Telomere dynamics: influence of lifestyle factors and associations with male reproductive quality

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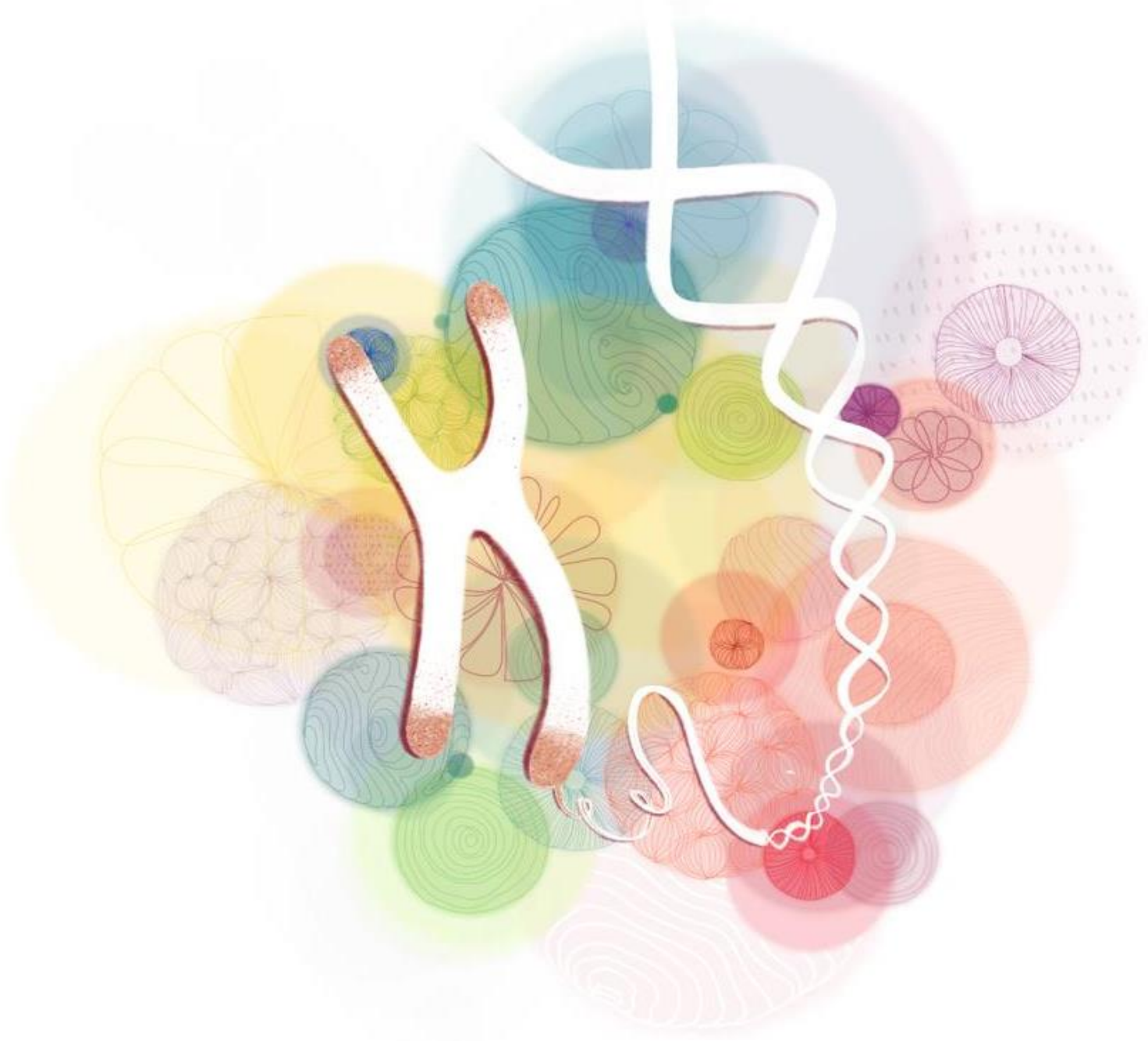

DOCTORAL THESIS
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Thesis supervised by Prof. Jordi Salas Salvadó and Dr. Silvia Canudas Puig

UNIVERSITAT ROVIRA i VIRGILI

Department of Biochemistry and Biotechnology

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I STATE that the present study, entitled "**Telomere dynamics: influence of lifestyle factors and associations with male reproductive quality**", presented by María Fernández de la Puente Cervera for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this university.

Reus, 30 August 2024

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Abstract

English

Telomeres are structures located at the end of chromosomes whose principal function is to protect and preserve genome integrity. In mammalian cells, these regions consist of the repetition in tandem of the sequence 5' TTAGGG 3'. These structures are elongated by an enzyme known as telomerase and stabilized by six proteins of the shelterin complex, which plays an important role in regulating the maintenance of the telomeric structure. Telomere attrition has been proposed as a hallmark of aging and has been associated with the onset of diseases such as obesity, infertility, and age-related diseases as metabolic syndrome (MetS), cardiovascular disease (CVD), diabetes, or cancer. Both non-modifiable factors, (e.g., sex, age, and genetic traits), and modifiable factors (e.g., diet, physical activity, smoking habits, or socioeconomic status (SES)) have been investigated in the context of telomere length (TL) modulation.

Several randomized controlled trials (RCTs) have analyzed the effect of lifestyle interventions on telomeres. However, scientific evidence from previous trials is limited regarding older populations with overweight or obesity and metabolic diseases. Therefore, the primary objective of this thesis was to evaluate the effect of a lifestyle intervention on TL after one and three years of the PREvención con Dieta MEDiterránea-Plus (PREDIMED-Plus) study. This ongoing lifestyle parallel-group, multi-center RCT included older Mediterranean participants with overweight or obesity and MetS for primary prevention of CVD. Participants in the control group followed an *ad-libitum* Mediterranean diet (MedDiet) and those allocated to the intervention group followed an energy-reduced MedDiet (erMedDiet) with physical activity promotion and behavioral support. For the present thesis, TL was measured in subsamples of participants at baseline and after one or three years of follow-up. Different statistical approaches were used to analyze the intervention effects on TL using data from this trial.

The results of the first pilot study revealed that the 69 PREDIMED-Plus participants included from the Reus center experienced a positive change in TL measured in peripheral blood mononuclear cells (PBMC) after one year of intervention. However, neither the intervention group nor the interaction between time and group were associated with changes in TL after one year. In addition, there were no differences in changes in plasma levels of 8-hydroxy-deoxyguanosine (8-OHdG) after one year of intervention. In the

second study, which included 317 participants from Reus and Navarra, women in the intervention group experienced a positive change in TL, measured in buffy coat, after three years of intervention compared to women in the control group. Furthermore, women in the intervention group had 83% lower odds of telomere shortening after the follow-up than women in the control group. These results were not observed in men or the population as a whole.

These findings support the benefits of MedDiet, together with physical activity promotion, behavioral support, and weight loss encouragement on TL in older adults over time. Although only women, but not men, seemed to take advantage of the PREDIMED-Plus intervention after a larger follow-up for slowing down telomere shortening, we should not dismiss the potential positive effect of this intervention on telomere homeostasis in men if analyzed in larger samples with longer follow-up.

Furthermore, short telomeres have been associated with impaired male reproductive quality determined by sperm parameters. Scientific evidence has reported that men with altered sperm quality parameters and a diagnosis of infertility have shorter sperm and leukocyte TL than men with normal sperm parameters or fertile men. Nevertheless, there is an important absence of studies including the general population and considering lifestyle factors to assess the relationship between TL and sperm quality.

In this context, the second aim of this doctoral thesis was to assess the cross-sectional associations between sperm and leukocyte TL and sperm quality parameters in 200 healthy volunteers of reproductive age in the framework of the Led-Fertyl study. Positive associations were found between TL measured in both cell types and sperm concentration and total count, independent of age, body mass index, and other potential confounding factors. These results suggest that sperm and leukocyte TL could be suitable candidates to be used as biomarkers of sperm quality to complement the conventional semen analysis.

In summary, and in light of the findings presented in this thesis in the context of one RCT and one cross-sectional study, telomeres and their dynamics are structures that could provide valuable information about a wide range of epidemiological studies. The measurement of TL can be very useful in understanding different biological pathways, as it can provide insights into different diseases. However, further research is necessary to delve into TL homeostasis and regulatory mechanisms.

Resumen

Castellano

Los telómeros son estructuras localizadas al final de los cromosomas cuya función principal es proteger y preservar la integridad del genoma. En las células de mamíferos, estas regiones consisten en la repetición en tándem de la secuencia 5' TTAGGG 3'. Estas estructuras son elongadas por una enzima conocida como telomerasa y son estabilizadas por seis proteínas que forman complejo shelterina, el cual desempeña un papel importante en la regulación y el mantenimiento de la estructura telomérica. El desgaste de los telómeros se ha propuesto como una de las huellas del envejecimiento y se ha asociado con la aparición de enfermedades como la obesidad, la infertilidad o enfermedades relacionadas con la edad como el síndrome metabólico (SM), las enfermedades cardiovasculares (ECV), la diabetes o el cáncer. En el contexto de la modulación de la longitud telomérica (LT), se han investigado tanto factores no modificables como el sexo, la edad y los rasgos genéticos, como factores modificables (por ejemplo, la dieta, la actividad física, el hábito tabáquico o el estatus socioeconómico).

Varios estudios han analizado el efecto de las intervenciones sobre el estilo de vida en los telómeros. Sin embargo, la evidencia científica de ensayos clínicos anteriores es limitada en lo que respecta a población de edad avanzada con sobrepeso u obesidad y enfermedades metabólicas. Por lo tanto, el primer objetivo de esta tesis fue evaluar el efecto de una intervención sobre el estilo de vida en la LT tras de uno y tres años del estudio PREvención con Dieta MEDiterránea-Plus (PREDIMED-Plus). Este ensayo clínico controlado, aleatorizado, multicéntrico y de grupos paralelos incluyó a participantes de la región mediterránea de edad avanzada con sobrepeso u obesidad y SM para la prevención primaria de la ECV. Los participantes del grupo de control siguieron una dieta mediterránea tradicional y los asignados al grupo de intervención siguieron una intervención basada en una dieta mediterránea con restricción calórica, promoción de la actividad física y apoyo conductual. Para la presente tesis, se midió la LT en submuestras de participantes al inicio del estudio y tras uno o tres años de seguimiento. Se utilizaron diferentes enfoques estadísticos para analizar los efectos de la intervención sobre la LT utilizando los datos de este estudio.

Los resultados del primer estudio piloto revelaron que los 69 participantes de PREDIMED-Plus incluidos del centro de Reus experimentaron un cambio positivo en la

LT medido en células mononucleares de sangre periférica tras un año de intervención. Sin embargo, ni el grupo de intervención, ni la interacción entre el tiempo y el grupo, se asociaron con cambios en la LT después de un año. Además, no se observaron diferencias en los cambios de los niveles plasmáticos de 8-hidroxi-deoxiguanosina (8-OHdG) tras un año de intervención. En el segundo estudio, que incluyó a 317 participantes de Reus y Navarra, las mujeres del grupo de intervención experimentaron un cambio positivo en la LT medido en células de la capa leucocitaria después de tres años de intervención, en comparación con las mujeres del grupo de control. Además, las mujeres del grupo de intervención presentaron un 83% menos de probabilidades de acortamiento de los telómeros que las mujeres del grupo de control tras el seguimiento. Estos resultados no se observaron ni en los hombres ni en el conjunto de la población.

Estos resultados respaldan los beneficios de la dieta mediterránea, junto con la promoción de la actividad física, el apoyo conductual y el fomento de la pérdida de peso, sobre la LT en adultos mayores a lo largo del tiempo. Aunque sólo las mujeres, pero no los hombres, parecieron beneficiarse de la intervención PREDIMED-Plus tras un seguimiento más amplio para ralentizar el acortamiento de los telómeros, no debemos descartar el posible efecto positivo de esta intervención sobre la homeostasis de los telómeros en los hombres si se analiza en un mayor número de muestras y durante un periodo más prolongado.

Además, los telómeros cortos se han asociado con una peor calidad reproductiva masculina determinada por los parámetros espermáticos. La evidencia científica ha demostrado que los hombres con una alteración en los parámetros de calidad espermática y con un diagnóstico de infertilidad tienen una LT medida en espermatozoides y leucocitos más corta que los hombres con parámetros espermáticos normales u hombres fértiles. Sin embargo, existe una importante ausencia de estudios que incluyan población general y que consideren factores de estilo de vida para evaluar la relación entre la LT y la calidad espermática.

En este contexto, el segundo objetivo de esta tesis doctoral fue evaluar las asociaciones transversales entre la LT de espermatozoides y leucocitos y los parámetros de calidad espermática en 200 voluntarios sanos en edad reproductiva en el marco del estudio Led-Fertyl. Se hallaron asociaciones positivas entre la LT medida en ambos tipos celulares y la concentración y el recuento total de espermatozoides, independientemente de la edad, el índice de masa corporal y otros posibles factores de confusión. Estos resultados sugieren que la LT medida en ambos tipos celulares podría considerarse candidatos

idóneos para ser utilizados como biomarcadores de la calidad espermática, complementando el análisis convencional del seminograma.

En resumen, y a la luz de los hallazgos incluidos en esta tesis en el contexto de un ensayo clínico controlado y aleatorizado y de un estudio transversal, los telómeros y su dinámica son estructuras que podrían proporcionar información valiosa en estudios epidemiológicos. La medición de la LT puede ser muy útil para comprender diferentes vías biológicas, ya que puede aportar información sobre diferentes enfermedades. Sin embargo, es necesario seguir investigando para profundizar en la homeostasis la LT y sus mecanismos reguladores.

Resum

Català

Els telòmers són estructures localitzades al final dels cromosomes. La seva funció principal és la de protegir i preservar la integritat del genoma. En les cèl·lules de mamífers, aquestes regions consisteixen en la repetició en tàndem de la seqüència 5' TTAGGG 3'. Aquestes estructures s´allarguen gràcies a un enzim conegut com telomerasa, i són estabilitzades per sis proteïnes que formen el complex shelterin, el qual exerceix un paper important en la regulació i el manteniment de l'estructura dels telòmers. El desgast dels telòmers s'ha proposat com una de les empremtes de l'envelliment, i la seva longitud s'ha associat amb l'aparició de malalties com l'obesitat, la infertilitat o malalties relacionades amb l'edat com la síndrome metabòlica (SM), les malalties cardiovasculars (ECV), la diabetis o el càncer. En el context de la modulació de la longitud telomèrica (LT), s'han investigat tant factors no modificables (per exemple, el sexe, l'edat i els trets genètics) com a factors modificables (per exemple, la dieta, l'activitat física, l'hàbit tabàquic o el nivell socioeconòmic).

Diversos estudis han analitzat l'efecte de intervencions sobre l'estil de vida en els telòmers. No obstant això, l'evidència científica d'assajos clínics previs és limitada pel que fa a la població d'edat avançada amb sobrepès o obesitat i malalties metabòliques. Per tant, el primer objectiu d'aquesta tesi va ser el d'avaluar l'efecte d'una intervenció sobre l'estil de vida en la LT després d'un i tres anys de seguiment en el marc de l'estudi Prevenció amb Dieta Mediterrània-Plus (PREDIMED-Plus). Aquest assaig clínic controlat, aleatoritzat, multicèntric i de grups paral·lels va incloure a participants de la regió mediterrània d'edat avançada amb sobrepès o obesitat i SM per a la prevenció primària de la ECV. Els participants del grup de control van seguir una dieta mediterrània tradicional, i els assignats al grup d'intervenció van seguir una dieta mediterrània amb restricció calòrica, promoció de l'activitat física i suport conductual. Per a la present tesi, es va mesurar la LT en submostres de participants a l'inici de l'estudi i després d'un o tres anys de seguiment. Es van utilitzar diferents enfocaments estadístics per a analitzar els efectes de la intervenció sobre la LT utilitzant les dades d'aquest estudi.

Els resultats del primer estudi pilot van revelar que els 69 participants de PREDIMED-Plus inclosos en el centre de Reus van experimentar un canvi positiu en la LT mesurada en cèl·lules mononuclears de sang perifèrica després d'un any d'intervenció. No obstant

això, ni el grup d'intervenció, ni la interacció entre el temps i el grup, es van associar amb canvis significatius en la LT al cap d'un any. A més, no es varen observar diferències en els canvis dels nivells plasmàtics de 8-hidroxi-deoxiguanosina (8-OHdG) després d'un any d'intervenció. En el segon estudi, que va incloure a 317 participants dels centres de Reus i Navarra, les dones del grup d'intervenció van experimentar un canvi positiu en la LT mesurada en cèl·lules de la capa leucocitària després de tres anys d'intervenció, en comparació amb les dones del grup de control. A més, les dones del grup d'intervenció van presentar un 83% menys de probabilitats d'escurçament dels telòmers que les dones del grup control, després del seguiment. Aquests resultats no es van observar ni en els homes ni en el conjunt de la població.

Aquests resultats recolzen els beneficis de la dieta mediterrània, juntament amb la promoció de l'activitat física, el suport conductual i el foment de la pèrdua de pes, sobre la LT en adults d'edat avançada al llarg del temps. Encara que només les dones, però no els homes, van semblar beneficiar-se de la intervenció PREDIMED-Plus després d'un seguiment més ampli per a alentir l'escurçament dels telòmers, no hem de descartar el possible efecte positiu d'aquesta intervenció sobre l'homeòstasi dels telòmers en els homes si s'analitza en un nombre superior d'individus que tinguin un seguiment més prolongat.

A més, els telòmers curts s'han associat amb una pitjor qualitat reproductiva masculina determinada pels paràmetres espermàtics. L'evidència científica ha demostrat que els homes amb una alteració en els paràmetres de qualitat espermàtica i amb un diagnòstic d'infertilitat tenen una LT en espermatozoides i leucòcits menor en comparació als homes amb paràmetres espermàtics normals o homes fèrtils. Malgrat tot, existeix una important absència d'estudis que incloguin població general i que considerin factors d'estil de vida a l'hora d'avaluar la relació entre la LT i la qualitat espermàtica.

En aquest context, el segon objectiu d'aquesta tesi doctoral va ser el d'avaluar les associacions transversals entre la LT d'espermatozoides i leucòcits amb els paràmetres de qualitat espermàtica de 200 voluntaris sans en edat reproductiva dins del el marc de l'estudi Led-Fertyl. Es van trobar associacions positives entre les mesures de LT en tots dos tipus cel·lulars i la concentració i el recompte total d'espermatozoides, independentment de l'efecte de l'edat, l'índex de massa corporal i altres possibles factors de confusió. Aquests resultats suggereixen que la mesura de la LT en tots dos tipus cel·lulars podrien ser considerats candidats idonis per a ser utilitzats com a

biomarcadors de la qualitat espermàtica, complementant l'anàlisi convencional del seminograma.

En resum, i a la llum de les troballes d'aquesta tesi en el context d'un assaig clínic controlat i aleatoritzat i d'un estudi transversal, els telòmers són estructures que podrien proporcionar informació valuosa en estudis epidemiològics. El mesurament de la LT podria ser molt útil per a comprendre diferents vies biològiques, ja que pot aportar informació sobre diferents malalties. No obstant això, és necessari continuar investigant per a aprofundir en l'estudi de l'homeòstasi de LT i els seus mecanismes reguladors.

Abbreviations

8-OHdG, 8-hydroxydeoxyguanosine

8-oxodG, 8-oxo-2'-deoxyguanosine

ALT, alternative lengthening of telomeres

ANCOVA, Analysis of covariance

ANOVA, Analysis of variance

BL, Blocker probes

BMI, Body mass index

bp, Base pair

CASA, Computer Aided Sperm Analysis

CE, Capture extenders

CP, Custom-designed probes

Ct, Cycle threshold

CV, Coefficients of variation

CVD, Cardiovascular disease

DNA, Deoxyribonucleic acid

dNTP, Deoxynucleotide triphosphate

erMedDiet, energy-reduced Mediterranean Diet

g, grams

GWAS, Genome-wide association study

HDL, High-density lipoprotein

HOS, Hypo-osmotic swelling

IFN, Interferon

IL, Interleukin

kcal, kilocalories

kg, kilograms

LDL, Low-density lipoprotein

LE, Label extenders

Led-Fertyl, Lifestyle and environmental determinants of seminogram and other male fertility-related parameters

m, meters

MedDiet, Mediterranean Diet

MetS, Metabolic Syndrome

MFI, Median fluorescence intensity

mg, miligrams

µL, microliters

MET, Metabolic equivalent task

MedDiet, Mediterranean Diet

MMqPCR, Monochrome Multiplex real-time PCR

mRNA, messenger RNA

ng, nanograms

NHANES, National Health and Nutrition Examination Survey

PBMC, Peripheral blood mononuclear cell

PBS, Phosphate-buffered saline

PCR, Polymerase Chain Reaction

POT, Protection of telomeres 1

PREDIMED-Plus, PRevención con Dieta MEDiterránea-Plus

QGP, QuantiGene Plex

qPCR, Quantitative Polymerase Chain Reaction

REGICOR, Registre Gironí del Cor

RCT, Randomized controlled trial

RNA, Ribonucleic acid

ROS, Reactive oxygen species

S, Single copy- gene

SAPE, Streptavidin-conjugated R-Phycoerythrin

SD, Standard deviations

SNP, Single nucleotide polymorphism

SES, Socioeconomic status

SUN, Seguimiento Universidad de Navarra

T, Telomere

Taq, Thermus aquaticus

TERC, Telomerase non-coding RNA component

TERT, Telomerase reverse transcriptase

TIN2, TRF1 interacting nuclear factor 2

TL, Telomere length

TNF-α, Tumor necrosis factor-alpha

TRF1, Telomere repeat binding factor 1

TRF2, Telomere repeat binding factor 2

T1D, Type I diabetes

T2D, Type II diabetes

WHO, World health organization

WBM, Working Bead Mix

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I. INTRODUCTION

1. Telomeres

1.1. History of telomeres

The first description of telomeres was provided in the 1930s, when a structure at the ends of chromosomes was identified in *Zea mays* and *Drosophila melanogaster* by Barbara McClintock and Hermann Muller, respectively[1,2]. The authors observed that these structures exhibited distinct behavior compared to induced broken ends. Muller defined these ends as being of critical importance for the prevention of chromosome end fusion and designated these structures, derived from the Greek, as '*telos'* (end) and '*meros'* (part). In the 1960s and 1970s, further studies contributed to a more precise definition and understanding of the role of telomeres. The Hayflick limit theory was proposed when it was discovered that human cells have a finite replicative potential of 40-60 cycles before becoming senescent[3,4]. This finding underscored the role of telomeres in cellular aging. During the same period, the "end-replication problem" was proposed when it was observed that with each cell division, a fragment of chromosomal deoxyribonucleic acid (DNA) was lost due to the inability of DNA polymerases to reach the ending region of chromosomes[5,6].

It was in 1978 when Elizabeth H. Blackburn and Joseph G. Gall characterized a repeating hexanucleotide sequence from the ribosomal DNA of the protozoan *Tetrahymena thermophila*[7]. Ten years later, in 1988, the human telomeric repeats were finally sequenced[8], thereby advancing the understanding of these structures.

1.2. Telomeric structure and function and the shelterin complex

Telomeres are structures located at the ends of chromosomes whose principal function is to protect and preserve genome integrity, thereby ensuring the health span and propagation of species. In mammalian cells, these non-coding DNA regions consist of tandem repeats of the sequence 5' TTAGGG 3', organized in a double-stranded manner with a single-stranded overhang at the 3' end, which is rich in guanine nucleotides[9]. The telomeric structure assumes a T-loop conformation when it folds back on itself and a D-loop when the 3' overhang invades a double-stranded region of the telomeric DNA sequence[10].

In mammalian cells, this looped structure is stabilized by a complex of proteins called shelterin, that binds telomeres with high specificity and protects them from the DNA damage response. Besides, this complex determines the structure of the telomere by being implicated in the T-loop generation, and it controls the synthesis of telomeric DNA by telomerase. Thus, this complex is essential for the protection and replication of chromosome ends[10,11].

Human shelterin is formed by six subunits: telomere repeat binding factors 1 and 2 (TRF1, TRF2), protection of telomeres 1 (POT1), TRF1 interacting nuclear factor 2 (TIN2), repressor activator protein 1 (RAP1) and TPP1[12]. Three subunits, TRF1, TRF2, and POT1 directly recognize TTAGGG repeats and are interconnected by TIN2, TPP1, and RAP1, forming the complex that allows cells to distinguish telomeres from sites of DNA damage. TIN2 is at the core of the complex, with binding sites to three subunits: TRF1 and TRF2, and TPP1, which binds to POT1, the single-strand overhang-binding protein[10,11].

