously controlled. Most previous studies used voluntary wheel-running, which is considerably less standardized.

Of course, there are also limitations that have to be considered when interpreting the present results. Our results and conclusion exclusively apply to healthy SD rats and cannot simply be translated to the situation in various diseases or other species. A study duration of ten months with rodents is rather long but may be insufficient to capture significant age-related effects on telomeres. Considering the life expectancy of SD rats, an observation period of two years might better reflect changes in telomere biology. However, towards the end of our study, some animals started developing tumours and thus had to be excluded from the analysis. This implies that for a longer study more animals would be needed in order to obtain a sufficient number of aged animals that are free from tumours and other relevant diseases. Furthermore, we performed only one measurement per animal and tissue type, which leaves room for random effects due to regional differences in RTL.

The exercise protocol applied was rather moderate and a more intensive regimen might have produced different results. However, with this protocol we aimed to mimic a common recreational activity pattern in adults. Energy intake and energy expenditure may have varied between individual animals and different groups. The lacking information on both factors adds some uncertainty to the interpretation of our results. In addition, solid organ tissues cannot be collected longitudinally. Therefore, we chose a cross-sectional study design. Also, the PCR-based method for RTL analysis harbours several limitations. As a relative method that requires the simultaneous measurement of a single copy reference gene, this approach is subject to greater technical variability than direct methods. Furthermore, it only provides an average TL across all telomeres. Information on the distribution of short and long telomeres can only be obtained from more sophisticated methods that are not feasible for the analysis of large sample sets.

Lastly, telomere-regulating genes were only analysed by mRNA expression, but not at protein level which might give more insights to the mechanisms behind the changes of TL.

Thus, future research should address the impact of lifestyle factors such as exercise and diet not only on the average TL but also on the shortest telomeres, and other markers of cell cycle arrest and cell senescence which might give a deeper understanding on the molecular mechanisms that lead to cell senescence and ageing. It is important also to focus on advanced adult age, where degenerative disease most frequently occur and explore the implications of these modifiable factors for aging and age-related diseases. Ultimately, these findings might be a valuable addition to the therapeutical approach in primary or secondary disease prevention.

Conclusions

In conclusion, the present in-vivo study conducted in healthy female SD rats questions the utility of RTL in PBMCs as a biomarker for the assessment of aging in an individual. Despite rigorous standardization of housing conditions, sample collection, and analytical procedures, excessive intra-, and inter-individual variability has been observed. Furthermore, RTL in PBMCs was not systematically correlated with solid organ tissues and no differences have been observed between young and old animals. In addition, modifiable lifestyle factors, such as obesity and exercise, do not have significant systemic effects on telomere shortening and the expression of telomere-regulating genes. Also, exercise and HFD do not show significant interaction. Any lifestyle-related effect on RTL and telomere-regulating genes in one tissue type does not allow conclusions on other tissue or cell types.

Despite the absence of a systemic effect on telomeres, long-term moderate exercise does alter the delicate equilibrium between oxidative and anti-oxidative compounds leading to a putative uncoupling of NOS with higher oxLDL and lower NOx concentrations. The alteration of NO bioavailability partly explains the variation observed in TL. However, these metabolic effects do not necessarily compromise the beneficial reduction of pro-inflammatory cytokines, such as TNF-*a*, IL-1*ß*, IL-2, MCP-1, MCP-3, and RANTES.

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Appendix

Review

Physical activity, a modulator of aging through effects on telomere biology

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ABSTRACT

Aging is a complex process that is not well understood but involves finite changes at the genetic and epigenetic level. Physical activity is a well-documented modulator of the physiological process of aging. It has been suggested that the beneficial health effects of regular exercise are at least partly mediated through its effects on telomeres and associated regulatory pathways. Telomeres, the region of repetitive nucleotide sequences functioning as a “cap” at the chromosomal ends, play an important role to protect genomic DNA from degradation. Telomeres of dividing cells progressively shorten with age. Leucocyte telomere length (TL) has been associated with age-related diseases. Epidemiologic evidence indicates a strong relationship between physical activity and TL. In addition, TL has also been shown to predict all-cause and cardiovascular mortality. Experimental studies support a functional link between aerobic exercise and telomere preservation through activation of telomerase, an enzyme that adds nucleotides to the telomeric ends. However, unresolved questions regarding exercise modalities, pathomechanistic aspects and analytical issues limit the interpretability of available data. This review provides an overview about the current knowledge in the area of telomere biology, aging and physical activity. Finally, the capabilities and limitations of available analytical methods are addressed.

INTRODUCTION

Over the last 200 years average life expectancy in developed countries has more than doubled and is now above 80 years [1]. In numerous studies this linear increase is suggested to rise to an average life span of 100 years or more [2–4]. This dramatic increase in life expectancy was largely driven by changes in lifestyle, sanitation and a continuous improvement of health care [5]. As a result, the major causes of death have shifted from infectious disease to chronic age-related conditions [6, 7]. Today, cardiovascular disease (CVD), cancer and respiratory disease are the most common causes of death worldwide [8, 9]. Other lifestyle and age-related conditions such as musculoskeletal disease, diabetes and dementia are also increasing rapidly and thus impact the number of disability-adjusted life years (DALYs), calculated in a population as the sum of the Years of Life Lost (YLL) due to premature mortality and the Years Lost due to Disability (YLD) [10, 11]. Therefore, strategies to promote healthy aging have gained great interest in developed societies.

The process of aging remains incompletely understood. A better understanding of the complex and interrelated biological mechanisms of aging would help to develop interventions that delay the aging process. Environmental and lifestyle factors, such as physical activity, nutrition, stress and smoking, are major determinants of the aging process [12]. In particular, regular exercise is a safe and cost-effective way to reduce morbidity and premature mortality [13]. However, the molecular mechanisms that mediate the beneficial effects of exercise are incompletely understood and remain an area of active research. In numerous observational and intervention studies the preservation of telomeres, the protective end-caps of all chromosomes, has been proposed as an appealing putative mechanism that contributes partially to the beneficial health effects of physical activity [14]. This review aims to provide an overview on the current knowledge in the area of telomere biology, aging and physical activity. In addition, the capabilities and limitations of available analytical methods will be addressed.

Structure and function of telomeres

The genetic information of eukaryotes is encoded in the deoxyribonucleic acid (DNA), which is packed in the chromosomes. With every division of mitotic cells a small fragment of DNA at the ends of every chromosome remains unreplicated due to a physiological phenomenon named the end-replication problem [15]. In order to prevent the loss of coding genetic information thousands of identical, non-coding oligonucleotides are attached to the ends of all chromosomes. These terminal non-coding DNA-regions are called telomeres. Human telomeres contain approximately 2,500 tandem copies of a simple hexanucleotide with the sequence 5'-$TTAGGG_n$-3' [16]. For most of its length, telomeric DNA is double stranded. However, the last portion of 30–100 base pairs (bp) at the 3'-end of the G-rich strand is single-stranded. This G-rich overhang at the 3'-end is essential for telomere maintenance and capping [17, 18]. Telomeres give rise to a complex three-dimensional structure limiting the access of telomerase and DNA damage repair (DDR) enzymes to the free ends of each DNA-strand. This three-dimensional structure is achieved through the binding of a highly abundant protein complex, named shelterin, to the telomeric hexanucleotide sequence 5'-$TTAGGG_n$-3'. The shelterin complex is composed of the following six-subunits (see Table 1): telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TRF1-interacting nuclear protein 2 (TIN2), telomeric overhang binding protein 1 (POT1), TIN2 and POT1 interacting protein 1 (TPP1), and repressor-activator protein 1 (RAP1). TRF1 and TRF2 interact with the double-stranded telomeric DNA, whereas POT1 associates with single-stranded telomeric DNA [19]. Through interactions with the shelterin proteins the terminal telomere section flips backwards resulting in the formation of a looped structure (t-loop). Furthermore, the shelterin proteins aid in displacing a short section of double-stranded telomeric DNA so that the single stranded G-rich overhang at the 3´end can be interposed. This structure is referred to as "D-loop" and protects the free end of the DNA strand from recognition as a strand break, which would induce inappropriate repair processes.

The interaction between the shelterin protein subunits is complex and has been investigated using mouse conditional knock-out cells for TRF1, TRF2, POT1, TPP1. It has been shown in several studies that shelterins prevent DNA damage response (DDR) activity at telomeres, chromosomal rearrangements and cell cycle arrest, thus demonstrating a role in maintaining telomere function and preserving genomic stability [17, 18, 29]. Through the binding of TRF1 and TRF2 to double-stranded telomeric $TTAGGG_n$ repeats RAP1, TIN2, TPP1 and POT1 can be recruited. TIN2 can bridge TRF1 and TRF2/RAP1 complexes by binding to both proteins simultaneously. Furthermore, TIN2 associates with the TPP1/POT1 heterodimer, which is typically bound to single-stranded TTAGGG repeats [19, 24]. These intimate interactions result in the formation of a "capped" loop [20, 30, 31].

Telomere length (TL) varies greatly between species [32]. At birth, every human individual has a specific TL that ranges between 5 to 15 kb [33]. Throughout life telomeres shorten continuously with a rate between 20-50 bp due to the end-replication phenomenon, oxidative stress and other modulating factors [15, 33]. However, telomere shortening rates and consequently also average TL vary amongst different tissue types, which is at least partly explained by tissue-specific proliferation rates [34, 35]. In dividing cells, the end replication problem is an important driver of telomere shortening that can be modified by other factors, such as oxidative stress or inflammation [33]. In postmitotic cells instead, oxidative stress can directly damage telomeric DNA and drive cells into senescence [36, 37]. The TL of peripheral blood leucocytes (LTL) has gained substantial interest as a potential marker of biological age [17]. Mean LTL in adults is approximately 11 kb and declines with an annual rate of 30-35 bp. Telomere attrition is most pronounced during the first two years of life, which are characterized by rapid somatic growth [34, 35, 38, 39]. The shortening of telomeres is not a unidirectional process since the reverse-transcriptase telomerase is capable of adding new hexanucleotides to

Table 1. Shelterin complex, subunits and functions.

Shelterin subunits	Function	References
Telomeric repeat binding factor 1 (TRF1)- binds to the canonical TTAGGG double-stranded telomeric repeats	Determines the structure of telomeric ends, it is implicated in the generation of t-loops, and it controls the synthesis of telomeric DNA by telomerase	de Lange [20]
Telomeric repeat binding factor 2 (TRF2)- TRF1 paralog	Implicated in telomere protection and telomere length homeostasis	Takai et al. [21]; Artandi et al. [22]; Palm et al. [23]
TRF1-interacting nuclear protein 2 (TIN2)- can bridge TRF1 and TRF2/RAP1 complex and can also recruit the TPP1/POT1 heterodimer	Responsible for the recruitment of other shelterins, therefore implicated in telomere protection	Lei et al. [24]
Telomeric overhang binding protein 1 (POT1)- associates with the single-stranded TTAGGG repeats	The telomere length maintenance is exerted through the interaction between POT1 and the reverse-transcriptase ribonucleoprotein telomerase	Baumann et al. [25]; Loayza et al. [26]
TIN2 and POT1 interacting protein 1 (TPP1)	Required for the recruitment of telomerase to the DNA	van Steensel et al. [27]
Repressor-activator protein 1 (RAP1)- 1:1 complex with TRF2	In addition to its telomeric function, also implicated in the upregulation of energy metabolism as a modulator of the NF-κB signalling pathway	de Lange [20]; Teo et al. [28]

telomeric ends [40, 41]. However, most somatic cells do not express telomerase. Detectable levels of telomerase activity can typically be found in germ line and embryonic stem cells, immune cells and in cancer cells [42, 43]. Human telomerase is made up of two main components: telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) endowed with a complementary sequence of telomeric DNA (3'-AUCCC-5'), which serves as a template for telomere elongation [44]. It is important to note that telomerase expression does not necessarily parallel enzyme activity [44]. In the brain for example, TERT is expressed without detectable telomerase activity (TA) [45]. In contrast, PI3K/Akt and other factors can modulate TA independently from TERT expression [46]. In humans, the telomerase enzyme complex is completed by several associated proteins, including dyskeratosis congenita 1 (DKC1) and NOP10 ribonucleoprotein (NOP10), which are essential for the maintenance of telomere integrity [47, 48].

In order to add new TTAGGG hexanucleotides the enzyme needs access to the telomere ends, which are hidden in the complex three-dimensional telomere structure [31]. Therefore, telomeres can change their conformational status between an 'open' state, where the enzyme has substrate access, and a "closed" state that prevents telomerase action [49]. Shelterin proteins play a key role in regulating the conformational state of telomeres and thus modulate TA [27, 50]. The low number of TRF1 and POT1 binding sites on short telomeres drives the formation of an open state. Whereas longer telomeres, with more TRF1 and POT1 binding sites, typically assume a closed configuration [26]. In this way, telomerase can be efficiently directed to the shortest telomeres within a cell, and sufficiently long telomeres will not undergo any inappropriate lengthening [51, 52].

The importance of TL and TA in the aging process has been described by Rudolph et al. demonstrating that age-dependent telomere shortening, and genetic instability are associated with shortened life span and a reduced regenerative potential [53]. Several genetic disorders with mutations in loci encoding for shelterin and telomerase subunits have also been described, all of which have been characterized by an accelerated rate of telomere attrition [54–56]. Higher rates of leucocytes telomere attrition are also associated with elevated risk of coronary artery disease, myocardial infarction, heart failure and stroke [57]. Additionally, changes in LTL, shelterin expression and function have been linked to structural changes in the thoracic aorta vessel wall and the myocardium [58, 59]. Furthermore, shorter LTL is related to the increased severity of CVD [60–62]. Many factors contribute to the shortening of telomeres including the genetic background [56, 63–65], gender [66], socioeconomic status and consequent stress perceived [58, 67, 68], dietary behaviour (i.e. antioxidant intake, alcohol consumption etc.) [69–73], body mass

index (BMI) [66, 74], smoking [66, 75] and physical inactivity [76].

Telomere length and telomerase activity – key mediators of mortality and morbidity

Based on the principles of telomere physiology explained earlier, it has been speculated that longer telomeres and high TA are beneficial for healthy aging [77]. In epidemiologic studies, adult men and women with shorter telomeres are characterized by higher mortality rate, which is nearly twice as high as in those with longer telomeres [78, 79]. It has been demonstrated that those with the shortest telomeres were characterized by a higher hazard ratio for all-cause mortality compared to those with the longest telomeres (1.66, 95%CI 1.09–2.53, p=0.018) [80]. In addition, a reduced LTL seems to indicate an existing or an elevated risk for future age-related disease such as CVD, type 2 diabetes mellitus (T2DM), neurodegenerative diseases, osteoporosis and premature aging syndromes [56, 57, 81–83]. Recent clinical association studies unveiled a correlation between leucocytes telomere attrition and clonal hematopoiesis of indeterminate potential (CHIP) [84]. During aging hematopoietic stem cells (hSC) start to accumulate somatic mutations. It can happen that through the accumulation of DNA damage one cell gains a competitive expansion advantage that gives rise to expanded clones of leucocytes with the same mutations. The prevalence of CHIP is very low in those aged <40 years, but can be found in >10% of those aged 70 years and in approximately 20% of octogenarians [84–87]. Individuals who harbour these mutated clones are at higher risk of hematological malignancies, but also several adverse cardiovascular outcomes [88]. In a whole-genome sequencing study, the strongest association of CHIP was found to be an 8 bp deletion in intron 3 of the TERT gene. Accordingly, TLs were observed to be significantly shortened in CHIP carriers [89]. Experimental studies demonstrate a delay in aging and an extended median life span in mice who have been genetically modified with constitutively expressed TERT compared to the respective controls [42, 43]. Moreover, telomerase reactivation reverses tissue degeneration in telomerase deficient mice [90]. On the contrary, constant expression of telomerase has been associated with carcinogenesis and is shown to have detrimental effects [91, 92]. Indeed, 85 to 90% of all human cancers have detectable TA [91]. A pivotal role of telomerase in cancer biology is further highlighted by the fact that inhibition of TA, in telomerase-positive human cancer cells, induces cell death and reduces tumour growth [93–96]. While constant unregulated TA, activation of oncogenes and/or silencing of tumor suppressor genes appears to drive tumour incidence and growth [97], a physiologically regulated telomerase activation appears to have beneficial health effects in mice and humans [59, 98, 99]. Therefore, substantial effort has been invested in the search for lifestyle factors that can modulate TA including nutrition or psychological stress. Based on existing data, also physical activity appears to be an effective way to induce telomerase and to preserve TL [59, 98, 100]. In the following sections, we review existing data on the effects of exercise on aging and in particular on telomere physiology.

Exercise, health and telomeres

Regular exercise is a well-established approach to reduce the risk of morbidity and premature mortality [13, 101]. Prospective cohort studies demonstrate that men and women who regularly exercise, have a 30% lower all-cause mortality risk than sedentary individuals [13, 101]. In the older persons the beneficial effects of regular physical activity (above 200 minutes a day) are even more pronounced reaching up to >40% mortality risk reduction [101–103]. Some studies have calculated that the gain of life years ranges between 2 to 4 years depending on the individual level of activity [104–108]. Despite strong evidence that supports beneficial health effects through regular exercise, comparability between individual studies is limited because of differences in the composition of study cohorts, exercise protocols and the duration of follow-up [104–108]. However, the pooled analysis of six major cohort studies including 632,091 participants with diverse ethnicity and an average age of 61 years showed that the effect of regular exercise on mortality is dose-dependent and already mild physical activity is associated with a significant reduction of mortality risk and a 1.8-year gain in life expectancy [13]. Metabolic equivalents (MET) are used to compare energy consumption between different activities dividing the actual energy expenditure of a given activity by the energy expenditure at rest [109]. Of note, even intermittent exercise sessions with a limited duration offer considerable health benefits, also in obese individuals and those with major risk factors [110]. The health effects of exercise are not only determined by the frequency and duration of training sessions, but also by the intensity. Vigorous exercise is more effective than mild or moderate exercise in improving cardiorespiratory fitness [111–113]. When adjusted to their specific needs and abilities, even in older individuals, regular physical activity attenuates the age-dependent decline in cardiorespiratory fitness [114], improves mobility and physical functioning [115], and reduces the risk of falls [116].

Besides a substantial reduction of mortality, regular exercise also reduces the incidence and progression of coronary heart disease, hypertension, stroke, diabetes,

metabolic syndrome, colon cancer, breast cancer, and depression [101]. When compared to inactive individuals, physically active adults exhibit better cardiorespiratory fitness and muscular strength, a healthier body mass and composition, and a favorable metabolic profile [101]. Furthermore, they report better quality of sleep and health-related quality of life [101]. In a 1-year randomized controlled study regular aerobic exercise (moderate-intensity aerobic exercise 3 days/week at 50–60% of the maximum heart rate reserve for week 1 to 7 and at 60–75% for the remainder of the program of 1 year) was shown to attenuate age-related brain atrophy and improve cognitive function in older individuals [117]. The authors reported that in 120 older persons aged 55–80 years, regular exercise improved memory function and age-related brain atrophy was reversed by approximately 1-2 years [117]. A key mechanism that mediates the neuronal effects of aerobic exercise is the secretion of neurotrophins, and in particular brain-derived neurotrophic factor (BDNF) [118–120].

Despite the existence of robust evidence for multiple health benefits of regular exercise, the underlying mechanisms are insufficiently understood. General key mechanisms that drive the process of aging include the accumulation of genetic damage, epigenetic modifications and shortening of telomeres [121]. It has been speculated that exercise can help preserve TL through the induction of telomerase [99, 122]. In the following section the scientific evidence addressing this concept is reviewed.

Exercise and telomere biology: animal studies

Although human studies suggest that regular exercise preserves telomeres, they are unable to unveil the underlying mechanisms. Animal models can help to close this gap as they allow to investigate on the mechanistic pathways. At present, only very few animal studies have been performed [59, 98, 123, 124]. It appears that telomeres of murine blood leucocytes and other cell types (e.g. myocardium, liver, aorta) also become shorter with advancing age [59, 98, 123]. However, this process is rather slow and may take between 12 to 18 months. For example, TL of blood leucocytes and cardiomyocytes was comparable in 3-week-old and 6-month-old C57/Bl6 mice, but was significantly reduced after 18 months [59, 98]. Interestingly, the myocardium of these exercising mice also showed increased telomerase and shelterin expression and a reduction of apoptosis and cell-cycle arrest [59, 98, 100]. Regular running exercise has been shown to attenuate the age-related erosion of TL in hepatocytes and cardiomyocytes of CAST/Ei J mice, a wild-derived inbred strain of mice, over a period of 1 year [123]. Moreover, in skeletal muscles and cardiomyocytes the age-related shortening of telomeres is accompanied by a decreased gene expression of the shelterin components TRF1 and TRF2 [123]. Chronic exercise can counteract the reduced expression of shelterins and thus aid to stabilize telomeres [123]. TRF1 and TRF2 protein content showed similar trends that failed to reach significance. In line with these results, Werner et al. reported a persistent up-regulation of cardiac telomere-stabilizing proteins TRF2 and TERT after 6 months of daily running exercise [98]. In parallel, the senescence-related proteins Chk2, p53, and p16 were down-regulated. Together, these effects lead to a substantial reduction of apoptotic cardiomyocytes in the heart of exercising mice. Regular running exercise also ameliorated the cardiotoxic effects of doxorubicin [98]. Overall, experimental studies suggest that the beneficial cardiac effects of regular exercise are primarily mediated by TERT, eNOS, and IGF-1.

Exercise-mediated telomere preservation and other beneficial health outcomes are most likely the result of a cumulative effect over an extended period of time. However, even a single bout of exercise has been shown to increase the protein levels of TRF1 and TRF2 as well as *Pot1a*, but not *Pot1b* gene expression [124]. These changes are accompanied by a greater expression of DNA-repair and -response genes (*Chk2* and *Ku80*) and greater protein content of phosphorylated p38 MAPK [124]. It has been speculated that the rapid increase in shelterin gene expression represents a direct adaptive reaction to the exercise stimulus, which depends on the duration, intensity and type of exercise [124]. In contrast, the fast increase in protein content is probably the result of improved proteostasis rather than increased mRNA translation. The rapid increase of shelterin expression in response to a single exercise session does not necessarily lead to a prompt increase in TA [124]. However, after three weeks of regular training, a persistent upregulation of myocardial TERT expression has been shown by Werner et al. [59, 98]. This activation of TERT appears to be essential for the cardioprotective effects of physical activity.