Each of these subunits plays a crucial role[10–12]:

- TRF1. Regulation of telomere length (TL) by binding to telomeric DNA and ensuring proper telomere maintenance.
- TRF2. Stabilization of the T-loop and protection of telomeres from the DNA damage response.
- TIN2. A central component that stabilizes the association of TRF1 and TRF2 with the telomere sequence by tethering POT1 to them and regulates telomere elongation by telomerase.
- POT1. It binds to the single-stranded overhang of telomeres and prevents activation of the DNA damage response.
- TPP1. It enhances the binding of POT1 to telomeres and facilitates the recruitment of telomerase to maintain TL.
- RAP1. It works in conjunction with TRF2 to modulate TL and contributes to the protective functions of the shelterin complex.

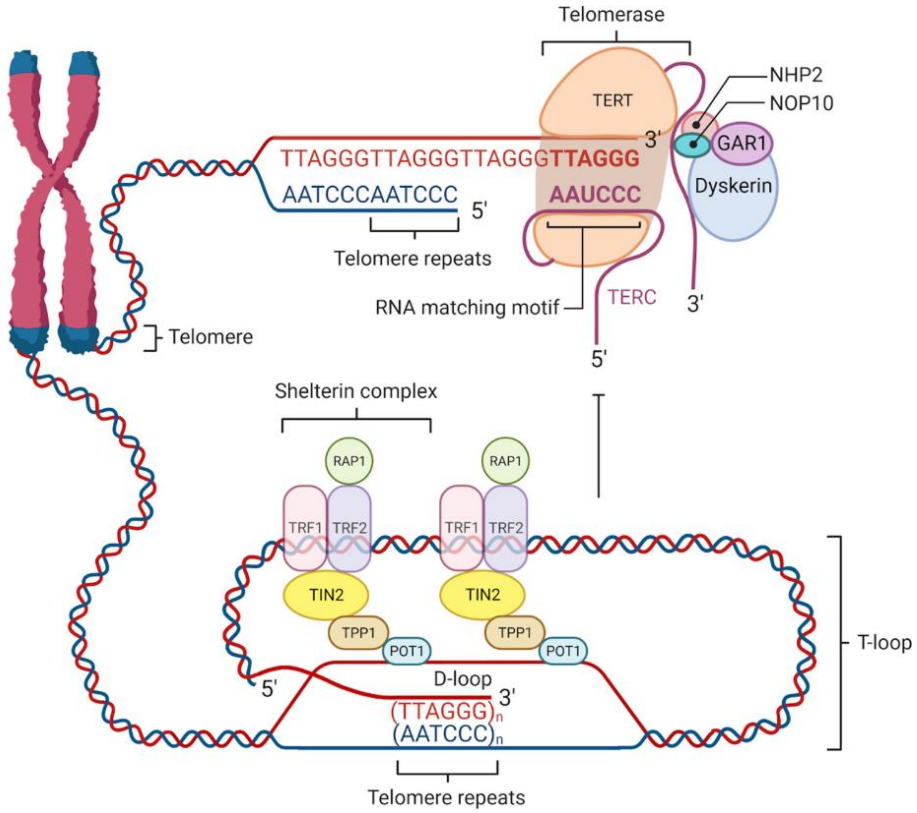

Figure 1. Telomere, shelterin, and telomerase complexes in human cells. Telomeres consist of the repetition in tandem of the sequence 5'TTAGGG3' in mammalian cells. These repeats are elongated by telomerase. The shelterin complex stabilizes the telomeric structure and regulates telomere length. Adapted from: Turner KJ, Vasu V, Griffin DK. Telomere Biology and Human Phenotype. Cells. 2019 Jan 19;8(1):73. DOI: 10.3390/cells8010073.

This structure constitutes a cap at the end of chromosomes (**Figure 1**) and prevents the loss of genes located near these ends[12]. Telomeres can be recognized as double-strand breaks when shelterin components are removed or dysfunctional, which then activates the DNA repair response and results in end-to-end fusions[13]. In addition, defects in shelterin proteins have been associated with the onset of different genetic disorders such as dyskeratosis congenita, a rare inherited bone marrow failure syndrome characterized by abnormally short telomeres[14]. These connections highlight the crucial role of the shelterin complex in cellular physiology and development, as well as its role in maintaining genomic stability and preventing disease.

1.3. Telomerase complex

A natural shortening of telomeres occurs when DNA polymerases are unable to complete the polymerization of the lagging strand at the end of chromosomes. This progressive shortening acts as a biological clock, triggering cellular senescence and/or apoptosis when telomeres become critically short. This shortening is compensated by the action of the ribonucleoprotein complex that elongates telomeres, named telomerase[15]. Telomerase

was discovered in 1985 by Carol Greider and Elizabeth Blackburn, who identified the terminal transferase activity of an enzyme, later known as telomerase, in *Tetrahymena thermophila*[16]. This groundbreaking discovery was recognized, along with Jack W. Szostak, with the Nobel Prize in Physiology or Medicine in 2009 for solving a fundamental problem in biology: how chromosomes can be fully replicated during cell division and how they are protected from degradation.

The human telomerase has a catalytic core comprising a telomerase reverse transcriptase (TERT) subunit and a non-coding ribonucleic acid (RNA) component (TERC/TR), which contains the template for the elongation of telomeric DNA[15].

The activity profile of telomerase is highly dependent on the cell type, therefore, not all cells can prevent senescence due to replication-dependent loss of telomeric repeats and it varies throughout life (**Figure 2**). In humans, TERT activity is notably present in cells with high proliferative potential, such as germ cells, embryonic stem cells, and cancer cells, to maintain their high replicative rate and TL. Stem cells, including hematopoietic stem cells, exhibit TERT activity which enables them to maintain their proliferative potential after birth, but it is insufficient to completely prevent telomere attrition throughout life[17].

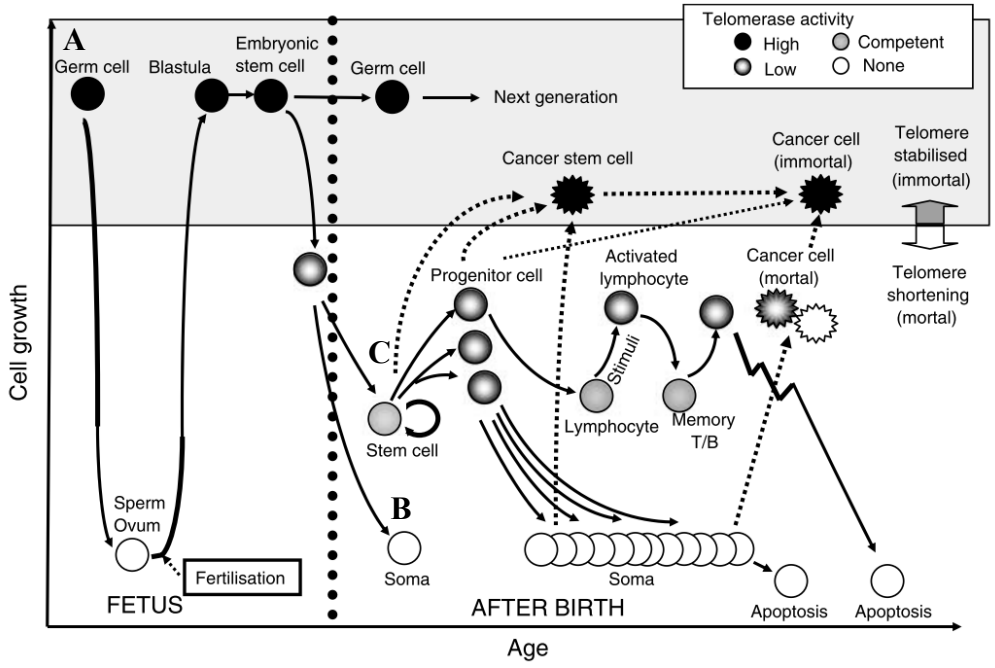

Figure 2. Telomerase activity profile. A) Germ and embryonic stem cells have high levels of telomerase activity due to their proliferative potential. **B)** In somatic cells, the activity of this enzyme decreases after birth. **C)** Stem cells have levels of telomerase activity to maintain the proliferative potential. Adapted from: Hiyama E, Hiyama K. Telomere and telomerase in stem cells. Br J Cancer. 2007 Apr 10;96(7):1020-4. DOI: 10.1038/sj.bjc.6603671.

In contrast, TERT activity decreases significantly and is generally repressed after birth in most normal somatic cells, leading to replicative senescence due to telomere shortening[17]. This decline in TERT activity contributes to the finite lifespan of somatic cells and is associated with cellular aging. While TERC is generally expressed in the majority of human cell types, both TERT and TERC are essential for telomerase enzymatic activity. However, the expression of TERC does not ensure telomerase functionality unless accompanied by TERT[18]. Accordingly, the regulation of TERT expression is a determining factor in the activity of telomerase across different cell types[19].

In humans, this complex is accompanied by accessory proteins including dyskerin (DKC1), nuclear protein family A member 2 (NHP2), nuclear protein family A member 3 (NOP10), pontin/reptin, TCAB1/WRAP53, and nuclear protein family A member 1 (GAR1). These proteins are involved in maintaining the correct telomerase conformation, producing functional telomerase, providing energy for recruitment and assembly, and ensuring the integrity and stability of the enzyme[20].

1.4. Telomere dynamics

Telomere dynamics encompass the complex processes of telomere length regulation and maintenance, and the biological implications of telomere shortening or lengthening within cells. These dynamics profoundly influence cellular aging, disease progression, and overall health outcomes. In humans, telomere dynamics vary depending on the developmental stage of the individual.

In leukocytes, often used as a proxy for TL in other tissues due to their high correlation[21], the rate of telomere loss is approximately 250 base pairs (bp) per year during infancy and early childhood (i.e., the first three years of life). This rate is higher than the shortening rate observed during young adulthood when it decreases to 30-50 bp/year[22], reflecting a slower pace of cellular turnover[23]. The elevated rate of cell replication observed during infancy may be indicative of an accelerated development and growth of the immune system, expanding the hematopoietic and progenitor stem cell pool. This provides a partial explanation for the differences in the shortening rates observed at different stages of infancy and childhood compared to adulthood and aging[22].

During later adulthood, leukocyte TL continues to decline, but at a slower age-dependent rate than during childhood[24]. Despite the presence of telomerase activity in hematopoietic stem cells, it is insufficient to fully offset telomere shortening caused by ongoing cellular

replication, which ultimately contributes to age-dependent leukocyte TL attrition[25]. Thus, this gradual shortening may be partially attributed to the self-renewal capacity of these hematopoietic stem cells, which replace progenitor pools lost due to senescence. Consequently, the TL of hematopoietic stem cells at birth could play a critical role in determining leukocyte TL and its subsequent attrition over the lifespan[26].

In germ cells, telomere dynamics differ from those observed in somatic cells and vary depending on sex. Male germ cells have high telomerase activity during the early stages of spermatogenesis. This activity is then gradually and slowly turned off during this process, with a delayed switching off observed in mature spermatozoa. This would prevent telomere shortening in male germ cells and maintain telomeres for the next generation, resulting in longer TL in male germ cells than in somatic cells[27,28]. During the initial stages of oogenesis, female germ cells also exhibit a peak in telomerase levels, which then undergo a faster decrease during oogenesis. The reduction in telomere length observed in oocytes appears to mirror the dynamics observed in somatic cells during the process of aging. Women have a fixed oocyte reserve, which is established during early fetal life. In contrast, male germ cells can constantly replicate during postnatal life, resulting in longer telomeres than oocytes[27]. Following fertilization, TL is either maintained or decreased from the zygote to the different cleavage stages of the embryo,

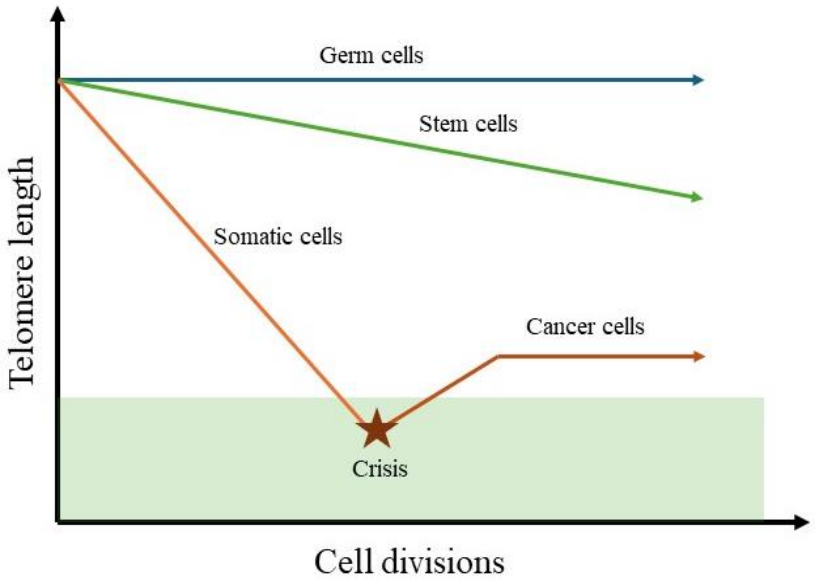

Figure 3. Telomere dynamics. After birth, germ and somatic cells preserve their telomere length by expressing telomerase. In contrast, somatic cells undergo telomere shortening with cell divisions. If senescence or apoptosis are bypassed at this point due to checkpoint failure, this could promote oncogenesis by telomerase activation. Source: thesis' author based on Toupance S, Fattet AJ, Thornton SN, Benetos A, Guéant JL, Koscinski I. Ovarian Telomerase and Female Fertility. Biomedicines. 2021 Jul 20;9(7):842. DOI: 10.3390/biomedicines9070842.

but it increases when the morula becomes a blastocyst due to an increase in the telomerase activity[29] (**Figure 3**).

Oxidative stress, inflammation, and telomere dynamics

Telomere dynamics are not only dependent on telomerase activity or the developmental stage of the individual. Indeed, oxidative stress and inflammation have been proposed as processes that could contribute to an accelerated shortening of telomeres. Furthermore, there are a number of modifiable and non-modifiable factors that could also influence TL, which are explained below.

Oxidative stress refers to the imbalance between the production of reactive oxygen species (ROS) and antioxidant mechanisms. ROS are highly unstable and reactive chemical species[30] that are formed endogenously as natural products of oxygen metabolism. These species act as second messengers in different signaling pathways, yet they can also damage cellular components, including telomeres, under imbalanced conditions[31]. The sources of ROS generation can also be exogenous, caused by ultraviolet radiation, environmental pollution, or lifestyle factors such as smoking, diet, or sedentary behavior[32].

Telomeres appear to be particularly susceptible to ROS, primarily due to their rich guanine sequence, which is prone to oxidation to 8-oxo-2'-deoxyguanosine (8-oxodG)[33]. These ROS preferentially target these regions, increasing the amount of 8-oxodG[33] and inducing DNA strand breaks. This results in telomere loss at a faster rate than normal conditions due to a collapse in replication[34]. In addition, oxidative damage affects telomerase activity by directly inhibiting its enzymatic function. These mechanisms collectively contribute to accelerated telomere shortening and cellular senescence, highlighting the intricate relationship between oxidative stress and telomere dynamics[35].

Chronic inflammation could exacerbate the shortening of telomeres by triggering an increase in hematopoietic stem cell replication to satisfy the demand for leukocytes necessary to overcome the inflammatory process. This increase in replication could induce accelerated shortening of telomeres and susceptibility to age-related diseases [25].

The inflammatory process implies the secretion of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukins (IL), or interferon (IFN) factors, among others. These cytokines can induce telomere shortening and dysfunction by regulating

telomere components, leading to accelerated cellular senescence. Additionally, telomere dysfunction would play an important role in initiating and perpetuating the inflammatory response, which in turn contributes to the onset of several diseases. Specifically, telomere dysfunction triggers cellular senescence, which subsequently stimulates the production and secretion of inflammatory factors[36].

1.5. Factors modulating telomere length

The factors identified as contributors or modulators of TL can be classified as non-modifiable and modifiable. Among the non-modifiable factors, age, sex, and genetic traits are the most extensively studied across scientific research. Modifiable factors can broadly include environmental and lifestyle factors. In the present thesis, the relationship between diet, physical activity, smoking habits, and socioeconomic traits with telomeres will be considered (**Figure 4**). These factors can act synergistically in changing telomere dynamics throughout life.

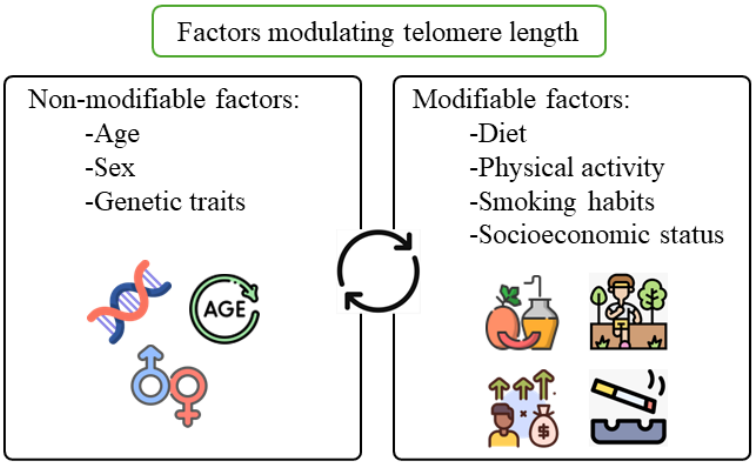

Figure 4. Factors modulating telomere length (TL). In the present thesis, the non-modifiable factors considered as modulators of TL will be age, sex, and genetic traits. Among the wide range of modifiable factors modulating TL, diet, physical activity, smoking habits, and socioeconomic status will be also considered. Source: thesis' author.

1.5.1. Non-modifiable factors

1.5.1.1. Age and aging

In 1990, Harley and collaborators observed a decrease in the length of telomeric DNA in human fibroblasts, a well-established *in vitro* model of cellular senescence. The authors concluded that telomeres must also undergo this shortening with age *in vivo*[37]. This finding suggested that TL can serve as a mitotic clock reflecting the replicative cell

history[38]. Subsequent evidence has delved into age as a significant contributing factor for telomere shortening in humans.

Epidemiological studies have consistently reported a progressive reduction of TL with age, showing an inverse relationship between chronological age and TL. A systematic review summarizing data from five longitudinal studies reported a reduction in leukocyte TL with mean age in well-being adults during the follow-up periods, which ranged from seven to 12 years[39]. An updated and more comprehensive meta-analysis comprising data from 743,019 individuals from cross-sectional and longitudinal studies concluded that TL, measured in blood in the majority of the studies, declines with chronological age. However, authors observed a non-linear trend, with the correlation between age and TL being weaker in adults than in children, likely due to higher replication rates during the first years of life[40]. Additional studies also reported a negative correlation or association between TL and chronological age[41,42].

While chronological age provides an important insight into the biological and functional state of a subject, it can differ from biological age and does not always reflect it due to comorbidities and other influencing factors, including those attributed to environmental or lifestyle habits. Given the significant variability among individuals in the appearance of age-related health complications[43], it is important to identify biomarkers to better understand the mechanisms related to the biological implications of aging beyond chronological age.

Aging is a multifactorial and irreversible process characterized by the deterioration and loss of physiological integrity and functionality. This results in molecular, cellular, and systemic alterations[44], which in turn lead to impairments across various systems (i.e., the cardiovascular, endocrine, reproductive, and nervous), as well as mechanical and structural abilities, psychosocial deficits, and an increased risk of diseases such as cardiovascular disease (CVD), cancer, or dementia[45]. The use of a composite of biomarkers of aging has been proposed as a means of accurately reflecting the complexity of aging, with TL being one of them considering its age-dependent decline[46]. In the recently updated review from López-Otín and colleagues[47], the role of telomeres and their attrition has been consolidated as one of the main hallmarks of aging and age-related diseases.

Short TL and its attrition rate, particularly from leukocytes, have been constantly proposed as biomarkers of biological age in epidemiological studies due to the association between TL and the onset of diseases related to the aging process, including CVD or metabolic syndrome (MetS), among others. However, some authors argue that the relationship between TL, age, and related diseases is more complex, as both short and long TL have been linked to an increased risk of different diseases. They therefore suggest that telomere research should be expanded, measuring TL in newborns and children to gain a deeper understanding of the relationship between TL and aging[48].

1.5.1.2. Sex

The role of sex in determining differences in TL has been investigated in several epidemiological studies across diverse populations. A meta-analysis of 36 cohorts conducted by Gardner and collaborators (2014) highlighted that women generally exhibit longer telomeres than men in age-adjusted analysis, with this difference being particularly evident in studies employing Southern Blot for TL determination[49]. Similarly, in the National Health and Nutrition Examination Survey (NHANES), a higher age-adjusted geometric mean of leukocyte TL in women than in men was reported[50]. Further studies have observed a faster decline in leukocyte TL with age in men compared to women[42,51–53], particularly after the age of 50[54].

In contrast, other studies did not report these sex differences in TL[55–57], or found that the differences disappeared in fully adjusted models that accounted for age, demographic, and health behaviors[58], or sex did not moderate the TL-age correlation found across the life course[40].

Several biological hypotheses have been proposed to gain a deeper understanding and to explain these results. These hypotheses are based on the action of estrogen. This hormone is present in premenopausal women and has been shown to have a beneficial effect on telomeres. Firstly, because it acts as an antioxidant, reducing oxidative stress by several mechanisms, including the inhibition of ROS formation[59]. This would enable a better oxidative state in women, with the consequent positive impact on TL.

Secondly, the potential effect of estrogens on telomerase expression and activity must be mentioned. In 1997, a pattern of telomerase activity was observed during the menstrual cycle in normal human endometrial samples. 95% of the samples collected during the proliferative phase presented telomerase activity, with levels ranging from moderate to

high. Although 47% of samples in the non-proliferative phase were telomerase positive, levels were lower. A telomerase regulation by sex hormones during the menstrual cycle was suggested[60]. It was subsequently observed that activation of the TERT promoter by estradiol was present in breast cancer cells[61] and ovarian epithelium cells[62].

Furthermore, estradiol has been shown to stimulate telomerase activity in normal peripheral blood lymphocytes from healthy volunteers. In this *in-vitro* study, authors also found that the enzymatic conversion of androgens to estrogens was the main explanation for the activation of telomerase observed by androgens[63]. In contrast, no direct regulation of TERT messenger RNA or protein expression by estradiol was observed in cultured peripheral blood mononuclear cells from normal volunteers aged between 27 and 62 years[64].

Although this hormonal advantage appears to contribute to the longer telomeres observed in women compared with men in some studies, further research is needed to elucidate the biological pathways.

1.5.1.3. Genetic traits

Genetic factors play a pivotal role in determining TL and can help to partially understand the variability observed among individuals. In this context, the heritability of TL has been investigated to establish the proportion of additive genetic variance relative to total variation. Besides, a number of genetic variants implicated in telomere regulation have been linked to an increased risk of several diseases, as well as to the onset of telomere biology disorders.

Inheritance component and parental age at conception

The inheritance pattern of leukocyte TL was meta-analyzed using data from six cohort studies involving 19,713 participants. The authors found a high estimated heritability of 70%, with stronger mother-offspring correlation observed[65]. In an updated meta-analysis, the heritability of telomeres in humans was estimated to be approximately 60%[66]. Other studies have reported that between 48%[67] and up to 64% of leukocyte TL is inherited in age and sex-adjusted models[68]. In addition, the estimated heritability of age-dependent leukocyte TL attrition has been estimated to be 28%[69].

Furthermore, the effect of paternal and maternal age at conception has been explored in the context of their relationship with offspring TL. In a meta-analysis, both paternal and

maternal age were observed to be positively associated with offspring TL. However, after adjusting for paternal age, maternal age was no longer associated with offspring TL, whereas paternal age remained significantly associated with TL after adding maternal age as a confounder[65]. A study that compared the relationship between parents' age at conception and offspring TL concluded that the major factor influencing offspring leukocyte TL was the father's age, with longer leukocyte TL observed in the offspring of older fathers[70]. Several studies have yielded consistent results in this regard, indicating a positive correlation between paternal age and offspring TL[71,72], whereas other authors did not find significant associations[73]. In the absence of pathology, male germ cells have longer TL than somatic cells due to the action of telomerase in elongating TL in the testes. As older fathers may be able to transmit longer TL to their offspring [74], this could be beneficial for the offspring's lifespan, particularly in relation to the onset of age-related diseases driven by telomere shortening.