Although existing evidence is rather limited, available data suggest that exercise induces an immediate short-lived regulatory response in shelterin mRNA expression, but only a continuous stimulation over an extended period of time results in a preservation of telomeres and delays cellular aging. Furthermore, regular exercise is directly involved in the establishment of an anti-apoptotic and anti-senescent cellular environment through up-regulation of genes implicated in the DNA damage response and repair, including *Ku70/Ku80* and down-regulation of *p16*, *p53* and *Chk2* [98, 123].

Interestingly, the myocardium of exercising mice showed increased telomerase and shelterin expression and a reduction in apoptosis and cell-cycle arrest [59, 98, 100].

Exercise and telomere biology: human studies

The first study to explore the relationship between exercise and TL in humans was conducted by Cherkas et al. In a cross-sectional survey of 2401 white men and women they showed that LTL was positively associated with higher physical activity levels [125]. Similar results were reported by Du et al. analyzing 7,813 adult women from the Nurses' Health Study, where even moderate amounts of activity were associated with longer telomeres [126]. In 5823 adult men and women of the National Health and Nutrition Examination Survey (NHANES 1999-2002) Tucker et al. showed that average LTL decreases by 15.6 bp per year of chronological age [127]. Individuals with higher levels of physical activity had substantially longer telomeres in peripheral blood leucocytes, corresponding to a gain of biological age of approximately 9 years [127]. All these epidemiologic studies are limited by their cross-sectional nature and the fact that physical activity is self-reported. However, several smaller studies support the concept of telomere preservation by regular exercise [128–130]. In a comparison of telomere biology in young and middle-aged endurance athletes with sedentary controls, Werner et al. demonstrated that regular endurance training is associated with a reduction in leucocyte telomere erosion [59]. In their study, LTL of middle-aged athletes was preserved at the level of young controls. In contrast, LTL of middle-aged controls was approximately 30-40% lower than in young controls and thus, demonstrating an age-related attenuation. The preservation of TL was confirmed by two independent methods, qPCR and flow-FISH. Furthermore, when compared with untrained individuals, athletes showed increased TA and expression of telomere-stabilizing shelterin proteins, such as TRF2. The effects on telomere biology were accompanied by a pronounced inhibition of the DNA damage checkpoint kinase (*Chk2*) and the regulators of cell-cycle progression and survival, termed *p16* and *p53* [59]. In line with these results, Denham et al. analysed LTL and the expression of telomere-regulating genes in 61 Australian endurance athletes and 61 healthy controls [51]. LTL in athletes was 7.1% (208-416 nucleotides (nt)) higher than in sedentary controls. In addition, athletes showed a higher expression of *TERT* and *TPP1* mRNA expression. Interestingly, resting heart rate emerged as an independent predictor of LTL, *TERT* and *TPP1* mRNA expression in this study. Denham et al. also showed that training volume determines the effect of exercise on telomere biology with the greatest effects seen in the most active athletes. A much smaller study from Østhus et al. showed greater LTL in older endurance athletes than in individuals of the same age with a medium level of activity [131]. However, young individuals with high and low activity levels showed no difference in LTL. On the molecular level telomere-associated genes, including *TERT*, *TERF2IP* (which encodes RAP1), Sirtuin-6 (*SIRT6*) and TATA-box binding protein (*TBP*) and miRNAs that target these genes are upregulated after a single running session of 30 minutes at 80% of peak oxygen uptake (VO2Peak). The analysis of white blood cells from 22 healthy male volunteers, immediately after and 60 min after exercise, showed that 56 miRNAs were differentially regulated post-exercise (FDR <0.05) and that 4 of these (miR-186, miR-181, miR-15a and miR-96) potentially target telomere-associated mRNA species [132].

Although cross-sectional observation studies suggest that regular exercise preserves TL through an activation of telomerase, experimental and prospective studies are necessary to proof causality. A recent study in 124 healthy previously inactive individuals explored the effects of regular endurance training, intensive interval training and resistance training over a period of 6 months [99]. Participants trained 3 times per week for 45 min. Compared to non-exercising controls, TA in blood mononuclear cells was up-regulated 2 to 3-fold in the endurance- and interval-training groups, but not in the resistance-training group. The activation of telomerase was accompanied by longer telomeres in lymphocytes, granulocytes, and leucocytes. In addition to this training study, Werner et al. also explored the effects of a single bout of exhaustive exercise using a stepwise ramp protocol on a treadmill. When compared to baseline, CD14+ and CD34+ leucocytes collected after exercise, exhibited increased TA, which was still measurable 24h-post exercise. IGF-1, a potential mediator of the exercise-induced activation of telomerase [59], showed a biphasic response. However, after the 6-month training program, IGF-1 was comparable to baseline levels. Furthermore, blood collection was performed from 48 hours to 7 days after the last exercise session. This suggests that whilst the exercise-induced effects on telomere biology are of short duration, any health benefit is the result of a cumulative effect achieved by regular training. The beneficial effects of long-term exercise on TL and TA have also been shown by Melk A et al. in 59 healthy middle-aged men with former sedentary lifestyles [133]. Besides the secretion of IGF-1, another putative hypothesis to explain the exercise-induced activation of telomerase with subsequent telomere elongation is the release of nitric oxide (NO) as a result of increased vascular shear stress [99]. Endothelial NO synthase and

TA appear to be linked in a signalling pathway that mediates vascular protection [59].

Despite robust evidence from cross-sectional and prospective intervention studies, not all previously published analyses support a relationship between exercise and telomere biology [134–139]. For example, a comparison of 17 marathon runners and 19 healthy sedentary controls reported no difference in LTL [136]. Similar findings were reported by Song et al. in 84 healthy volunteers [135]. Finally, in a cross-sectional and longitudinal analyses of 582 older adults, Soares-Miranda et al. found no consistent relationship between physical activity and LTL [137]. Only some general functional measures, such as walking distance and "chair test performance", were cross-sectionally related to LTL. In addition, changes in leisure-time activity and in "chair test performance" altered the change in LTL over time. Results from the "Berlin Aging Study" suggest that, in adult men aged over 61 years, long periods of physical activity are necessary for the prevention of telomere shortening (at least 10 years), with intensive sports activities having the greatest effect [140]. This concept is confirmed by a study from Lane et al. where former elite athletes were found to have comparable LTL to age-matched, sedentary individuals [141].

Some researchers suggest that the relationship between LTL and exercise is U-shaped [51, 142, 143]. For example, Savela et al. analysed physical activity levels, LTL and the proportion of short telomeres in 204 randomly selected survivors of the "Helsinki Businessman Study". Moderate physical activity was associated with the longest mean LTL. A cross-sectional comparison of endurance athletes and healthy controls provides additional support that moderate amounts of exercise training protects against biological aging, while higher amounts may not elicit additional benefits [51].

In summary, the evidence implies that the protective effects of exercise require a rather long-time span and continuity in order to become evident.

Differential effects of exercise modalities on telomere biology

As reported above, it is not clear whether exercise can preserve or increase TL. The controversial results may be explained, in part, by the fact that "exercise" is a general term that includes many different types of physical activities, such as running, swimming, dancing, weightlifting, ball sports and others. Therefore, the question arises whether different exercise modalities exert differential effects on telomeres? Most existing studies have investigated the effects of endurance exercise [59, 128, 144], in particular running and cycling, or mixed exercise regimens [126, 129]. However, in most epidemiologic studies, physical activity was self-reported [125, 145, 146]. To date, only one study directly compared the effects of different exercise modalities on telomere biology [99]. This randomized controlled trial showed that only endurance and high-intensive interval training, but not resistance training, increased TA and LTL in middle-aged healthy individuals. All intervention groups performed 3 exercise sessions per week with a duration of 45 min for 6 months. In an analysis of the NHANES (1999-2001), different types of self-reported leisure time activities were assessed, and only moderate/vigorous physical activity was significantly associated with LTL [147]. A lack of resistance training to preserve TL has also been observed in a small cross-sectional study that compared power lifters with healthy, active individuals with no history of strength training [148]. In summary, there is insufficient data to judge if different training modalities exert differential effects on telomeres, telomerase and shelterin expression. However, existing studies suggest that aerobic endurance exercise, but not resistance training, is helpful to preserve TL, at least in leucocytes.

Mechanistic considerations

Besides the preservation of telomeres, several other mechanisms have been proposed to contribute to the anti-aging effects of physical activity (Figure 1). Regular endurance exercise over 5 months improved mitochondrial biogenesis and morphology in skeletal muscles and other organs including lungs and heart in mtDNA mutator mice (animals with accelerated rates of mitochondrial DNA mutation). As a result, exercise delayed the age-related degeneration process of multiple organs, increased mobility, and attenuated telomere shortening [149, 150]. As noted, exercise contributes to an increased shelterin expression via upregulation of p38 MAPK and a subsequent regulation of several transcription factors [123], including the upstream transcription factors of the PGC-1α gene. PGC-1α is a pleiotropic protein involved in cellular energy metabolism [151, 152] that has also been linked to aging [153]. During endurance exercise and caloric restriction, PGC-1α is activated by adenosine monophosphate-activated protein kinase (AMPK), accumulates in the nucleus through sirtuin 1-dependent deacetylation and acts as a co-activator for other transcription factors [153, 154] including nuclear respiratory factor 1 (NRF-1), a regulator of mitochondrial biogenesis [155, 156]. Age-dependent telomere shortening contributes to mitochondrial and genomic DNA damage via activation of p53 and down-regulation of PGC-1α/β [157]. Recently, de Carvalho Cunha et al. have demonstrated that exercise regulates *p53* and *Chk2* in an intensity-

dependent fashion, with high intensity endurance exercise being more effective in downregulating *p53* than low intensity exercise [158]. Moreover, high intensity training appears to be more effective in enhancing antioxidant defense, AMPK and PGC-1α expression [159].

Today, only very few animal studies have explored the mechanisms behind the exercise-mediated preservation of telomeres [59, 98, 123, 124]. Although these studies seem to confirm the results obtained in human studies, many mechanistic aspects remain to be clarified. Therefore, future research is needed to improve our understanding on the effects of exercise on telomere biology and genomic aging.

Analytical aspects

Despite robust evidence linking leucocyte telomere shortening with aging and age-related diseases, the measurement of LTL is not yet used clinically. Several unresolved pre-analytical, analytical and post-analytical aspects have hampered the transition of this promising marker from research laboratories into routine diagnostics. From a pre-analytical point of view the pronounced inter-individual variability of LTL [78, 160] and leucocyte telomere shortening [161] complicate a meaningful interpretation of individual results. Aviv et al. have shown that amongst young adults LTL changes between -240 and +12 bp per year. As previously discussed, telomere shortening throughout life is not a

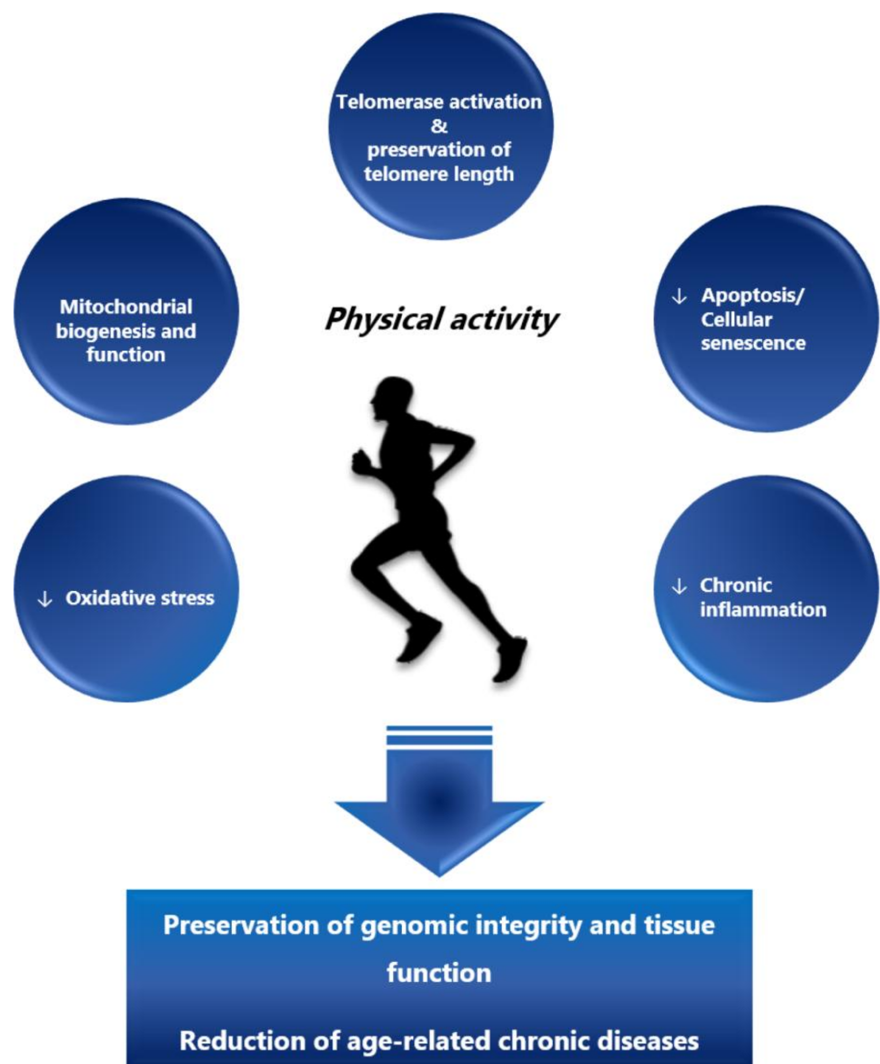

Figure 1. The beneficial effects of regular physical activity. Regular physical activity exerts its beneficial effects through activation of telomerase, preservation of telomere length and improved mitochondrial biogenesis and function. On the cellular level these effects lead to the reduction of apoptosis, cellular senescence and oxidative stress, lowering the subsequent multi-system chronic inflammation. In summary, regular physical activity is a means to preserve genomic integrity and tissue function and reduce the onset of age-related chronic diseases.

linear process instead it is most pronounced during the period of rapid somatic growth in the first two years of life [34, 35, 38, 39]. In addition, young individuals with either longer or shorter than the average TL tend to maintain that classification throughout the rest of their life [162, 163]. Together, these unresolved pre-analytical issues have prevented a consensus amongst researchers and clinicians as to when measurement of TL is meaningful and might provide a benefit for the individual.

Another major concern is the appropriate sample matrix. Variable proliferation rates lead to vastly different TLs amongst several tissue types [35]. In different organs, from the same individual, TL can differ by factor 6 and more [35]. It also appears that TL within the same organ varies substantially, and consequently results depend on the site of sample collection. Only few studies have investigated the distribution of TL in different organs from the same donor. Perhaps normalizing LTL for TL of a post-mitotic tissue like fat or skeletal muscles might provide a better understanding of leucocyte telomere dynamics during aging [164]. Dlouha et al. measured telomere length in twelve human tissues (peripheral blood leukocytes, liver, kidney, heart, intercostal skeletal muscle, subcutaneous and abdominal fat) from dead human donors with a wide age range (29 weeks to 88 years). They found an inverse relationship between relative telomere length (rTL) and donor age, with the longest rTL detected in the youngest [35]. TL was significantly higher in blood compared to the majority of tissues but not different compared to adipose and renal tissue. The largest interindividual variability was observed in leucocytes and kidney [35]. Nonetheless, these results were confounded by the small number of donors and their variable health status. Up until now, little is known about the effect of injury and physical activity on telomere dynamics in human skeletal muscle. A recent study assessed whether aerobic capacity was associated with TL in skeletal muscles and leucocytes and whether TL is associated in these two tissues, across a wide age range (18–87 years). The findings support a correlation between LTL and mean skeletal muscles telomere length indicating that individuals with short (or long) telomeres in one tissue also display short (or long) telomeres in another tissue. However, skeletal muscle TL was not associated with age, and aerobic capacity was not associated with longer telomeres in either leukocytes or skeletal muscles [139]. Therefore, more studies are needed to consolidate our knowledge about tissue specific differences in telomere dynamics.

To avoid invasive sample collection and regional variability of TL in solid organ tissues, blood leucocytes have been proposed as an alternative matrix for telomere analysis. Blood can easily be collected multiple times and LTL, at least theoretically, mirrors telomere dynamics in hematopoietic stem cells (hSC) and is an index of hSC reserve [165, 166]. However, blood leucocytes represent a heterogeneous cell population including monocytes, granulocytes and lymphocytes. The composition of this population is highly variable depending on stressors i.e. exercise, nutrition, smoking, psychological stress and others. These stressors can trigger a redistribution of leucocytes from immune reservoirs to the circulation and peripheral tissues [167]. As a result, the percentage of neutrophil granulocytes can range from 40 to 70% of the entire leucocyte count. Compared to many other cell types, neutrophils have a very short lifespan of 1-3 days. Therefore, it is not surprising that LTL exhibits by far the highest intra- and inter-individual variability amongst all sample types [35]. Conditions, such as CHIP, which arise from leucocyte precursor cells, may also influence the distribution of LTL and thus hamper the interpretation of LTL results. None withstanding the potential association between LTL and CHIP, which is primarily based on observational data, variable telomere attrition rates between individuals and amongst different solid tissues remain a major issue when interpreting the results of TL measurements. Therefore, more experimental data are needed to consolidate our knowledge about the relationship between TL in leucocytes and different solid tissues in the context of CHIP and other TL modifying conditions [168]. In summary, our present knowledge is insufficient to judge the validity of LTL as marker of biological age and as prognostic tool for poor outcomes and shorter DALYs in clinical settings. Furthermore, it is not clear how telomere dynamics of peripheral blood leucocytes reflect pathophysiological changes in individual organs.

Besides the aforementioned pre-analytical issues, there are also analytical aspects that hamper a wider use of TL analysis. Existing methods are quantitative PCR (qPCR), Terminal Restriction Fragment (TRF) analysis by Southern blot, fluorescence in situ hybridization coupled with flow cytometry (flow-FISH), Single Telomere Length assay (STELA), Universal STELA, and Telomere Shortest Length Assay (TeSLA). Although all these methods analyse TL, the information they provide is substantially different and the results are not directly comparable [169]. Briefly, the qPCR assay is most frequently used in epidemiologic studies because it is easy to perform, requires small amounts of DNA and allows high throughput. The method provides a relative TL (T) compared to a single copy gene (S) and results are expressed as a T/S ratio. Information about the distribution of short and long telomeres, as well as differences between individual chromosomes and cells cannot be obtained. TRF is considered the "gold standard" for TL analysis that measures the intensity of telomere smears to determine an average TL. However,

applicability of this method is limited by the requirement of large amounts of DNA (approx. 3 µg) and a relatively laborious and time-consuming assay procedure, additionally with this technique very short telomeres (approx. 2 kb or less) are difficult to detect. Although reproducibility within the same laboratory can be rather good, results cannot easily be compared between laboratories. However, commercial TRF kits are now available and may help to improve inter-laboratory comparability. TL of peripheral blood leucocytes can also be measured by fluorescence in-situ hybridization (FISH) based methods. FISH based methods produce very reliable results, but are laborious and require expensive instrumentation [169]. Q-FISH expresses TL as relative fluorescence units. With the help of TRF measured standards absolute TLs can be derived. With this technique it has been shown that the shortest telomeres determine cell viability and chromosome stability [170–172]. Reliable measurement of the shortest telomeres might open new possibilities for the assessment of biological age, the determination of individual risk for age-related degenerative disease and patient management. Finally, TeSLA assay, requires only small amounts (<1µg) of DNA and allows the unbiased measurement of TL distribution [173]. A wider use of TeSLA is hampered by its low throughput. Furthermore, very long telomeres, such as in inbred strains of mice, are not captured by this method. For a more comprehensive overview on the various techniques we refer to a recent review from Lai et al. [169]. Yet, for the measurement of TA the commonly used assay remains the Telomere-Repeat Amplification Protocol (TRAP), a two-step procedure composed of telomerase mediated primer extension and PCR-based detection of extended products. This method has been further adapted to combine TRAP and droplet digital PCR (ddTRAP), thus increasing the sensitivity, repeatability and throughput of the assay. The specifics of the latter are reviewed by Ludlow at et al. [174, 175]. More laborious and not clinically used methods to detect TA include PCR-free assays such as electrochemical assays, optical assays, and signal-transduction assays. However, all of them must be optimized to improve throughput and sensitivity and need special instrumentation to be performed [176].

In summary, telomere length and TA are almost exclusively measured in research laboratories. Sample matrix and analytical procedure should be carefully chosen for the intended use, and analyses should be performed by sufficiently trained staff.

CONCLUSIONS

Telomere research has gained much attention in the previous decade for its potential use and promise as a future therapeutic target, disease management and measurement of genomic aging. Interventions, such as physical activity that target the deleterious processes of aging have concomitantly created interest in the area of lifestyle and aging related research. Largely, the available physical activity data do not exclude that an association between regular exercise and TL exists. However, to date, the observed results from human studies are skewed largely by associations and observational or cross-sectional data. In light of the limited data, available evidence suggests altogether, that regular, and consistent physical activity over an extended period of time may assist with preservation of telomeres and cellular aging. Nevertheless, conflicting and a lack of consistent findings from the existing evidence, and particularly from the few available mechanistic studies means there is much more to explore and understand, prior to measurements such as TL will be adopted clinically.

Considering the above, future research should be focused on 1) developing more experimental data to further elucidate and confirm the relationship and mechanistic pathways between physical activity, aging and telomere biology, 2) investigating the effects of the use of different exercise modalities and intensities on telomeres and 3) further determining if these effects are tissue-specific or systemic.

AUTHOR CONTRIBUTIONS

Authors contributing to the presented manuscript writing and editing: MDS: manuscript conception, writing and editing; CS, MK, IL, GD: manuscript writing, editing and final approval; HJG, MH: manuscript conception, writing, editing and final approval.

CONFLICTS OF INTEREST

No conflicts of interest to declare.