Genetic variants and telomere biology disorders

Genome-wide association studies (GWASs) have identified genetic variants associated with TL regulation that could affect the onset of several diseases. For example, single nucleotide polymorphisms (SNPs) in the *TERC* locus associated with longer leukocyte TL have been associated with an increased risk of colorectal cancer or multiple sclerosis, whereas SNPs in *TERT* locus associated with shorter leukocyte TL have been associated with a higher risk of idiopathic pulmonary fibrosis. A genetic risk score based on seven SNPs from different genes (including *TERT* and *TERC*) revealed a significant association of the allele associated with shorter TL with an increased risk of coronary artery disease [75].

Telomere biology disorders are a heterogeneous group of diseases caused by genetic mutations that affect telomere maintenance[76]. Dyskeratosis Congenita is a rare inherited disorder linked to germline mutations in genes responsible for telomere maintenance, leading to critically short telomeres. The first mutation was identified in the dyskerin gene (*DKC1*) on the X chromosome, leading to a reduction in telomerase activity. This discovery marked the first link between mutations in telomere biology genes and human disorders. Currently, it is known that at least 18 genes related to telomerase and shelterin components can be involved in this disease. Individuals with this disorder have very short TL for their age, resulting in a spectrum of clinical manifestations, including bone marrow failure, pulmonary fibrosis, liver disease, and an increased predisposition to cancer. These

manifestations, as well as the severity of the disease, depend on the affected gene and mode of inheritance[14].

1.5.2. Modifiable factors

It is evident that non-modifiable factors are not sufficient to explain and deeply understand telomere dynamics and the variability among individuals. It is important to consider environmental, nutritional, and lifestyle factors when investigating TL.

1.5.2.1. Diet

In light of the implications of nutrition on health, there has been a notable increase in scientific interest in the potential associations or effects of diet and its components on telomere homeostasis. Traditionally, diet has been understood in the context of each individual nutrient or food group and their isolated effect on health. However, in recent years, a new perspective has emerged, considering diet as a combination and variety of nutrients and foods that interact synergistically with each other and that better reflect the cumulative effects of the entire dietary pattern on health.

A substantial body of research has been conducted to examine the relationship between nutrients, foods, dietary patterns, and TL in diverse populations. A comprehensive systematic review examined the impact of global nutrition on telomere health, synthesizing results from observational cohort studies and randomized controlled trials (RCTs) in adult populations. This review delved into the relationship between micro- and macronutrients, food groups, dietary patterns and TL. The authors concluded that high adherence to healthy dietary patterns, such as the Mediterranean Diet (MedDiet), which includes the introduction of certain micronutrients and food groups as fruits and vegetables, could protect the length of telomeres and potentially prevent accelerated telomere shortening[77].

Food groups and TL

In children and adolescents, a higher consumption of dairy products, simple sugars[78], cereals and white bread[79], and sugar-sweetened beverages[80] was negatively associated with leukocyte TL. Conversely, a positive association was observed between the consumption of fish, nuts and seeds, olives, fruits and vegetables [78], and legumes[79] with leukocytes or whole blood cell TL.

In the case of studies conducted in adults, data from 5,448 participants in the NHANES study revealed positive associations between fruit and vegetable consumption, evaluated together or separately, with leukocyte TL, whereas legume and potato consumption were not associated with TL[81]. Furthermore, research indicates that the intake of processed meat, but not of unprocessed red meat, is negatively associated with leukocyte TL[82], and ultra-processed food consumption is associated with a double risk of short TL, measured in saliva samples[83]. The intake of added fructose from processed foods and beverages was also associated with a shorter leukocyte TL, a trend not observed for natural fructose from fruits, vegetables, and honey[84]. However, the consumption of several food groups, including fruits, vegetables, nuts, or meat, has not been correlated with sperm TL[85].

Dietary patterns and TL

The exploration of dietary patterns and their relationship with TL has increased in importance because it offers valuable guidance for nutritional strategies aimed at enhancing lifespan and health. Cross-sectional analyses of the NHANES data revealed a positive association between the oxidative balance score -which considers diet and lifestyle factors- and leukocyte TL in women but not in men. The authors found that these associations remained consistent when dietary factors were evaluated separately from the other lifestyle factors[86].

Adherence to the traditional diet, comparable to the MedDiet, has been positively associated with leukocyte TL[87]. In agreement with this, adherence to an *a priori* Mediterranean-style diet and an *a posteriori* "Prudent" pattern was positively associated with leukocyte TL, whereas an inverse association was identified between the *a posteriori* "Meat" pattern and TL among 422,797 participants in the UK Biobank[88]. The highest adherence to a Mediterranean dietary pattern was also observed to be positively associated with saliva cell TL in the 886 participants from the Seguimiento Universidad de Navarra (SUN) project. Conversely, no association was found between the Western diet and TL[89]. In the case of TL measured in sperm cells, a healthy diet consumption has also been correlated with longer sperm TL[90].

By contrast, a cross-sectional study including Chinese adults aged 65 or above reported no associations between several dietary patterns (i.e., Diet Quality Index, the Dietary Approaches to Stop Hypertension, the Mediterranean-DASH Intervention for

Neurodegenerative Delay Diet, the Mediterranean diet score, the Okinawan score, or the Hong Kong diet pattern score) and TL[91].

A meta-analysis of cross-sectional studies was conducted to investigate the associations between MedDiet and TL. In this instance, the authors found positive associations between higher adherence to MedDiet and longer TL. However, these results were significant only in age-adjusted models and in women[92]. A further meta-analysis of five clinical trials found no effect of different diets on TL, with a high degree of heterogeneity between studies due to the considerable variability in the time of intervention and the type of diet followed by the participants (i.e. energy-reduced diet, omega-3 supplementation, hyperproteic diet, or MedDiet)[93].

The available evidence suggests that adherence to healthy diets such as the MedDiet, or a similar dietary pattern based on the consumption of similar foods, could be an effective and suitable dietary approach to protect against accelerated telomere shortening and to maintain good health status. This dietary pattern is characterized by a high consumption of plant foods (vegetables, fruits, cereals, potatoes, and nuts), a high intake of olive oil as the principal source of fat, a moderate intake of dairy products and eggs, a low to moderate consumption of fish and poultry, and restricted amounts of red meat and processed meat. The combination and interaction of these food groups provide an important source of antioxidant and anti-inflammatory components that may impact metabolic and molecular mechanisms, helping to maintain telomere homeostasis, with the consequent benefit for health outcomes such as obesity, MetS, CVD, type 2 diabetes (T2D), or cancer[94].

1.5.2.2. Physical activity

It is becoming increasingly clear that regular physical activity has emerged as a key lifestyle factor in maintaining a healthy lifestyle, delaying the onset of chronic and age-related diseases, and enhancing healthy aging[95]. The beneficial effect of physical activity has been observed in both healthy individuals and patients, with a notable reduction in pain and disability, as well as an improvement in overall function, physiological health, and mental wellbeing[96].

Evidence from a meta-analysis of studies and trials conducted in healthy individuals revealed very low certainty about the effect of exercise or physical activity on leukocyte TL[97]. When only RCTs were meta-analyzed, exercise for more than six months, specifically aerobic exercise, was associated with a significant improvement in TL[98].

In contrast, an updated meta-analysis of seven additional controlled trials, involving only healthy adults, found no global effect of exercise on TL measured in blood and saliva cells. Nevertheless, the subgroup analysis indicated that high-intensity interval training, defined as brief, intermittent, and vigorous exercise followed by periods of low-intensity recovery, offers an advantage in terms of greater TL. This trend was not observed for resistance or aerobic exercise[99]. While no correlation was reported in one study between physical activity and sperm TL[85], the group of participants with higher exercise frequency was found to have longer sperm TL in a recent study[90].

A recent meta-analysis of trials reporting the effect of lifestyle interventions on leukocyte TL included 12 studies with an intervention based on physical activity and diet. Results from the analysis revealed that participants allocated to the control group of the trials experienced a non-significant decrease in TL through the follow-up, while TL from individuals in the lifestyle intervention groups based on diet and physical activity significantly increased at the end of the follow-up. Differences between groups in TL changes revealed that TL decreased less in participants from the intervention groups compared to controls[100].

An improvement in the oxidative and inflammatory status of the individual as a result of physical activity and exercise may contribute to the beneficial relationship or effect on TL reported in some studies, which may lead to telomere maintenance[101]. While scientific research in this area has yielded contradictory results, a better understanding of the relationship between physical activity and TL could provide valuable insights into how to promote healthy life and aging and mitigate accelerated telomere attrition. Nowadays, evidence in relation to physical activity and TL suggests that it is crucial to integrate regular physical activity into our daily routines on a global scale to ensure cellular health and longevity.

1.5.2.3. Smoking habits

Tobacco is the second most commonly used psychoactive substance worldwide. In 2020, 22.3% of the global population used tobacco, according to the WHO[102]. The major consequences of smoking are damage to the skin, mouth, respiratory system, heart, bones, and reproductive system. It also increases the risk of CVD, cancer, and overall mortality[103].

It is widely acknowledged that smoking is an unhealthy habit and a risk factor due to exposure to a harmful mixture of chemicals that impact health by inducing oxidative stress through ROS production and causing lipids, proteins, and DNA damage[104].

Active smoking has been proposed as one of the significant contributors to accelerated leukocyte telomere attrition, with its effect increasing the yearly attrition rate by more than threefold[105]. In this context, a meta-analysis of 30 cross-sectional, cohort and case-control studies demonstrated the detrimental effect of cigarette smoking on TL in humans, measured primarily in leukocytes and blood samples. Current smokers exhibited shorter TL compared to non-smokers and former smokers. Besides, an inverse but weak association was found between the number of packs of cigarettes smoked per year and TL[106]. Consistent results have reported a negative association between smoking status and blood TL[107], and between the number of cigarettes consumed with leukocyte TL[108].

However, contradictory results have also been reported. For example, no associations were observed between smoking status and sperm TL[85,90], and in participants from the NHANES study, no differences in leukocyte TL were found between current, former, and never smokers[108].

1.5.2.4. Socioeconomic status

Socioeconomic status (SES) has emerged as a factor influencing various health outcomes, including the dynamics of TL. SES encompasses a range of elements, including income, educational attainment, occupation, and social standing. Collectively, these elements impact an individual's access to resources, stress levels, and overall lifestyle[109]. In addition, factors previously mentioned as diet and lifestyle habits significantly interact with the relationship between SES and TL.

In a study involving 506 healthy participants aged 63 years, longer leukocyte TL was associated with higher educational attainment, but not with occupation or income[110]. These results were replicated in another study involving 4,441 women, in which lymphocyte TL was positively related to educational level, while not to social class[111]. Similarly, among the 2,599 participants in the Health, Aging and Body Composition study, leukocyte TL was positively associated with education, while income was not[112].

Zhou and collaborators (2020) did not identify a relationship between leukocyte TL and income. However, men with greater education had longer telomeres compared to men

with lower educational attainment, a result not observed in women[113]. In an observational longitudinal study, faster age-related telomere attrition was observed in individuals with lower income, while no association was found between TL and level of education[55].

2. Crosstalk between different pathologies and telomere length

There is a growing body of evidence that links TL with a range of human diseases, including obesity, infertility, and age-related diseases such as MetS or CVD, certain cancers, and diabetes. By examining the role of TL in the onset of these diseases, as well as the impact of these diseases in TL homeostasis, we can gain deeper insights into the underlying mechanisms and pathways. This knowledge could provide valuable information regarding the progression and prognosis of these conditions.

2.1. Obesity

Over the past decades, dietary recommendations have evolved globally in response to the increased range of food options and eating habits that are now available. An unhealthy or imbalanced diet, with lower consumption of foods such as fruits, vegetables, or legumes and increased consumption of ultra-processed foods, can lead to a population consuming more added sugars, trans fats, or salt and less fiber, vitamins, antioxidants, and minerals. This, combined with reduced physical activity, increased phone or computer time, and additional unhealthy factors, may have a dramatic impact on health because it can contribute to overweight and obesity[114].

Obesity is defined as a state of abnormal or excessive adiposity that represents a health risk due to its association with an increased risk of chronic diseases such as CVD, diabetes, or cancer. Obesity results from excessive caloric intake in relation to caloric expenditure over time, which leads to an energy imbalance[115]. Global rates of obesity in 2022 increased up to 8% in children and adolescents and 16% in adults, with higher prevalence in economically developed countries[116]. BMI is the most commonly used measurement of overweight or obesity in epidemiology due to its simplicity of assessment, making it an ideal metric for epidemiological studies. A person has overweight if the BMI is between 25 and 29.9 kg/m² and has obesity if the BMI is equal to or greater than 30 kg/m² [117].

One meta-analysis of 87 cross-sectional studies reported negative associations between BMI and TL, measured in leukocytes in the vast majority of the articles. The analysis included data from 146,114 individuals aged from 18 to over 75 years. In the age- and sex-adjusted model, each unit increase in BMI corresponded to an absolute TL decrease of 3.99 base pairs[118]. Pediatric obesity has also been associated with blood, saliva, or leukocyte TL, as shorter TL was observed in children with overweight or obesity compared to normal-weight participants[119]. Shorter sperm TL has been observed in participants with obesity compared to normal-weight individuals, with sperm TL negatively correlated with BMI[120].

Other indices of obesity and body composition (e.g., hip and waist circumference, waist-to-height ratio, or body fat, among others) have been negatively correlated[121] or inversely associated[122] with TL from whole blood or buffy coat samples. In a recent study, the weight-adjusted-waist index was associated with TL but followed an inverted U-shaped curve[123], while in the NHANES population, a higher visceral adiposity index was negatively associated with leukocyte TL when the index value was less than 2.84[124]. Moreover, in a prospective cohort study of women from China, participants who gained weight by more than 15% had shorter TL compared to participants who lost weight, maintained their weight, or gained less weight[122].

The relationship between obesity and TL has also been investigated in relation to adipose tissue. It has been reported that TL from subcutaneous and visceral adipose tissue was shorter in samples from individuals with overweight or obesity compared to samples from individuals without obesity[125,126]. Besides, evidence from surgical interventions in patients with severe obesity has shown that after morbid obesity surgery, changes in body weight towards weight loss[127] or greater loss of BMI ($\geq$ 10 kg/m^2)[128] were positively associated with significant increases in white blood cell TL.

Conversely, shorter telomeres could serve as a risk factor for elevated adipose tissue accumulation. In children, shorter leukocyte TL at ages 4 and 5 was associated with obesity at age 9[129]. Likewise, in older adults, leukocyte TL was negatively associated with subcutaneous and total body fat at baseline, but not with obesity status, BMI, or visceral fat[130].

Obesity is linked to chronic inflammation, caused by the secretion of proinflammatory cytokines and acute-phase proteins, such as C-reactive protein, as well as the infiltration

of adipose tissue by immune cells. These mechanisms contribute to the low-grade inflammation that occurs in adipose tissue and peripherally. In addition, oxidative stress can also contribute to the induction of inflammation and be involved in the development of obesity[131]. Conversely, obesity could be considered a contributor to oxidative stress[132]. It has been observed that individuals with obesity have higher levels of oxidative or inflammatory markers[133], and even higher ROS levels in sperm cells[120], compared to normal-weight individuals. This could result in a disruption to telomere homeostasis. Nevertheless, whether telomere attrition could increase obesity risk or is the result of the obesity status needs further study.

Although obesity affects all ages, it shares pathophysiological mechanisms at multiple levels with aging, including genomic instability, altered mitochondrial function, weakened immune systems, systemic inflammation, and shortened telomeres. Obesity also accelerates cellular aging, intensifying the burden of diseases and promoting the early onset of conditions typically associated with aging[134,135].

2.2. Age-related diseases

2.2.1. Metabolic syndrome (MetS)

MetS comprises several metabolic conditions such as central or abdominal obesity, high blood pressure, elevated fasting glucose, high triglyceride levels, and low HDL cholesterol levels. Each of the conditions in this cluster is considered a risk factor for CVD, but when an individual has three or more, the probability of developing CVD or diabetes increases[136]. Indeed, it has been reported that having MetS increases up to three times the risk of CVD and all-cause mortality[137]. The risk of developing other health issues as cognitive decline, T2D, or frailty also increases in the presence of MetS[31]. The prevalence of MetS is dependent on the criteria applied for its diagnosis, but globally, it is estimated to be up to 31%, with higher rates in developed countries with higher levels of income[138].

In the context of the relationship between MetS and TL, shorter whole blood TL has been observed in men and women with MetS compared to non-MetS participants[139,140], and in individuals aged between 61 and 70 years with uncontrolled MetS compared to age-matched healthy controls[141]. Results from a meta-analysis reported that leukocyte TL was shorter in individuals with hypertension than in normotensive controls[142]. In fact, a higher number of MetS components[139,143], including higher waist circumference, fasting glucose

or triglycerides, and lower HDL cholesterol[144], were associated with shorter leukocyte TL. An association has been observed between TL reduction and higher odds of having MetS[144] and, conversely, the presence of MetS has been associated with higher odds of having shorter TL[140]. In a longitudinal approach, MetS components such as higher waist-hip ratio, blood pressure, and serum triglyceride levels were related to an increased rate of telomere attrition, whereas higher HDL cholesterol levels were related to lower attrition in adults[105]. However, other studies did not find any association between leukocyte TL and MetS score in adults[145] or reported longer leukocyte TL associated with a higher number of MetS components[146].

The presence of MetS implies a metabolic overload manifested in each component of the syndrome. This could result in complications due to alterations in the antioxidant mechanisms, mitochondrial dysfunction, or organelle stress, with the subsequent production of ROS and cytokines[31,147]. This increases the pro-oxidant and inflammatory state in the body, which could impact telomere homeostasis and cause accelerated attrition. However, the interaction between metabolic disturbance and TL could be bidirectional since accelerated telomere shortening could contribute to cellular senescence and further impair the metabolic condition of patients with MetS.

2.2.2. Cardiovascular disease (CVD)

CVD is a group of diseases of the heart and blood vessels that, only in 2019, resulted in 17.9 million deaths, representing the leading cause of death worldwide with 32% of all deaths[148]. It is crucial to maintain optimal cardiovascular health to ensure adequate oxygenation of the blood supply to the body, but this process is naturally compromised with age[149]. These conditions include heart failure and myocardial infarction, stroke, coronary artery disease, arrhythmia, or peripheral artery disease, among others[148].

It has been reported that individuals with CVD have shorter TL than healthy subjects. This has been observed in stroke patients when TL was analyzed in quartiles in one study[150], and in men, but not in women, included in another study[151]. Furthermore, it has been observed that patients with coronary artery disease had baseline TL comparable to control subjects who were six years older[152]. In fact, a meta-analysis evaluating the relationship between TL and coronary artery disease in 5,150 patients and 9,341 controls reported shorter leukocyte TL in CVD patients than in controls. The subgroup analysis revealed that these results were more evident in men and individuals aged under 65

years[153]. Furthermore, leukocyte TL in healthy offspring of parents with a family history of coronary disease or myocardial infarction was shorter than offspring TL from parents with no history of CVD[154].

Several studies have identified short TL as a risk factor for the development of different CVDs. A meta-analysis of 25,340 participants reported that participants with shortened TL were at higher risk of stroke[155]. In subsequent studies, shorter TL was associated with higher odds of stroke[150,151], severe stroke phenotype[150], and a higher risk of coronary artery disease[152]. In contrast, the results of a prospective study conducted on 14,916 healthy men followed over six years showed no association between leukocyte TL and the risk of ischemic stroke[156]. A recent Mendelian Randomization study, with data from 472,174 adults from the UK Biobank, found that individuals with genetically longer TL traits had lower odds of coronary atherosclerosis, myocardial infarction, ischemic heart disease, and stroke. Conversely, longer TL was not associated with heart failure, atrial fibrillation, or cardiac death[157].

One of the primary causes of the development and progression of CVDs is insulin resistance, inflammation, and the imbalance between oxidant formation and antioxidant defenses. These processes are particularly prevalent in individuals of advanced age. A meta-analysis of 14 case-control and prospective studies including data from 810 patients with CVDs (e.g., coronary artery disease, stroke, peripheral artery disease, and atherosclerosis) and 1,106 controls revealed that urine and plasma levels of 8-Hydroxy-2-deoxyguanosine (8-OHdG), a commonly used marker of oxidative DNA damage, were significantly higher in CVD patients than in controls[158].

Inflammatory processes have been shown to play a significant role in the development of CVD because inflammation contributes to the formation of atherosclerosis, which is one of the primary manifestations of several CVDs, such as stroke or coronary artery disease[159]. Indeed, different pro-inflammatory cytokines, including IL-6, IL-18, and TNF-α, have been linked to an elevated risk of non-fatal myocardial infarction and coronary artery disease mortality[160].

In the context of this oxidative and inflammatory status, leukocyte telomere shortening may occur due to cell replication needed to overcome inflammation. This could lead to cell senescence and dysfunction, which, in turn, may contribute to the onset of CVDs. Similarly, shortened TL in hematopoietic stem cells could restrict the number and

functionality of endothelial progenitor cells required for vascular repair mechanisms, leading to endothelial dysfunction and facilitating atherosclerosis. Several cardiovascular and telomere shortening risk factors attributed to lifestyle or other diseases can accelerate and aggravate this situation by reducing TL[161,162].

2.2.3. Diabetes

Diabetes is characterized by chronic hyperglycemia due to heterogeneous metabolic disorders. Type I diabetes (T1D) is an autoimmune disease that results in impaired production of insulin due to the destruction of β-cells in

the pancreas. T2D, which is the most common form of diabetes in adults, involves insulin resistance or dysfunction in insulin production[163]. Complications of diabetes include CVDs such as coronary heart disease or stroke, chronic kidney disease, retinopathy, and neuropathy, among others[164]. Currently, 537 million individuals aged 20 to 79 years have diabetes, and it is estimated that this number will increase to 783 million by 2045[165].

A meta-analysis of case-control and cohort studies reported that shorter leukocyte TL was associated with higher odds of T2D[166], a result that was consistent with another meta-analysis of three prospective cohort studies[167]. Additionally, individuals with this disease had shorter TL than controls, with the difference being more evident for T2D individuals[168].

The relationship between TL and diabetes can be understood in the context of hyperglycemia, oxidative status, inflammation, and cellular senescence. Oxidative stress is a common feature in diabetes, where elevated glucose levels lead to increased production of ROS. As previously mentioned, this oxidative damage not only shortens telomeres but also, in individuals with diabetes, impairs β-cells, reducing insulin secretion. Additionally, chronic inflammation, prevalent in diabetic patients, accelerates telomere shortening through the secretion of cytokines that enhance oxidative stress and directly damage telomeric DNA. Moreover, telomere shortening due to hyperglycemia and oxidative stress could lead to β-cell senescence, compromising tissue regeneration and repair while reducing insulin secretion and glucose tolerance. This situation may exacerbate diabetes development and progression[169,170].

2.2.4. Cancer

Cancer is a large and heterogeneous group of diseases that can originate in almost any organ or tissue of the body when abnormal cells proliferate in an uncontrolled manner. Cancer is the second leading cause of death globally, resulting in 9.6 million deaths in 2018. In men, the most prevalent types of cancer are lung, prostate, colorectal, stomach, and liver cancer. Among women, the most common cancer types are breast, colorectal, lung, cervical, and thyroid cancer[171]. The incidence of cancer rises with age; older individuals are particularly vulnerable due to the cumulative effects of aging on cellular processes and the progressive telomere shortening associated with aging[172].

The relationship between TL and cancer is a complex trait since scientific evidence has associated both short and long telomeres with various cancer risks. A meta-analysis of 62 studies, including 23,379 cases and 68,792 controls, found no overall association between short blood cell TL and the risk of cancer. However, in cancer-specific analyses, short TL was associated with an increased risk of gastrointestinal, head and neck cancers, whereas short TL was associated with a lower risk of lung cancer in prospective studies[173]. In contrast, a more recent meta-analysis of 73 retrospective and prospective articles reported that longer leukocyte TL was associated with an increased risk of melanoma, prostate, and hematological cancers, and with a decreased risk of gastric and esophageal cancers. In this study, no association between TL and the risk of breast cancer was found[174].

Critically short telomeres can result in chromosomal instability, which in turn can lead to extensive genetic rearrangements and mutations. When senescence or apoptosis is bypassed at this point due to checkpoint failure, it could promote oncogenesis by enabling the characteristic accumulation of genetic lesions and alterations. Environmental factors and unhealthy habits can exacerbate cancer progression through oxidative stress, genomic alterations, and accelerated telomere shortening [175].