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Article 1

Telomere length in leucocytes and solid tissues of young and aged rats 2 3

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Abstract: (1) Background: telomeres are protective nucleoprotein structures at the end of chromosomes that shorten with age. Telomere length (TL) in peripheral blood mononuclear cells (PBMCs) has been proposed as surrogate marker for TL in the entire organism. Solid evidence that supports this concept is lacking; (2) Methods: relative TL (RTL) was measured in PBMCS and multiple solid tissues from 24 young (4 months) and 24 aged (14 months) Sprague-Dawley (SD) rats. The mRNA expression of telomerase (TERT) and shelterin proteins TERF-1 and TERF-2 was also measured; (3) Results: mean RTL in PBMCs and solid tissues of young rats ranged from 0.64±0.26 in large intestine to 1.07 ± 0.22 in skeletal muscle. RTL in PBMCs correlated with that in kidney (*r=0.315, p=0.008*), skeletal muscle (*r=0.276, p=0.022*), liver (*r=0.269, p=0.033*), large intestine (*r=-0.463, p=7.035E*$^{-5}$) and aorta (*r=-0.273, p=0.028*). A significant difference of RTL between young and aged animals was only observed in aorta (0.98±0.15 vs 0.76±0.11, *p=1.987E-6*), lung (0.76±0.14 vs 0.85±0.14, *p=0.024*) and visceral fat (0.83±0.14 vs 0.92±0.15, *p=0.44*). The expression of TERT significantly differed between the tested organs with highest levels in liver and kidney. Age-related differences of TERT expression were found in PBMCs, skeletal muscle and visceral fat. mRNA expression of TERF-1 and TERF-2 was tissue specific with highest levels in liver. Age-related differences in TERF-1 and TERF-2 expression were inconsistent; (4) Conclusions: the present study questions the utility of RTL in PBMCs as a biomarker for the individual assessment of aging. 10–26

Keywords: telomeres; telomerase; shelterin; aging; Sprague Dawley rats 27 28

1. Introduction 29

Individuals age at remarkably different rates so that the health status and functional impairment can vary widely at the same chronological age [1]. Telomere length (TL) has been proposed as a biomarker of biological age that may assist to estimate individual trajectories of aging. Telomeres are protective nucleoprotein structures at the ends of eukaryotic chromosomes that are of critical importance for the preservation of our genome [2-4]. They are composed by multiple repeats of short non-coding DNA sequences and associated proteins known as shelterins. Depending on species, the basic telomeric DNA motif is six to eight base pairs long and can be found in up to several thousand copies per telomere [5]. Telomeric DNA is double-stranded for most of its length with a short single-stranded overhang at the 3'-OH end. Telomeric DNA and shelterin proteins form a unique three-dimensional structure, which is essential for the protective function of telomeres. With the help of shelterins, telomeric DNA folds backward forming a loop structure (t-loop) that allows the single stranded DNA-overhang to invade double-stranded telomeric DNA. Together, this prevents DNA repair systems from mistaking telomeric ends as DNA strand brakes and from inappropriate attempts to repair them. 30–44

The progressive impairment of genomic integrity and genomic instability are key drivers of the aging process [6,7]. With every cell division, telomeres shorten a little bit due to incomplete replication of the DNA lagging strand. In addition, accidental damage can also cause telomere shortening. This age-related shortening progressively compromises the three-dimensional structure of telomeres and once a critical threshold is reached, cells can no longer divide [8,9]. Because of their progressive shortening, telomeres are considered a molecular clock of aging.

However, telomere shortening is not a linear process. Human and animal studies have repeatedly shown that lifestyle factors, such as obesity, physical activity, smoking, psychological stress, and sleep, modify the rate of telomere shortening. Furthermore, these factors are related to the risk of many age-related diseases and mortality. Telomerase (TERT), an enzyme that elongates telomeres, and shelterin proteins are key regulators of the telomere shortening rate. Previous studies suggest that many lifestyle factors modify the telomere shortening rate through an altered expression of these proteins.

The unique role of telomeres in the aging process has led to the idea that TL could be a useful surrogate marker of biological age. In fact, human and animal studies have shown that in age-related diseases, such as atherosclerosis, diabetes and rheumatic diseases, TL is reduced in the affected tissues [6,10-13]. As the analysis of TL in solid tissues requires an invasive biopsy, TL in peripheral blood leucocytes (LTL) has been proposed as surrogate marker for TL in other tissues. However, solid evidence that supports this concept is largely lacking. A rather small study by Dlouha *et al.* analysed TL in twelve human tissues from deceased donors [14]. TL differed by factor six between different organs and LTL was only correlated to TL in liver and muscles. Besides a rather small number of cases, this study is strongly limited by a very wide age range of the donors (29 weeks to 88 years). Also, Hiam *et al.* reported a weak correlation between LTL and skeletal muscle TL in healthy men. However, skeletal muscle TL was not associated with age [15]. To date, the strongest evidence that supports LTL as a suitable surrogate marker for TL in other tissues is provided by the large scale GTEx project [16]. Although this study is of cross-sectional nature, the results suggest that LTL shortens at a comparable rate as other somatic cells [16]. However, specimen collection was not standardized in this study, individuals were very rather heterogeneous and information on their health status has not been obtained. A systematic mapping of TL in multiple organs and tissues of the same individual has not been performed as yet. Moreover, a structured analysis of age-related changes of TL in multiple tissues is also lacking.

Animal studies are useful to investigate age-related changes of telomeres in multiple organs of the same organism [17]. The few existing studies have focused on specific tissue types, such as leucocytes, myocardium, liver, and aorta. Similar to humans, the telomeres of murine blood leukocytes and other cell types (i.e., myocardium, liver, aorta) shorten with age [18-20], but this process may take more than a year [18,19]. Moreover, in skeletal muscle and cardiomyocytes the age-related shortening of telomeres is accompanied by a decreased gene expression of the shelterin proteins TERF-1 and TERF-2 [20].
To address the existing gap of knowledge, the present study aimed to explore if LTL is a suitable marker of biological age that represents TL in other organs of the same organism. For this purpose, we performed a structured analysis of TL in multiple tissues from young and aged Sprague-Dawley (SD) rats. In addition, we studied RNA expression of telomerase (TERT) and selected shelterins (TERF-1 and TERF-2).

2. Results

2.1. Distribution of RTL in blood leucocytes and solid organ tissues

RTL showed pronounced interindividual variability across all tissues with greatest scatter in large intestine, spleen, and brain (Figure 1). The young animals had a mean RTL in PBMCs of 0.88 ± 0.15. Mean RTLs of the solid tissues ranged from 0.64 ± 0.26 in large intestine to 1.07 ± 0.22 in skeletal muscle. Liver, skeletal muscle, aorta and kidney had significantly higher mean RTLs than PBMCs. In contrast, RTL in large intestine and lung was lower than in PBMCs. Aged animals exhibited a comparable distribution of RTL across organs. Similar to young animals, mean RTL in aorta and large intestine was significantly lower than in PBMC, whereas in lung RTL was significantly higher (Figure 1).

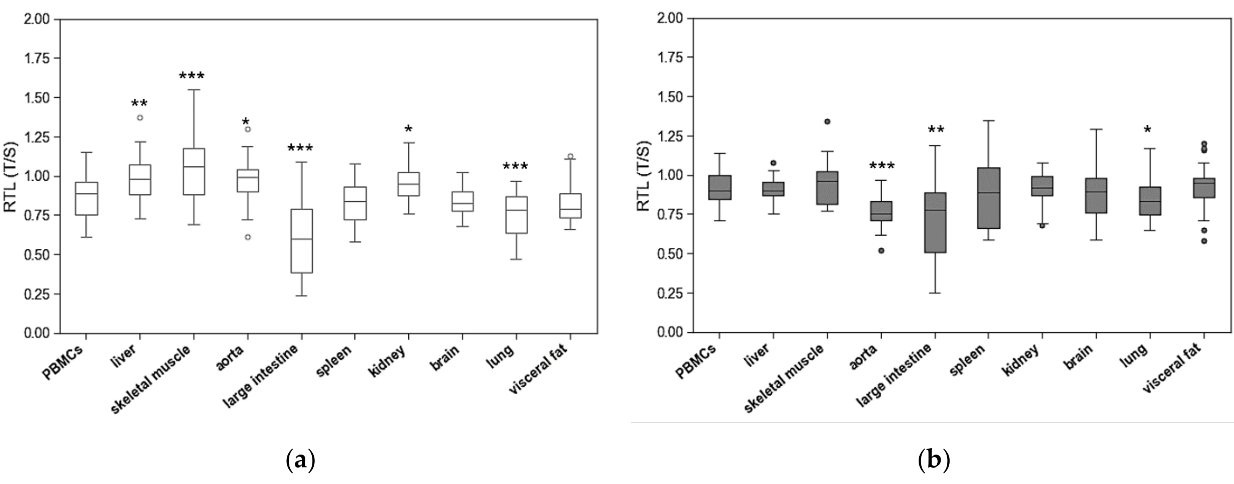

Figure 1. Relative telomere length (RTL) of peripheral blood mononuclear cells (PBMCs) compared to RTL of 9 different solid organs (including liver, skeletal muscle, aorta, large intestine, spleen, kidney, brain, lung, visceral fat) isolated from: (**a**) young and (**b**) adult rats. RTL is expressed as ratio of average telomere length to the reference gene GAPDH. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. PBMCs RTL.

To test the hypothesis that RTL in PBMCs represents the situation in other organs, RTL from PBMCs and solid organs were correlated in young and aged animals. Figure 2 shows that there was no consistent correlation between RTL in PBMCs and solid organs. Significant positive correlations were found between RTL in PBMCs and liver, skeletal muscle, and kidney. Inverse correlations were found between RTL in PBMCs and RTL in large intestine and aorta. RTL of all other tissues was not correlated to that of PBMCs.

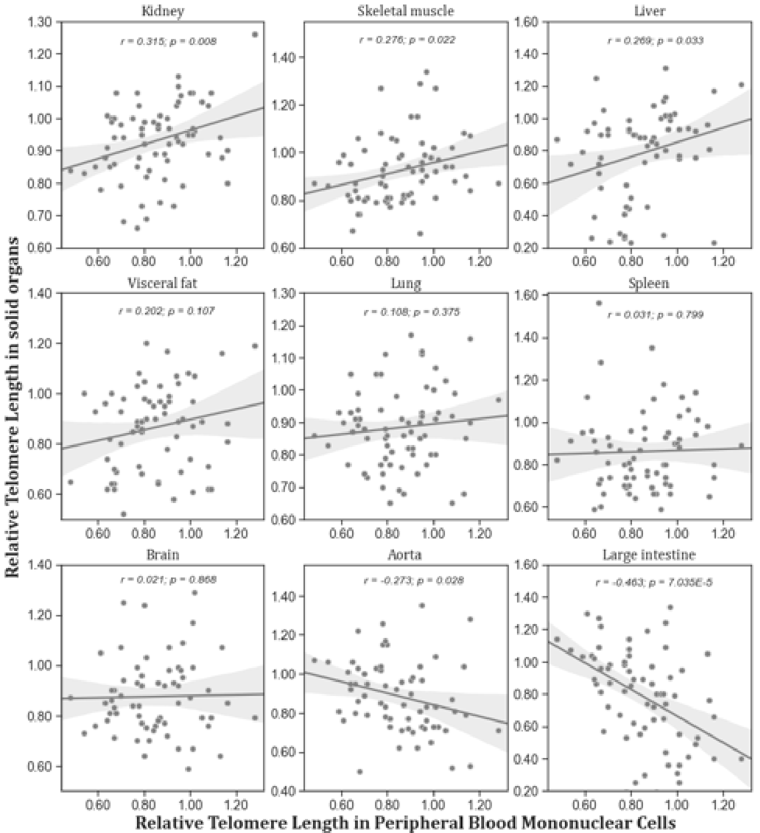

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Figure 2. Correlation between RTL in PBMCs and RTL in different organs isolated from adult rats (n = 72). *r* – Pearson correlation coefficient, *p* – p value. The figure also shows in grey the regression line and 95% confidence interval. 117 118

2.2. Age-related changes of RTL 119

To identify age-related differences of RTL in PBMCs and solid tissues, we compared RTL of aged and young animals in all these matrices (Figure 3). The only organ with lower RTL in aged animals was aorta. Lung and visceral fat tissue showed higher RTL in aged animals. All other organs had comparable RTL in both age groups. 120 121 122 123

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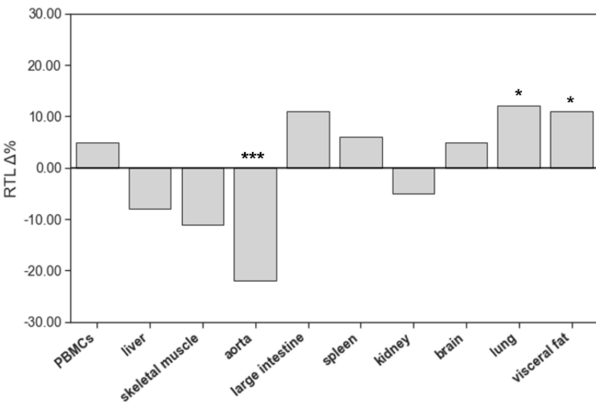

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Figure 3. The impact of age on relative telomere length (RTL) of different tissue types. The boxes represent the delta% variation of RTL in adults compared to young. * $p < 0.05$; *** $p < 0.001$ vs. young. 126 127

2.3. Telomerase and Shelterin mRNA expression from different tissue types in young and adult SD rats 128 129

The expression of TERT markedly differed between the tested organs with highest levels in liver and kidney (Figure 4). In the liver, TERT mRNA expression was 40 times higher than in spleen and lung tissue. 130 131 132

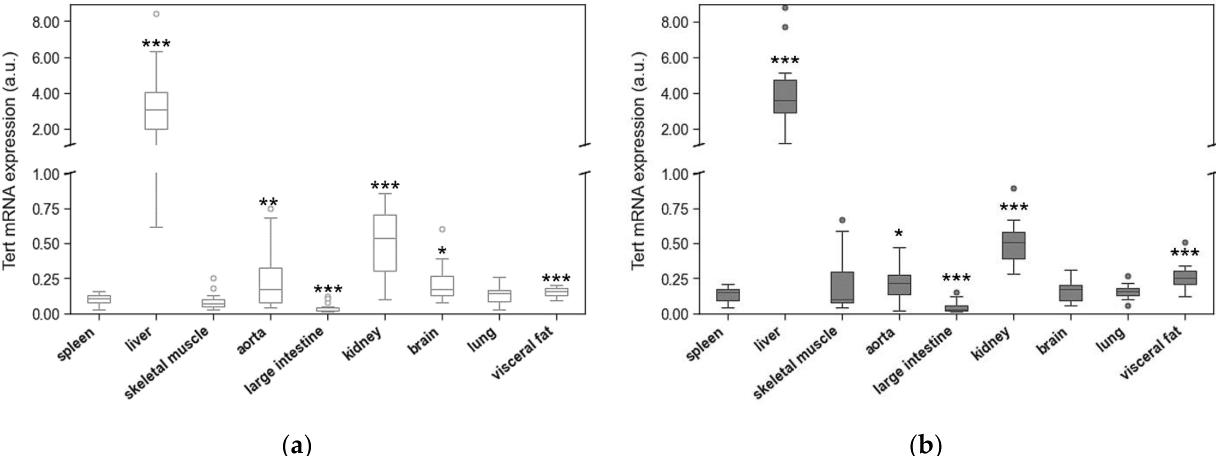


Figure 4. Tert mRNA expression of spleen compared to Tert mRNA expression of 8 different solid organs (including liver, skeletal muscle, aorta, large intestine, kidney, brain, lung, visceral fat) isolated from: (**a**) young and (**b**) adult rats. Tert mRNA expression is shown in arbitrary units. * p < 0.05; ** p < 0.01; *** p < 0.001 vs. spleen Tert mRNA expression.

Age-related differences of TERT expression were only found in spleen, skeletal muscle, and visceral fat (Figure 5).

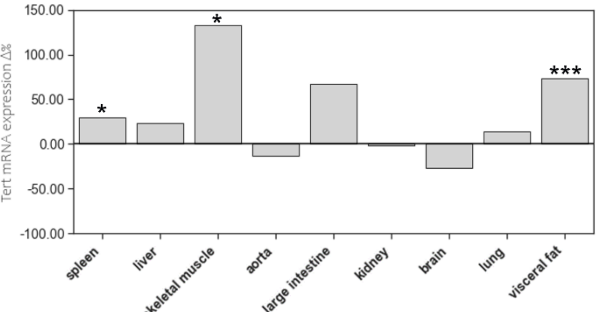
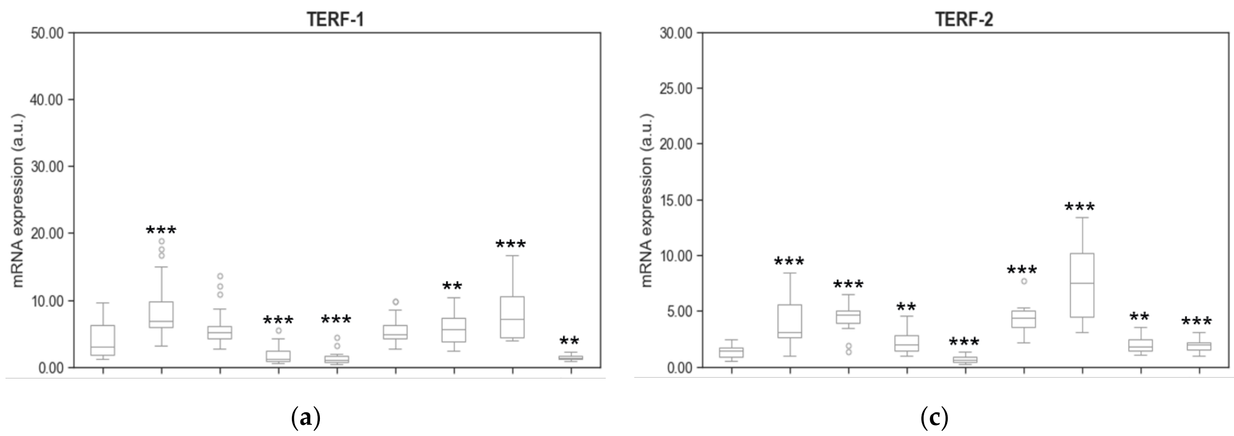
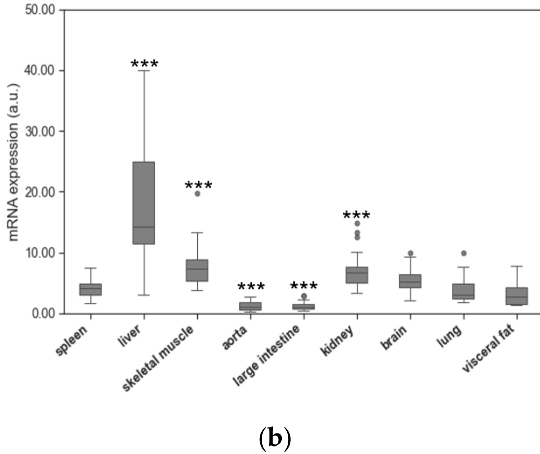


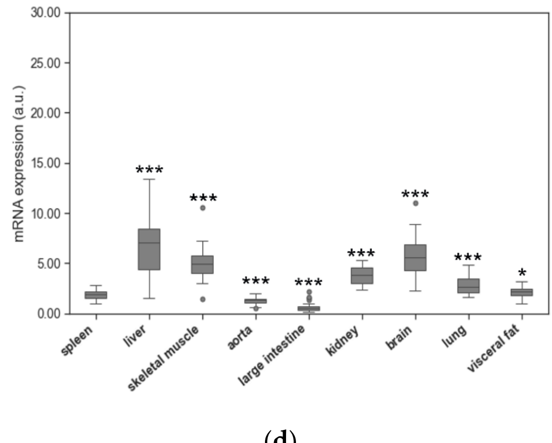
Figure 5. The impact of age on Tert mRNA expression of different tissue types. The boxes represent the delta% variation of Tert mRNA expression in adults compared to young. * p < 0.05; *** p < 0.001 vs. young.

The mRNA expression of TERF-1 and TERF-2 was tissue specific showing pronounced variation between the different organs and spleen. The highest expression levels of both genes were found in liver (Figure 6). In addition, TERF-2 was highly expressed in brain.





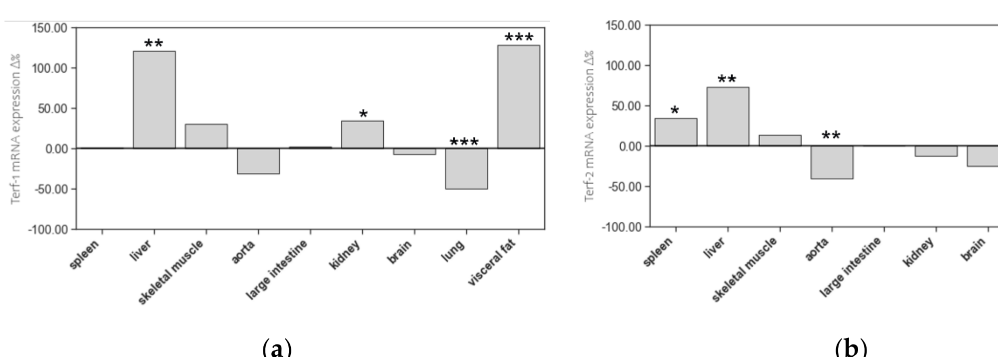
(**b**)



(**d**)

Figure 6. Spleen Terf-1 mRNA expression compared to Terf-1 mRNA expression of 8 different solid organs (including liver, skeletal muscle, aorta, large intestine, kidney, brain, lung, visceral fat) isolated from: (**a**) young and (**b**) adult rats. Spleen Terf-2 mRNA expression compared to Terf-2 mRNA expression of 8 different solid organs (including liver, skeletal muscle, aorta, large intestine, kidney, brain, lung, visceral fat) isolated from: (**c**) young and (**d**) adult rats. Terf-1 and Terf-2 mRNA expression is shown in arbitrary units. * p < 0.05; ** p < 0.01; *** p < 0.001 vs. spleen Terf-1 or Terf-2 mRNA expression.