Telomerase reactivation, which occurs in 85-90% of cancers, can be triggered by this genomic instability caused by critically short telomeres, perpetuating cells with high chromosomal instability and contributing to cancer growth[176]. It has been observed that alterations in the *TERT* gene and its regulation induce telomerase activation, enabling unlimited proliferation capacity and allowing cells to escape from senescence through telomere lengthening[19]. Additionally, some cancers that do not upregulate telomerase undergo the alternative lengthening of telomeres (ALT) pathway, a mechanism that could

also facilitate telomere maintenance in cancer cells[177]. Furthermore, longer TL could lead to larger replicative capacity before senescence, the accumulation of cells with mutations, and the onset of malignant transformation[172]. Consequently, both excessively short and long telomeres can be associated with and could contribute to cancer development[175].

2.3. Infertility

The following systematic review and meta-analysis conducted in the context of this thesis has explored the association between TL and sperm quality or infertility conditions.

Chapter 1

Is telomere length a biomarker of sperm quality? A systematic review and meta-analysis of observational studies.

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Overview of this publication

What is already known?

- Telomere function has been investigated in the context of infertility due to the apparently important role of these structures in fertilization.
- Sperm TL has been associated with several reproductive outcomes in different studies.
- Evidence from the associations between somatic and germ cells TL and sperm quality parameters has increased in the last years.

What does this study add?

- A systematic review and meta-analysis of cross-sectional and case-control studies evaluating the correlations and/or associations between sperm and leukocyte TL with sperm quality parameters.
- Individuals with oligozoospermia (impaired sperm count or concentration) and infertility had shorter leukocyte and sperm TL than normozoospermic or fertile men.
- It is important to evaluate the relationship between TL and sperm quality parameters according to a seminogram abnormality or a fertility diagnosis, separately.

What is the main conclusion?

- Sperm and leukocyte TL has the potential to be used as a biomarker of sperm quality and male infertility.

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ORIGINAL ARTICLE

Is telomere length a biomarker of sperm quality? A systematic review and meta-analysis of observational studies

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Abstract

Background: Telomeres are essential for the integrity of chromosome ends during cell division and their involvement in different processes linked to aging has been established. These chromosome components are involved in spermatogenesis and seem to play an important role in fertilization and embryo development. Telomere length is shortened with each cell division. Recently, short sperm telomere length has been proposed as a potential biomarker of male infertility.

Objectives: To conduct a systematic review and meta-analysis of studies exploring the association between spermatozoa and/or leukocyte telomere length with sperm quality parameters and different infertility conditions.

Material and methods: A systematic review and meta-analysis was conducted with studies from Medline-PUBMED and Cochrane Library databases until May 2022. Eligible studies included cohort, cross-sectional and case–control studies, and telomere length in spermatozoa and/or leukocytes cells was defined as the exposure. Semen quality parameters or infertility conditions (e.g., oligozoospermia, asthenozoospermia, teratozoospermia, or other spermatogenic impairment combinations) were defined as the outcomes.

Results: Twenty-three observational studies were included. In the qualitative analysis, high heterogeneity was observed between studies regarding the associations between telomere length and semen parameters in different normozoospermic/fertile and oligozoospermic/infertile populations. In the meta-analysis, spermatozoa and leukocyte telomere length were shorter in infertile individuals than in fertile individuals (mean difference [95% confidence interval]: −1.43 [−1.66 to −1.21], p-value <0.001 and −1.67 [−2.02 to −1.31], p-value <0.001, respectively). Moreover, in terms of sperm telomere length, these differences were also significant between individuals with a normal seminogram and individuals with a low quantity of spermatozoa in the ejaculate (−0.97 [−1.32, −0.61], p-value <0.001).

Conclusion: The current systematic review and meta-analysis suggests the potential role of spermatozoa or leukocyte telomere length as a reliable biomarker of semen

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quality, which may help distinguish between infertility conditions beyond the routine semen analysis.

KEYWORDS
biomarker, leukocyte, spermatozoa, sperm quality, telomere length

1 | INTRODUCTION

Infertility is a disease of the reproductive system leading to the failure to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse, according to the World Health Organization (WHO).[1] Currently, infertility affects 8%–12% of the world's population and over the last years this condition has increased.[2] Globally, of couples affected by infertility, male factors are responsible for 40%–50% of cases.[3] Research on infertility is therefore important and quality of life and mental health of populations may also benefit.[4]

Oxidation and inflammation processes have a recognized influence on sperm quality parameters and consequently on male fertility because of the susceptibility of spermatozoa to oxidative stress (OS).[5] The pathological characteristics of oxidative molecules, such as reactive oxygen species (ROS), can lead to impairments in the male reproductive system resulting from an imbalance between oxidant production and antioxidant capacity. A comparative study showed that seminal ROS concentrations were higher in infertile men than in healthy sperm donors and in infertile men with all abnormal sperm parameters than in infertile men with normal sperm parameters.[6] Therefore, investigating the biological mechanisms implicated in infertility beyond conventional sperm parameter analysis and identifying reliable biomarkers of infertility is of clinical relevance.

Telomeres are repetitive DNA sequences and specialized proteins at the end of the eukaryote chromosome, whose main function is to maintain genome integrity.[7] With each cell division under normal conditions, a small fragment of telomeric DNA is lost, leading to the activation of a DNA damage response that induces replicative senescence, anticipating the onset of age-related diseases.[8] Telomeric structures are susceptible to oxidation processes because of their high guanine content, thus leading to accelerated telomere shortening.[9] Telomere length (TL) is mainly regulated by a reverse transcriptase called telomerase, which adds 5′-TTAGGG-3′ sequences in tandem[10] and whose activity decreases progressively during embryonic differentiation in somatic tissues. However, in male germ cells, this activity is maintained until spermatogenesis, resulting in longer telomeres in spermatozoa than other cell types.[10]

TL has been consolidated as a hallmark of processes linked to aging such as oxidation, inflammation, epigenetic regulation, or mitochondrial dysfunction, among others.[11] Furthermore, in recent decades, telomere function has been investigated as a potential biomarker of infertility because of its apparently important role in fertilization and embryo development.[12] A recent systematic review and meta-analysis of observational studies evaluating sperm TL as a biomarker of embryonic development revealed that higher sperm TL was associated with a higher probability of pregnancy but not with fertilization rate.[13] However, the study has design limitations in the evaluation of the associations between sperm TL and sperm quality.

Recently, observational studies measuring TL as a marker of infertility in men[14,15] or evaluating the relationship between TL and spermatozoa-related parameters[16,17] have increased. The main aim of the present study is to conduct a systematic review and meta-analysis of: (a) cross-sectional studies exploring the association between spermatozoa and/or leukocyte TL with sperm quality parameters, and (b) case–control studies comparing TL in populations with different semen abnormalities or (in)fertility. Our main hypothesis is that TL is a biomarker of semen quality and male infertility and can complement the analysis of other parameters predicting semen quality.

2 | METHODS

2.1 | Protocol and registration

The protocol of the systematic review and meta-analysis was registered in the international prospective register of systematic reviews PROSPERO (https://www.crd.york.ac.uk/prospero) with the code CRD42021227690.

2.2 | Literature search strategy

A literature search of human studies published in English was carried out in both MEDLINE-PubMed and Cochrane Library databases from the earliest available indexing year until May 2022. In order to obtain a reference list of the articles we performed a systematic search of two subsets of Medical Subject Heading terms and keywords: the first subset comprised telomere-related terms (telomere OR telomere shortening OR telomere homeostasis OR telomerase OR telomere length OR telomerase activity OR telomere maintenance) and the second subset comprised keywords related to seminogram alterations or infertility (spermatozoa OR spermatogenesis OR sperm motility OR sperm count OR sperm maturation OR sperm capacitation OR semen OR semen analysis OR infertility, male OR oligospermia OR aspermia OR asthenozoospermia OR azoospermia OR teratozoospermia OR sperm OR semen quality OR oligozoospermia OR oligoasthenozoospermia OR oligoasthenoteratozoospermia OR male fertility OR sperm dysfunction OR spermatogenesis OR protamine deficiency OR

sperm parameters OR sperm DNA fragmentation OR sperm DNA damage OR varicocoele OR non-obstructive azoospermia OR erectile dysfunction OR sperm DNA extraction OR spermatozoa abnormality OR sperm chromosomal abnormalities). In Supporting Information S1 the complete search strategy is available.

2.3 | Eligibility criteria and study selection

In a preliminary screening, two independent researchers screened titles and abstracts for eligibility (M. F., C. V. H.), and the discrepancies were re-evaluated by two other authors (A. S.-H. and S. C.). Eligible studies included in the systematic review and meta-analysis were those with a cohort design and cross-sectional and case–control studies. Articles defining TL in spermatozoa and/or leukocyte cells as exposure, and semen quality parameters (volume, ejaculate pH, total sperm count or concentration, sperm vitality, sperm motility, sperm morphology) or seminogram alterations (oligospermia, aspermia, asthenozoospermia, azoospermia, teratozoospermia, oligozoospermia, oligoasthenozoospermia, oligoasthenoteratozoospermia, varicocoele, non-obstructive azoospermia) as outcomes were included in this systematic review and meta-analysis when the number of studies was three or more. Studies including data regarding sperm DNA fragmentation, sperm DNA damage or protamine deficiency were included in this systematic review with these endpoints as secondary outcomes. Study exclusion criteria were as follows: ecological, retrospective, methodological or case report studies; review or meta-analysis articles; animal or in vitro studies; studies without describing TL as exposure and without semen quality parameters or fertility outcomes as endpoints. Finally, non-original articles (letters, commentaries, viewpoints, summaries, editorials), abstracts, symposium presentations or invited lectures, guidelines or scientific statements, and special articles were also excluded.

2.4 | Data extraction

A standardized model was used to extract the information from each study: title, type of publication, first author, journal and year of publication, study design and period, sample size and participant's disease status, age, country of origin and city or place of recruitment, endpoint data, statistical analysis performed, and main conclusions.

The main exposure of this study, TL, can be reported in relative (telomere [T] to single-copy gene [S] sequence [T/S] ratio) or absolute (bp) units. TL was mainly reported in relative units, so we contacted the corresponding authors of studies reporting absolute units to transform them into relative units to standardize data.

2.5 | Study quality assessment

The National Heart, Lung, and Blood Quality Assessment Tool for observational cohort and cross-sectional and case–control studies was used to evaluate the risk of bias of the articles included in this systematic review and meta-analysis (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools; accessed on 6 March, 2023). Two independent researchers (M. F., C. V–H.) assessed the risk of bias, and the discrepancies were re-evaluated by two other authors (A. S-H. and S. C.). The studies were categorized as good, fair or poor depending on the overall quality score. Cross-sectional studies with a score between 0 and 5 points and case–control studies with a score between 0 and 4 were considered low-quality and therefore excluded.

2.6 | Statistical and sensitivity analysis

When three or more articles analyzed the same exposure and outcome, the results were meta-analyzed. Meta-analyses were conducted using the *meta* package for R 4.2.2 statistical software and Review Manager 5.4 in accordance with Cochrane guidelines.[18] The mean difference (MD) and 95% confidence interval (CI) were computed from the mean and standard deviation (SD) extracted in each study included. If other data distribution values (e.g., median, standard error of mean, or interquartile range) were presented in the original studies, they were recalculated to mean and SD. Values were obtained by two authors (M. F. and C. V. H.) and checked by another author (A. S–H.). Fixed effect models were used to obtain summary MD and 95% CI of the studies analyzed. The statistical significance level was set at $p < 0.05$ (two-tailed). Chi-square tests and the I^2 index were used to evaluate heterogeneity between studies, and in this case, the significance level was set at $p < 0.1$. I^2 values <50% were considered moderate, ≥50% to <75% were considered substantial, and ≥75% were considered of considerable heterogeneity. To evaluate the robustness of our findings, sensitivity analyses using random effect models were performed.

3 | RESULTS

3.1 | Article selection

Study selection, identification, screening, inclusion and exclusion processes are summarized in Figure 1. Following a primary search, screening of the titles and abstracts of 681 articles led to 37 studies that were eligible for inclusion. At this point, two studies not in English or unavailable for download were excluded. Following full-text screening, 12 studies were excluded for (a) only including processed sperm data (density gradient centrifugation and/or swim-up); (b) being in vitro experiments; (c) including a specific selected population highly exposed to pollutants; or (d) being reviews or hypothesis analysis articles. Finally, 23 cross-sectional or case–control studies were included for analyses.

3.2 | Study characteristics

The characteristics of the cross-sectional and case–control studies included in the analyses are presented in Tables 1 and 2, respectively. The studies were conducted mainly in China ($n = 3$), the UK ($n = 1$), India

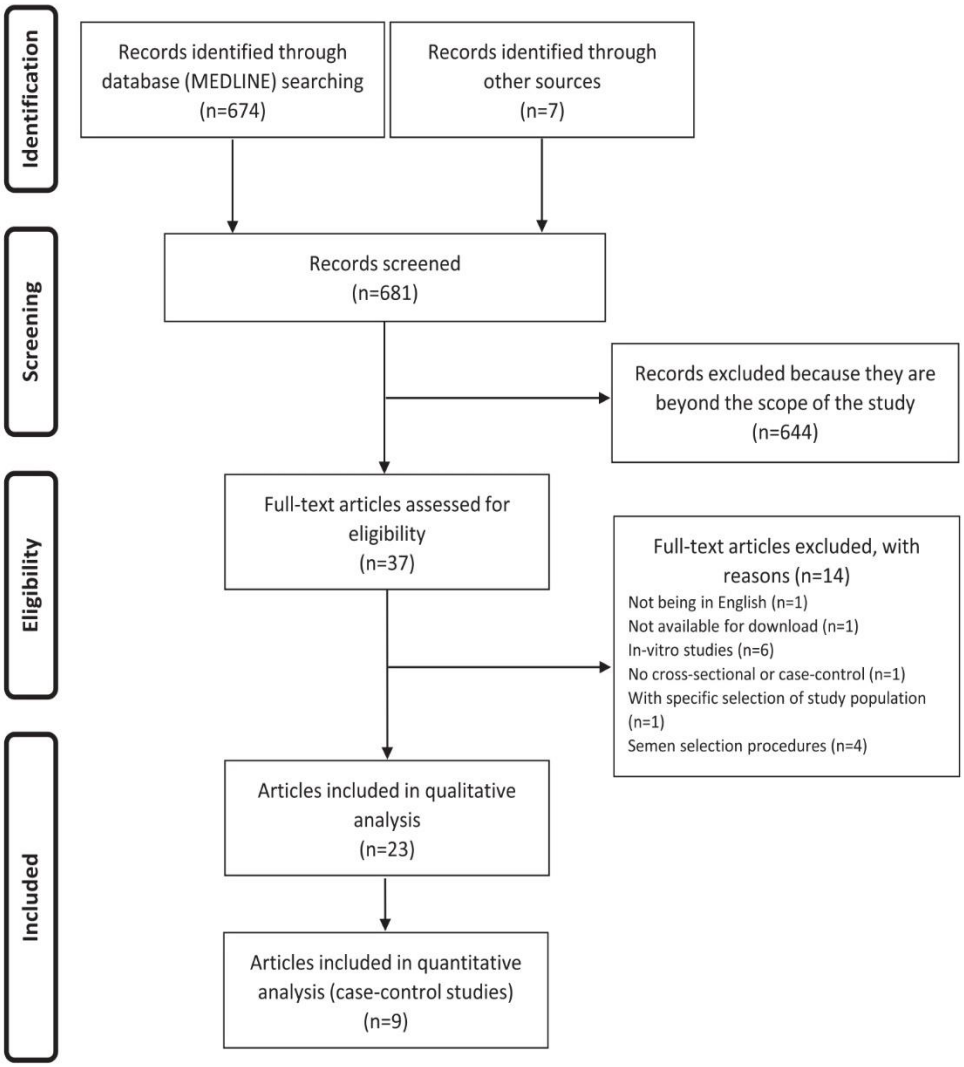

FIGURE 1 Flowchart of the literature search and selection process.

($n = 3$), Iran ($n = 5$), Israel ($n = 1$), Italy ($n = 5$), France ($n = 1$), and Spain ($n = 4$) with sample sizes ranging from 20 to 239 participants in the case of cross-sectional studies, and from 20 to 866 for case–control studies. Fertile and/or infertile populations were included with ages ranging from 18 to 52 years.

3.3 | Qualitative analysis

3.3.1 | Results of cross-sectional studies

A total of 17 cross-sectional studies were selected for the qualitative analysis. Four articles included fertile men only, four included infertile men, and 9 included both.

Fertile or normozoospermic men

The study of the relationship between sperm TL and sperm parameters in fertile or normozoospermic populations revealed contradictory results among the papers. In relation to sperm vitality and motility, Rocca et al.[19] reported positive correlations between sperm TL and vitality and progressive motility in 100 normozoospermic subjects. No associations between sperm TL and motility were reported in the other two studies including semen samples from 65 normozoospermic and from 60 donor participants.[16,20] A positive correlation between sperm TL and sperm concentration was reported by Torra-Massana et al.,[20] but this association was not reported in the other two studies.[16,19] Regarding other spermatozoa-related parameters, no associations were observed between sperm TL and volume, count or morphology in any of the three aforementioned studies. A study by Balmori et al.[21] only observed a positive correlation between sperm TL and sperm count, as well as total progressive motility, in a group of 20 normozoospermic participants under 25 years of age.

Concerning secondary outcomes, a negative correlation between sperm TL and sperm DNA fragmentation, and a positive correlation with normal sperm protamination were reported.[19]

Infertile population

In relation to semen parameters, the study performed by Lafuente et al.[17] showed a positive correlation between sperm TL measured before sperm selection and percentage of immotile spermatozoa in 42 infertile participants. The investigators also reported a negative correlation between sperm TL and progressive motility.[17] However, Zhao

TABLE 1 Summary of the cross-sectional studies investigating the association between sperm telomere length (STL) and/or leukocyte telomere length (LTL) and sperm quality parameters or fertility outcomes.

Reference	Location	Population studied	Age (years)	Cell type	Primary outcomes	Secondary outcomes
Fertile or normozoospermic populations						
Rocca et al.[19]	Italy	100 normozoospermic fertile participants	34.0 (8.6)	STL	Semen parameters (volume, total count, concentration, progressive motility, vitality, and morphology)	Sperm DNA fragmentation and normal protamination
Berneau et al.[16]	UK	66 normozoospermic participants	35.5 (4.5); [25–45]	STL	Semen parameters (volume, total count, concentration, progressive and grade A motility, and immotile spermatozoa)	No
Torra-Massana et al.[20]	Spain	60 donor participants	24.3 (5.0); [18–35]	STL	Sperm parameters (concentration and motility)	No
Balmori et al.[21]	Spain	20 normozoospermic subjects	21.2 (2.4)	STL	Semen parameters (total count and total progressive motility)	No
Infertile populations						
Lafuente et al.[17]	Spain	42 infertile patients	NR	STL	Sperm parameters (concentration, progressive motility, and immotile spermatozoa)	Sperm DNA fragmentation
Thilagavathi et al.[24]	India	25 iRPL subjects	33.2 (5.2)	LTL	Sperm parameters (pH, total count, and motility)	Sperm DNA fragmentation
Zhao et al.[22]	China	150 normozoospermic infertile subjects	31.8 (6.1)	STL	Semen parameters (volume, total count, concentration, progressive motility, and normal morphology)	Sperm DNA fragmentation
Sun et al.[23]	China	105 infertile subjects	31.2 (6.1)	STL	Semen parameters (total count)	No
Fertile and infertile or normozoospermic and oligozoospermic populations						
Ferlin et al.[14]	Italy	61 normozoospermic and 20 oligozoospermic subjects	[18–19]	STL and LTL	Semen parameters (total count)	No
Cariati et al.[15]	Italy	54 normozoospermic and 19 oligozoospermic	Normozoospermic 39.4 (5.5); oligozoospermic 39.3 (5.3); [31–52]	STL	Semen parameters (total count, motility, and normal morphology)	No
Thilagavathi et al.[26]	India	32 idiopathic infertile and 25 fertile participants	NR	STL	Semen parameters (pH, volume, total count, motility, and normal morphology)	Sperm DNA fragmentation
Amirzadegan et al.[25]	Iran	10 fertile and 10 oligozoospermic subjects	Fertile: 35.5 (5.6); oligozoospermic: 40.3 (3.8)	STL and LTL	Semen parameters (total count, concentration, motility, abnormal morphology)	Sperm DNA fragmentation, protamine deficiency

(Continues)

TABLE 1 (Continued)

Reference	Location	Population studied	Age (years)	Cell type	Primary outcomes	Secondary outcomes
Darmishonnejad et al.[27]	Iran	10 fertile and 10 infertile subjects	Fertile: 40.1 (3.1); infertile: 38.1 (4.2)	STL and LTL	Semen parameters (total count, concentration, motility, and abnormal morphology)	No
Darmishonnejad et al.[28]	Iran	19 fertile and 38 infertile subjects	Fertile: 40.5 (3.8); infertile 32.6 (6.6); [20–50]	STL	Semen parameters (concentration, motility, and abnormal morphology)	Sperm DNA fragmentation, protamine deficiency
Mishra et al.[32]	India	102 fertile participants and 112 infertile	Fertile: 32.2 (4.0); infertile:31.7 (4.4); [18–45]	STL	No	No
Tahamtan et al.[30]	Iran	20 fertile and 18 infertile varicocoele patients	Fertile: 41.4 (3.6), varicocoele: 28.5 (5.5)	STL and LTL	Semen parameters (sperm count, concentration, motility, and abnormal sperm morphology)	Sperm DNA fragmentation, protamine deficiency
Gentiluomo et al.[29]	Italy	239 participants	34.8 (7.5)	STL	Semen parameters (total count, concentration, motility, morphology)	No

Note: The studies are ordered as: fertile population, infertile population, and fertile and infertile population. Age is given as mean (SD), or [range] where such data are available.
Abbreviations: iRPL, idiopathic recurrent pregnancy loss; NR, not reported.

et al.[22] found a significant but positive correlation between sperm TL and progressive motility in unprocessed semen samples of 150 normozoospermic infertile patients. Lafuente et al.[17] reported a negative correlation between sperm TL and sperm concentration. Nevertheless, this association was not significant in the case of sperm concentration or morphology in the analysis performed by Zhao et al.[22] A positive correlation between sperm TL and sperm count was reported in two of the four studies that have evaluated this endpoint.[22,23]

On the other hand, Thilagavathi et al.[24] did not report any significant association between leukocyte TL and sperm count or motility in 25 men from couples with a history of idiopathic recurrent pregnancy loss.

Three studies investigated the associations between sperm TL and secondary outcomes in infertile populations. While Zhao et al.[22] reported a negative correlation between sperm TL and sperm DNA fragmentation index, no association between these two parameters was reported by Lafuente et al.[17] Besides, no significant correlations between leukocyte TL and sperm DNA fragmentation index were observed.[24]

Populations including a mix of fertile and infertile or normozoospermic and oligozoospermic subjects

Nine studies have analyzed the association between sperm TL and sperm quality parameters in populations including fertile and infertile or normozoospermic and oligozoospermic subjects. A positive correlation between sperm TL and sperm count was found in 81 subjects (61 normozoospermic and 20 idiopathic oligozoospermic).[14] This was also true in a similar population including 54 normozoospermic and 19 oligozoospermic adults,[15] as well as in the study performed by Amirzadegan et al.,[25] in which 10 fertile and 10 oligozoospermic men were included. On the contrary, in a group of 32 idiopathic infertile men and 25 fertile controls, sperm TL and sperm count were not correlated.[26] No associations were reported between sperm TL and motility or morphology in the aforementioned studies.[15,25,26] Two studies reported a positive correlation between sperm TL and sperm concentration,[25,27] but Darmishonnejad et al.[27] did not observe correlations between sperm TL and sperm count, motility, or abnormal morphology in 10 fertile and 10 infertile subjects. In Darmishonnejad et al.'s study,[28] no associations between relative sperm TL and different semen-related parameters (concentration, motility, and abnormal morphology) in 38 infertile and 19 fertile participants mixed together were found. Besides, Gentiluomo et al.'s study,[29] performed in 239 participants, revealed no associations between sperm TL and semen-related parameters (concentration, total number, motility, and morphology). Only Tahamtan et al.[30] reported a positive correlation between sperm TL and spermatozoa motility in 20 fertile and 18 infertile men with grade II or III varicocoele. In relation to leukocyte TL, positive correlations with concentration and sperm count were also shown in two studies.[25,30] Amirzadegan et al.[25] also reported negative associations between leukocyte TL and abnormal morphology. In contrast, leukocyte TL was not significantly related to sperm count[14] or motility.[25]

TABLE 2 Summary of case–control results investigating the differences on sperm telomere length (STL) and/or leukocyte telomere length (LTL) between normozoospermic (controls) and oligozoospermic (cases) or fertile (controls) and infertile (cases) subjects.

Reference	Location	Population studied	Age (years)	Cell type	Main analyses
Normozoospermic (controls) and oligozoospermic (cases) subjects					
Ferlin et al.[14]	Italy	61 normozoospermic and 20 oligozoospermic subjects	[18–19]	STL and LTL	Differences in STL and LTL between normozoospermic and oligozoospermic men
Cariati et al.[15]	Italy	54 normozoospermic and 19 oligozoospermic subjects	Normozoospermic 39.4 (5.5); oligozoospermic 39.3 (5.3); [31–52]	STL	Differences in STL between normozoospermic and oligozoospermic men
Amirzadegan et al.[25]	Iran	10 fertile and 10 oligozoospermic subjects	Fertile: 35.5 (5.6); oligozoospermic: 40.3 (3.8)	STL and LTL	Differences in STL and LTL between fertile and oligozoospermic men
Balmori et al.[21]	Spain	Younger group: 20 normozoospermic and 17 oligozoospermic Older group: 20 normozoospermic and 20 oligozoospermic	Younger group: 20 normozoospermic, 21.2 (2.4) and 17 oligozoospermic, 21.4 (2.3) Group ≥40 years: 20 normozoospermic, 43.3 (3.4) and 20 oligozoospermic, 43.6 (4.0)	STL	Differences in STL between normozoospermic and oligozoospermic men under 25 years and between normozoospermic and oligozoospermic men over 40 years
Fertile (controls) and infertile (cases) populations					
Mishra et al.[32]	India	102 fertile participants and 112 infertile patients	Fertile: 32.2 (4.0); infertile: 31.7 (4.4); [18–45]	STL	Differences in STL between fertile and infertile men
Darmishonnejad et al.[28]	Iran	19 fertile and 38 infertile subjects	Fertile: 40.5 (3.8); infertile 32.6 (6.6); [20–50]	STL and LTL	Differences in STL between fertile and infertile men
Berby et al.[34]	France	20 control men and 30 infertile patients	Control: 35.1 (5.7); infertile: 35.2 (8.0)	STL	Differences in STL between control and infertile men
Rocca et al.[33]	Italy	30 healthy controls and 35 men undergoing ART	Control: 36.1 (6.8); ART group: 39.6 (5.4)	STL	Differences in STL between control and ART men
Thilagavathi et al.[26]	India	32 idiopathic infertile and 25 fertile men	NR	STL	Differences in STL between fertile and idiopathic infertile men
Darmishonnejad et al.[27]	Iran	10 fertile and 10 infertile patients	Fertile: 40.1 (3.1); infertile: 38.1 (4.2)	STL and LTL	Differences in STL between fertile and infertile men
Biron-Shental et al.[31]	Israel	10 fertile and 16 sub-fertile subjects requiring ICSI	Fertile: 36.5 (7.0); sub-fertile: 37.4 (5.0)	STL	Differences in STL between fertile and sub-fertile subjects
Yang et al.[37]	China	270 normal men, 247 with obstructive azoospermia and 349 with non-obstructive azoospermia patients	Normal: 33.0 (29.0–38.0); OA: 27.0 (25.0–31.0); NOA: 27.0 (25.0–31.0)	LTL	Differences in LTL between normal, OA and NOA men
Thilagavathi et al.[24]	India	20 fertile controls and 25 iRPL patients	Controls: 31.5 (5.3); iRPL subjects: 33.2 (5.2)	LTL	Differences in STL between fertile and iRPL patients
Tahamtan et al.[30]	Iran	20 fertile controls and 18 infertile subjects with grade II or III varicocoele	Fertile: 41.4 (3.6); varicocoele: 28.5 (5.5)	STL and LTL	Differences in STL between fertile and varicocoele patients
Lara-Cerrillo et al.[35]	Spain	12 fertile donors and 20 patients with unilateral or bilateral grade II or higher varicocoele	17–44	STL	Differences in STL between fertile controls, before and after surgery in varicocoele patients
Heidary et al.[36]	Iran	30 fertile participants and 30 idiopathic non-obstructive azoospermic patients	35.4 (4.5)	LTL	Differences in LTL between fertile controls and idiopathic non-obstructive azoospermic men

Note: Age is given as mean (SD), [range], or median (25th–75th percentile) where such data are available.
Abbreviations: ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection; iRPL, idiopathic recurrent pregnancy loss; NOA, non-obstructive azoospermia; NR, not reported; OA, obstructive azoospermia.

With respect to secondary outcomes, one study did not observe a significant relationship between sperm TL and sperm DNA fragmentation.[26] However, in three other articles, negative correlations between sperm TL and DNA fragmentation[25,28,30] were observed. In addition, two studies reported negative correlations between spermatozoa or leukocyte TL and sperm protamination deficiencies.[25,30]

3.3.2 | Results of case–control studies

Sixteen studies evaluated the differences in spermatozoa and leukocyte TL between controls and cases.

Differences in sperm TL between normozoospermic (controls) and oligozoospermic (cases) subjects

Three studies clearly showed that the normozoospermic men had significantly longer telomeres than the oligozoospermic patients.[14,15,25] Similarly, Balmori et al.[21] reported significant differences (in the same direction) in sperm TL between older normozoospermic and older oligozoospermic participants, between younger normozoospermic and older normozoospermic participants and between younger oligozoospermic and older normozoospermic participants.

Differences in sperm TL between fertile (controls) and infertile (cases) subjects

Four studies reported significantly longer sperm TL in fertile men than in infertile men,[26–28,31] even after adjustment for age.[32] Besides, Rocca et al.[33] observed longer sperm TL in 30 controls than in 35 men who underwent their first assisted reproductive procedure. On the contrary, Berby et al.[34] reported no differences between control men and infertile men. While significant differences in sperm TL were observed between 18 infertile men with grade II or III varicocoele and 20 fertile men,[30] no differences were reported by Lara-Cerrillo et al.[35] between 12 fertile donors and 20 patients before microsurgical varicocelectomy (MV) and after MV.

Differences in leukocyte TL between fertile and infertile or normozoospermic and oligozoospermic subjects

Regarding TL in leukocytes, three studies reported lower leukocyte TL in infertile patients than in fertile men.[24,28,36] Similarly, longer leukocyte TL in 10 fertile men than in 10 oligozoospermic patients was reported by Amirzadegan et al.[25] On the other hand, Yang et al.[37] reported shorter leukocyte TL in 349 non-obstructive azoospermia patients than in 247 obstructive azoospermia patients and 270 normospermic men, and results from the Tahamtan et al.[30] study showed significantly lower leukocyte TL in men with infertility resulting from varicocoele than fertile controls. Only two studies found no differences in leukocyte TL between normozoospermic and oligozoospermic men[14] or between fertile and infertile subjects.[27]

3.4 | Quantitative analysis

The relatively high number of case–control studies providing sufficient data and the homogeneity between them led us to conduct three meta-analyses to test the associations between TL in male sexual cells (spermatozoa) and somatic cells (leukocytes) and different case and control populations. No quantitative analysis was carried out with the cross-sectional studies because of the heterogeneity and insufficient data from these.

3.4.1 | Infertile versus fertile populations

Sperm telomere length

Data from five studies were meta-analyzed to test the associations between sperm TL and fertility group. In summary, the infertile group of patients had significantly shorter sperm TL than the fertile group (MD [95% CI]: −1.43 [−1.66, −1.21], p-value < 0.001). However, there was evidence of considerable interstudy heterogeneity (I^2 = 94%, p-value < 0.001) (Figure 2A). The sensitivity analysis was consistent with the primary analysis (Figure S1A).

Leukocyte telomere length

Analyzing data from four different studies testing the associations between leukocyte TL and fertility group showed that the infertile group had significantly shorter leukocyte TL than the fertile group (MD [95% CI]: −1.67 [−2.02, −1.31], p-value < 0.001). This comparison displayed a considerable interstudy heterogeneity (I^2 = 86%, p-value < 0.001) (Figure 2B). The results were consistent with those of the sensitivity analysis (Figure S1A).

3.4.2 | Oligozoospermic versus normozoospermic populations

Sperm telomere length

Data from three studies were meta-analyzed to test associations between sperm TL and the WHO 2010 reference limits in sperm concentration or total sperm count; oligozoospermic versus normozoospermic patients. In summary, the oligozoospermic group of patients had shorter sperm TL than the normozoospermic group (MD [95% CI]: −0.97 [−1.32, −0.61], p-value < 0.001). Interstudy heterogeneity was non-significant in this evaluation (I^2 = 0, p-value = 0.51) (Figure 3). The sensitivity analysis result was identical to the primary analysis (Figure S2).

4 | DISCUSSION

This systematic review and meta-analysis of observational studies provides the most comprehensive and up-to-date analysis of the associations between spermatozoa and leukocyte TL and sperm quality

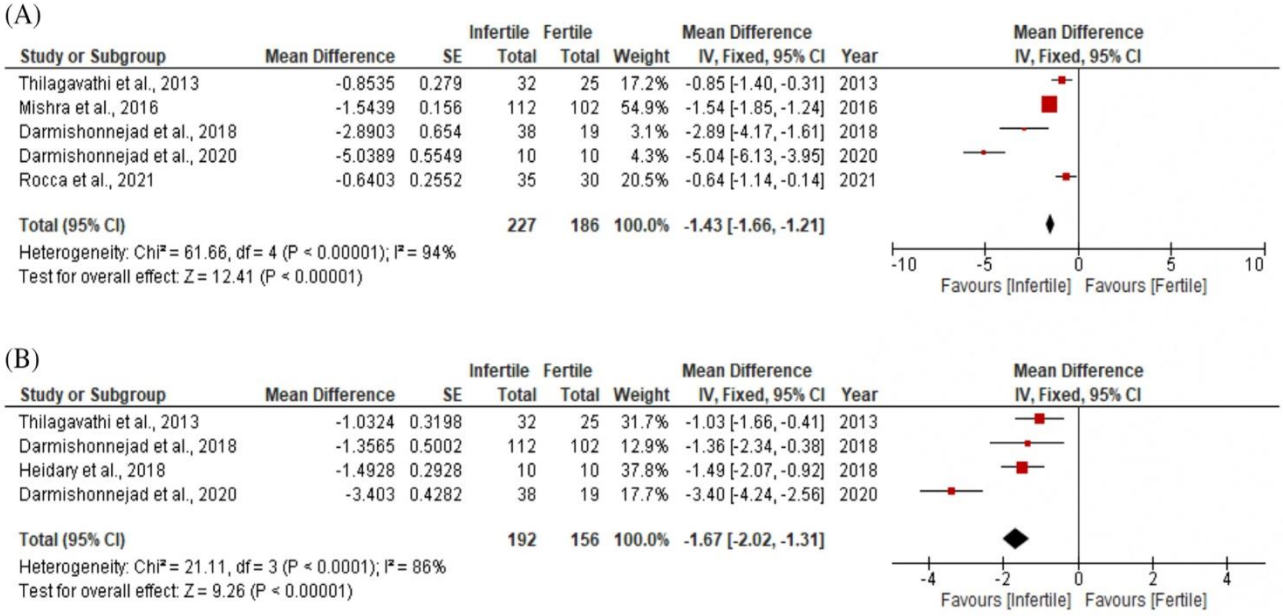

FIGURE 2 Forest plot of mean differences (MD) and 95% confidence intervals (CI) for studies evaluating the association between (A) sperm telomere length and (B) leukocyte telomere length in infertile versus fertile participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represent the pooled MD and 95% CI. Overall estimates were obtained using fixed-effect models. An MD value <0 indicates a negative association between telomere length and fertility group; infertile group had lower telomere length than fertile group.

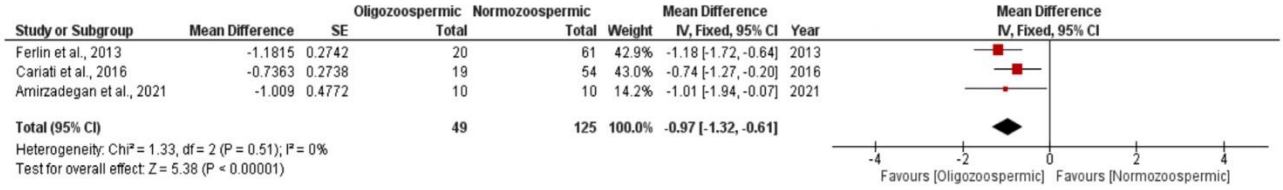

FIGURE 3 Forest plot of mean differences (MD) and 95% confidence intervals (CI) for studies evaluating the association between sperm telomere length in oligozoospermic versus normozoospermic participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represent the pooled MD and 95% CI. Overall estimates were obtained using fixed-effect models. An MD value <0 indicates a negative association between telomere length and normal seminogram result; oligozoospermic group had lower telomere length than normozoospermic group.

parameters and male fertility to date. Our qualitative results showed high heterogeneity in methodology evaluation and contradictory outcomes, making the identification of clear patterns difficult. However, when data were comparable, the quantitative analysis revealed that spermatozoa and leukocyte TL are shorter in infertile than in fertile men. Moreover, these differences, in terms of sperm TL, are also significant between men with a normal seminogram and those with a low quantity of ejaculate spermatozoa. This systematic review and meta-analysis suggests the potential role of spermatozoa and leukocyte TL as a biomarker of semen quality, which may help distinguish between different spermatogenic alterations beyond the routine semen analysis. These results suggest that spermatozoa and leukocyte TL may also be relevant biomarkers to help discriminate fertility potential in cases with sperm quality parameters that are within or close to the threshold values established by the WHO.

To the best of our knowledge, only Yuan et al.[13] performed a systematic review and meta-analysis evaluating the role of sperm TL as biomarker of male infertility and embryonic development. However, only the data from studies that they meta-analyzed were summarized, the qualitative results were omitted, and important limitations to their meta-analysis should be highlighted. For example, in the main analysis, all normozoospermic individuals were considered as fertile population and all oligozoospermic men as the infertile population without considering their fertility status. However, in a secondary analysis, they appropriately compared fertile men versus unexplained infertile men and reported similar results. Although the MD and 95% CI were completely different, they also concluded that sperm TL is shorter in infertile than in fertile men.

In our study, we also compared sperm TL between normozoospermic and oligozoospermic men, and it was shorter in the oligozoospermic population. It is important to mention that in the study performed by Yuan et al., on some occasions, untransformed data to mean and SD were incorrectly included in their meta-analysis. However, in our study we carefully estimated for each meta-analyzed study the mean and SD

values using the original data. Finally, in our study, we analyzed not only studies measuring sperm TL but also those measuring TL in leukocytes, and our results also revealed that infertile men had shorter TL in leukocytes than fertile men.

Although this was not the scope of our study, in the Yuan et al. systematic review and meta-analysis, the capacity of sperm TL to predict the success of pregnancy outcome and embryo development was also explored. A relationship between longer sperm TL and clinical pregnancy was reported, whereas higher fertilization probabilities were not observed in men with higher sperm TL. We recognize that in light of medical and social concerns, identifying biomarkers not only of sperm quality but also of infertility and reproductive outcomes is essential. This will provide valuable insights into possible clinical implications.

Two other studies not included in Yuan et al.'s meta-analysis should also be mentioned. First, in normozoospermic male partners of couples undergoing assisted reproductive technology (ART) treatments, Berneau et al.[16] reported positive significant associations between sperm TL and the percentage of fertilization rate. Second, regarding embryological parameters, sperm TL was positively associated with good embryo quality and transplantable embryo rates in the men of couples undergoing in vitro fertilization (IVF).[38] On the contrary, no associations between sperm TL and embryological parameters (e.g., good embryo cleavage, implantation rates or biochemical, clinical and ongoing pregnancy rates) were found.[16] Likewise, no associations were observed with clinical pregnancy and fertilization rates.[38] These contradictory results might be explained by differences in the study design, population studied, and/or unconsidered factors related to female partners, suggesting that evidence exploring the associations between sperm TL and clinical and embryological outcomes is still needed.

The effect of parental age at conception has been studied because offspring conceived by older parents have been found to have longer telomeres. Kimura et al.[39] confirmed that paternal age was positively associated with leukocyte TL from the offspring in four cohorts, and this may be related to telomere characteristics in paternal spermatozoa. Longer sperm TL was observed in older than younger men. Moreover, when comparing the effect of maternal and paternal age at conception, the major determinant of offspring leukocyte TL was paternal age, with longer leukocyte TL observed in the offspring of older fathers.[40] Ferlin et al.[14] also explored the relationship between maternal and paternal age and offspring sperm TL and reported longer sperm TL in the offspring of older fathers and mothers. However, because there was a high correlation between paternal and maternal ages, which contributed the most could not be determined. The fact that paternal age may act as a determining factor of human telomere dynamics because the inheritance of TL to the offspring demonstrates the important role of these structures in reproduction. Therefore, sperm selection procedures for spermatozoa with longer telomeres in ART practices may play a role in IVF success, but this must be extensively explored. Unfortunately, we could not evaluate this because the studies did not report maternal and paternal age at conception.

Several methods are currently available for TL measurement and quantification, each with its own advantages and limitations. These methods can be broadly categorized into four groups: hybridization, polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), and mixed methods (e.g., hybridization/PCR combination), each providing different information about TL. Terminal restriction fragment analysis, the "gold standard," is based on hybridization techniques, and it is considered more accurate in providing absolute TL measurements. However, it requires large amounts of genomic DNA and laborious processes, making it less practical for large-scale population studies. PCR-based methods have become the most popular method for large-scale studies because of their high-throughput capability and simplicity and because small amounts of DNA are required. These methods determine the ratio between the telomere (T) and single-copy gene (S) signals, providing a proportional measure of relative average TL. Nevertheless, these PCR-based methods often exhibit difficulties in standardization. Methods based on FISH (e.g., quantitative FISH, flow FISH) are also used to determine TL. Because of their low detection limit capacity, these techniques are particularly useful for detecting short telomeres, which are highly indicative of cellular senescence. These procedures can be suitable for large-scale studies to determine TL on fixed lymphocytes but are time consuming and limited to specialized laboratories. Additionally, other techniques, such as single telomere length analysis or telomere shortest length assay, are also used to quantify the shortest telomeres but with low throughput.[41]

This study has several strengths that should be highlighted. This is the first comprehensive systematic review and meta-analysis that has separately meta-analyzed spermatozoa and leukocyte TL differentiating between fertility conditions and seminogram alterations: fertile versus infertile and normozoospermic versus oligozoospermic individuals. Second, in the meta-analysis, we only included studies measuring TL by quantitative PCR as a mode of standardizing the results. Third, in this systematic review and meta-analysis, we did not include studies using sperm selection procedures as we understand that TL measurement would not be representative of the whole sperm population. However, our work also has some limitations that should be considered. Relatively few studies evaluating spermatozoa and leukocyte TL as a biomarker of infertility have been carried out. Besides, we could not meta-analyze cross-sectional data because of the lack of information provided despite trying to obtain this through contact with the corresponding authors. Unfortunately, because of the limited number of published studies, we cannot conduct a sensitivity analysis to consider differences between the populations of the studies included with regard to age, sample size, or other factors that could affect TL. Finally, it is worth mentioning that although positive associations were observed between TL and conventional sperm quality parameters, its translation into better reproductive outcomes (e.g., clinical pregnancy or live birth) remains unanswered and deserves further studies.

In conclusion, our study presents a global review of the evidence available regarding the associations between TL and semen quality parameters and differences in TL between different populations based on their seminogram abnormalities or fertility. Our results suggest that TL has the potential to be used as a biomarker of sperm quality and male infertility. However further and larger studies are warranted in the future to increase the certainty of evidence of a potential relationship between TL, semen quality and fertility. Finally, if TL is a proxy of

sperm quality or fertility potential (or vice versa) should be tested using molecular and in vitro experiments.

AUTHOR CONTRIBUTIONS

Conceptualization: María Fernández de la Puente, Albert Salas-Huetos, Cristina Valle-Hita, Silvia Canudas, and Jordi Salas-Salvadó. *Data curation*: María Fernández de la Puente and Cristina Valle-Hita. *Formal analysis*: María Fernández de la Puente, Albert Salas-Huetos, and Cristina Valle-Hita. *Investigation*: María Fernández de la Puente, Albert Salas-Huetos, and Cristina Valle-Hita. *Methodology*: María Fernández de la Puente, Albert Salas-Huetos, and Cristina Valle-Hita. *Visualization*: María Fernández de la Puente and Albert Salas-Huetos. *Writing original draft*: María Fernández de la Puente. *Writing review and editing*: Albert Salas-Huetos, Cristina Valle-Hita, Nancy Babio, Michelle M. Murphy, Silvia Canudas, and Jordi Salas-Salvadó. *Funding acquisition*: Jordi Salas-Salvadó. *Supervision*: Albert Salas-Huetos, Nancy Babio, Michelle M. Murphy, Silvia Canudas, and Jordi Salas-Salvadó. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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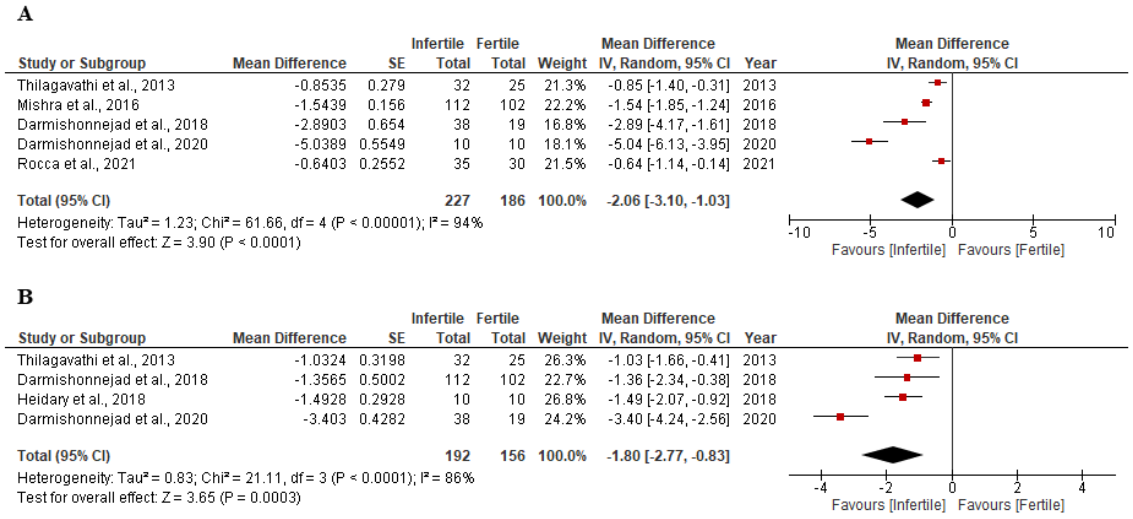
SUPPLEMENTAL INFORMATION

Supplemental Information 1. Complete search strategy for the literature published between the earliest available online indexing year and May 2022 in the MEDLINE-Pubmed database.