Age-related differences in mRNA expression of these two shelterins were inconsistent. In aged animals, TERF-1 showed higher mRNA expression levels in liver, kidney, and visceral fat but lower levels in lung. TERF-2 expression was higher in spleen, liver, and lung of aged animals, whereas aorta showed a lower expression level (Figure 7).



(**a**) (**b**)

Figure 7. the impact of age on mRNA expression of (**a**) Terf-1 and (**b**) Terf-2 in different tissue types. The boxes represent the delta% variation of mRNA expression of Terf-1 and Terf-2 in adults compared to young. * p < 0.05; ** p < 0.01; *** p < 0.001 vs. young.

3. Discussion

The systematic mapping of RTL in young and old rats showed substantial differences between organs. These differences were accompanied by pronounced interindividual variability of RTL in all tissues. Surprisingly, the distribution of RTL across different organs was comparable in young and aged animals without a systemic age-related reduction. Furthermore, correlation analyses showed no consistent association between RTL in PBMCs and solid tissues. Together, these results question the utility of RTL of PBMCs as a valid biomarker that represents the aging process in the entire organism.

The present study provides robust evidence for tissue specific RTLs. In SD rats, the longest and the shortest telomeres were found in skeletal muscle and large intestine, respectively, with a 2-fold difference. To date, similar studies have not been performed in rodents.

Human studies that compared RTL between organs also found significant differences of up to 2.5-fold [14,16]. In a rather small study by Dlouha et al, leucocytes showed the longest telomeres, whereas liver, brain and skin exhibited the shortest telomeres [14]. However, this study analysed only samples from twelve individuals with a very wide age-range of 29 weeks to 88 years, which limits the robustness of the results. In a much larger study of 6391 tissue samples from 952 donors, Demanelis *et al.* found the shortest telomeres in leucocytes and the longest ones in testis, skeletal muscle, and colon [16]. The organ distribution of RTL in rats and humans seems to differ substantially, which is probably due to species-effects. Despite, rigorous standardization of age, genetic background and housing conditions, the animals of the present study displayed great interindividual variation in RTL, which was similar to that observed in the human studies discussed before.

In contrast to current concepts, RTL in PBMCs was positively correlated to kidney, skeletal muscle, and liver but not to any of the other tissue types. In some tissues, such as large intestine and aorta, we even observed an inverse correlation. However, our results are aligned to those from others, Dlouha *et al.* also found a significant correlation of RTL in leucocytes with that in liver and muscle [14]. Moreover, in blood leucocytes and skeletal muscle biopsies of 93 healthy men between 18–87 years of age, Hiam *et al.* showed a weak, but significant, correlation of RTL [15]. In the large scale GTEx project, Demanelis *et al.* reported that RTLs were positively correlated amongst different tissues and that whole blood RTL was a reasonable surrogate for RTL in most tissues [16]. Inverse associations, as observed by Dlouha *et al.* in humans and us in rats, may thus be chance findings that are primarily due to the marked interindividual variability of RTL in most tissues. Based on previous studies and the present result it appears that RTL in peripheral blood cells is not a suitable biomarker to judge RTL in other organs of the same individual. However, in large cohorts there may be a robust association between RTL in peripheral blood cells and most other tissue types, which makes this an interesting marker for epidemiologic aging studies.

Another observation of the present study was that in most tissues RTL was comparable in young adults and aged animals. This contrasts the concept of telomere shortening with advancing age [21-24]. However, existing longitudinal studies measured RTL exclusively in leucocytes [21,23-29]. In the large scale GTEx project, Demanelis *et al.* also found an age-related reduction of RTL in most tissues [16]. Here we performed a systematic analysis of RTL in multiple organs of young and aged animals that were kept under standardized conditions. Only in aortic tissue RTL was significantly lower in aged than juvenile young animals. In lung and visceral fat RTL increased with advancing age. The absence of telomere shortening in our rats could be related to their age. SD rats have a life-expectancy of 2.5 to 3.5 years. In the present study, animals were sacrificed at 14 months of age, which corresponds to mid-adult age. Therefore, it cannot be excluded that a longer aging period would have yielded a different result. Previous work from Werner *et al.* supports a rather slow shortening of telomeres in leucocytes and cardiomyocytes of C57/Bl6 mice [18,19]. In these animals, a significant reduction of telomere length took up to 18 months. Large scale human studies have also provided robust evidence that longitudinal changes of RTL vary substantially between individuals [21,22,24]. For example, amongst 4053 adults that were analysed at baseline and again after 7-8 years, LTL decreased in 66.3%, did not change in 11.2% and increased in 22.5% [24]. Such heterogeneous results may be due to multiple individual factors that influence telomere dynamics, such as stress, nutrition, physical activity, smoking and others [11,30-40]. In the present animal study, most of these factors were rigorously controlled. Moreover, we analysed 10 different tissues without seeing a systematic effect. Together with existing human studies, the present results indicate that neither RTL in PBMCs nor in solid tissues is a useful marker to monitor aging on an

individual level. Only in cohorts of substantial size, age-related changes of RTL can reliably be shown reliably.

It is important to consider that existing studies have measured telomere length primarily by qPCR, which provides an average RTL across all cells and chromosomes in the sample [41]. However, there is substantial evidence that only the shortest telomeres trigger DNA damage responses and induce senescence. Information on the shortest telomeres can only be obtained by much more sophisticated methods, such as Telomere Shortest Length Assay (TeSLA) or Quantitative Fluorescence In-Situ Hybridization (Q-FISH). The characteristics and caveats of available methods for the analysis of telomere length have been summarized by Aubert G *et al.* and Lai TP *et al.* [41,42]. It can be speculated that the use of a different method that provides information on the distribution of short and long telomeres might have given a different result. However, such methods are not established in our laboratory, require particular expertise and are not feasible for the analysis of large sample sets. Another important aspect is that RTL may vary within the same organ depending on the site of specimen collection. In most organs the distribution of specific cell populations is not homogenous so that regional differences may impact the results.

An organ specific regulation of telomeric function is further supported by the mRNA expression analyses of TERT, TERF-1 and TERF-2. Liver tissue showed by far the highest TERT mRNA expression. However, this phenomenon was not accompanied by markedly longer telomeres. Considering that hepatocytes divide rapidly, it can be assumed that the high expression of TERT is needed to prevent excessive telomere shortening in these cells. Also, TERF-1 and TERF-2 are highly expressed in hepatic tissue supporting a tissue specific maintenance of telomeres in the liver. In contrast, terminally differentiated tissues, such as skeletal muscle and brain, are characterized by a low expression level of TERT and TERF-1, but a rather high expression of TERF-2. TERF-2 is pivotal for t-loop formation and aids the invasion of the single-stranded telomeric DNA overhang into the double-stranded telomeric region upstream. Loss of TERF-2 has been shown to prevent t-loop formation and leads to excessive telomere shortening with premature cell death [43]. It can be speculated that the high TERF-2 expression in terminally differentiated tissues reflects the particular need to protect their telomeres from DDR.

In the present study, we have not seen systematic age-related differences in mRNA expression of these proteins. TERT expression was slightly higher in spleen, skeletal muscle, and visceral fat of the aged animals, but not in any of the other tissues. When considering the interindividual variability of TERT expression in most tissues, the physiological relevance of these differences in questionable. However, TERF-1 and TERF-2 expression was markedly higher in aged liver tissue, which further supports the concept of an organ specific maintenance of telomere homeostasis in rapidly dividing hepatocytes. In addition, TERF-2 expression was higher in spleen and lung tissue of aged animals, which also have a great capacity of renewal. Interestingly, aortic tissue showed a substantially lower TERF-2 expression in aged animals, which was paralleled by a reduction in RTL.

There are several limitations that have to be considered when interpreting the present results. A study duration of ten months with rodents is rather long but may be insufficient to capture significant age-related effects on telomeres. Considering the life expectancy of SD rats, an observation period of two years might better reflect changes in telomere biology. However, towards the end of our study, some animals started developing tumours and thus had to be excluded from the analysis. This implies that for a longer study more animals would be needed in order to obtain a sufficient number of aged animals that are free from tumours and other relevant diseases. Furthermore, we performed only one measurement per animal and tissue type, which leaves room for random effects due to regional differences in RTL. Furthermore, solid organ tissues cannot be collected

longitudinally. Therefore, we chose a cross-sectional study design. Through a robust number of animals per group we aimed to compensate for most of these influencing factors.

4. Materials and Methods

4.1. Animal model

Forty-eight female Sprague Dawley (SD) rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France) at four months of age. All animals were fed a standard chow-based diet and kept on a 12h/12h light/dark cycle at the core facility animal housing at the Medical University of Graz (Austria). Temperature was maintained between 22 and 25°C. Humidity ranged between 55 to 58%. After one week of acclimatization, half of the animals were sacrificed at young age (n=24). The other half was euthanatized after ten months. At the time of scarification, blood was collected by heart puncture into plasma-EDTA and serum tubes (Sarstedt, Nümbrecht, Germany) under deep isoflurane anaesthesia (Forane, Abbott, Austria). After centrifugation at 2000g for 12 min at room temperature, plasma and serum samples were aliquoted and stored at −80°C until batched analysis. Immediately after blood collection, the following organs were explanted and snap frozen in liquid nitrogen: liver, skeletal muscle, heart, aorta, large intestine, spleen, kidney, brain, lung, visceral fat. Subsequently, all tissue samples were stored together deep-frozen at −80°C until analysis.

4.2. Analysis of relative telomere length (RTL) in PBMCs and solid organs

After diluting 100µl of whole blood with 100µl of dH_2O, DNA was isolated with the MagNA Pure LC instrument (Roche, Austria) using the Total Nucleic Isolation Kit (Roche, Austria). Subsequently, relative telomere length (RTL) of peripheral blood mononuclear cells (PBMCs) was measured by quantitative real-time PCR (qPCR) using a protocol developed by Cawthon [44].

This assay quantifies the ratio of average TL (T) to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as single copy reference gene (S). The single copy gene is used as amplification control for each sample and to determine the number of genome copies per sample. All qPCR analyses were performed on Thermocycler CFX384 TouchTM (Biorad, Germany) instrument using the following primers:

- Telomere Forward: 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′;
- Telomere Reverse: 3′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-5′;
- GAPDH Forward: 5′-CACCTAGACAAGGATGCAGAG-3′;
- GAPDH Reverse: 3′-GCATGACTGGAGGAATCACA-5′.

All primers have been purchased from Eurofins Genomics, Austria. Each run included a standard curve made by dilutions of isolated and pooled rat DNA from 21 different blood samples, to determine the quantity of the targeted templates. RTL has been calculated as the ratio of telomere quantity to single copy reference gene quantity (T/S ratio).

RTL in solid organs was analysed with the same method described before. For this purpose, approximately 10mg of tissue were homogenised in 300µl Magna Pure Lysis Buffer (Roche, Austria) using the MagnaLyser (Roche, Austria). Subsequently, the DNA was isolated and quantified using the same procedure as for blood leucocytes

4.3. The mRNA expression analyses in blood cells and solid organs

TERT, TERF-1, and TERF-2 gene expression was analysed in RNA extracts of all solid organs. As blood leucocytes were used up for the measurement of RTL, they were not available for mRNA expression analyses. Therefore, mRNA expression in spleen was used as reference because the organ belongs to the lymphatic system and is rich in leucocytes.

From each organ, 10mg of tissue were homogenised in 300µl Magna Pure Lysis Buffer (Roche, Austria) using the MagnaLyser (Roche, Austria). RNA was extracted from these homogenates with the Total Nucleic Isolation Kit (Roche, Austria) on a MagNA Pure LC instrument (Roche, Austria). Subsequently, the mRNA in these extracts was transcribed into cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Germany). Finally, mRNA expression of TERT, TERF-1, and TERF-2 was analysed by qPCR with TaqMan probes (Life Technologies dba Invitrogen, United States). The expression of each target gene expression was calculated with the ΔΔCT method using β-actin as reference gene. The sequences of the probes used were as follows:

- B-actin: 5'-CTTCCTTCCTGGGTATGGAATCCTG-3';
- Tert: 5′-ATCGAGCAGAGCATCTCCATGAATG-3′;
- Terf-1: 5′-AAAACAGACATGGCTTTGGGAAGAA-3′;
- Terf-2: 5′-GAGAAAATTTAGACTGTTCCTTTGA-3′.

4.4. Statistical analyses

Results are shown as mean ± standard deviations. Group differences were assessed using the two-tailed Student's t test for dependent or independent samples or the Mann-Whitney U test depending on the distribution of the data. Correlations between variables were determined by linear regression analysis according to Pearson (r, Pearson Correlation coefficient; p, univariate ANOVA). p values of < 0.05 were considered statistically significant. Data were plotted using Python programming language with Jupyter Notebook within the data science package Anaconda3 for Windows. IBM SPSS v. 26 for Windows was used for explorative data analysis and a level of acceptance of the null hypothesis was set at p = 0.05.

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5. Conclusions

In conclusion, the present study questions the utility of RTL in PBMCs as a biomarker for the individual assessment of aging. Despite rigorous standardization of housing conditions, sample collection and analytical procedures, excessive intra- and interindividual variability has been observed. Furthermore, RTL in PBMCs was not systematically correlated with solid organ tissues and no differences have been observed between young and old animals. Thus, future studies should focus on the analysis of older animals and measurement of the shortest telomeres.

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Data Availability Statement: The datasets generated during and/or analysed during the current study are based on the work for a PhD thesis and therefore are not publicly available but are available from the corresponding author on reasonable request.

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Influences of Long-Term Exercise and High-Fat Diet on Age-Related Telomere Shortening in Rats

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Abstract: (1) Obesity and exercise are believed to modify age-related telomere shortening by regulating telomerase and shelterins. Existing studies are inconsistent and limited to peripheral blood mononuclear cells (PBMCs) and selected solid tissues. (2) Female Sprague Dawley (SD) rats received either standard diet (ND) or high-fat diet (HFD). For 10 months, half of the animals from both diet groups performed 30 min running at 30 cm/s on five consecutive days followed by two days of rest (exeND, exeHFD). The remaining animals served as sedentary controls (coND, coHFD). Relative telomere length (RTL) and mRNA expression of telomerase (TERT) and the shelterins TERF-1 and TERF-2 were mapped in PBMCs and nine solid tissues. (3) At study end, coND and coHFD animals showed comparable RTL in most tissues with no systematic differences in TERT, TERF-1 and TERF-2 expression. Only visceral fat of coHFD animals showed reduced RTL and lower expression of TERT, TERF-1 and TERF-2. Exercise had heterogeneous effects on RTL in exeND and exeHFD animals with longer telomeres in aorta and large intestine, but shorter telomeres in PBMCs and liver. Telomere-regulating genes showed inconsistent expression patterns. (4) In conclusion, regular exercise or HFD cannot systematically modify RTL by regulating the expression of telomerase and shelterins.

Keywords: telomeres; telomerase; shelterin; moderate exercise; high-fat diet; Sprague Dawley rats

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1. Introduction

The shortening of telomeres, protective nucleoprotein structures at the end of all chromosomes, is a hallmark of aging that compromises genomic integrity and alters the expression of many genes [1]. Due to the inability of the DNA polymerase to fully replicate the 3′ end of chromosomes, telomeres progressively shorten with every cell division until they reach a critical threshold below which they lose their DNA-protecting properties and send cells into senescence or apoptosis [2]. Numerous studies have shown that short and dysfunctional telomeres are linked to premature atherosclerosis, diabetes, and hypertension [3–7]. Furthermore, telomere length is inversely related to mortality risk [8–11].

Aging is an individual process that can be influenced by modifiable lifestyle factors, such as physical activity, nutrition, stress, sleep, smoking and others [12–21]. Physical inactivity and obesity are established triggers of metabolic dysfunction, chronic inflammation, and oxidative stress, which increase the risk of atherosclerosis, diabetes, hypertension, dementia, and other age-related diseases [22,23]. Based on previous studies, it has been speculated that physical inactivity and obesity also accelerate telomere attrition and promote telomeric dysfunction [24]. Conversely, it has been proposed that regular exercise and a balanced diet promote healthy aging not only through beneficial effects on body composition, metabolic function, vascular function, blood pressure, inflammatory processes, and mental stress [25], but also through the preservation of telomere length and function [13,26–30]. It has further been hypothesized that lifestyle-induced effects on telomeres are mediated through telomere-regulating proteins, such as telomerase and

shelterins [16,18,19,21,27,31]. Telomerase can counteract telomere shortening by adding new hexanucleotides to the telomeric ends. With the help of shelterins, a complex of six individual proteins, telomeres assume a unique three-dimensional structure that is essential for their function. Upon binding of the shelterin complex to the TTAGGG motif, telomeric DNA folds backward forming a structure known as t-loop [32,33]. A breakdown of the t-loop structure, called telomere uncapping, represents a critical mechanism that promotes age-related vascular dysfunctions, cellular senescence and inflammation beyond telomere shorting [34].

Obesity and physical inactivity are highly prevalent in modern societies [35]. According to the World Health Organization (WHO), approximately 30% of the global population is obese [36] and the Centre of Disease Control in the US has estimated an overall prevalence of physical inactivity in the US of approximately 25% [37]. Despite intensive research activities, the mechanisms that mediate the increased risk of chronic degenerative diseases in obese and inactive individuals are incompletely understood. Previous studies have nurtured the idea that both of these lifestyle factors could increase disease risk and mortality through an enhancement of telomere shortening that compromises genomic integrity [38]. However, the results of observational studies are controversial, and experimental evidence that establishes a mechanistic link between obesity, physical inactivity and accelerated telomere shortening is largely lacking. Several observational studies showed an inverse relationship between telomere length (TL) in leucocytes (LTL) and BMI [39,40], whereas others found the opposite [41] or no significant association [42,43]. Inverse correlations have also been reported for LTL and different indices of body composition, such as body fat content, waist circumference, waist-to-hip ratio, and nuchal fat thickness [43–50]. In contrast, two mouse models of obesity and metabolic syndrome failed to show accelerated telomere shortening despite an upregulation of telomerase and senescence-associated genes, such as checkpoint kinase 2 (*Chk2*), *p53*, and *p21* [22,23].

Considering that exercise is a highly cost-effective way to improve health and to prolong life [51–55], obese individuals are often prescribed a physical activity program with moderate endurance exercise, such as walking or cycling [56]. Observational studies have reported higher LTL in exercising individuals of different age groups and different activity levels [15,17,18,57,58]. However, available prospective and interventional studies provide conflicting results. In a 5-year longitudinal study by Soares-Miranda L et al., physical activity and physical performance were unrelated to LTL [59]. In contrast, Werner et al., showed increased LTL and an upregulation of telomerase and telomere repeat binding factor (TRF) 2 after 6 months of aerobic endurance training or high intensity training [17,18]. The results from animal studies are also inconclusive. While Ludlow et al. showed a preservation of TL in cardiomyocytes and hepatocytes of exercising mice [16,17,21,60], Werner et al. did not find differences between cardiac TL of exercising and sedentary mice [16,17,21,60]. Regardless of potential effects on TL, exercise seems to alter the expression telomere-regulating proteins, such as telomerase and shelterins [60].

Whether or not obesity and physical activity are causally related to telomere length and the expression of telomere-regulating proteins remains elusive. Furthermore, previous studies are limited to analyses of TL in leucocytes, myocardium, skeletal muscle, and liver. Additionally, potential interactions between the consumption of a hypercaloric diet and regular exercise has not been studied systematically. This aspect is of particular relevance as exercise is often used to compensate bad eating habits and to treat obesity. Therefore, the present study aimed to address this gap of knowledge by mapping TL in leucocytes and 9 solid tissues from aged sedentary rats that were fed for 10 months either a normal chow-based diet (ND) or a synthetic high-fat diet (HFD). In order to explore potential interactions between the consumption of HFD and exercise, half of the animals from both groups performed regular treadmill running with moderate intensity.

2. Materials and Methods

2.1. Animal Model

Ninety-six female Sprague Dawley (SD) rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France) at four months of age. The animals were kept in groups of three animals per cage under constant conditions on a 12 h/12 h light/dark cycle at the core facility animal housing at the Medical University of Graz (Austria). Temperature was maintained between 22 and 25°C. Humidity ranged between 55 to 58%. After one week of acclimatization, the animals were randomly assigned to receive either a standard diet (ND) (Altromin, Lage, Germany) with 3230 kcal/kg and 11% fat or a custom-designed beef-tallow high-fat diet (HFD), rich in saturated fatty acids (SFA), in particular C16:0 and C18:0, with 5150 kcal/kg and 60% fat (Table 1; ssniff, Soest, Germany). Food and tap water were provided ad libitum.

Table 1. Organ weight in female SD rats after 10 months of treadmill exercise.

Organs	Measurement	ND		HFD	
		Sedentary $n = 22$	Exercising $n = 22$	Sedentary $n = 16$	Exercising $n = 12$
heart	average weight	1.31 ± 0.21	1.24 ± 0.11	1.40 ± 0.14	1.46 ± 0.19 ###
	normalized weight	0.28 ± 0.04	0.27 ± 0.03	0.30 ± 0.03	0.31 ± 0.03 ###
spleen	average weight	0.98 ± 0.16	0.97 ± 0.15	1.20 ± 0.16 ###	1.18 ± 0.23 ##
	normalized weight	0.21 ± 0.03	0.21 ± 0.03	0.24 ± 0.07	0.25 ± 0.04 ###
liver	average weight	12.53 ± 1.72	12.50 ± 1.80	14.03 ± 2.36 #	15.16 ± 4.40 #
	normalized weight	2.67 ± 0.31	2.56 ± 0.68	2.98 ± 0.52 #	3.28 ± 0.89 #
visceral fat	average weight	13.20 ± 5.26	10.46 ± 4.48	40.13 ± 12.81 ###	39.46 ± 23.20 ###
	normalized weight	0.03 ± 0.01	0.03 ± 0.01	0.08 ± 0.018 ###	0.07 ± 0.03 ###

Organ weight is given in grams. The weights of heart, spleen, and liver were normalized to total tibia length (cm), while visceral fat weight was normalized to body weight (g). Data are presented as mean ± SD; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the appropriate normal diet control group with the two-tailed Student's *t*-test for independent samples.