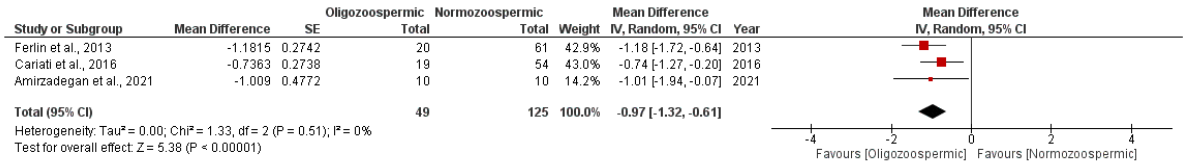
Search terms:

"(""telomere""[MeSH Terms] OR ""telomere shortening""[MeSH Terms] OR ""telomere homeostasis""[MeSH Terms] OR ""telomerase""[MeSH Terms] OR (""telomere""[All Fields] OR ""telomere shortening""[All Fields] OR ""telomere homeostasis""[All Fields] OR ""telomer*""[All Fields] OR ""telomere length""[All Fields] OR ""telomerase""[All Fields] OR ""telomerase activity""[All Fields] OR ""telomere maintenance""[All Fields])) AND (""spermatozoa""[MeSH Terms] OR ""spermatogenesis""[MeSH Terms] OR ""sperm motility""[MeSH Terms] OR ""sperm count""[MeSH Terms] OR ""sperm maturation""[MeSH Terms] OR ""sperm capacitation""[MeSH Terms] OR ""semen""[MeSH Terms] OR ""semen analysis""[MeSH Terms] OR ""infertility, male""[MeSH Terms] OR ""oligospermia""[MeSH Terms] OR ""aspermia""[MeSH Terms] OR ""asthenozoospermia""[MeSH Terms] OR ""azoospermia""[MeSH Terms] OR ""teratozoospermia""[MeSH Terms] OR (""sperm""[All Fields] OR ""sperm*""[All Fields] OR ""sperm motility""[All Fields] OR ""sperm count""[All Fields] OR ""semen""[All Fields] OR ""semen""[All Fields] OR ""semen analysis""[All Fields] OR ""semen quality""[All Fields] OR ""oligospermia""[All Fields] OR ""aspermia""[All Fields] OR ""azoospermia""[All Fields] OR ""asthenozoospermia""[All Fields] OR ""teratozoospermia""[All Fields] OR ""oligozoospermia""[All Fields] OR ""oligoasthenozoospermia""[All Fields] OR ""oligoasthenoteratozoospermia""[All Fields] OR ""male fertility""[All Fields] OR ""sperm dysfunction""[All Fields] OR

""spermatogenesis""[All Fields] OR ""protamine deficiency""[All Fields] OR ""sperm parameters""[All Fields] OR ""sperm dna fragmentation""[All Fields] OR ""sperm dna damage""[All Fields] OR ""varicocele""[All Fields] OR ""non obstructive azoospermia""[All Fields] OR ""erectile dysfunction""[All Fields] OR ""sperm dna extraction""[All Fields] OR ""spermatozoa abnormality""[All Fields] OR ""sperm chromosomal abnormalities""[All Fields]))"



Supplemental Figure 1. Sensitivity analysis. Forest plot of mean differences (MD) and 95% of confidence intervals (CI) for studies evaluating the association between **(A)** Sperm Telomere Length and **(B)** Leukocyte Telomere Length in infertile vs. fertile participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represents the pooled MD and 95% CI. Overall estimates were obtained using random-effect models. A MD value <0 indicates a negative association between telomere length and fertility group; infertile group had lower telomere length than fertile group.



Supplemental Figure 2. Sensitivity analysis. Forest plot of mean differences (MD) and 95% of confidence intervals (CI) for studies evaluating the association between Sperm Telomere Length in oligozoospermic vs. normozoospermic participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represents the pooled MD and 95% CI. Overall estimates were obtained using random-effect models. A MD value <0 indicates a negative association between telomere length and normal seminogram result; oligozoospermic group had lower telomere length than normozoospermic group.

II. JUSTIFICATION

Telomeres are essential for maintaining and preserving genome integrity and stability. Scientific evidence has demonstrated that these structures and their dynamics are influenced by a variety of modifiable and non-modifiable factors, including age and sex, genetics, lifestyle, and environmental exposures. Understanding these influences is crucial, as dysfunctional telomeres could lead to an accelerated onset of different diseases.

In the context of telomere scientific research, among the different modifiable factors that have been explored, diet and physical activity have emerged as key contributors to TL modulation. Healthy dietary patterns have been linked with longer TL. In particular, the MedDiet has been shown to be positively associated with TL. Besides, several studies have reported a beneficial effect of physical activity on telomeres in different populations. Thus, the combined action of these two factors over time could provide an advantage, not only in terms of global health but also in addressing telomere maintenance.

While some trials have focused on the impact of intervention programs on telomere dynamics[100], there is a limited number of studies conducted in older populations with overweight or obesity. Given the potential for obesity to have a significant negative impact on telomeres, it is important to delve into how an intervention aimed at improving lifestyle habits to reduce weight loss in the context of individuals with obesity could influence telomere dynamics. Furthermore, examining telomere length in the context of aging could provide valuable insights into the complex process of aging and related conditions, such as MetS, diabetes, CVD, or infertility.

Therefore, **the first objective** of this doctoral thesis was to provide new evidence on the potential effect of an intensive lifestyle intervention based on an *ad-libitum* MedDiet or an energy-reduced MedDiet (erMedDiet) with physical activity promotion and behavioral support on TL. To conduct this investigation, data from the PREDIMED-Plus study was used, an RCT that included older adults with overweight or obesity, MetS, and at high risk of CVD.

On the other hand, in the last few years, TL has emerged as a candidate to be a biomarker for reproductive health. In this regard, scientific evidence has been gathered to examine the associations between somatic and germ cell TL and reproductive quality, with the aim of gaining insights into fertility. The total fertility rate, defined as the number of births per woman, has drastically decreased worldwide between the 1950s and 2022 and male factors account for nearly half of the cases of couples affected by infertility. In light of

the potential significance of the inheritance component associated with TL, evidence has highlighted the role of telomeres in determining sperm quality beyond the conventional parameters.

However, there is a significant gap in the existing literature regarding the associations between TL and sperm quality parameters in the general population. This is because most of the studies have been conducted using data from individuals attending fertility clinics. Furthermore, the current studies have not accounted for the influence of lifestyle factors that can significantly modulate the relationship between TL and reproductive quality.

In view of the absence of evidence currently available**, the second main aim** of this doctoral thesis was to investigate the associations between sperm and leukocyte TL and sperm quality outcomes in the general population of reproductive age, with consideration given to lifestyle habits.

III. HYPOTHESIS AND OBJECTIVES

Hypothesis 1: A healthy lifestyle could be beneficial in terms of telomere lengthening and reducing oxidative stress.

- **Objective 1:** To evaluate the effect of an intensive lifestyle intervention based on an erMedDiet, physical activity promotion, and behavioral support on TL and 8-OHdG levels after one year of follow-up, compared to an *ad-libitum* MedDiet, in non-diabetic Mediterranean older adults who had overweight or obesity, MetS and were at high risk of CVD.

Hypothesis 2: An intensive lifestyle intervention could increase TL after three years of follow-up in individuals at high risk of CVD.

- **Objective 2:** To investigate whether a lifestyle intervention comprising an erMedDiet together with physical activity promotion and behavioral support might play a role in the prevention of telomere shortening in non-smoking Mediterranean older population with overweight or obesity, MetS and at high cardiovascular risk, compared to participants following a traditional MedDiet without weight-loss encouragement, in the context of a RCT.

Hypothesis 3: Sperm and leukocyte TL might be positively associated with better sperm quality parameters in healthy individuals.

- **Objective 3**: To assess the associations between sperm and leukocyte TL and sperm quality outcomes in 200 healthy volunteers of reproductive age from the general population.

IV. MATERIALS AND METHODS

1. PREDIMED-Plus study

1.1. Study objectives

The PRevención con Dieta MEDiterránea-Plus (PREDIMED-Plus) study, the largest nutritional research challenge in Spain, is an ongoing parallel-group single-blind randomized controlled trial conducted in 23 Spanish recruiting centers. The primary objective of this trial is to evaluate the effect of a long-term intensive lifestyle intervention (intervention group) aimed at weight loss through a caloric reduced MedDiet, promotion of physical activity, and behavioral therapy on the primary prevention of CVD in adults with overweight or obesity. Besides, the second main objective is to evaluate the effect of the intervention on the long-term maintenance of weight loss. This intervention is compared to another (control group) whose participants are advised to follow usual care and received recommendations on an ad libitum MedDiet. This trial was registered at the International Standard Randomized Controlled Trial (ISRCT) with the number 89898870 (https://www.isrctn.com/ISRCTN89898870, registration date: 24 July 2014).

The secondary aim of the PREDIMED-Plus trial comprises the evaluation of the effects of lifestyle interventions on T2D and its complications, cancer incidence, bone fractures, neurodegenerative diseases, unipolar depression, or other endpoints related to obesity or MetS. The trial also considers the effect of the interventions on several intermediate outcomes such as nutrient intake, blood pressure, kidney function, biomarkers such as lipid profile, C-reactive protein, or hemoglobin A1c levels, and cognitive function, quality of life or psychopathological symptoms.

1.2. Study population

Participants in the PREDIMED-Plus study were recruited between October 2013 and December 2016 at primary health centers associated with hospital or university recruiting centers. The inclusion criteria were as follows:

- Women aged 60-75 years and men aged 55-75 years.
- With overweight or obesity, defined as a BMI of 27-40 kg/m^2.
- Without a documented history of CVD at enrollment (except heart failure New York Heart Association class I and II or valvular heart disease).

- And who met at least 3 components of MetS (according to the criteria from the International Diabetes Federation, National Heart, Lung, and Blood Institute and the American Heart Association)[178].

If the following criteria were present, individuals were excluded:

- Illiteracy, inability or unwillingness to provide written informed consent or communicate with study staff.
- Documented history of previous CVD (angina, myocardial infarction, coronary revascularization procedures, stroke, symptomatic peripheral artery disease that required surgery or was diagnosed with vascular imaging techniques, ventricular arrhythmia, uncontrolled atrial fibrillation, congestive heart failure, hypertrophic cardiomyopathy, and history of aortic aneurysm).
- Institutionalization (the participant is a permanent or long-stay resident in a nursing home).
- Active malignant cancer or history of malignancy within the last 5 years (except non-melanoma skin cancer).
- Inability to follow the recommended diet or to perform physical activity.
- A low predicted likelihood to change dietary habits according to the Prochaska and DiClemente Stages of Change Model[179].
- Inclusion in another weight loss program with weight loss objectives (> 5 kg) in the 6 months before the selection visit.
- History of surgical procedures for weight loss or intention to undergo bariatric surgery in the next 12 months.
- History of small or large bowel resection or inflammatory bowel disease.
- Obesity of unknown endocrine origin, except for treated hypothyroidism.
- Food allergy to any component of the MedDiet.
- Immunodeficiency or HIV-positive status.
- Cirrhosis or liver failure.
- Serious psychiatric disorders, including schizophrenia, bipolar disorder, eating disorders, and depression with hospitalization within the last 6 months.
- Any severe co-morbidity condition with less than 24 months' life expectancy.
- Alcohol abuse or addiction, considered as a total daily alcohol intake above 50 g or drug abuse within the past 6 months.
- History of major organ transplantation.
- Concurrent therapy with immunosuppressive drugs or cytotoxic agents.

- Current treatment with systemic corticosteroids.
- Current use of weight loss medication.
- Concurrent participation in another randomized clinical trial.
- Patients with an acute infection or inflammation, as pneumonia, were allowed to participate in the study 3 months after resolution of such condition.
- Any other condition that might interfere with adherence to the study protocol.

Following a 4-week run-in period consisting of three screening visits to evaluate the adherence of potentially eligible participants to the study procedures, candidates were evaluated according to the aforementioned inclusion and exclusion criteria. A total of 6,874 participants were selected and randomized to either the intervention group or the control group. All participants provided written informed consent. The study protocol was approved in accordance with the ethical standards of the Declaration of Helsinki.

1.3. Recruitment and randomization

The participants' recruitment was done by medical doctors from primary care centers associated with the recruiting centers. The demographic data and eligibility criteria of the candidates were collected from the medical records at the primary care centers at a pre-screening evaluation stage before being contacted. Candidates were interviewed by telephone, informed about the objectives and characteristics of the study, and invited to attend a screening visit at the corresponding recruiting center.

At the first screening visit, candidates were given the inclusion and exclusion criteria questionnaire. If they were eligible and willing to participate, they were given the study information sheet and asked to sign a written informed consent. Information on anthropometric measurements and blood pressure was also collected. Dietitians gave instructions and asked participants to complete the 3-day food record questionnaire, a leisure-time physical activity questionnaire, and a self-measurement chart for self-recording their weight, waist circumference, and hip circumference. Candidates were requested to return the completed questionnaires at the third screening visit. After 2 weeks, at the second screening visit, participants were contacted by telephone call to assess their change in anthropometric measurements. At the third screening visit, candidates who had fully attended the previous visits, met the eligibility criteria, correctly completed the administered questionnaires, and correctly self-reported the measurements were selected for the baseline visit.

After the third screening visit, eligible participants were randomly assigned to one of the trial groups in a 1:1 ratio using a centrally-controlled, computer-generated random-number system. Participants were assigned with stratification by center, sex, and age group (<65, 65-70, >70 years). The system then automatically assigns the participants to their respective groups.

Intervention group

The basic cornerstones of the intensive program followed by the intervention group were an erMedDiet, the promotion of physical activity, and behavioral therapy.

During the first six-month period, participants were encouraged to replace one meal with a low-calorie food based on the culinary traditions of the MedDiet. The main goal for this phase was to achieve an average body weight reduction of 8% and a reduction in waist circumference of more than 5%. All participants attended six individual sessions and six group sessions. Dietitians conducted motivational interviewing sessions if the weight loss targets were not met. In the subsequent six months, participants attended one individual and group session each month, with telephone reinforcement calls from the dietitians. After this first year of follow-up and until the sixth year of intervention, participants attended one quarterly individual session, one monthly group session and received two quarterly telephone calls.

In relation to the diet followed by the intervention group, the main aim was to replace food such as sugar-sweetened beverages, fast food, refined grain cereals or sweets with key components of the MedDiet (e.g., virgin olive oil, nuts, fruits, vegetables, whole grains…) in order to reduce the energy intake by 600 kcal/day, in alignment with the basal metabolic rate and physical activity level of the participants. The recommended intakes of total animal fat, protein and carbohydrate were 35-40%, 20% and 40-45% of total calories, respectively. Furthermore, the recommended intake of cholesterol was 300 mg or less per day and 30-35 g of fiber per day.

Participants allocated in the intensive intervention group received counseling to comply with the 17 objectives from the 17-item questionnaire that evaluates the adherence to the erMedDiet[180].

The second component of the intervention program was physical activity. Physical exercise recommendations were based on a gradual increase of exercise level to at least

45 minutes per day (6 days per week) after the first 6-month phase of intervention. The activities included moderate-intensity aerobic exercises (e.g., walking or equivalent) and resistance training. According to personal preferences, dietitians adapted the recommended activities and encouraged participants to perform different exercises with the same metabolic equivalence of tasks. Their progress was monitored.

Thirdly, participants were supported and provided with tools and strategies to help them to avoid consuming high-calorie foods, performing sedentary activities, and losing control of their food intake in stressful or anxious situations. Participants were also instructed to exercise self-control.

Control group

Participants allocated in the usual care group were advised to follow a traditional MedDiet and general lifestyle habits to manage the MetS. At the start of the trial, participants attended a group session and an individual session where dietitians provided them with recipes, menus, and descriptions of the components of the MedDiet, avoiding instructions on calorie restriction, how to lose weight or physical exercise. At the initial visit and each 6-month group session, dietitians provided recommendations on how to follow the MedDiet.

The 14-item adherence questionnaire was the intervention tool for the control group to assess the participants' adherence to the energy un-restricted MedDiet[181], and the 17-item questionnaire was also used to enable comparisons.

1.4. Data collection

Anthropometric measurements

Weight, height, and waist circumference were recorded with the participants wearing light clothing and without shoes or accessories. Waist circumference was measured at the midpoint between the lowest rib and the iliac crest. BMI was calculated by dividing weight (kg) by the square of the height (m). These measurements were taken at baseline, 6 months, and at the annual visits, in duplicate by dietitians.

Blood pressure

This parameter was measured in triplicate by a semiautomatic oscillometer (Omron HEM-705CP, Netherlands) with the participant seated. This measurement was taken at baseline, 6 months, and at the annual visits by trained dietitians.

Blood samples and biochemical determinations

Blood samples were collected at each recruiting center, with the participant in fasting conditions and by the nursing staff. Conventional biochemical determinations were performed according to standard hospital or primary care center procedures, including fasting plasma glucose, HDL, LDL and total cholesterol, and glycated hemoglobin, among others. Serum, EDTA, and citrate plasma samples were processed, isolated, and then stored at -80°C in an ultra-low-temperature freezer at the recruiting centers. Additionally, buffy coat samples were isolated from EDTA plasma tubes after a centrifugation step at 1700g for 15 minutes at room temperature and were also stored at -80ºC in 500 µl aliquots. Fasting blood samples were collected at baseline, 6 months and 12 months, 3 years, 5 years, 7 years, and final follow-up visits.

Oxidative stress measurement

This parameter was only assessed in the samples of participants included in the first chapter of this thesis in which 8-OHdG levels were measured at baseline and after one year of follow-up.

To measure oxidative stress, the levels of 8-OHdG were analyzed using an OxiSelect™ Oxidative DNA damage ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA), as previously used by our group[182]. Through this competitive enzyme immunoassay, a quantitative measure of 8-OHdG from plasma samples was determined by comparing their absorbance with that of a known 8-OHdG standard curve provided with the kit. Briefly:

- The plate was incubated with the 8-OHdG Conjugate over night at 4ºC.
- The excess of coated solution was removed, and the plate was blocked with 200 µL of Assay Diluent for one hour at room temperature.
- A volume of 20 µL of plasma samples was mixed with 180 µL of Assay Diluent and 50 µL of this mixture were added to each well after removing the excess of Assay Diluent. The incubation occurred at room temperature for 10 minutes on an orbital shaker.

- Then, 50 µL of the diluted Anti-8-OHdG Antibody were added to each well and incubated at room temperature for 1 hour on an orbital shaker.
- After washing the plate 3 times with Wash Buffer, 100 µL of the diluted Secondary Antibody-Enzyme Conjugate were added to all wells and incubated at room temperature for 1 hour on an orbital shaker.
- After washing the plate 3 times with Wash Buffer, 100 µL of Substrate Solution were added to each well and incubated at room temperature on an orbital shaker.
- To stop the enzyme reaction 100 µL of Stop Solution were added into each well.

Finally, a conventional ELISA plate reader (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA, USA) was used to monitor the colorimetric substrate at a wavelength of 450 nm. All samples were measured in duplicate and those with a coefficient of variation greater than 2.5% were re-analyzed.

Questionnaires

Diet

A validated 143-item[183] food frequency questionnaire was administered at baseline and at each annual visit to evaluate the diet of each participant. Trained dietitians asked the participants to complete the questionnaire in relation to the last year. The answers, ranging from never or almost never to more than six times per day, were then converted to grams or milliliters per day using the standard portion size of each item. Then, the Spanish food composition tables were used to calculate total energy intake, nutrients, and food groups[184,185]. As previously mentioned, and to assess dietary adherence, a 17-item erMedDiet questionnaire was administered to both groups and a 14-item questionnaire was administered to the control group.

Physical activity

Participants completed the validated Registre Gironí del Cor (REGICOR) Short Physical Activity Questionnaire for the adult population adapted from the Minnesota leisure-time physical activity questionnaire[186]. This questionnaire was used to record the type of activity, frequency (number of days) and duration (minutes per day). The summed product of frequency and duration divided by 30 was used to estimate total physical activity in minutes per day. Then, the intensity of exercise expressed in metabolic equivalent task (MET) was obtained based on the 2015 Compendium of Physical Activity[187] and

categorized in light (< 4.0 MET), moderate (4 ± 5.5 MET), and vigorous (≥ 6.0 MET). Recommendations were made for aerobic physical activity, strength training and activities to improve balance and flexibility, depending on the participants' physical status.

Table 1. General data collection per visit in the PREDIMED-Plus study used for this thesis

	Screening	Baseline	Year 1	Year 3
Eligibility questionnaire	X			
Anthropometric measurements	X	X	X	X
General questionnaire		X	X	X
143-item FFQ		X	X	X
17-item erMedDiet questionnaire		X	X	X
Physical activity questionnaire	X	X	X	X
Blood sample collection		X	X	X

Anthropometric measurements included weight, height, waist and hip circumference.
Abbreviations: FFQ, food frequency questionnaire; erMedDiet, energy-reduced Mediterranean Diet.

1.5. Study population for the present thesis

Two different PREDIMED-Plus sub-populations were selected to study the primary outcome of the present thesis: telomere length.

Chapter 1: Modulation of telomere length by Mediterranean diet, caloric restriction, and exercise: Results from PREDIMED-Plus Study

In this first pilot sub-study, a total of 80 non-diabetic participants from the PREDIMED-Plus Reus center were randomly selected from those randomized to the control group and the intervention group in a 1:1 ratio considering age, sex, and BMI. Due to the lack of baseline or 1-year follow-up DNA samples from 2 participants in the control group and 9 participants in the intervention group, a total of 69 subjects with available DNA were included in the analysis.

Chapter 2: Effect of a 3-year lifestyle intervention on telomere length in participants from PREDIMED-Plus: A randomized trial

From the 1088 eligible participants from the Reus and Navarra centers with available buffy coat samples at baseline and after 3 years of the study, 322 non-smoking participants were randomly selected in a 1:1 ratio according to age, sex, and BMI. After excluding 5

samples with a low TL quality rate, a final sample size of 317 participants was included in the analysis, 159 individuals from the intervention group and 158 individuals from the control group.

2. Led-Fertyl study

2.1. Study objectives

The Led-Fertyl (Lifestyle and Environmental Determinants of Seminogram and Other Male Fertility-Related Parameters) study is an ongoing cross-sectional study being conducted in Reus, the first Spanish recruiting center. Currently, a center in Poland has begun recruitment, while one center in Spain and another in the UK are in the process of completing the documentation required to start recruiting participants.

The primary objective of this study is to identify and quantify dietary and lifestyle determinants associated with sperm quality by analyzing the potential mechanisms implicated such as adiposity, insulin resistance, oxidation and inflammation, DNA damage, and cellular aging that contribute to the risk of male infertility.

The secondary aims of the Led-Fertyl study comprise:

- The evaluation of possible related mechanisms explaining the association between diet and other lifestyle determinants with semen parameters, including cellular aging parameters, DNA methylation, leukocyte and sperm TL, telomerase activity, and sperm PRM1:PRM2 ratio.
- The assessment of possible dietary determinants and diet quality on semen parameters, including semen pH and volume, total sperm count and concentration, total and progressive motility, vitality, and morphology.
- The determination of the exposure of persistent and non-persistent pollutants in the body and quantify or estimate their absorption, distribution, metabolism, and excretion in the body as endocrine disruptors and their impact on the hormonal system.

2.2. Study population

The recruitment of the first 200 Led-Fertyl participants started in February 2021 and finished in April 2023. The inclusion criteria were men aged between 18 and 40 years

from the general population, with good health status, and who have previously signed the informed consent. If the following criteria were present, individuals were excluded:

- Illiteracy, inability or unwillingness to provide written informed consent or communicate with study staff.
- Institutionalization (the participant is a permanent or long-stay resident in a nursing home).
- History of resection of the small or large intestine or inflammatory bowel diseases.
- History of major organ transplantation.
- Some type of disease in the reproductive history or vasectomy.
- Documented history of previous CVD: angina, myocardial infarction, coronary revascularization procedures; cerebrovascular accident (ischemic or hemorrhagic, including transient ischemic attacks); symptomatic peripheral arterial disease that required surgery or was diagnosed with vascular imaging techniques; ventricular arrhythmia; uncontrolled atrial fibrillation; congestive heart failure (New York Heart Association Class III or IV); hypertrophic cardiomyopathy; and history of aortic aneurysm ≥ 5.5 cm in diameter or aortic aneurysm surgery.
- Concurrent therapy with immunosuppressive drugs, with cytotoxic agents or treatment with systemic corticosteroids.
- Use of medications associated with sperm disorders: β calcium channel blockers, alpha-adrenergic blockers, anti-epilepsy, or antiretroviral.
- Excessive weight loss (more than 5 kg in the last month).
- Cirrhosis or liver failure.
- Liver, kidney, thyroid, or other endocrine diseases assessed by medical history, a complete physical examination, and laboratory tests.
- Immunodeficiency or seropositive status or hepatitis B / C.
- Participants with an acute infection or inflammation, such as pneumonia, will be able to participate in the study 3 months after their condition resolution.
- Alcoholism, or drug abuse.
- Active malignant cancer or a history of malignancy within the past 5 years (except non-melanoma skin cancer).
- Severe psychiatric disorders: schizophrenia, bipolar disorder, eating disorders or depression with hospitalization in the last 6 months.

- Any condition of severe comorbidity (situation of suffering from two or more diseases at the same time) with less than 24 months of life expectancy.
- Any other condition that may interfere with compliance with the study protocol.

Volunteers were evaluated according to the aforementioned inclusion and exclusion criteria. A total of 224 participants were selected; however, 24 were excluded due to an absence of relevant information for the study. All participants signed an online and written informed consent. The study protocol was approved by the Institut d'Investigació Sanitària Pere i Virgili Ethics Committee (Reference: CEIM: 181/2019) and the study was conducted according to the ethical standards laid down in the Declaration of Helsinki.

2.3. Study recruitment and design

The participants' recruitment was done by dissemination days at Rovira i Virgili University and in different cities and villages of Catalonia (Spain). Flyers and posters were exhibited in establishments, pharmacies, hospitals, and associated primary healthcare centers, and through social media and newspapers.