2.2. Experimental Design and Treatment

Animals were randomly allocated to following 4 groups, each consisting of 24 animals: coND, exeND, coHFD and exeHFD. coND and exeND animals were fed with ND for the entire study period, whereas coHFD and exeHFD received HFD. The animals from exeND and exeHFD groups performed a 10-month exercise program consisting of 30 min of forced running on a treadmill (Panlab, Barcelona, Spain) on five consecutive days followed by 2 days of rest. The running speed was constant and set at 30 cm/s. The training protocol was based on previous experimental studies [61–64]. The animals in the coND and coHFD groups did not exercise and had no access to a running wheel. These animals were used as sedentary controls.

2.3. Euthanasia and Sample Preparation

At the time of scarification, blood was collected by heart puncture into plasma-EDTA and serum tubes (Sarstedt, Nümbrecht, Germany) under deep isoflurane anaesthesia (Forane, Abbott, Austria). After centrifugation at 2000× *g* for 12 min at room temperature, plasma and serum samples were aliquoted and stored at −80°C until batched analysis. Immediately after blood collection, the following organs were explanted and snap frozen in liquid nitrogen: liver, skeletal muscle, heart, aorta, large intestine, spleen, kidney, brain, lung, visceral fat. Subsequently, all tissue samples were stored together deep-frozen at −80°C until analysis. Exclusion criteria were the development of illnesses or tumours during the intervention period.

2.4. Analysis of Relative Telomere Length (RTL) in PBMCs and Solid Organs

After diluting 100 µL of whole blood with 100 µL of dH_2O, DNA was isolated with the MagNA Pure LC instrument (Roche, Austria) using the Total Nucleic Isolation Kit (Roche, Austria). Subsequently, relative telomere length (RTL) of peripheral blood mononuclear cells (PBMCs) was measured by quantitative real-time PCR (qPCR) using a protocol developed by Cawthon [65]. This assay quantifies the ratio of average TL (T) to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as single copy reference gene (S). The single copy gene is used as amplification control for each sample and to determine the number of genome copies per sample. All qPCR analyses were performed on a Thermocycler CFX384 TouchTM (Biorad, Feldkirchen, Germany) instrument using the following primers:

1. Telomere For: 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′;
2. Telomere Rev: 3′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-5′;
3. GAPDH For: 5′-CACCTAGACAAGGATGCAGAG-3′;
4. GAPDH Rev: 3′-GCATGACTGGAGGAATCACA-5′.

All primers have been purchased from Eurofins Genomics, Austria. Each run included a standard curve made by dilutions of isolated and pooled rat DNA from 21 different blood samples, to determine the quantity of the targeted templates. RTL has been calculated as the ratio of telomere quantity to single copy reference gene quantity (T/S ratio).

RTL in solid organs was analysed with the same method described before. For this purpose, approximately 10 mg of tissue were homogenised in 300 µL Magna Pure Lysis Buffer (Roche, Wien, Austria) using the MagnaLyser (Roche, Wien, Austria). Subsequently, the DNA was isolated and quantified using the same procedure as for blood leucocytes.

2.5. The mRNA Expression Analyses in Blood Cells and Solid Organs

TERT, TERF-1, and TERF-2 gene expression was analysed in RNA extracts of all solid organs. As blood leucocytes were used up for the measurement of RTL, they were not available for mRNA expression analyses. Therefore, mRNA expression in spleen was used as reference because the organ belongs to the lymphatic system and is rich in leucocytes. From each organ, 10 mg of tissue were homogenised in 300 µL Magna Pure Lysis Buffer (Roche, Wien, Austria) using the MagnaLyser (Roche, Wien, Austria). RNA was extracted from these homogenates with the Total Nucleic Isolation Kit (Roche, Wien, Austria) on a MagNA Pure LC instrument (Roche, Wien, Austria). Subsequently, the mRNA in these extracts was transcribed into cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Finally, mRNA expression of TERT, TERF-1, and TERF-2 was analysed by qPCR with TaqMan probes (Life Technologies dba Invitrogen, Waltham, MA, USA). The expression of each target gene expression was calculated with the ΔΔCT method using β-actin as reference gene. The sequences of the probes used were as follows:

5. B-actin: 5′-CTTCCTTCCTGGGTATGGAATCCTG-3′;
6. Tert: 5′-ATCGAGCAGAGCATCTCCATGAATG-3′;
7. Terf-1: 5′-AAAACAGACATGGCTTTGGGAAGAA-3′;
8. Terf-2: 5′-GAGAAAATTTAGACTGTTCCTTTGA-3′.

2.6. Statistical Analyses

Results are shown as mean ± standard deviations (SD). Qualitative variables such as tumor abundance and type were assessed with the Fisher's exact test or the Chi-squared test. Group differences were assessed using the two-tailed Student's *t* test for dependent or independent samples or the Mann–Whitney U test depending on the distribution of the data. Group comparisons with three or more groups were analysed using the two-way ANOVA or the Kruskall–Wallis test for independent samples. Correlations between variables were determined by linear regression analysis according to Pearson (r, Pearson Correlation coefficient; p, univariate ANOVA). Data were plotted using Python programming language with Jupyter Notebook within the data science package Anaconda3 for Windows. IBM

SPSS v. 26 for Windows was used for explorative data analysis and a level of acceptance of the null hypothesis was set at $p = 0.05$.

3. Results

3.1. Characterization of the Animal Model

From the 96 rats, 6 were excluded prior to the end of the study due to general health issues. A total 18 animals developed benign tumours and, thus, were excluded from the final analysis. Tumours were more frequent in animals on HFD rather than on ND (16 vs. 2 rats, $p = 1.289 \times 10^{-4}$). The tumours in the HFD animals were of heterogeneous nature compared to the ND group ($p < 0.001$), as masses were found in breasts, ovaries, and abdomen of obese animals. Regular exercise did not significantly change tumor incidence in both diet groups (coND vs. exeND, $p = 0.975$; coHFD vs. exeHFD, $p = 0.347$) nor tumor diversity in the HFD group ($p = 0.197$). After exclusion of dropouts, 72 eligible animals were included in the final statistical analyses.

At study end, the animals in the two HFD groups had a significantly higher body weight than those in the ND groups (Figure 1). Median body weight between ND and HFD differed by 115 g in sedentary animals and by 90 g in exercising animals. In line with this finding, also the weight of individual organs and tissues, such as heart, liver, and visceral fat, was significantly higher in HFD animals (Table 1).

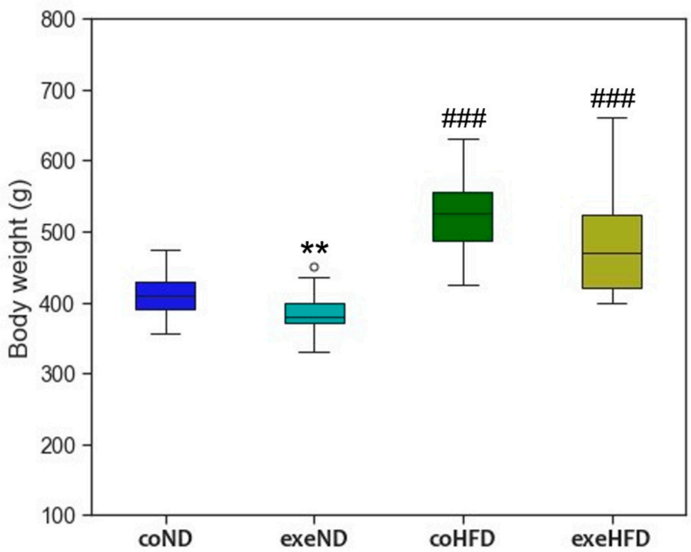

Figure 1. Box and Whisker plot of the body weight at the end of the 10 months study period. Outliers are shown as white circles above the box plots. The two-tailed Student's *t*-test was used for group comparison of independent samples. ** $p < 0.01$ compared to appropriate sedentary control group; ### $p < 0.001$ compared to appropriate normal diet control group.

The exercise protocol was well tolerated by the animals of both diet groups. Body weight of exeND animals was significantly lower than that of coND animals ($p < 0.01$), whereas coHFD and exeHFD animals showed no difference. In the factorial ANOVA, the main effects of diet and exercise on body weight were significant with F (1, 67) = 80.92, $p = 3.89 \times 10^{-13}$, and F (1, 67) = 8.29, $p = 0.005$, respectively. There was no significant interaction between diet and exercise, F (1, 67) = 0.138, $p = 0.712$. Regular exercise induced a higher organ weight of heart and liver in HFD animals, but not in ND animals (Table 1).

3.2. Influence of HFD on RTL and the Expression of Telomere-Regulating Genes in Different Tissues

When compared to ND, 10 months of HFD consumption had no systematic effect on RTL across different organs. In visceral fat RTL was significantly lower in coHFD animals than in coND animals. In contrast, renal RTL was slightly higher in coHFD than in coND

animals. All other solid tissues and PBMCs showed comparable RTL between the two diet groups. (Figure 2).

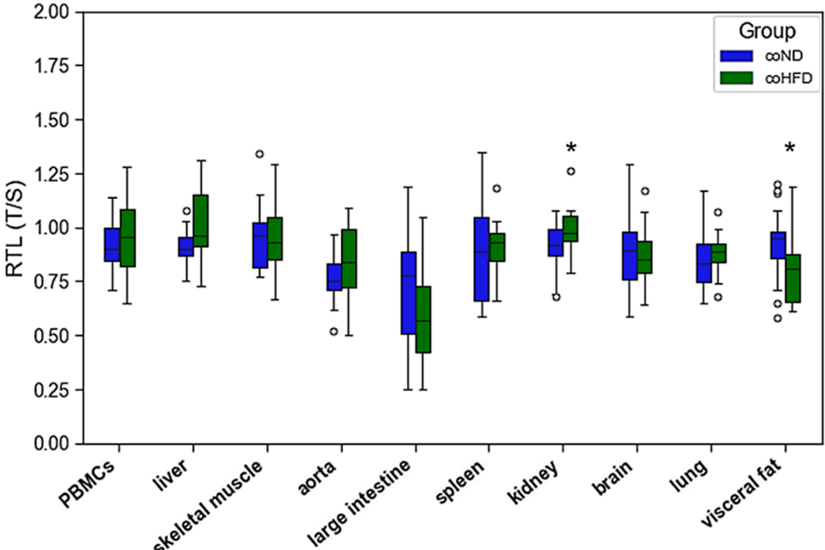

Figure 2. Distribution of RTL in PBMCs and nine solid organs isolated from lean (coND) and obese rats (coHFD). Outliers are shown as white circles above the box plots. RTL is expressed as ratio of average telomere length to the reference gene GAPDH. The two-tailed Student's t-test or the Mann–Whitney U-test were used for group comparison of independent samples. * $p < 0.05$ vs. coND.

TERT mRNA expression varied substantially between tissues with highest expression levels in liver and kidney. The consumption of HFD did not result in a systematic difference of TERT mRNA expression across different organs (Figure 3). Spleen, large intestine, and kidney showed higher TERT mRNA expression levels in coHFD than in coND animals, whereas in visceral fat a lower TERT mRNA expression was observed.

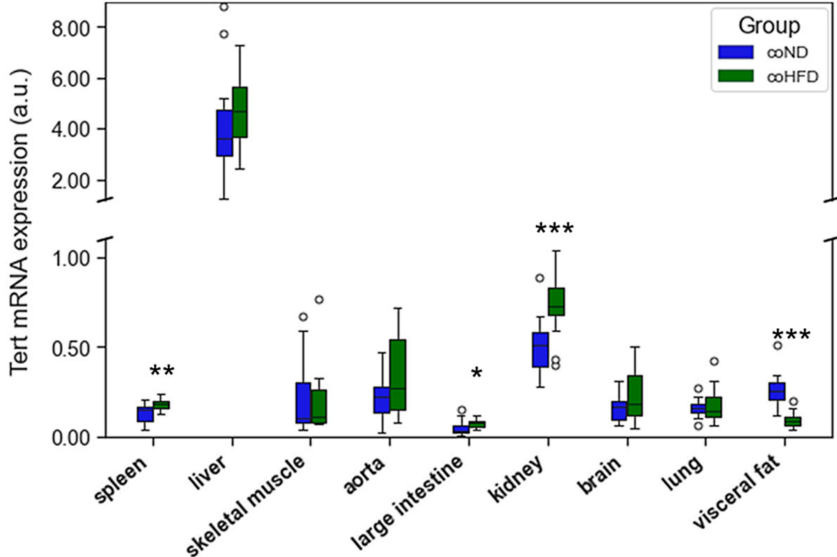

Figure 3. Differences in the TERT gene expression isolated from different solid organs of lean (coND) and obese rats (coHFD). Outliers are shown as white circles above the box plots. TERT mRNA expression is shown in arbitrary units. The two-tailed Student's t-test or the Mann–Whitney U-test were used for group comparison of independent samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. coND.

Similar to TERT, also mRNA expression of the two shelterins, TERF-1 and TERF-2, varied substantially between tissues. The consumption of HFD upregulated TERF-1 and TERF-2 mRNA expression in 3 (Figure 4a) and 5 (Figure 4b) out of nine tissues, respectively. In contrast, a reduced mRNA expression of both shelterins was seen in only one and two tissues, respectively. A simultaneous upregulation of TERF-1 and TERF-2 in coHFD animals was found in skeletal muscle, aorta, and large intestine. In contrast, visceral fat showed a lower mRNA expression of both shelterins in these animals. Furthermore, TERF-1 was markedly reduced in the liver of coHFD rats, whereas TERF-2 was increased in spleen and kidney but decreased in lung tissue.

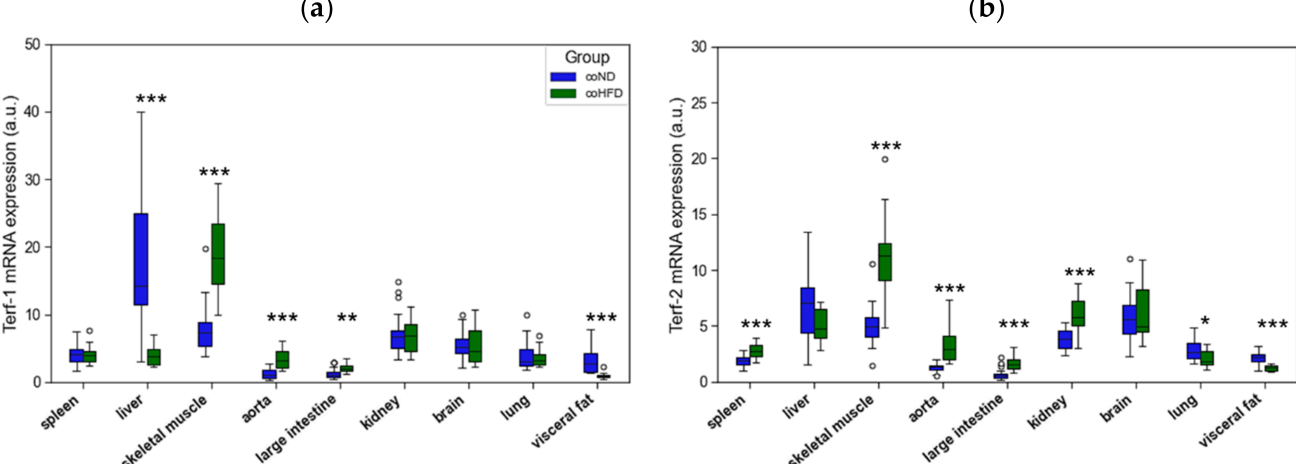

Figure 4. Differences in the gene expression of (**a**) TERF-1, and (**b**) TERF-2 isolated from different solid organs of lean (coND) and obese rats (coHFD). Outliers are shown as white circles above the box plots. TERF-1, and TERF-2 mRNA expression is shown in arbitrary units. The two-tailed Student's *t*-test or the Mann–Whitney U-test were used for group comparison of independent samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. conD.

Combining all the differences described before in an effect matrix, it becomes apparent that only in visceral fat HFD consistently reduces RTL and the mRNA expression of telomere-regulating genes (Figure S1). Instead, in four out of nine tissues mRNA expression of one or more telomere-regulating genes was increased without a change in RTL.

3.3. Influence of Exercise on RTL and the Expression of Telomere-Regulating Genes in Different Tissues

Ten months of regular treadmill running had heterogeneous effects on RTL in different tissues with significantly longer telomeres in aorta and large intestine tissue, but shorter telomeres in PBMCs and liver RTL (Figure 5). In all other tissues, RTL did not significantly differ between sedentary and exercising animals. Of note, the simultaneous administration of HFD did not substantially change this pattern.

Exercise had vastly different effects on mRNA expression of TERT, TERF-1 and TERF-2 in different tissues. In some tissues, but not all, HFD altered the exercise-induced effects observed in ND animals. TERT mRNA expression was increased in spleen, liver, kidney, and lung of exeND animals compared to coND animals (Figure 6a). Conversely, in exeHFD animals, TERT expression in large intestine and kidney was significantly lower than in coHFD, whereas spleen, liver and lung showed no differences (Figure 6b).

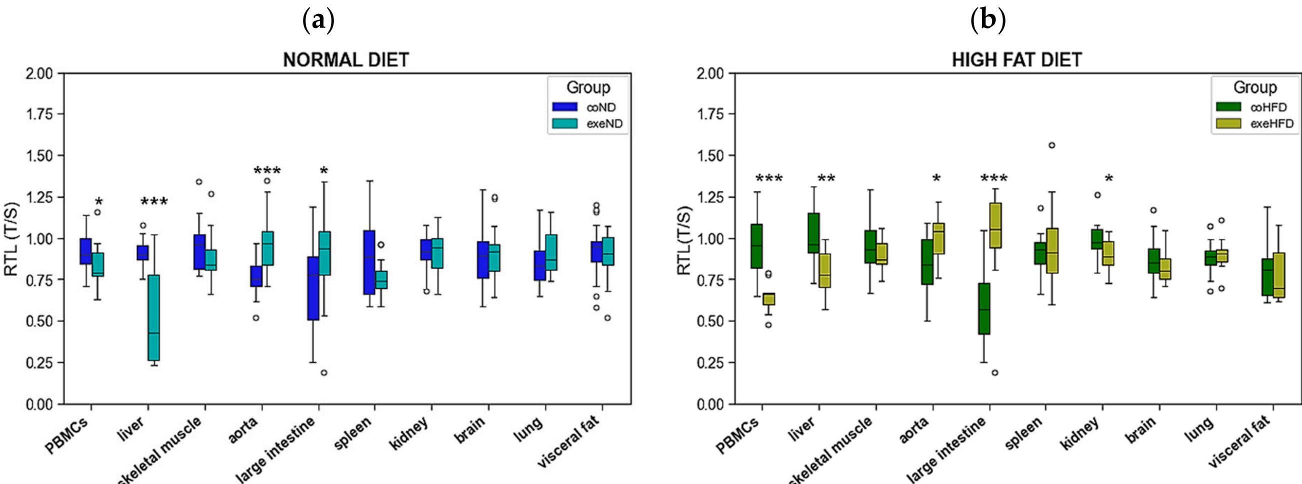

Figure 5. Comparison of RTL in PBMCs and nine solid organs isolated from exercising and sedentary SD rats that received normal diet or HFD for 10 months. (**a**) sedentary (coND) vs. exercising (exeND) animals on ND, (**b**) sedentary (coHFD) vs. exercising (exeHFD) animals on HFD. Outliers are shown as white circles above the box plots. RTL is expressed as ratio of average telomere length to the reference gene GAPDH. The two-tailed Student's t-test or the Mann–Whitney U-test were used for group comparison of independent samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. respective sedentary controls.

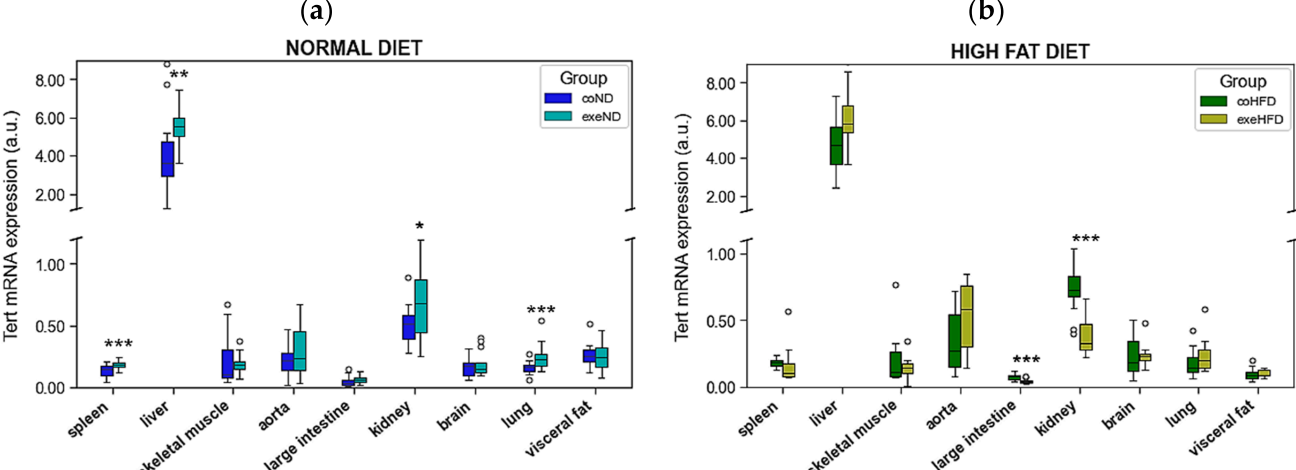

Figure 6. Comparison of TERT expression in nine solid organs from exercising and sedentary SD rats that received either normal diet or HFD for 10 months. (**a**) sedentary lean animals (coND) vs. exercising lean animals (exeND), (**b**) sedentary obese animals (coHFD) vs. exercising obese animals (exeHFD). Outliers are shown as white circles above the box plots. TERT mRNA expression is shown in arbitrary units. The two-tailed Student's *t*-test or the Mann–Whitney U-test were used for group comparison of independent samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. respective sedentary controls.

TERF-1 mRNA expression was significantly reduced in liver, lung, and visceral fat, but increased in skeletal muscle, aorta, and large intestine of exeND rats when compared to coND animals (Figure 7a). In exeHFD animals instead, TERF-1 mRNA expression was increased in spleen, liver, large intestine, and kidney, but reduced in skeletal muscle when compared to coHFD animals (Figure 7c).

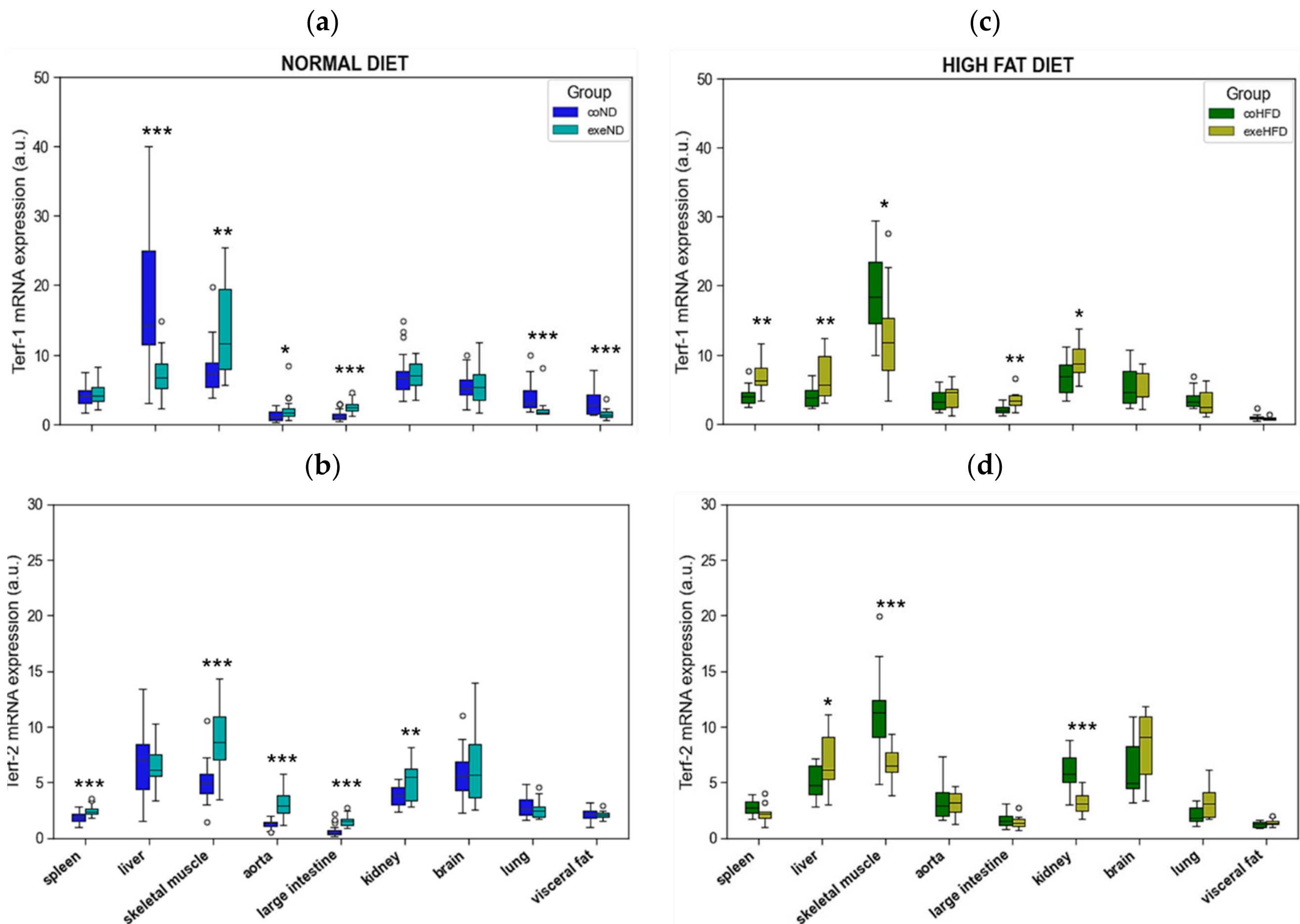

Figure 7. Comparison of TERF-1 and TERF-2 expression in nine solid organs from exercising and sedentary SD rats that received either normal diet or HFD for 10 months. (**a**) TERF-1 in sedentary lean animals (coND) vs. exercising lean animals (exeND), (**b**) TERF-2 in sedentary lean animals (coND) vs. exercising lean animals (exeND) (**c**) TERF-1 in sedentary obese animals (coHFD) vs. exercising obese animals (exeHFD), (**d**) TERF-2 in sedentary obese animals (coHFD) vs. exercising obese animals (exeHFD). Outliers are shown as white circles above the box plots. TERF-1 and TERF-2 mRNA expression is shown in arbitrary units. The two-tailed Student's *t*-test or the Mann–Whitney U-test were used for group comparison of independent samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. respective sedentary controls.

TERF-2 mRNA expression was significantly increased in five out of nine solid organs of exeND animals when compared to coND rats (Figure 7b), namely spleen, skeletal muscle, aorta, large intestine, and kidney. In exercising obese animals instead, TERF-2 mRNA expression was profoundly different with a higher expression level in liver, but reduced expression levels in skeletal muscle and kidney in exeHFD rats when compared to coHFD animals (Figure 7d).

Summarizing all the results from sedentary and exercising lean animals in the effect matrix (left column), it becomes clear that only in aorta and large intestine an increase in one or more telomere-regulating genes was associated with an increase in RTL (Figure S1). All other differences in mRNA expression of telomere-regulating genes were unrelated to RTL. Similar to exercise, also HFD failed to induce systematic effects on RTL and telomere-regulating genes. Only in kidney and visceral fat of obese sedentary animals, did RTL and telomere-regulating genes show changes directed in the same way. However, the changes in both tissues pointed in opposite directions. An interaction between HFD and exercise was only observed in kidneys, where exercising obese rats exhibited a similar RTL to sedentary

lean controls (Figure S1). Additionally, both exercising groups show reductions in hepatic RTL, but inconsistent changes in the hepatic expression of telomere-regulating genes.

3.4. Correlation Analysis

To further explore our hypothesis that lifestyle factors can modify RTL through regulatory effects on the expression of telomere-regulating genes, we performed correlation analyses that included the animals from all four groups. Figure 8 illustrates that RTL was not consistently correlated to any of the telomere-regulating genes. For example, in kidney ($R = 0.337$; $p = 0.004$) and visceral fat ($R = 0.337$; $p = 0.004$) RTL and TERT mRNA expression were positively correlated, whereas large intestine ($R = -0.252$; $p = 0.036$) and spleen ($R = -0.263$; $p = 0.028$) showed the opposite. Likewise, RTL and TERF-2 were positively correlated in aorta ($R = 0.373$; $p = 0.002$), kidney ($R = 0.318$; $p = 0.007$) and visceral fat ($R = 0.332$; $p = 0.007$), but negatively correlated in liver ($R = -0.247$; $p = 0.053$).

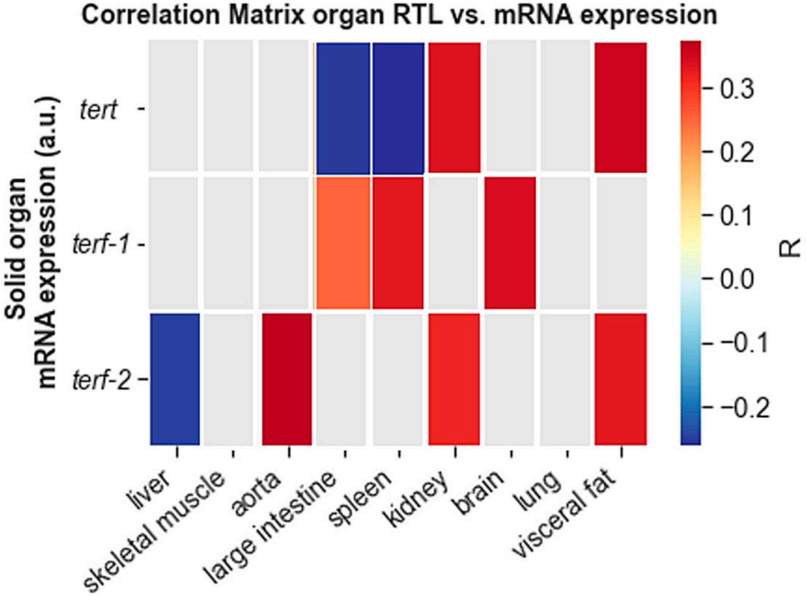

Figure 8. Correlation matrix for RTL and telomere-regulating genes in 9 solid tissue types. The colour bar on the right side of the figure shows the Pearson correlation coefficient as R. Only significant correlations are shown in colour ($p \leq 0.05$). Grey boxes indicate the lack of significant correlation.

4. Discussion

Here, we show for the first time that neither regular exercise nor the consumption of HFD have a systematic effect on RTL in solid tissues and PBMCs of SD rats. In fact, most tissues had comparable RTL in the respective intervention and control groups. Additionally, dual stimulation by feeding HFD to exercising animals did not change this result. Nevertheless, some tissues exhibited significantly higher RTL after 10 months of HFD (kidney) or exercise (aorta and small intestine), whereas other tissues showed reduced RTL upon HFD (visceral fat) or exercise (PBMCs and liver). These differences were not accompanied by a consistent mRNA expression pattern of the respective telomere-regulating genes *tert*, *terf-1* and *terf-2*. Therefore, the present results do not support the hypothesis that regular moderate endurance exercise or prolonged exposure to a diet rich in saturated lipids influence RTL through the expression of telomerase and shelterins.

The comprehensive mapping of RTL and related telomere-regulating genes after long-term exposure to HFD and exercise significantly expands existing knowledge on the influence of modifiable lifestyle factors on age-related telomere shortening. Previous in vivo studies have mostly focused on RTL in specific cells or tissue types, such as PBMCs or myocardium [16,17,21,60]. The results are rather inconsistent. Ludlow et al. showed in wild type derived short telomere mice (CAST/Ei) that 1 year of voluntary running in

a running wheel preserved TL in myocardium and liver, but not in skeletal muscle [60]. Similar to the present study, these effects were not accompanied by consistent alterations of telomere-regulating genes that would explain these effects. In contrast, after 3 weeks of voluntary wheel running, Werner et al. reported an upregulation of telomerase activity (TA) in murine aorta and PBMCs, and an increased aortic gene expression of TERF-2. Additionally, senescence-associated genes, such as Chk2, p53, and p21, were lowered in the aorta of exercising animals. However, the increased expression of these telomere-regulating genes did not result in a significant difference of aortic TL after 6 months of exercise when compared to inactive controls [17]. The TL results reported by the two studies of Ludlow et al. and Werner et al. are not in line with our findings, where 10 months of regular moderate running exercise reduced RTL in PBMCs and liver, whereas aorta and large intestine showed a significant increase.

The inconsistencies between existing exercise studies in animals may, at least partly, be explained by differences in the animal models used. Our results were obtained in SD rats, whereas previous studies worked with CAST/Ei [60] and C57/Bl6 mice [17]. Additionally, the duration of exercise varied amongst existing studies between a few weeks and one year, which further limits comparability. In addition, a greater group size with 22 coND and 22 exeND animals provides robustness to our results. The studies from Ludlow et al. and Werner et al. were performed with no more than 10 animals per group, which limits statistical power and leaves more room for random effects. A major strength of the present study is strict standardization of the exercise intervention, which consisted in forced treadmill running for 30 min at fixed speed on 5 consecutive days per week. The efficacy of this intervention is evidenced by a significantly lower body weight at the time of scarification. In contrast, most previous studies used voluntary wheel running, which is not standardized.

Similar to the exercise studies discussed before, mouse models of obesity and metabolic syndrome also failed to show accelerated telomere shortening despite an upregulation of Chk2, p53, and p21 [22,23]. For example, feeding mice for 60 weeks with a high-fat/high-sucrose diet induced obesity and metabolic dysfunction, but did not accelerate LTL shortening [23]. With advancing obesity, the animals were physically less active, which should have amplified potentially adverse effects of obesity. Additionally, in genetically modified rats with metabolic syndrome, Takahashi et al. showed comparable myocardial TL than in wild-type controls [22]. At the same time, telomerase expression and TA were upregulated together with the senescence-associated genes Chk2, p53, and p21. These results are in line with our present study, showing similar RTL in PBMCs, liver, aorta, and skeletal muscle after 10 months of HFD or ND. Additionally, large intestine, spleen, brain, and lung showed comparable RTL in the two groups. In our model neither exercise nor HFD induced a consistent expression pattern of telomere-regulating genes, namely *tert*, *terf-1* and *terf-2*. Only kidney and visceral fat showed significant differences in RTL, but in opposite directions. Similar differences were detected for the expression of *tert* and *terf-2*. However, the relevance of these effects is questionable as TERT, TERF-1 and TERF-2 were also altered in several other tissues of HFD animals without affecting the respective RTL. Furthermore, most existing studies reported effects of exercise and obesity on telomerase and shelterins, but often this was not associated with changes in RTL. In line with existing data, correlation analyses in the present study showed inconsistent correlations between RTL and mRNA expression levels of the three telomere-regulating genes. Altogether, these results question the pathophysiological relevance of such observations.

A unique aspect of the present study is the combination of exercise and HFD. In modern societies, people often try to compensate adverse nutritional habits with exercise, but the efficacy of this approach is not well documented. Our results show that such an approach does produce a different outcome than exercise alone. Specifically, RTL was lower in PBMCs, liver and kidney of exercising animals on HFD, but higher in aorta and large intestine. Despite a comparable pattern of RTL in the different organs of exercising animals on normal diet and HFD, incongruent results were registered for the mRNA expression of

tert, *terf-1* and *terf-2*. For example, *terf-1* and *terf-2* were both increased in skeletal muscle of exercising animals on ND but decreased in exercising animals on HFD. However, both groups showed comparable RTL in this tissue. In line with this argument, also correlation analyses that included all 72 animals did not show consistent correlations between RTL and the expression level of telomerase or shelterins. For example, an inverse correlation between RTL and *tert* was seen in spleen and large intestine, whereas kidney and visceral fat showed the opposite. In all other tissues both parameters were not correlated.

This present animal study does not support the results from human studies showing a reduced LTL in obese people [39,40] and a preservation of TL upon regular endurance exercise [17]. Although some studies do not support an inverse relationship between TL and obesity [41–43], a recent meta-analysis calculated a significantly lower LTL in obese individuals than in normal-weight individuals [49]. Moreover, LTL was inversely correlated with BMI, body fat content, waist circumference, waist-to-hip ratio, and nuchal fat thickness. However, the observational character of the studies included impedes any conclusion towards causality. Additional insights can be gained from longitudinal observation studies that assessed LTL in obese patients before and after bariatric surgery [66]. Available results indicate an improvement in LTL after >2 years, probably due to an improvement in inflammation and oxidative stress. However, only a small number such studies has been published, with rather heterogenous design and outcome. Human studies that investigated LTL in exercising and sedentary individuals are also inconsistent. Several observational studies have shown higher LTL in exercising individuals of different age groups and activity levels [15,17,18,57,58]. Additional support from prospective observation and intervention studies is strongly limited. Soares-Miranda L et al. performed serial blood collections over a 5-year period in 582 older US adults and found no significant association between physical activity, physical performance, and LTL [59]. In contrast, Werner et al. reported an increase in LTL, TA, and TERF-2 expression after 6 months of aerobic endurance training or high intensity training, which was not seen in controls [17,18].

A general downside of existing human studies is the limitation of TL analyses to blood leucocytes, which impedes conclusions about TL in solid tissues of obese and lean individuals. However, previous results from our group have shown that LTL does not provide reliable information on TL in other tissues [67]. While RTL in some tissues, exhibit a positive correlation with LTL, others show the opposite. Additionally, RTL in young and aged SD rats did not systematically change.

The present results should be interpreted with caution keeping in mind the strengths and limitation of this study. A rather large number of animals per group and a strictly standardized exercise intervention provide robustness to the results. In addition, the intervention period was quite long. However, results from Werner et al. suggest, that up to 18 months may be needed to observe a significant reduction in TL [16]. SD rats have an average life expectancy of 2 years so that our animals were sacrificed at advanced adult age, but they cannot be regarded old. The exercise protocol applied was rather moderate and a more intensive regimen might have produced different results. However, with this protocol we aimed to mimic a common recreational activity pattern in adults. Energy intake and energy expenditure may have varied between individual animals and different groups. The lacking information on both factors adds some uncertainty to the interpretation of our results. Another important limitation is the RT-qPCR method that has been used for the measurement of RTL. This method gives an average TL across all cells and chromosomes but does not provide information on the percentage of very short and long telomeres. There is some evidence that the percentage of very short telomeres rather than average TL is associated with aging and age-related disease [68]. However, determination of the shortest telomeres requires highly sophisticated and cumbersome methods, such as Telomere Shortest Length Assay (TeSLA) [69]. In addition, these methods are difficult to standardize and not suitable for high throughput analysis. As we had planned to analyze more than 1000 samples, these assays were deemed not feasible for our purpose. Lastly, telomere-regulating genes were only analysed by mRNA expression, but not at protein

level. Although mRNA expression and protein analyses may give discordant results, we do not feel that this limits the overall meaning of our results. The absence of systemic effects on RTL in PBMCs and solid tissues and the highly inconsistent mRNA expression pattern of telomerase and shelterins limit the potential scope of these factors as relevant mediators of telomere effects induced by exercise and diet.

5. Conclusions

In summary, the present in vivo study does not provide evidence that modifiable lifestyle factors, such as obesity and exercise, have significant systemic effects on telomere shortening and the expression of telomere-regulating genes. Additionally, exercise and HFD do not show significant interaction. Any lifestyle-related effect on RTL and telomere-regulating genes in one tissue type does not allow conclusions on other tissues or cell types. Future research should address the impact of exercise and diet on the shortest telomeres and explore their role for aging and degenerative disease. Moreover, future studies on the effects of lifestyle factors on telomere length and telomere function should focus on advanced adult age, where degenerative disease most frequently occurs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11101605/s1, Figure S1: Effects matrix that visualizes the effects of exercise, HFD, and the interaction of both lifestyle factors on RTL and the mRNA expression of telomere-regulating genes.

Author Contributions: Conceptualization, M.H., H.-J.G. and G.A.; methodology, M.D.S., G.A. and H.-J.G.; software, H.-J.G. and M.D.S.; validation, W.R. and H.-J.G.; formal analysis, M.D.S., W.R. and H.-J.G.; investigation, M.H., H.-J.G., M.D.S. and G.A.; resources, M.D.S., H.-J.G. and G.A.; data curation, M.D.S.; writing—original draft preparation, M.D.S.; writing—review and editing, M.H., H.-J.G., W.R. and G.A.; visualization, M.D.S.; supervision, M.H. and H.-J.G.; project administration, M.H.; funding acquisition, M.H. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data underlying the findings reported in this manuscript are provided as part of the article. The raw data that are not already presented in the figures are available from the corresponding author upon reasonable request.

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ORIGINAL CONTRIBUTION

The effects of long-term moderate exercise and Western-type diet on oxidative/nitrosative stress, serum lipids and cytokines in female Sprague Dawley rats

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Abstract

Purpose Regular exercise reduces obesity and the risk of cardiovascular disease. However, health-promoting benefits of physical activity are commonly associated with increased inflammation and oxidative stress. Here, we tested whether constant moderate exercise is able to prevent or attenuate the oxidative/nitrosative stress, inflammation, and serum lipids in lean and obese rats.

Methods Four-month-old female Sprague Dawley rats received standard or a high-fat diet. Animals were subjected to a physical activity protocol, consisting of 30 min forced treadmill exercise for 5 consecutive days per week during 10 months. Baseline and sedentary (non-exercised) rats were used as controls. Lipids, oxidized low-density lipoprotein cholesterol, nitric oxide metabolites, and pro- and anti-inflammatory markers were measured in blood collected upon euthanasia.

Results At variance to young baseline control rats, 14-month-old animals fed normal diet had increased plasma lipid levels, including total cholesterol and triglycerides, which were further elevated in rats that consumed a high-fat diet. While treadmill exercise did not lower the amount of serum lipids in standard diet group, forced physical activity reduced non-high-density lipoprotein cholesterol in response to high-fat diet feeding. Exercised rats fed standard diet or high-fat diet had lower abundancy of nitric oxide metabolites, which coincided with increased levels of oxidized low-density lipoprotein cholesterol. Accordingly, the amount of nitric oxide metabolites correlated inversely with oxidized low-density lipoprotein cholesterol and homo-arginine. Exercise significantly reduced inflammatory cytokines in high-fat diet fed rats only.

Conclusion Our study suggests that regular exercise alters the equilibrium between oxidative and anti-oxidative compounds and reduces pro-inflammatory cytokines.

Keywords Long-term moderate exercise · Western-type diet · Oxidized LDL · Nitric oxide · Nitric oxide synthase · Sprague Dawley rats

Introduction

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in Western societies and developing countries [1]. Modifiable risk factors, such as obesity and sedentarism are highly prevalent in patients with CVD, and both can be improved by safe and effective lifestyle interventions [2]. Such lifestyle factors are diet and exercise that affect the concentration of low-density lipoproteins (LDL), which are known metabolic driver of CVD, such as atherosclerosis [3–5]. In addition, it is well established that elevated concentrations of LDL cholesterol (LDL-C) promote atherosclerosis and increase the risk of non-fatal and lethal CVD events. LDL-C is subjected to oxidation, resulting in

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the formation of oxidized LDL (oxLDL), which is thought to be more aggressive than non-oxidized LDL in inducing atherogenesis [6, 7].