Candidates contacted staff by telephone, email, or face-to-face expressing their willingness to participate. A preliminary screening was conducted by telephone to inform potential participants about the study's objectives and characteristics. In this first telephone call, potential participants were asked to provide their age, social security information, and any severe disorder or disease they may have. After this short screening, selected candidates were provided with an online questionnaire containing the study information sheet and the aforementioned inclusion and exclusion criteria. They were then asked to sign an online informed consent. Volunteers who met the inclusion criteria, did not meet the exclusion criteria, and who provided signed consent were included.

Four online questionnaires were sent by email and used to collect information on socio-demographic and lifestyle data, leisure-time physical activity habits, personal history, and erectile function data. After completing these questionnaires, dietitians provided instructions and asked the participants to complete the food frequency questionnaire and the adherence to the MedDiet questionnaire by telephone call. Volunteers who had correctly completed the administered questionnaires attended the only face-to-face visit to measure anthropometric parameters and collect biological samples in Sant Joan de Reus hospital.

2.4. Data collection

Anthropometric measurements

Weight, height, and waist circumference were recorded with the participants wearing light clothing and without shoes or accessories. Waist circumference was measured at the midpoint between the lowest rib and the iliac crest. BMI was calculated by dividing weight (kg) by the square of the height (m).

Blood pressure

This parameter was measured in duplicate with an interval of 5 minutes by a semiautomatic oscillometer (Omron HEM-705CP, Netherlands) and with the participant seated.

Blood samples and biochemical determinations

Blood samples were collected during the face-to-face visit by the nursing staff and with the participant in fasting conditions. Conventional biochemical determinations were performed in accordance with standard hospital procedures. In addition, serum, EDTA, and citrate plasma tubes were centrifuged, isolated, and then stored at -80°C. Leukocyte samples were isolated from of EDTA and citrate plasma tubes via centrifugation at 1700g for 15 minutes at room temperature. The same volume of phosphate-buffered saline (PBS) 1X was added to the resulting samples that were then stored at -80°C in an ultra-low-temperature freezer in 500 µl aliquots.

Semen samples

Semen samples were obtained through masturbation after 3 or more days of sexual abstinence and deposited into sterile standard polypropylene containers. Conventional semen analysis was performed following the WHO (2010) guidelines[188]. After 20-30 minutes of liquefaction at 37ºC, the following semen macroscopic parameters were analyzed:

- Volume: 1.5 ml tubes were filled with 1 ml of semen to calculate the volume.
- pH: indicator strips (Thermo Fisher Scientific, Waltham, MA, USA) from 4.5 to 10 were used.

Then, an Olympus CX43 phase-contrast microscope (Olympus Corporation, Tokyo, Japan) was used in conjunction with the Computer Aided Sperm Analysis (CASA) SCA®

System 6.5.0.67 version (Microptic, Barcelona, Spain) to assess the following microscopic characteristics of spermatozoa:

- Sperm concentration. Millions of spermatozoa per ml were calculated by the software using the 10X phase-contrast objective. Special Leja slides optimized for SCA® were used.
- Sperm total count. Millions of spermatozoa per ejaculate were obtained by multiplying sperm concentration and semen volume.
- Sperm motility. A total of 200 spermatozoa were analyzed using the 10X phase-contrast objective. The CASA SCA® software classifies each cell according to the type of motility in progressive, non-progressive, and no motility. Then, total motility was calculated by adding progressive and non-progressive motilities. The same special Leja slides were used.
- Sperm vitality. A hypo-osmotic swelling (HOS) solution was prepared with 0.735 grams of sodium citrate dihydrate and 1.351 grams of D-fructose dissolved in 100 ml of purified water. A total of 100 µl of semen were added to aliquots containing 1 ml of the HOS solution. After an incubation of 30 minutes at 37ºC, 10 µl of the mixture were transferred to a clean slide and covered with a coverslip to evaluate 200 spermatozoa. Live cells were identified by changes in the cell shape, as indicated by the coiling of the tail. The number of vital and non-vital cells was quantified using the software manual counter using the 60X objective.
- Sperm morphology. Smears with 10 µl of semen were prepared on slides for staining using the Hemacolor protocol (Merck KGaA, Darmstadt, Germany. Spermatozoa were observed with the 60X objective, and 200 cells were classified by the CASA SCA® software as normal or abnormal. Sperm abnormalities can be found in the head, mid-piece, tail or combined abnormalities. Analyzed slides were then stored at 4ºC.

Questionnaires

Online questionnaires

A total of 4 questionnaires were administered by email:

- Socio-demographic questionnaire. Data were collected on socio-demographic information, including age, civil status, level of education, occupation, and monthly income.

- Personal background questionnaire. Information about general reproductive and medical history and personal care was requested. Additionally, volunteers were asked to provide information related to smoking status and frequency of consumption of toxic substances.
- International Index of Erectile Function questionnaire. A validated multidimensional scale to collect this information was used[189]. Information related to frequency, difficulty or satisfaction during sexual activity was asked.
- Physical activity questionnaire. Participants completed the Regicor Short Physical Activity questionnaire[186].

Diet

A validated 143-item food frequency questionnaire was used to evaluate the diet of each participant by trained dietitians over the previous year[183]. Answers were then converted to grams or milliliters per day using the standard portion size of each item. Then, the Spanish food composition tables were used to calculate total energy intake, nutrients, and food groups[184,185]. To assess dietary adherence, a validated 14-item MedDiet questionnaire was used[181].

3. Telomere length determination

3.1. DNA extraction

For genomic DNA extraction, different cell types were used according to the sample availability.

For the second chapter of the present thesis, genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) by PureLinkTM Genomic DNA Mini Kit (Invitrogen, Madrid, Spain), according to the manufacturer's instructions. PBMCs were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation within 6 hours of drawing blood.

Genomic DNA was extracted from the frozen buffy coats for the third chapter. The extraction was performed using the Maxwell RSC Blood DNA kit (Promega, Madison WI, USA) with the Maxwell RSC Instrument, according to the manufacturer's recommendations.

Finally, for the fourth chapter, genomic DNA was isolated from frozen sperm and leukocyte samples DNA using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations.

After the extraction, DNA concentration and purity was verified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and samples were frozen at -20ºC for the following analyses.

3.2. Measurement techniques

For each chapter of the present thesis, a different technique was used to measure the length of telomeres:

Table 2. Telomere length measurement techniques used in each chapter of this thesis.

Chapter	Technique
Second	QuantiGene Plex chemistry on Luminex technology
Third	Monochrome Multiplex real-time Polymerase Chain Reaction
Fourth	Quantitative Polymerase Chain Reaction

3.2.1. Luminex technology

Technique principle

The QuantiGene Plex (QGP) DNA Assay method (Thermo Fisher Scientific, Waltham, MA, USA) is a multiplexed probe-based non-PCR method. For the present thesis, it has been used to measure the abundance of the telomere repeat sequence using the Luminex technology. This technique uses microbeads with custom-designed probes (CP) to capture DNA molecules. Two specific probes are used for each target sequence (**Figure 5**):

- Capture extenders (CE): this probe has a complementary part to the capture probe and a second part complementary to the target DNA sequence.
- Label extenders (LE): this probe has a sequence complementary to the target DNA molecule and another part that is complementary to a pre-amplifier sequence.

The target DNA sequence hybridizes the target-specific sequences of CE, LE, and blocker probes (BL) and, consequently, is immobilized in the capture bead. In our case, we used both CE and LE probes for the telomeric region that were designed to target the "TTAGGG" repeats. The DNA amplification molecules, called pre-amplifiers, partially overlap with the LE and bind with multiple biotinylated amplifiers. Each pre-amplifier

consists of multiple hybridization regions where biotinylated label probes will bind. These biotinylated probes will also bind Streptavidin-conjugated R-Phycoerythrin (SAPE) resulting in fluorescent signals that are read on a Luminex flow cytometer. The signal is reported as median fluorescence intensity (MFI), and it is proportional to the number of target DNA molecules present in the sample[190].

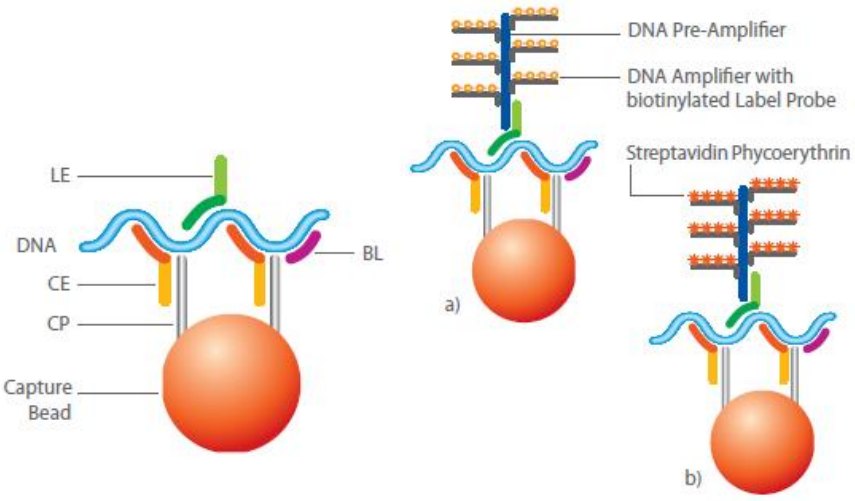


Figure 5. Diagram of the assay components. BL, Blocker probes; CE, Capture Extenders; CP, Capture Probe; LE, Label Extender. Adapted from the QuantiGene Plex DNA Assay User Manual, Affymetrix.

To minimize errors that can lead to sample-to-sample variations that could affect the results, a reference single gene is used as an internal reference to normalize the results. These single genes, called housekeeping genes, are simultaneously amplified with the target and should be expressed at a constant level among tissues and in different experimental conditions[191].

QGP Technique protocol

On a first day, all steps were followed to capture the target DNA:

- For each well, the neutralizing genomic DNA Mix was prepared by mixing 40 µl of sample, diluted to 1.25ng/ µl, or of standard DNA, 18 µl of pre-warmed Lysis Mixture, 5 µl of Probe Set and µl of 2.5 M NaOH solution. After 30 minutes of incubation at room temperature, 12 µl of Neutralization Buffer per well were added.
- To prepare the Working Bead Mix (WBM) 1.8 µl of Nuclease-free water, 15 µl of Lysis Mixture, 2 µl of Blocking reagent, 0.2 µl of Proteinase K, and 1 µl of Capture Beads were mixed per well.

- A total of 20 µl of the WBM and 80 µl of the neutralized DNA were transferred to each well of the 96-well Hybridization Plate and incubated 18-22 hours at 54ºC ± 1ºC at 600 rpm in a VorTemp Shaking Incubator (Thermo Fisher Scientific, Waltham, MA, USA).

On a second day, signal amplification and detection of the target DNA were performed following these steps:

- After removing the Hybridization plate from the shaker and transferring the hybridization mixture to the Magnetic Separation Plate, the unbound sample was washed away, not allowing the plate to dry.
- DNA Pre-Amplifier hybridization. The DNA Pre-Amplifier Working Reagent was prepared by mixing 36 µl of DNA Pre-Amplifier and 12 mL of Amplifier Diluent and a total of 100 µl of it were added to each well. After a 1-minute shaking at 800 rpm at room temperature, the Magnetic Plate was incubated for 1 hour at 50 ºC ± 1ºC at 600 rpm.
- DNA Amplifier hybridization. The DNA Amplifier Working Reagent was prepared by mixing 36 µl of DNA Amplifier and 12 mL of Amplifier Diluent. After washing the plate, a total of 100 µl Amplifier WR were added to each well. After a 1-minute shaking at 800 rpm at room temperature, the Magnetic Plate was incubated for 1 hour at 50 ºC ± 1ºC at 600 rpm.
- Label Probe hybridization. The Label Probe Working Reagent was prepared by mixing 36 µl of Label Probe and 12 mL of Label Probe Diluent. After washing the plate, a total of 100 µl Label Probe WR were added to each well. After a 1-minute shaking at 800 rpm at room temperature, the Magnetic Plate was incubated for 1 hour at 50 ºC ± 1ºC at 600 rpm.
- SAPE binding. The SAPE Working Reagent was prepared by mixing 36 µl of SAPE and 12 mL of SAPE Diluent. After washing the plate, a total of 100 µl SAPE WR were added to each well. After a 1-minute shaking at 800 rpm at room temperature, the Magnetic Plate was incubated for 30 minutes at 600 rpm at room temperature.
- Signal detection. The Magnetic Plate was 3 times washed with 200 µl of SAPE Wash Buffer, and a total of 130 µl of SAPE Wash Buffer were added to each well. After 2-3 minutes of shaking at 800 rpm, the plate was read immediately in a Luminex flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA).

To avoid errors and to normalize data, the following controls were added to each plate:

- Assay background. Two wells contained all the assay components except for the sample. The result from this control is used to determine data that is below the limit of detection.
- Control or reference sample. A control sample of the same DNA was included in duplicate as a standard (300 ng to 0.4 ng in 3-fold dilutions).
- Normalization gene. *ALK* gene was used as the housekeeping gene.

In addition, repeated measures samples were run in the same batch, and subjects with samples having a coefficient of variation higher than 5% were fully reanalyzed.

3.2.2. Quantitative Polymerase Chain Reaction

Technique principle

Polymerase Chain Reaction (PCR) technique was an innovation by Kary Mullis in the 1980s[192]. This technique is based on the action of the thermostable enzyme Thermus aquaticus (Taq) DNA polymerase that, thanks to its 5'-3' polymerase and exonuclease activities, is able to synthesize complementary strands from a target DNA sequence. This sequence acts as the template strand from which copies will be exponentially generated. PCR requires short synthetic oligonucleotides, known as primers, from which the polymerase will add deoxynucleotide triphosphates (dNTPs) and amplify the target sequence. The target DNA sequence is then amplified by multiple cycles of primer-guided DNA synthesis. Each PCR cycle consists of 3 steps: double strand DNA denaturation at 95ºC, annealing of the primers to the target sequence at around 55ºC and extension at 72ºC. These cycles consisting of a series of temperature changes are repeated approximately 30 times[191,193].

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) is a later development of the traditional PCR method. It allows for the amplification and quantification of DNA sequences thanks to the addition of double-stranded DNA-intercalating agents or hydrolysis probes, which ultimately produce fluorescence when excited with an appropriate wavelength[191,193]. In this thesis, the SYBR® Green dye was used. This dye molecule intercalates into the double-stranded DNA product during polymerization, the fluorescence signal increases and is measured

at the end of the extension step of each cycle. The increase in fluorescence is detected and directly related to the generated product that will be quantified[191].

A reference single-copy gene is used as an internal reference to normalize the results. This standardization system allows results to be reported as a ratio by dividing the expression of the gene of interest, in this case, telomeric DNA, by the expression of the single-copy gene (T/S ratio), which must have stable expression.

qPCR technique protocol

A qPCR approach was performed following and adaptation of O'Callaghan protocol[194] using the human 36B4 as single-copy gene. Samples were amplified in two PCRs: the first to determine the cycle threshold (Ct) value for telomere (T) amplification and the second to determine the Ct value for 36B4 single-copy gene using the corresponding HPLC-purified primers (Merck KGaA, Darmstadt, Germany) (**Table 3**).

Table 3. Primer characteristics for telomere length quantification in chapter four.

Name	Size (bp)	Sequence (5' to 3')
PCR primers		
teloF	39	5' CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT 3'
teloR	39	5' GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT 3'
36B4F	23	5' CAGCAAGTGGGAAGGTGTAATCC 3'
36B4R	25	5' CCCATTCTATCATCAACGGGTACAA 3'
Standards		
Telomere	84	5' (TTAGGG)14 3'
36B4	75	5' CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAAGGCCAG GACTCGTTTGTACCCGTTGATGATAGAATGGG 3'

Bp, base pairs. The primers were purchased to Merck KGaA (Darmstadt, Germany).

To complete a final volume of 20 µL per reaction well, a volume of 9 µL of genomic DNA (5 ng/µL), 1 µL of Telo/36B4 primers at a concentration of 2 µM and 10 µL of a master mix from the commercial SYBR® Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) kit were added. This kit contains an optimized buffer including an AmpliTaq® Polymerase engineered with a hot-start mechanism for high specificity and stability; a SYBR® GreenER™ dye to detect double-stranded DNA and yield a bright fluorescence signal; uracil-DNA glycosylase to avoid carryover contamination; a blend of dNTPs; and a ROX™ dye for increase precision and normalize signals. The qPCRs

were performed in 96-well plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

A six-point standard curve was included in each plate using a 10-fold dilution series of known quantities of telomere/36B4 standards for relative quantification. The standard curve linearity agreement was $R^2 > 0.97$. In addition, as assay background, two wells contained all the assay components except for the sample, which was substituted by purified water.

For quality control, each sample was run in triplicate for telomeres and duplicates for 36B4 to evaluate consistency. To account for plate-to-plate variations and to evaluate the variation between triplicates or duplicates, the inter-assay and intra-assay coefficients of variation (CV) were calculated, respectively. In all cases, the intra-assay CV was less than 10% and the inter-assay CV was less than 15%. Samples corresponding to the same participants (leukocyte and sperm) were run on the same plate.

Data collection after amplification was carried out by the CFX Maestro™ Software (Bio-Rad Laboratories, Hercules, CA, USA), which records the increased fluorescence signal of each cycle. The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold line exceeding the background level. It is inversely proportional to the amount of target sample; thus, lower Ct means a greater amount of sample. A calibration curve was obtained using the Ct of the six-point standard curve and the logarithm of the standard concentrations, one for the telomere plate and one for 36B4. Then, the Ct of each sample was extrapolated using the calibration curves, and the relative amount of telomere and 36B4 for each sample was calculated using the antilogarithm. Finally, the relative quantity of telomeres was calculated as telomere to single-copy gene ratio (T/S ratio).

Monochrome Multiplex real-time PCR (MMqPCR)

The Monochrome Multiplex real-time PCR (MMqPCR) is an improved variant of the qPCR that allows to amplify and quantify the product of amplification using multiple fluorogenic probes for the discrimination of multiple PCR products in a single well. This approach reduces variability and sample requirements[195], thereby reducing costs and increasing efficiency. In our study, telomeric DNA and a single-copy gene (albumin) are amplified in the same reaction.

The Ct values for the albumin are collected at a temperature above the melting temperature of the telomere sequence. To raise the melting temperature of the single-copy gene, GC-clamps are placed on the 5' ends of the primers. Accordingly, the two signals are provided at different temperatures[196]. **Figure 6** shows the amplification protocol of the MMqPCR.

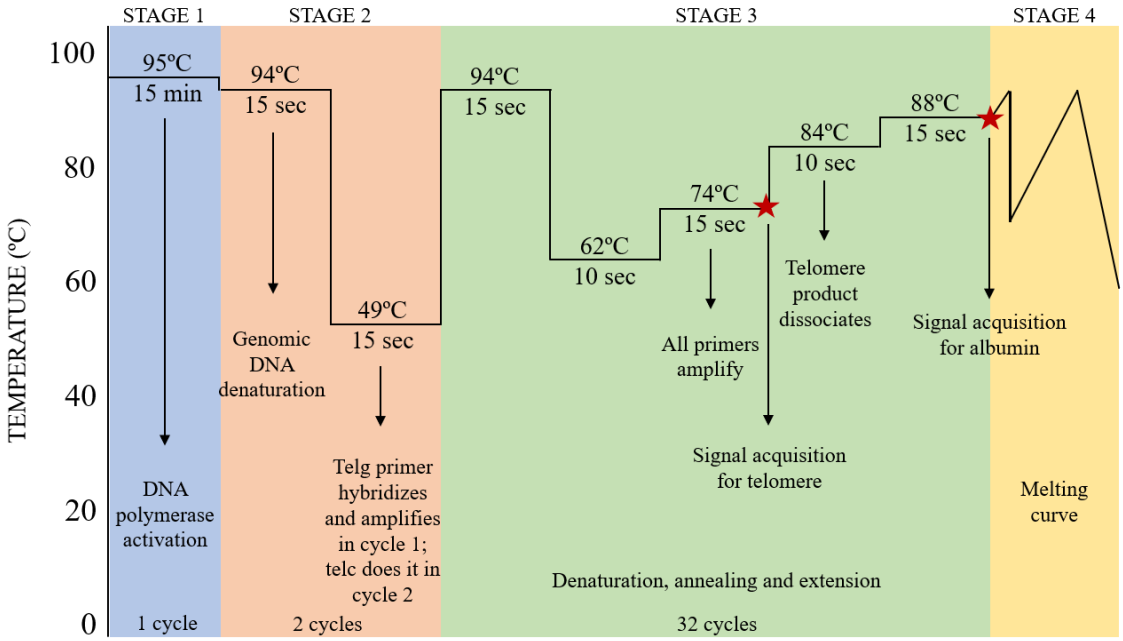


Figure 6. Monochrome Multiplex real-time PCR thermal cycling for telomere length measurement. Source: thesis' author based on Tarik M, Ramakrishnan L, Sachdev HS, Tandon N, Roy A, Bhargava SK, Pandey RM. Validation of quantitative polymerase chain reaction with Southern blot method for telomere length analysis. Future Sci OA. 2018 Jan 18;4(4): FSO282. Doi: 10.4155/fsoa-2017-0115. PMID: 29682317; PMCID: PMC5905642.

MMqPCR technique protocol

TL was measured with a MMqPCR approach following Cawthon's method[196] (2009) using albumin as the single-copy gene. A pair of HPLC-purified primers were used complementary to the telomere sequence and a primers pair for albumin were included to correct for variations in the quantification of telomeric DNA. The primer's sequences are shown in **Table 4**.

Table 4. Primer characteristics for telomere length quantification in chapter three.		
Name	**Size (bp)**	**Sequence (5' to 3')**
Telg	40	5'ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT3'
Telc	42	5' TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA3'
Albu	48	5' CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG3'
Albd	47	5' GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT3'
Bp, base pairs. The primers were purchased by Merck (Darmstadt, Germany).		

In each reaction well, a final volume of 10 µL was added containing 10ng of genomic DNA (2 µL), 0.9 µL of all pairs of primers at a concentration of 900nM, 1.2 µL of ultra-pure water, and 5 µL of a master mix from the commercial QuantiTect SYBR Green PCR kit (QIAGEN, Hilden, Germany). This optimized kit includes a HotStartTaq DNA Polymerase that provides a hot start (15 minutes at 95ºC) for the prevention of nonspecific product formation during the initial phase; a QuantiTect SYBR Green PCR Buffer with balanced combination of NH_4^+ and K^+ ions for promotion of specific primer annealing, high sensitivity, and specificity to ensure reliable results; dNTP mix; SYBER Green I dye, which yields a strong fluorescence signal by binding double-stranded DNA for high sensitive quantification and ROX dye for normalization of fluorescent signals. The MMqPCR was performed using 384-well plates in a CFX384 Touch-Real-time PCR system (Bio-Rad Laboratories, Hercules, California, United States).

Again, a seven-point standard curve made from reference DNA samples was included in each plate using a 2-fold dilution series of DNA, ranging from 150 to 2.34 ng/mL. Standard curves with a linearity agreement $R^2 > 0.99$ were accepted in this case.

For quality control, each sample was run in triplicate and rerun when the variation resulted above 10%. Samples corresponding to different time points (baseline and after 3 years of intervention) from the same participant were run on the same plate. To account for plate-to-plate variations and to evaluate the variation between triplicates, the inter-assay and intra-assay coefficients of variation (CV) were calculated. In all analyses, the intra-assay CV was less than 10% and the inter-assay CV was less than 15%.

The CFX Maestro™ Software (Bio-Rad Laboratories, Hercules, CA, USA) was also used and the same aforementioned process was followed for data collection after amplification and relative quantity of telomeres calculation (T/S ratio). As previously explained, two

standard curves were obtained for each plate, one for telomere and one for albumin signals. The Ct of each sample was extrapolated using the calibration curves and the relative amount of telomere and albumin was calculated using the antilogarithm. Again, the relative quantity of telomeres was calculated as telomere to single-copy gene ratio (T/S ratio).

4. Statistical analyses

The statistical methods performed in each chapter of the present thesis are detailed in the results section. Here a brief explanation is stated.

First chapter

1. PREDIMED-Plus database used: June 2020.
2. Descriptive data were shown as means ± standard deviations (SD) for continuous variables and number and percentage (%) for categorical variables.
3. Differences between groups for baseline and 1-year changes in general characteristics were analyzed through Chi-squared test for categorical variables and one-way Analysis of Variance (ANOVA) for continuous variables.
4. The main outcomes, TL and 8-OHdG plasma levels were log2 transformed for normal distribution.
5. Linear Mixed Models were used to examine the associations of changes in TL and 8-OHdG plasma levels according to the intervention group, time, and its interaction (intervention group x time). We examined potential interactions with age, sex, and BMI by including interaction terms and comparing models using ANOVA.
6. We accounted for intra-cluster correlations, taking into account the members of the same household who were randomized together. Analyses were performed with RStudio (The R Foundation, Vienna, Austria) and statistical significance was set at P value <0.05.