Elevated oxidative stress characterized by an increased generation of radical oxygen species (ROS) is a common manifestation in patients with CVD, obesity, and diabetes mellitus. These highly reactive compounds can modify many biomarkers including LDL-C [8–10]. The modification of LDL-C by oxidation promotes the receptor-mediated uptake of oxLDL by macrophages, thereby causing cholesterol accumulation in the vasculature. Previous studies in humans and animals indicate that Western diet and physical inactivity induce free radical production, which may enhance the susceptibility of LDL to oxidation [3, 4, 11]. One of the principal pathways involved in the adaptation to exercise is nitric oxide (NO) signalling. NO triggers vasodilation and, thus, can mitigate the high shear stress during exercise. Furthermore, NO is involved in several other physiological and pathological processes, such as cell inflammation and adhesion as well as angiogenesis [12–15]. NO is synthetised by three isoforms of the enzyme NO synthase (NOS), including neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial isoform (eNOS or NOS-3). All of them are constitutively but not exclusively expressed in the cardiovascular system [10]. In particular, eNOS requires dimerization to maintain its normal function but under certain conditions the reduction of molecular oxygen by eNOS is not coupled anymore with the oxidation of the substrate L-arginine (L-arg), thus resulting in the production of superoxide instead of NO [10]. The main eNOS uncoupling motifs are the cofactor of NO synthesis, tetrahydrobiopterin (BH4), reduced bioavailability of L-arg, or high levels of the endogenous NOS inhibitor, asymmetrical dimethyl-arginine (ADMA) [8].

Although regular exercise is proven to reduce CVD risk and promote health benefits [12, 16–19], constant physical activity also increases the production of free radicals and oxidative stress [4, 9]. This raises the question as to whether exercise-induced oxidative stress is beneficial or detrimental. In this regard, obese and normal-weight human adults have reportedly comparable oxLDL concentrations [20], whereas other studies showed that body weight reduction after the bariatric surgery or regular moderate exercise decrease oxLDL [21, 22]. In contrast, a single intensive exercise session appears to increase the susceptibility of LDL and other lipoproteins to oxidation in healthy adults [3, 4]. Thus far, a single in vivo study explored the effects of HFD and regular physical activity on plasma lipids, oxidative/nitrosative stress and LDL oxidation. Specifically, Elmas et al. showed that rats consuming a HFD exhibit increased oxidative stress in aortic and myocardial tissue [2]. Regular exercise appears to modify the balance of antioxidants and oxidants as well as NO metabolism in these tissues. However, it remains elusive whether constant moderate exercise is able to prevent or attenuate the oxidative/nitrosative stress, inflammation, and serum lipids in lean and obese rats.

In the present study, we investigated the impact of HFD and regular moderate exercise on the oxidative/nitrosative stress, serum lipids and cytokines. For this purpose, blood samples from young and old Sprague Dawley female rats that were subjected to forced treadmill exercise sessions for 10 months in combination with a HFD or ND were analysed.

Materials and methods

Animal model

Four-month-old healthy female SD rats ($n=120$) with an average body weight of approximately 300 g were purchased from Janvier Labs (France) and kept in groups of three animals per cage under constant conditions on a 12 h light and 12 h dark cycle in the institutional animal facility. The decision to work with female animals aimed to avoid gender effects and to reduce the risk of dropouts due to aggressive behaviour between animals. After 1 week of acclimatization, the animals were randomly assigned to receive a standard diet (ND) (Altromin, Germany) with 3.23 kcal/kg and 11% fat or a custom-designed beef-tallow high-fat diet (HFD), rich in saturated fatty acids (SFA), in particular C16:0 and C18:0, with 5.15 kcal/kg and 60% fat (Table 1; ssniff, Germany). Saturated fatty acids (SFA) and mono-unsaturated fatty acids (MUFA) are present in a ratio of 1:1. While the ratio of SFA and poly-unsaturated fatty acids (PUFA)

Table 1 Composition of the high-fat diet

Fatty acids		Vitamins	
Saturated fatty acids	%	Antioxidant	Per kg
C 12:0	0.04	Vitamin A	15,000 IU
C 14:0	1.18	Vitamin E	150 mg
C 16:0	8.27		
C 17:0	0.38	Others	
C 18:0	6.06	Vitamin D_3	1500 IU
C 20:0	0.04	Vitamin K (as MNB)	20 mg
		Thiamine (B_1)	25 mg
Mono-unsaturated fatty acids		Riboflavin (B_2)	16 mg
C 16:1	1.33	Pyridoxine (B_6)	16 mg
C 18:1	12.29	Cobalamin (B_{12})	30 µg
		Nicotinic acid	47 mg
Poly-unsaturated fatty acids		Pantothenic acid	55 mg
C 18:2	2.53	Folic acid	16 mg
C 18:3	0.34	Biotin	300 µg
		Choline	920 mg

Fatty acids and antioxidant vitamins content

is close to 5:1. The HFD composition was based on previous studies [23–25]. Food and tap water were provided ad libitum.

Experimental design and treatment

Animals were randomly allocated to 5 groups, each consisting of 24 animals. The rats in group 1 were sacrificed after the acclimatization period and served as a baseline control (BL). Ninety-six animals were divided in a 1:1 ratio, fed ND or HFD and subjected to the 10-month study protocol as follows: half of the rats fed ND or HFD performed 30-min running exercise sessions on a treadmill (Panlab, Spain) on 5 consecutive days (indicate at what time of the day) followed by 2 days of rest. The running speed was constant and set at 30 cm/s. The training protocol was based on previous experimental studies [26–29]. The animals that did not exercise throughout the entire study period were used as sedentary controls.

Euthanasia and sample preparation

At the end of the 10-month study period, blood was drawn by heart puncture under deep isoflurane anaesthesia (Forane, Abbott, Austria). Also for the baseline animals, the blood was collected only once at the time of sacrification. Blood and plasma were collected using S-Monovette Serum-Gel tubes and S-Monovette Plasma-EDTA tubes (Sarstedt, Nümbrecht, Germany), respectively. Samples were centrifuged at 2000 g for 12 min at room temperature, aliquoted and stored at − 80 °C until the analysis. Blood collections and consequently serum analyses were performed in a non-fasting state.

Evaluation of the oxidative/nitrosative stress

The circulating oxLDL concentration was determined in serum (100 µl) with a rat-specific commercial Sandwich ELISA kit (USCN Life Sciences, Texas) according to the manufacturer's instructions. This assay uses rat polyclonal antibodies against oxLDL and has a measurement range between 31.2 and 2000 pg/ml. In this range, intra- and inter-assay imprecision is below < 12%. NO was estimated in serum (100 µl) by measuring the degradation products nitrite (NO_2^-) and nitrate (NO_3^-) using a commercial photometric method (NO quantification kit, Active Motif, California) on a FlexStation3 (Molecular devices, California). In addition, plasma concentrations of homo-arginine (h-arg), ADMA and symmetrical dimethyl-arginine (SDMA) were quantified by a reverse-phase high-pressure liquid chromatography (HPLC) method as described previously [30, 31].

Assessment of systemic inflammatory markers

A profile of 22 inflammatory markers and chemokines, including Regulated and Normal T-cell Expressed and Secreted (RANTES), eotaxin, macrophage inflammatory protein 1α (MIP-1 α), monocyte chemoattractant proteins 1 and 3 (MCP-1 and 3), tumour necrosis factor α (TNF-α), interferon γ (IFN-γ), IFN-γ-inducible protein (IP-10), and interleukins (IL-1β, IL-2, IL-5, IL-6, IL-10, IL-12, IL-17) were determined in 25 µl of serum with a preconfigured multiplex immunoassay kit (ThermoFisher Scientific, Austria) using the BioPlexTM 200 detection system (Bio-Rad, Austria). The activity of matrix metalloproteinases (MMPs) was measured through an enzymatic reaction using a Mca-PLGL-Dpa-AR-NH_2-fluorogenic peptide substrate (R&D Systems, Canada). Serum (90 µl) was incubated with the diluted working solution (10 µl) for 20 min at room temperature. The fluorescent signal was detected at 320 nm excitation and 405 emission wavelength using the photometer FLUOstar OPTIMA (BMG Labtech, Germany).

Assessment of lipid metabolism and adipocytokines

The serum lipid profile was determined on fully automated Olympus AU640 analyser (Olympus, Hamburg, Germany) using commercial assays. Briefly, total cholesterol (TC), triglycerides (TG), phospholipids (PL), non-esterified fatty acids (NEFA), and HDL cholesterol (HDL-C; homogeneous assay) were measured using enzymatic methods and reagents from Diasys (Holzheim, Germany). The instrument was calibrated using secondary standards from Roche Diagnostics (Mannheim, Germany; for TC, TG) and Dyasis (Holzheim, Germany; for FC, PL). Insulin growth factor-1 (IGF-1), leptin and adiponectin were evaluated in serum by commercial sandwich ELISAs (Demeditec Diagnostics Gmbh, Germany) according to the manufacturer's instructions. Finally, resistin was quantified using a sandwich enzyme immunoassay from BioVendor—Laboratorní medicína a.s. (Brno, Czech Republic).

Statistical analyses

Data are presented as means ± standard deviations. The differences between groups were assessed using two-tailed Student's t test for dependent or independent samples or Mann–Whitney U test depending on the distribution of the data. Correlations between variables were determined by linear regression analysis according to Pearson (r, Pearson correlation coefficient; p, univariate ANOVA). p value of < 0.05 was considered statistically significant. Analyses

were performed by explorative data analyses using SPSS for Windows (IBM© SPSS© Statistics, version 25).

Results

Exclusion criteria and body weight

From the 120 rats included in the study, 6 rats had to be sacrificed before the end of exercise protocol because of illness, while additional 18 animals developed tumours and, thus, were excluded from the analysis (Fig. 1) [32]. Tumours were more frequent in animals fed high-fat than standard diet (16 vs. 2 rats). At the end of the study, 96 animals were included in the analysis. All animals had significantly increased body weight as compared to the baseline group (Fig. 2a, $p < 0.001$). However, this increase in body weight was markedly higher in response to HFD than ND feeding. Also organ weights increased with age and HFD (Table 2).

The exercise protocol was well tolerated in all animals and reduced the weight gain in the ND animals ($p < 0.01$), but not in HFD animals. Moreover, regular moderate exercise induced an increase of myocardial and hepatic weight in HFD animals, but not in ND animals.

Lipid profile

Serum lipids are established risk factors of atherosclerosis and CVD that are affected by lifestyle factors, such as diet and physical activity [3–5]. Therefore, we first explored the effects of HFD and exercise on serum lipids. After 10 months of intervention, average TC and TG concentrations were significantly higher compared to baseline, regardless of diet and exercise (Fig. 2b, c). HFD animals showed additional alterations of the lipid profile beyond simple age-related changes. TG and NEFA were significantly higher than in ND animals, whereas non-HDL-C was lower (Fig. 2c, d, f). Exercise induced a decrease of TC and HDL-C in HFD, but not ND animals (Fig. 2b, e). All other lipid parameters were comparable between sedentary and exercising animals in both diet groups.

In an attempt to explore potential mechanism that mediate the changes in plasma lipids, correlation analyses have been performed. At study end, body weight was positively correlated with TG ($r = 0.598$; $p < 0.001$),

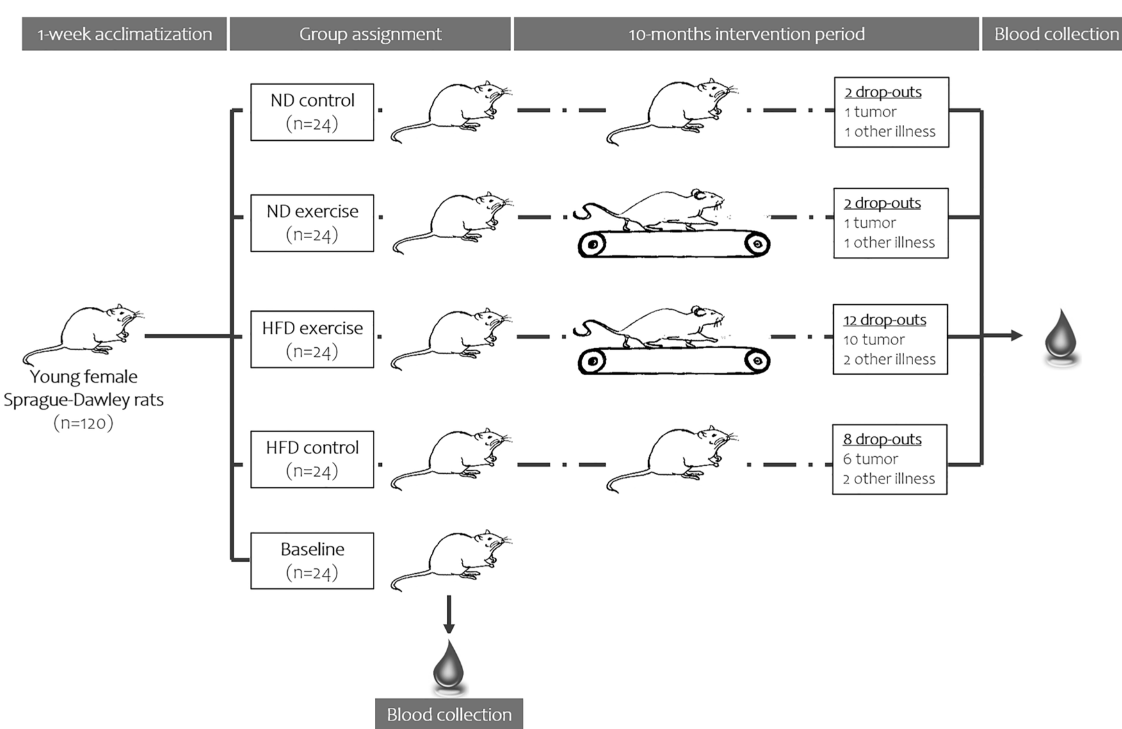


Fig. 1 The experimental design. One-hundred and twenty young female Sprague Dawley rats were randomly allocated into 5 groups, each consisting of 24 animals. The animals in the baseline control group were euthanized after the acclimatization period. The remain of ninety-six animals were divided in a 1:1 ratio and fed ND or HFD and subjected to a 10-month study protocol as follows: half of the rats fed ND or HFD performed 30-min running exercise sessions on a treadmill. Six animals (2 vs. 4 rats fed ND and HFD, respectively) died prior to the end of the study and eighteen more (2 vs. 16 rats under ND and HFD) had to be excluded due to the development of tumours

Fig. 2 Box and Whisker Blot of the body weight (**a**) and the lipid profile (**b**–**f**) after the 10 months study period. $^{**}p<0.01$ compared to appropriate sedentary control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to appropriate normal diet control group; $^{£££}p<0.001$ compared to baseline control group. The study groups have been abbreviated as follows: baseline group (BL), control normal diet (coND), exercise normal diet (exeND), control high fat diet (coHFD) and exercise high-fat diet (exeHFD)

NEFA ($r=0.238$; $p=0.025$) and non-HDL-C ($r=0.271$; $p=0.010$), whereas TC ($r=-0.291$; $p=0.006$) and HDL-C ($r=-0.365$; $p<0.001$) were inversely correlated to adiponectin.

Oxidative/nitrosative stress

Advanced age, obesity and physical inactivity are known to modulate the equilibrium between oxidant and antioxidant

Table 2 Organ weight and serum biomarkers in female Sprague Dawley rats after 10 months of treadmill exercise

	Baseline $n=24$	Normal diet group		High-fat diet group	
		Control $n=22$	Exercise $n=22$	Control $n=16$	Exercise $n=12$
Organ weight					
Heart					
Average weight	1.18 ± 0.12	1.31 ± 0.21£	1.24 ± 0.11	1.40 ± 0.14£££	1.46 ± 0.19###£££
Normalized weight	0.26 ± 0.03	0.28 ± 0.04£	0.27 ± 0.03	0.30 ± 0.03£££	0.31 ± 0.03###£££
Liver					
Average weight	2.39 ± 0.25	12.53 ± 1.72£££	12.50 ± 1.80 ££	14.03 ± 2.36#£££	15.16 ± 4.40#£££
Normalized weight	2.39 ± 0.25	2.67 ± 0.31££	2.56 ± 0.68	2.98 ± 0.52#£££	3.28 ± 0.89#£££
Visceral fat					
Average weight	5.81 ± 2.07	13.20 ± 5.26£££	10.46 ± 4.48£££	40.13 ± 12.81###£££	39.46 ± 23.20###£££
Normalized weight	0.01 ± 0.00	0.02 ± 0.01£££	0.02 ± 0.01£££	0.07 ± 0.02###£££	0.07 ± 0.04###£££
Biochemical analyses					
SDMA	0.43 ± 0.08	0.27 ± 0.11£££	0.28 ± 0.08£££	0.33 ± 0.04#£££	0.20 ± 0.04***#£££
Resistin	14.97 ± 5.15	8.16 ± 3.45£££	7.72 ± 2.94£££	7.78 ± 3.86£££	7.49 ± 3.99£££
Eotaxin (CCL11)	230.53 ± 46.15	420.79 ± 104.54£££	335.76 ± 94.67**£££	372.79 ± 143.41£££	294.85 ± 150.66
MIP-1α (CCL3)	18.42 ± 5.05	18.96 ± 5.06	22.47 ± 9.27	25.68 ± 16.33	17.66 ± 4.07*
IFN-γ	38.36 ± 11.58	46.20 ± 31.04	44.22 ± 28.37	42.11 ± 22.80	53.36 ± 39.07
IP-10 (CXCL10)	109.25 ± 33.01	153 ± 35.70£££	163.78 ± 46.04£££	125.05 ± 51.60	118.30 ± 23.12##
IL-5	79.43 ± 29.89	31.59 ± 17.50£££	38.57 ± 18.05£££	43.41 ± 17.29 #£££	37.27 ± 21.91*£££
IL-6	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
IL-10	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
IL-12	58.62 ± 17.45	58.07 ± 27.91	121.53 ± 83.54**£££	52.18 ± 25.04	45.72 ± 21.98
IL-17	21.79 ± 8.02	18.73 ± 11.90	22.45 ± 15.95	11.28 ± 3.91£££	16.37 ± 11.40
MMPs activity	10,881 ± 1177	12,136 ± 1488££	14,092 ± 1832***£££	11,876 ± 2026	9966 ± 2943###

The organ weight is given in grams; organ weight is normalized to total tibia length (cm). SDMA is expressed in µM, while resistin in ng/ml. All other cytokines and chemokines are expressed in pg/ml. Data are presented as mean ± standard deviation

*$p<0.05$

**$p<0.01$

***$p<0.001$ compared to appropriate sedentary control group

#$p<0.05$

##$p<0.01$

###$p<0.001$ compared to the appropriate normal diet control group

£$p<0.05$

££$p<0.01$

£££$p<0.001$ compared to the baseline control group

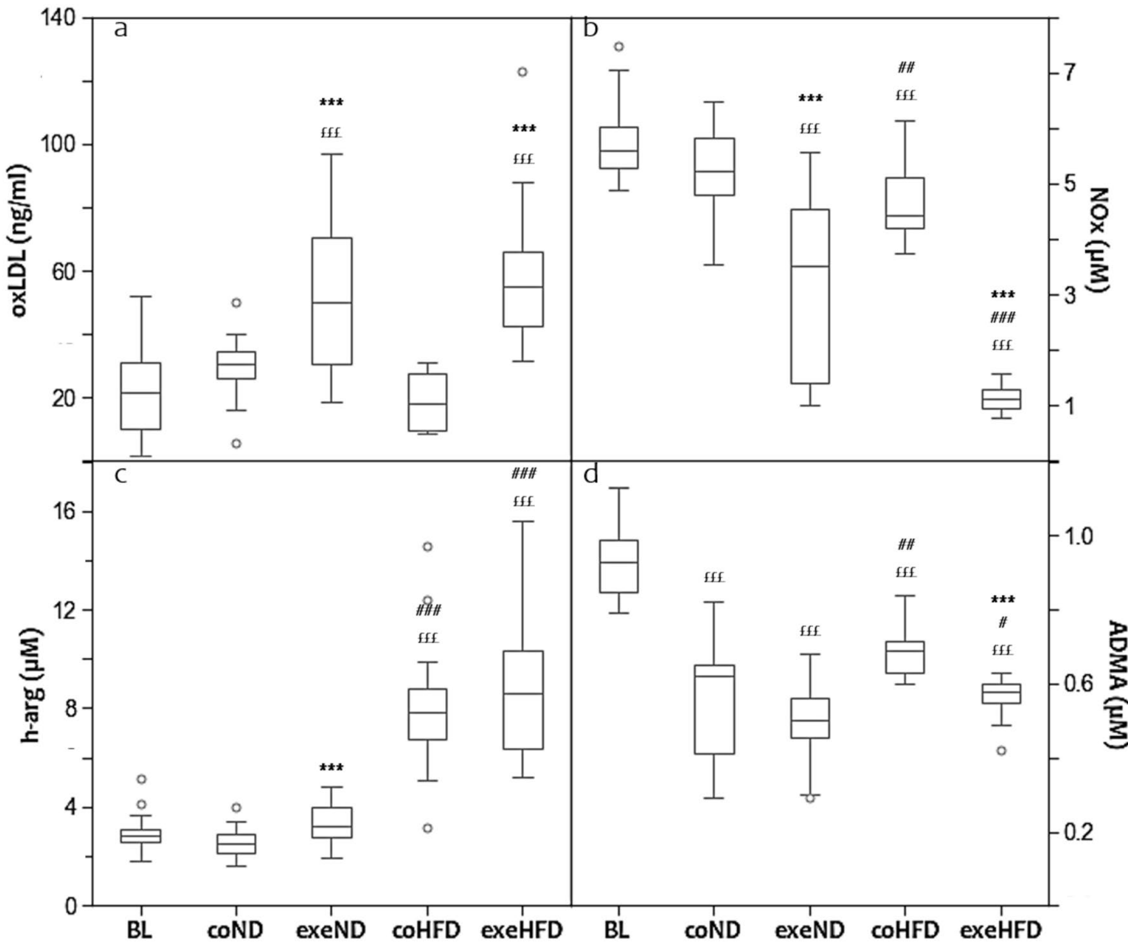


Fig. 3 Box and Whisker Blot of oxidized LDL (**a**). $^{***}p<0.001$ compared to appropriate sedentary control group; $^{£££}p<0.001$ compared to baseline control group. Box and Whisker Blot of nitric oxide metabolites (**b**). $^{***}p<0.001$ compared to appropriate sedentary control group; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to appropriate normal diet control group; $^{£££}p<0.001$ compared to baseline control group. Box and Whisker Blot of homo-arginine and ADMA (**c**–**d**). $^{***}p<0.001$ compared to appropriate sedentary control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to appropriate normal diet control group; $^{£££}p<0.001$ compared to baseline control group

compounds, with consequent alterations of lipid peroxidation and NOS function [8–10]. Therefore, we analysed the effects of HFD and exercise on oxLDL and NOx. Figure 3 shows that age and diet had a substantial effect neither on oxLDL nor on NOx (Fig. 3a, b). Exercise instead altered both biomarkers significantly. After 10 months of regular training, oxLDL was increased in ND and HFD by 44% and 68%, respectively. In contrast, NOx was markedly lower in exercising animals with the lowest concentrations in the HFD group.

To corroborate the NOx results, we also measured the non-proteogenic amino acid h-arg, a substrate of NOS for the production of NO, and ADMA, a competitive inhibitor of NOS. h-arg was significantly higher in HFD animals than in ND and baseline controls (Fig. 3c). Exercise increased h-arg in ND animals, but not in HFD animals. The serum concentrations of ADMA were highest at baseline and decreased with age. This decrease was less pronounced in HFD than in ND animals. Exercise reduced ADMA in the HFD group, but not in the ND group (Fig. 3d, $p<0.001$).

Considering that oxidative stress and NOS function are linked with each other, we performed linear regression analyses that showed a strong inverse association between oxLDL and NOx (Fig. 4a). An inverse relationship was also found between h-arg and NOx (Fig. 4b, $r=-0.453$; $p<0.001$). ADMA and SDMA were positively related to NOx with (ADMA *vs.* NOx, $r=0.401$; $p<0.001$) and (SDMA *vs.* NOx, $r=0.459$; $p<0.001$), but inversely associated with oxLDL with ($r=-0.338$; $p<0.001$) and ($r=-0.274$; $p=0.009$), respectively.

Effects of physical activity and diet on cytokines and chemokines

Dyslipidaemia and oxidative/nitrosative stress are established drivers of chronic systemic inflammation, an

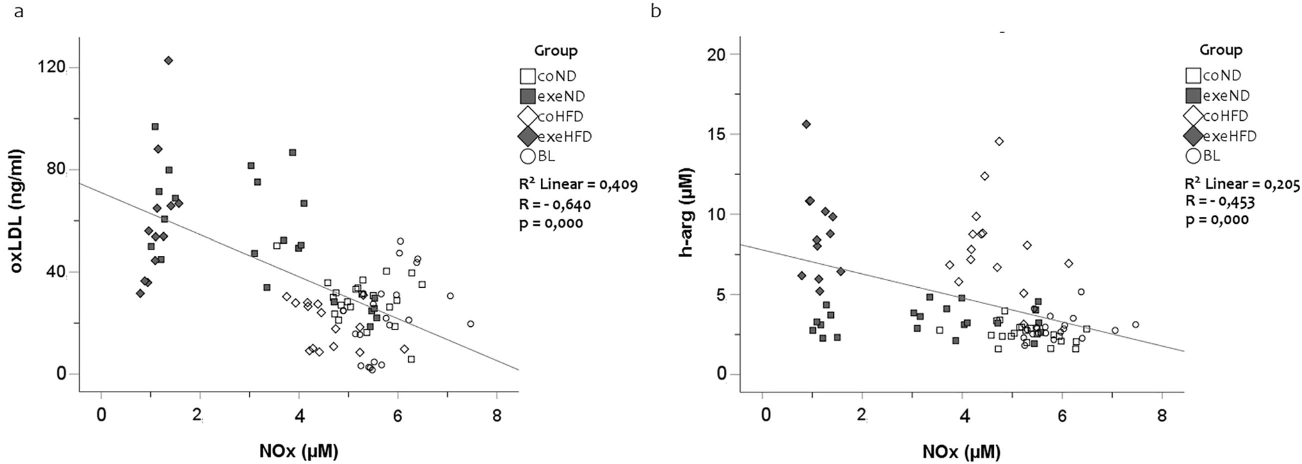


Fig. 4 Simple Scatter Dot Plot of the linear regression analysis with $r = -0.640$ and $p = 0.000$ between oxidized LDL-C and nitric oxide metabolites (**a**) and $r = -0.453$ and $p = 0.000$ between h-arg and nitric oxide metabolites (**b**)

important factor in the pathogenesis of atherosclerosis [6, 7]. To study the immunological response of HFD and physical activity, we analysed a broad panel of pro- and anti-inflammatory cytokines (Fig. 5). Regular treadmill exercise reduced the serum concentrations of the pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-2 in rats consuming HFD, but not those fed ND (Fig. 5b, e, f). All other cytokines were not significantly affected by exercise. Age and diet alone had no significant effects on the serum concentrations of the measured pro-and anti-inflammatory cytokines (Table 2).

Chemokines are secreted signalling proteins that mediate the migration of immune cells in response to pro-inflammatory cytokines [33]. In the present study, several pro-inflammatory chemokines, including RANTES, MCP-1 and 3, were measured. In 14-month-old sedentary ND and HFD animals, the average serum concentrations of RANTES and MCP-3 were significantly higher than in young baseline controls (Fig. 5a, d). Regular exercise attenuated this age-related increase irrespective of diet. MCP-1 showed similar trends, but due to a greater inter-individual variability of this marker, significant effects were present only in HFD animals (Fig. 5c).

Adipocytokines

Adipocytokines, such as adiponectin, leptin, and IGF-1 are key regulators of energy metabolism and fat stores that are centrally involved in the pathomechanistic sequaele of adipositas and obesity [34, 35]. Furthermore, they have immune-modulatory effects [36]. In the present study, age, diet, and exercise had profound effects on the serum concentrations of IGF-1, adiponectin, and leptin (Fig. 6). At the end of the 10-month protocol, IGF-1 concentration was approximately 50% lower than in young baseline controls, irrespective of diet (Fig. 6a). Exercising animals fed HFD, had slightly higher IGF-1 concentrations than their respective controls. In contrast, leptin was markedly higher in old than in young animals (Fig. 6c). The age-related increase in serum leptin was substantially amplified by HFD. Exercise reduced serum leptin concentrations slightly in both diet groups. Adiponectin was not affected by age and exercise in ND animals, but increased in HFD animals (Fig. 6b). This increase was greater in exercising HFD animals. Resistin decreased with age in HFD and ND animals ($p < 0.001$). In both dietary groups, exercising animals had even lower resistin serum concentrations than sedentary counterparts.

Discussion

The present study shows that long-term moderate exercise reduces the body weight gain and NOx but increases oxLDL in normal-weight and obese rats. Administering HFD to sedentary animals resulted in a marked increase in body weight gain and triglycerides; however, it failed to systematically alter oxLDL, NOx or any other lipid profile parameter.

In the SD rats used in this project, long-term moderate running exercise did not change TC, HDL-C, and non-HDL-C. In HFD fed animals, TG was slightly reduced by exercise. Furthermore, exercise increased oxLDL in both dietary groups. These results are in contrast to most previous studies in humans and rodents where regular moderate endurance exercise reduced TG, TC and LDL-C, whereas HDL-C increased [2, 26, 37–39]. However, the results of individual studies may vary substantially [4, 37, 40] and comparability between studies is limited by different experimental settings. For example, Elmas et al. analysed the

Fig. 5 Box and Whisker Blot of the panel of cytokines and chemokines (**a**–**f**). $*p<0.05$, $**p<0.01$ compared to appropriate sedentary control group; $^{\#}p<0.05$ compared to appropriate normal diet control group. $^{£}p<0.05$, $^{££}p<0.01$, $^{£££}p<0.001$ compared to baseline control group

effects of a 6-week exercise intervention in SD rats where animals were forced to swim for 1 h/d on 5 days/week [2]. Animals that received a high-fat diet showed a decrease in TC and TG when compared to sedentary controls. In our study, the administration of a HFD increased TG, but not TC. Furthermore, exercise did not show a modulatory effect on TG, TC, or non-HDL-C. When compared to the present results, the lipid concentrations reported by Elmas et al. are quite different. These contrasting results may be explained the use of male animals that were considerably younger at the time of sacrification. Furthermore, the present study used a HFD with a different lipid composition and a less vigorous exercise intervention. Analytical differences may also have contributed to the different results. In our laboratory, all methods are strictly controlled by internal and external quality controls. Furthermore, the lipid concentrations that

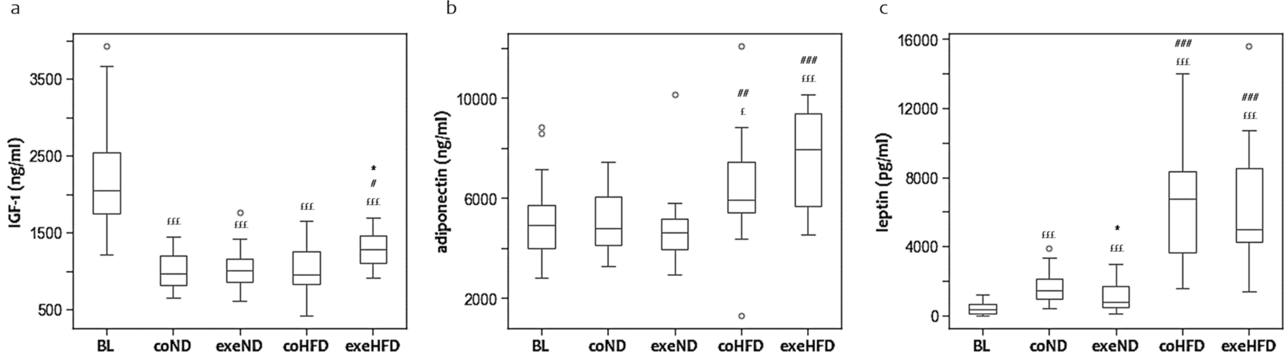


Fig. 6 Box and Whisker Blot of IGF-1 (**a**), adiponectin (**b**) and leptin (**c**). $*p<0.05$ compared to appropriate sedentary control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to appropriate normal diet control group. $^{£}p<0.05$, $^{£££}p<0.001$ compared to baseline control group

we obtained in young and old control animals are in line with a previous study in SD rats [11].

The HFD used in this study is particularly rich in SFA and MUFA and thus induces an imbalance of lipoprotein metabolism with consequent alterations of plasma lipoproteins. According to the manufacturer, this diet aims to mimic the situation in humans who follow a typical Western-type dietary regimen and live in an obesogenic environment. Furthermore, it reliably induces obesity and metabolic syndrome in mice and rats. Differences in the dietary lipid composition may explain differences in plasma lipoproteins and oxidative–nitrosative stress markers observed in other HFD intervention studies in rodents [2, 23, 41, 42]. The current National Cholesterol Education Program (NCEP) and American Heart Association (AHA) dietary guidelines recommend limiting fat intake to 30–40% of the total dietary calories [43]. However, increasing fat intake inside the recommended range may already have adverse effects on the lipid profile with increasing TC and LDL-C concentrations [43]. In contrast, reducing fat intake to 20% or less can also be troublesome due to a reduction of LDL-C and HDL-C and a contemporary rise in TG. This combination is typically associated with the formation of small and dense LDL particles with a high atherogenic potential [43]. With the aim to promote a healthier LDL/HDL ratio, the original AHA Step I fat recommendation advices for a 1:1:1 proportion of SFA: MUFA: PUFA in the diet.

In old animals the lipid profile was substantially different from that of young animals with markedly higher TC and TG concentrations. Moreover, HDL-C, non-HDL-C and NEFA were higher in old than in young animals. Such age-related changes of the lipid profile are expected and have been described by others before [44]. With the HFD used in this study, the concentrations of TG and NEFA increased, whereas all other parameters of the lipid profile remained unchanged. Previous studies that treated rodents with HFD reported mixed results. While some studies found increasing concentrations of TC, TG and LDL-C [2, 25, 41, 44] others did not [11]. However, a direct comparison of these studies is limited due to differences in study design and composition of the diets. For example, in 4–8-week-old Wistar rats the administration of HFD for 4–8 weeks resulted in increased body weight and adipose tissue weight, TC, TG, LDL-C concentrations [23, 42]. However, both studies did not include baseline measurements, which impedes a longitudinal evaluation of age-related effects. Zelzer et al. treated adult female SD rats for 12 weeks with a HFD comparable to the one used in the present study [11]. This intervention did not result in different TC or TG concentrations. Also, HDL-C and non-HDL-C were comparable between controls and HFD-treated animals. The limited comparability of different animal models is not surprising and has already been shown before [44]. Similar to animal models, also human studies that compared the lipid profile of obese and non-obese individuals yielded heterogeneous results [20, 21, 45]. Most existing studies showed higher TC, TG, LDL-C, and small dense LDL-C (sdLDL-C) concentrations in obese individuals when compared to normal weight controls. However, for HDL-C, inconsistent results have been reported [21, 45]. A controlled dietary intervention study by Egert et al. demonstrated that the substitution of a high-fat diet rich in saturated fatty acids with either a high-fat or a low-fat diet rich in mono-unsaturated fatty acids ameliorated the lipid profile, reducing TC, LDL-C/HDL-C ratio, LDL-C size and its susceptibility to oxidation [46].

A main finding of our study is the exercise-induced increase in oxLDL that is accompanied by a reduction of NO. Elevated oxidative stress is a common condition in sedentary and obese individuals that increases the generation of ROS and leads to the modification of many biochemical targets [8–10] including LDL-C. OxLDL is supposed to be more aggressive than non-oxidized LDL in driving inflammation, atherogenesis, and ultimately the risk of CVD events [5–7]. Nevertheless, exercise-induced ROS production

seems to protect cells against oxidation by maintaining the cellular oxidant-antioxidant homeostasis [47, 48]. Furthermore, regular physical activity improves blood pressure control through an increased production of NO and other vasoactive substances [10]. In the present study, however, long-term moderate running exercise was associated with an increase in oxLDL and a reduction in NOx. While the increase in oxLDL was comparable in both dietary groups, the reduction in NOx was more pronounced in HFD animals. These results are in agreement with previous studies demonstrating an increased susceptibility of LDL-C and other lipoproteins to oxidation after a single intensive exercise session [3, 4]. Furthermore, in another study by Zelzer et al., the administration of a similar HFD to rats increased several markers of oxidative stress, such as malondialdehyde, but not oxLDL [11]. While Zelzer et al. measured oxLDL by ELISA, the other studies analysed lipoprotein oxidation indirectly [3, 4], which limits the comparability of results. When interpreting the modification of oxidative stress biomarkers by physical activity, it should be considered that chronic exercise as well as high-intensity training can increase oxidative stress through several mechanisms including increased mitochondrial oxygen consumption and activation of oxidase enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major source of ROS [9]. This might at least partially explain the exercise-induced increase in oxLDL that we observed in the present study. Additional support for this hypothesis comes from the reduction in NOx by regular exercise. Under normal circumstances, the potent vasodilator NO is released during the conversion of L-arginine to L-citrulline. This reaction is catalysed by NOS. However, under certain circumstances, NOS can also produce superoxide ($O2^{*-}$), which reacts avidly with vascular $NO^{\cdot}$ to form peroxynitrite ($ONOO^{-}$). This metabolite is capable of impairing NOS dimerization and function [8, 49], which is called NOS-uncoupling. Such an uncoupling of NOS can occur in the absence of either L-arg or BH4, and increased concentrations of ADMA, a competitive inhibitor of NOS. In the present study, HFD and regular exercise increased h-arg, another substrate of NOS for the production of NO. Although h-arg competes with L-arg for NOS-binding sites, it seems to be a less efficient substrate for NO synthesis. According to März et al., the role of h-arg in NO metabolism is still insufficiently understood [50]. Some studies support the hypothesis that h-arg might increase arginine bioavailability by inhibition of the enzyme arginase, which competes with NOS for the utilization of the key substrate L-arg [50, 51]. If this is correct, the substantial increase in h-arg is likely to exert a protective effect under HFD. This concept is supported by a previous study showing that h-arg supplementation ameliorates blood glucose in mice on HFD [52]. The reduction of ADMA and SDMA observed in the present study further supports the beneficial effect of h-arg in HFD animals. However, h-arg competes with L-arg in more than one way. They both utilize the same transport system for cell entry, and high extracellular h-arg concentrations will result in reduced L-arg uptake [53]. This might at least partially explain the exercise-induced reduction of NOx. The inverse relationship between oxLDL and NOx strongly supports the concept of NOS uncoupling in exercising animals. Considering that NOx levels were lower in non-exercising HFD animals than in non-exercising ND animals, HFD consumption seems to have an independent NO-reducing effect, which is amplified by regular exercise. The very low NOx concentration in exercising HFD animals further supports the concept of an additive NO-reducing effect of HFD and exercise.

In this project, we also investigated the influence of regular exercise on the adipokine, cytokine and chemokine profile in both dietary groups. Obesity is typically associated with an increased secretion of adipokines and a mild tissue inflammation [36]. As expected, our HFD animals showed markedly higher leptin and adiponectin serum concentrations than ND animals regardless of exercise, whereas IGF-1 and resistin were comparable in both dietary groups. Insulin was not considered as blood was collected non-fasting. Exercise was effective in reducing leptin in ND, but not HFD, animals suggesting that the HFD overwhelmed the effect of exercise. The mild increase in serum adiponectin in exeHFD animals might simply reflect the weight loss in these animals. Interestingly, the HFD used in this study had only minor pro-inflammatory effects inducing increased serum concentrations only for MCP-1 and IL-5. Other pro-inflammatory cytokines, such as TNF-α, IFN-γ or IL-6 were comparable between the two dietary groups.

Consistent with previous studies [26, 54], our exercise protocol significantly reduced the serum concentrations of TNF-α, IL-1β, IL-2, MCP-1, MCP-3 and RANTES in HFD animals. MCP-3 and RANTES were also decreased in the exeND group. Both chemokines regulate the migration and infiltration of monocytes and macrophage into solid tissues [33]. The present results are in line with recent findings from Rocha-Rodrigues et al. showing that regular physical activity reduces inflammation in response to HFD administration [26]. Borst et al. have shown that in the context of obesity, visceral fat derived resistin, TNF-α and several other interleukins contribute to insulin resistance [35], whereas weight loss or visceral fat removal decrease serum IL-6 and increase the insulin sensitizing hormone adiponectin [34, 35]. The exercise-induced increase of IP-10, IL-12, and MMP activity in ND animals might reflect a mild activation of cellular immunity and tissue remodelling in response to exercise, which is masked in the HFD animals.

The present study has several strengths and limitations that should be considered when interpreting the results. The rather long intervention period with sufficiently sized groups

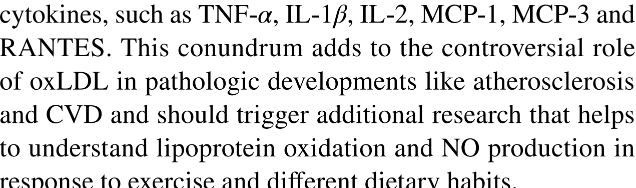
allows robust conclusions about the effects of the different diets and physical activity as important modifiable lifestyle factors that impact CVD risk. Furthermore, our exercise protocol was well controlled and imitated a realistic activity regimen that would normally be considered as healthy. Another strength of this study is the use of validated and strictly quality controlled methods for the measurement of serum lipids. The unlimited access of the animals to food allowed them to compensate the exercise-related increase in energy expenditure through higher food consumption. Due to the unlimited access to food, we cannot account for natural occurring differences in food intake. Furthermore, we lack metabolic studies and blood collections were performed in a non-fasting state. Although this may have an impact on the results of several metabolic biomarkers, we intentionally decided to perform non-fasting blood collections to avoid unwanted psychological stress in the animals. It should also be mentioned that our method for the measurement of TG does not distinguish between TG and free glycerol. However, the blood concentration of free glycerol is <1 mg/dL, which accounts for approximately 10 mg/dl of triglycerides [55]. Considering that in the SD rats used in this study TG concentrations ranged between 100 and 400 mg/dl, the unintentional detection of free glycerol does not represent a relevant confounder of our results.

Another weakness of our study is the lack of mechanistic information on the effects of exercise on plasma lipids. Considering that body weight was strongly correlated with TG, it can be speculated that the higher fatty acid intake in HFD animals, was the main driver of differences in plasma TG. The HFD used in this study contains approximately 60% of SFA and MUFA, which are incorporated into TG. Exercise instead, induced only minor differences in body weight and plasma lipids. This is not surprising, as our exercise regimen was rather moderate. Previous studies have shown that exercise induces beneficial effects on plasma lipids and lipoprotein lipase activity only above a certain threshold of energy expenditure [56, 57]. Another putative mediator of plasma lipids could be adiponectin, which is known to increase energy expenditure through fatty acid oxidation in target organs, such as liver and skeletal muscle [58]. This theory is supported by the inverse correlations of TC and HDL-C with adiponectin.

Conclusion

In summary, long-term moderate exercise may alter the delicate equilibrium between oxidative and anti-oxidative compounds leading to an uncoupling of NOS with higher oxLDL and lower NOx concentrations.

However, these metabolic effects do not necessarily compromise the beneficial reduction of pro-inflammatory



cytokines, such as TNF-α, IL-1β, IL-2, MCP-1, MCP-3 and RANTES. This conundrum adds to the controversial role of oxLDL in pathologic developments like atherosclerosis and CVD and should trigger additional research that helps to understand lipoprotein oxidation and NO production in response to exercise and different dietary habits.

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Authors' contributions Conceived and designed the experiments: MH, H-JG, GA; performed the experiments: MDS, H-JG, GA, SZ, AM, HS; data analysis and assessment: MDS, H-JG, GA; wrote the original draft of the paper: MDS; reviewed and edited the paper: H-JG, MH; contribution to the critical appraisal of the paper: GA, MK, SZ, AM, HS, SS, H-JG, MH.

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Availability of data and materials All data and materials as well as software application or custom code support their published claims and comply with field standards.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that no conflict of interests influenced the work reported in this paper.

Ethics approval The study was approved by the responsible national ethics committee (GZ: 66.010/0070-V/3b/2018) and performed in accordance with the guidelines of the Animal Care and Use Committee of the Ministry of Science and Research, Vienna, Austria.

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