Second chapter

1. PREDIMED-Plus database used: January 2022.
2. Descriptive data were shown as means ± SD for continuous variables and number and percentage (%) for categorical variables.

3. Differences between intervention groups for baseline and 3-year changes in general characteristics were analyzed through Chi-squared test for categorical variables and ANOVA for continuous variables.
4. The primary outcome was TL changes (ΔTL), obtained as 3-year minus baseline values.
5. Analysis of Covariance (ANCOVA) models were run to test the effect of the intervention groups on TL changes. Besides, multivariable-adjusted logistic regression models were performed to estimate the risk for accelerated telomere shortening, defined as ΔTL ≤ percentile 20, after 3 years by intervention group.
6. The likelihood ratio test was used to examine interactions between the intervention group and sex for TL changes after the intervention in both the linear (ANCOVA) and the logistic models.
7. All analyses were performed with the Stata 14.2 software program (StataCorp LP, College Station, TX, USA) and RStudio and statistical significance was set at *P* value <0.05.

Third chapter

1. Led-Fertyl database used: January 2024.
2. The Kolmogorov–Smirnov test was used to assess the normal distribution of the variables.
3. Means ± SD or median [P25 – P75] for continuous variables and number (%) for categorical variables were reported. Differences across tertiles of sperm or leukocyte TL were analyzed by ANOVA or Kruskal–Wallis test according to normal or skewed distributions, respectively. To compare categorical variables across TL tertiles, the Chi-square test was used.
4. Multivariable linear regression models adjusted for several potential confounders were fitted to test the associations between sperm and leukocyte TL and sperm parameters.
5. The Stata 14.2 software program (StataCorp LP, College Station, TX, USA) was used for all analyses, and a *P* value <0.05 was considered statistically significant.

V. RESULTS

Chapter 2

Modulation of Telomere Length by Mediterranean Diet, Caloric Restriction, and Exercise: Results from PREDIMED-Plus Study.

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Overview of this publication

What is already known?

- Telomere attrition has been associated with aging since telomeres shorten with age and are associated with the onset of age-related diseases.
- Several healthy and unhealthy lifestyle factors have been associated with longer and shorter TL, respectively. However, evidence from RCTs in older adults is limited.

What does this study add?

- Either participants following a traditional MedDiet or participants following an intensive lifestyle intervention (i.e., erMedDiet, physical activity promotion, and weight loss advice), both experienced positive changes in TL after one year of intervention, with no differences between groups. Nevertheless, there were no differences between groups in changes in 8-OHdG plasma levels after one year of intervention.

What are the main conclusions?

- The MedDiet could have an important role in preventing telomere shortening after one year of intervention, whereas calorie restriction and exercise promotion did not provide this additional advantage.

antioxidants

MDPI

Article

Modulation of Telomere Length by Mediterranean Diet, Caloric Restriction, and Exercise: Results from PREDIMED-Plus Study

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Abstract: Telomere length (TL) has been associated with aging and is determined by lifestyle. However, the mechanisms by which a dietary pattern such as the Mediterranean diet (MedDiet) affects TL homeostasis are still unknown. Our aim was to analyse the effect of an energy-restricted MedDiet with physical activity promotion (intervention group) versus an unrestricted-caloric MedDiet with no weight-loss advice (control group) on TL and 8-hydroxydeoxyguanosine (8-OHdG) plasma levels. In total, 80 non-diabetic participants with metabolic syndrome were randomly selected from the PREDIMED (PREvención con DIeta MEDiterránea)-Plus-Reus study. TL was measured by a hybridisation method and 8-OHdG levels by ELISA at baseline and after one year of intervention. Linear mixed models (LMM)—raw and after adjusting for potential confounders—were used to examine the associations between TL or 8-OHdG plasma levels by intervention group and/or time. A total of 69 subjects with available DNA samples were included in the analyses. A significant β-coefficient was found for time towards increasing values through the year of follow-up for TL (unadjusted β of 0.740 (95% CI: 0.529 to 0.951), and multivariable model β of 0.700 (95% CI: 0.477 to 0.922)). No significant βs were found, neither for the intervention group nor for the interaction between the intervention group and time. Regarding 8-OHdG plasma levels, no significant βs were found for the intervention group, time, and its interaction. Our results suggest that MedDiet could have an important role in preventing telomere shortening, but calorie restriction and exercise promotion did not provide an additional advantage concerning telomere length after one year of MedDiet intervention.

Keywords: Mediterranean diet; telomere length; 8-hydroxydeoxyguanosine (8-OHdG)

1. Introduction

Aging is defined as an irreversible deterioration based on a physiological integrity decrease affecting most living systems. Several hallmarks are followed by this progressive loss of function such as telomere attrition, genomic damage, epigenetic changes, etc. [1,2]. Telomeres, the ends of our linear genomic DNA, are structures responsible for maintaining the stability of eukaryote chromosomes [3]. In mammals, the 5′-TTAGGG-3′ tandem repeated sequence that defines telomeres is highly conserved [4]. Following cell division, telomeres shorten to a critical level, triggering replicative senescence, which is a key factor

of cellular aging [5]. Telomerase is the enzymatic ribonucleoprotein complex that adds 'TTAGGG' repeats, allowing telomere elongation [6]. The regulation of the telomerase activity has been shown to be essential to maintain a healthy overall status through the regulation of telomere lengthening [7].

In humans, it has been suggested that the rate of telomere shortening is both age- and tissue dependent [8]. Lifestyle factors (e.g., diet, physical activity, alcohol intake, and smoking) have been considered important determinants of telomere length maintenance. Adherence to a healthy lifestyle was positively associated with a lengthening of the leukocyte telomeres [9], whereas an unhealthy lifestyle with a negative impact on oxidative stress, inflammation, blood pressure, and insulin resistance, may have an accelerated telomere shortening effect related to chronic aging diseases [10].

During the last years, different dietary approaches have been suggested to have beneficial effects on telomere homeostasis since nutritional and behavioural strategies have been shown to influence telomere length (reviewed in [11,12]). Mediterranean diet has largely demonstrated cardiovascular benefits (reviewed in [13,14]) but also may have additional beneficial effects on telomere length. In fact, the available evidence derived from a systematic review of cross-sectional and prospective studies suggests that some antioxidant nutrients, the consumption of fruits, vegetables, and seeds (nuts, grains, and coffee), and Mediterranean diet (MedDiet) adherence are mainly associated with longer telomeres, suggesting a protective effect of some plant-based food compounds in the prevention of telomere shortening [15].

In a recent systematic review and meta-analysis of cross-sectional studies, higher MedDiet adherence was associated with longer telomeres [16]. However, no association was found between these two variables in the Helsinki Birth Cohort Study (HBCS), the only 10-year prospective cohort study conducted until now in a large Finnish middle-aged population [17].

To date, only one randomised controlled trial (RCT) has evaluated the effect of the MedDiet on telomere shortening after 5 y of follow-up, showing no effect of the MedDiet supplemented with extra virgin olive oil or nuts on telomere length changes when compared with a control group advised to follow a low-fat diet [18].

In order to delve deeper into the study of MedDiet as a key factor for the prevention of aging processes related to telomere length and/or 8-hydroxydeoxyguanosine (8-OHdG) plasma levels, we designed this study to evaluate the effect of an intensive lifestyle intervention in individuals with cardiometabolic risk, with weight-loss encouragement based on an energy-reduced MedDiet, compared with usual care, and participants were advised to follow a MedDiet without energy restriction on telomere length and 8-OHdG plasma levels in the framework of the PREDIMED (PREvención con DIeta MEDiterránea)-Plus study after one year of follow-up.

2. Materials and Methods

2.1. Study Design

This pilot study was conducted in the context of the PREDIMED-Plus trial, a 6-year parallel-group, multicentre RCT involving 6874 participants recruited in 23 Spanish recruiting centres. The PREDIMED-Plus protocol has been detailed elsewhere [19], and it was registered at the International Standard Randomised Controlled Trial (ISRCT; http://www.isrctn.com/ISRCTN89898870; accessed date: 11 October 2021) with number 89898870 (Registration date: 24 July 2014). However, the current report is aimed to evaluate the effect of the intensive lifestyle intervention on genomic DNA telomere length in comparison with usual care after 1-year follow-up. This analysis represents a nested sub-study performed in the local centre of Reus (PREDIMED-Plus-Reus). The local institutional review board approved the study protocol, and all participants provided written informed consent.

2.2. Study Subjects

In this sub-study conducted with participants of the PREDIMED-Plus Reus centre, a total of 80 non-diabetic participants with metabolic syndrome were randomly selected from those randomised to the control group (n = 40) and the intervention group (n = 40). Due to the lack of baseline or 1-year follow-up samples from 2 participants of the control group and 9 of the intervention group, a total of 69 subjects were included in the analysis. Participants allocated to the intervention group followed an energy-restricted MedDiet (erMedDiet) and physical activity promotion with specific weight loss objectives and individualised behavioural support. Participants in the control group were aimed to maintain an unrestricted caloric MedDiet with no advice on weight loss strategies.

Eligible participants were women aged 60–75 years and men aged 55–75 years with no documented history of cardiovascular disease at enrolment, who were overweight or obese (BMI 27–40 kg/m^2), and who had at least three components of the metabolic syndrome. The main exclusion criteria were (1) active malignant cancer or history of malignancy within the last 5 years; (2) inability to follow the recommended diet or to perform physical activities; (3) history of surgical procedures for weight loss or intention to undergo bariatric surgery; (4) history of bowel resection or inflammatory bowel disease; (5) obesity of unknown endocrine origin; (6) food allergy to any component of the MedDiet; (7) immunodeficiency or HIV-positive status; (8) cirrhosis or liver failure; (9) serious psychiatric disorders; (10) severe co-morbidity condition; (11) alcohol or drug abuse; (12) history of major organ transplantation; (13) type 2 diabetes; (14) therapy with immunosuppressive drugs, cytotoxic agents, treatment with systemic corticosteroids, use of weight loss medication, etc. [20].

2.3. Blood Samples and DNA Extraction

Blood samples were collected after an overnight fast (10 h) at baseline and after a 1-year intervention period. Aliquots were stored at −80 °C, and measurements of the levels of serum total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), and triglycerides were conducted using routine enzymatic methods. Low-density lipoprotein cholesterol (LDL-cholesterol) concentration was calculated by the Friedwald formula. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation within 6 h of drawing blood. Genomic DNA was extracted from PBMCs by PureLink™ Genomic DNA Mini Kit (Invitrogen, Madrid, Spain).

2.4. Telomere Length Determination

Telomere length was measured with the use of a QuantiGene Plex DNA assay method (Termo Fisher Scientific, Madrid, Spain) using custom-designed probes to measure the abundance of the telomere repeat sequence [21]. The fluorescence signal after hybridisation was read on a Luminex flow cytometer (Termo Fisher Scientific, Madrid, Spain). Signal was reported as median fluorescence intensity (MFI), and it is proportional to the number of target DNA molecules present in the sample. ALK was used for reference single gen [21]. The assay was carried out in a 96-well plate with a fixed amount of 50 ng of genomic DNA, and all samples were measured in duplicate, and repeated measures samples were run in the same batch. Subjects with samples having a coefficient of variation higher than 5% were fully reanalysed.

2.5. Measurement of 8-Hydroxydeoxyguanosine Plasma Levels

Levels of 8-OHdG were analysed at baseline and after a 1-year follow-up period in plasma samples. A volume of 20 µL of plasma sample was used for the quantitative measurement of 8-OHdG with the use of an OxiSelect Oxidative DNA damage ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA, EEUU). according to the manufacturer's instructions. An ELISA conventional plate reader (Fluoroskan Ascent; Thermo Fisher Scientific, Madrid, Spain) was used to monitor the colorimetric substrate at a wavelength of 450 nm. All samples were measured in duplicate and those with a coefficient of variation

greater than 2.5% were reanalysed. Samples from the same subject were included in the same plate.

2.6. Assessment of Covariates

Body weight, waist circumference, and height were measured twice at baseline and after one year of follow-up, with the subjects wearing no shoes and light clothes. Body mass index (BMI) was calculated at the beginning and at the end of the 1-year period as weight (kg) divided by the square of height (m^2). Blood pressure was measured using a validated semiautomatic oscillometer (Omron HEM-705CP, Hoofddorp, The Netherlands) in the non-dominant arm and after 5 min of rest in-between measurements. Except for blood pressure determined in triplicate, the remaining anthropometric variables were determined in duplicate, and the mean of these measurements was used. Dietitians used a validated food frequency questionnaire to estimate dietary consumption [22] and a 17-point validated tool [23] to calculate the energy-restricted MedDiet adherence score (erMedDiet score). Leisure-time physical activity was assessed using the validated REGICOR questionnaire [24] (including questions to collect information on the type of activity, frequency (number of days), and duration (min/day)).

2.7. Statistical Analyses

Descriptive data of participants at baseline and differences during the intervention periods are shown as means (SD) for continuous variables, and number (%) for categorical variables. Descriptive analysis was conducted by using the chi-squared test for categorical variables and ANOVA for continuous variables. Telomere length and 8-OHdG plasma levels data were first transformed by applying log2.

We used linear mixed models with a random intercept for each participant and an unstructured correlation matrix to examine the associations of changes in telomere length and 8-OHdG plasma levels according to the intervention group, time, and its interaction (intervention group x time). In all models, we used robust variance estimators to account for intra-cluster correlations, taking into account the members of the same household who were randomised together. We adjusted for the following fixed covariates: sex (men/women), age (continuous), education (primary/secondary/university), and smoking (never/former/current); and time-varying covariates: BMI (continuous), total energy intake (continuous), 17-point erMedDiet score (continuous), physical activity (continuous), and alcohol intake (continuous). We examined potential interactions with age, sex, and BMI by including interaction terms and comparing models using ANOVA. All models are presented as crude (unadjusted) and fully adjusted. All analyses were performed with R version 6.3.0. (The R Foundation, Vienna, Austria) and packages "tableone" [25], "lme4" [26] and "lmerTest" [27]. Statistical significance was set at p value <0.05.

3. Results

3.1. Baseline Characteristics of Control and Intervention Group Participants

This pilot sub-study was conducted in a total of 69 PREDIMED-Plus participants. Baseline participants' characteristics according to the intervention group are presented in Table 1. No significant baseline differences between intervention groups were found for sex, age, weight, BMI, number of metabolic syndrome components, obesity, or prediabetes status at baseline.

3.2. Food Consumption

Baseline and 1-year changes in food consumption are shown in Table 2. Participants in the intervention group experienced a significant decrease in the total energy intake ($p = 0.013$), while those in the control group experienced a non-significant decrease. During the intervention, subjects in the control group increased the intake of monounsaturated and polyunsaturated fatty acids ($p = 0.001$ and $p = 0.02$, respectively) and decreased the intake of saturated fatty acids ($p = 0.013$), whereas participants in the intervention

group increased the levels of monounsaturated and polyunsaturated fatty acids, proteins and fibre ($p \leq 0.001$, $p \leq 0.001$, $p \leq 0.001$ and $p \leq 0.001$, respectively) and reduced the intake of carbohydrates ($p \leq 0.001$). Participants in the intervention group increased the consumption of vegetables, legumes, nuts, and whole-grain cereals ($p = 0.003$, $p \leq 0.001$, and $p \leq 0.001$, $p = 0.001$, respectively) and decreased the consumption of sugary drinks and refined cereals ($p = 0.039$ and $p \leq 0.001$, respectively), while in the control group, participants increased the consumption of virgin olive oil and nuts ($p = 0.03$ and $p \leq 0.001$, respectively). Neither control group nor intervention group significantly changed the consumption of fruits ($p = 0.713$ and $p = 0.768$, respectively).

Table 1. Baseline characteristics of the subjects at randomisation before the start of the study.

Characteristics	Control Group ($n = 38$)	Intervention Group ($n = 31$)	p-Value
Age (years)	64.8 (5.1)	64.3 (5.1)	0.655
Male, *n* (%)	17 (44.7)	12 (38.7)	0.795
Weight (kg)	85.8 (13.1)	88.8 (14.2)	0.360
BMI (kg/m^2)	32.1 (3.4)	33.1 (3.6)	0.254
Waist circumference (cm)	104.1 (10.4)	107.3 (8.4)	0.174
Number of metabolic syndrome components (%)			0.940
≤3 Components	23 (60.5)	18 (58.1)	
4 Components	10 (26.3)	8 (25.8)	
5 Components	5 (13.2)	5 (16.1)	
Obese status			0.333
Overweight (BMI ≥25 kg/m^2) (%)	11 (28.9)	5 (16.1)	
Obesity (BMI ≥30 kg/m^2) (%)	27 (71.1)	26 (83.9)	
Prediabetes * (%)	21 (55.3)	13 (41.9)	0.390
Dyslipidaemia (%)	28 (73.7)	15 (48.4)	0.056
High blood pressure (%)	29 (76.3)	25 (80.6)	0.888
Depression (%)	16 (42.1)	7 (22.6)	0.146
Smokers (%)			0.407
Current	4 (10.5)	4 (12.9)	
Former	17 (44.7)	9 (29.0)	
Never	17 (44.7)	18 (58.1)	
Educational level (%)			0.404
Primary school	24 (63.2)	16 (51.6)	
High school or bachelor	8 (21.1)	12 (38.7)	
University	3 (7.9)	1 (3.2)	
Higher degree	3 (7.9)	2 (6.5)	
Medications use (%)			
Lipid-Lowering drugs			
Statin	18 (47.4)	10 (32.3)	0.408
Other lipid-lowering drugs	2 (5.3)	4 (12.9)	0.387
Hypotensive drugs			
Renin direct inhibitor	2 (5.3)	3 (9.7)	0.813
Angiotensin receptor blocker	8 (21.1)	8 (25.8)	0.660
Angiotensin converting enzyme inhibitor	14 (36.8)	12 (38.7)	0.742
Thiazide drugs ‡	18 (47.4)	13 (41.9)	0.750

Data are shown as means (standard deviation) or number (%); * prediabetes was defined as fasting plasma glucose of 100–125 mg/dL (5.6–6.9 mmol/L) or glycated haemoglobin (HbA1c) of 5.7–6.4% (39–47 mmol/mol). ‡ Thiazide drugs include thiazides and thiazide-like diuretics. p values for differences between groups by ANOVA or chi-squared test, as appropriate. Abbreviations: BMI, body mass index.

Table 2. Baseline and 1-year changes in energy and nutrient intake, total fat, and key food items by treatment groups.

	Control Group (n = 38)		Intervention Group (n = 31)		
	Baseline	**Change**	**Baseline**	**Change**	***p*-Value**
Total energy intake (kcal/day)	2483.43 (602.53)	−72.44 (417.88)	2568.71 (543.51)	−334.94 (449.36) ¥	0.015
Protein (%)	15.54 (2.26)	0.27 (1.61)	15.78 (2.18)	2.14 (2.23) ¥	<0.001
Carbohydrate (%)	40.95 (4.98)	−1.37 (4.09)	41.40 (5.57)	−7.13 (6.12) ¥	<0.001
Fibre (g/d)	25.00 (9.48)	2.60 (7.08)	24.94 (6.45)	6.24 (7.37) ¥	0.041
Total fat (%)	40.02 (3.92)	1.58 (4.22)	39.87 (3.61)	5.16 (5.22) ¥	0.002
Monounsaturated fatty acids (%)	21.22 (3.10)	2.31 (3.44) ¥	21.06 (2.54)	4.82 (3.72) ¥	0.005
Polyunsaturated fatty acids (%)	6.31 (1.19)	0.88 (1.54) ¥	6.64 (1.20)	1.70 (1.38) ¥	0.025
Saturated fatty acids (%)	9.94 (1.58)	−0,85 (7.10) ¥	9.54 (1.55)	−0.31 (1.46)	0.166
Key food items					
Vegetables (g/d)	270.41 (105.07)	35.23 (104.99)	294.36 (86.35)	85.00 (137.87) ¥	0.093
Fruit (g/d)	341.49 (155.03)	11.18 (137.47)	355.50 (146.97)	9.84 (138.12)	0.968
Legumes (g/d)	19.38 (9.81)	0.45 (12.68)	17.65 (9.60)	11.21 (10.62) ¥	<0.001
Cereals (g/d)	160.01 (95.63)	9.03 (85.15)	180.09 (76.76)	−55.45 (84.81) ¥	0.003
Dairy products (g/d)	294.45 (188.68)	−47.26 (140.66)	244.13 (134.57)	6.85 (141.57)	0.118
Meat products (g/d)	154.46 (41.27)	−12.52 (36.33)	166.95 (59.29)	−21.20 (48.53)	0.399
Fish and seafood (g/d)	94.28 (43.08)	1.64 (51.53)	111.76 (44.65)	8.95 (45.28)	0.539
Nuts (g/d)	9.86 (10.91)	13.45 (12.98) ¥	14.06 (10.80)	18.13 (11.66) ¥	0.124
Red wine (ml/d)	59.16 (111.43)	−27.66 (61.46)	51.75 (76.98)	20.98 (91.30)	0.010
Virgin olive oil (g/d)	37.76 (19.48)	9.13 (14.85) ¥	41.98 (16.19)	6.08 (17.81)	0.440
Sugary drinks (ml/d)	31.80 (54.06)	−10.55 (67.35)	22.24 (41.27)	−16.83 (43.45) ¥	0.655
Sugar-free drinks (ml/d)	24.01 (89.13)	2.66 (65.32)	32.26 (179.61)	−30.91 (174.57)	0.277
Refined cereals (g/day)	118.15 (79.61)	−0.73 (63.42)	155.21 (94.56)	−97.18 (103.28) ¥	<0.001
Whole-grain cereals (g/day)	39.84 (93.80)	9.93 (81.45)	20.96 (41.31)	40.96 (47.70) ¥	0.065

Data are shown as means (SD) for continuous variables or number (%) for categorical variables for baseline characteristics. ¥ means statistical significance within a group. p values for differences between groups by ANOVA or chi-squared test, as appropriate.

A higher decrease in the consumption of total energy and carbohydrate (p = 0.015 and $p \leq 0.001$, respectively) and a higher increase in monounsaturated (p = 0.005) and polyunsaturated fatty acids (p = 0.025), protein intake ($p \leq 0.001$), and fibre (p = 0.041) were shown in participants of the intervention group, compared with the control group. No significant differences in changes between groups were found in the case of saturated fatty acids intake. Significant differences in changes between groups were found in the consumption of legumes ($p \leq 0.001$), refined cereals ($p \leq 0.001$),) and red wine (p = 0.01). Compared with participants in the control group, those in the intervention group showed a higher increase in the consumption of legumes. A decrease in the consumption of refined cereals and an increase in red wine intake in the intervention group, compared with the control group, was observed. No significant differences in changes between groups were observed in relation to the consumption of vegetables, fruits, dairy products, meat, sea and seafood, virgin olive oil, nuts, sugar-sweetened or sugar-free drinks, and whole-grain cereals.

3.3. Anthropometric Measurements and Biochemical Parameters

Table 3 shows baseline and 1-year changes in anthropometric measurements, biochemical parameters, telomere length, and 8-OHdG plasma levels. After 1-year of follow-up, a significant reduction in BMI and waist circumference was observed in participants of the intervention group (p = 0.033 and p = 0.001, respectively), while participants in the control group showed no variation in these anthropometric measurements. Subjects in both intervention groups showed a decrease in triglycerides levels; however, this decrease was only significant in the case of the intervention group (p = 0.026). No significant changes in relation to the levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, plasma glucose, HbA1c, or 8-OHdG plasma levels were observed, neither in participants in the control group nor in those in the intervention group. Participants in the intervention group experienced a significant increase in the total leisure-time physical activity from baseline

($p = 0.02$), while those in the control group experienced a non-significant decrease. Subjects in both the control and intervention groups experienced a significant increase in telomere length ($p \leq 0.001$ and $p = 0.001$, respectively).

Table 3. Baseline and 1-year changes in anthropometric measurements, biochemical parameters, telomere length, and 8-OHdG plasma levels by treatment groups.

	Control Group (n = 38)		Intervention Group (n = 31)		
	Baseline	**Change**	**Baseline**	**Change**	***p*-Value**
Anthropometric measurements					
Body weight (kg)	85.76 (13.08)	−0.20 (2.18)	88.79 (14.15)	−5.51 (3.12)	<0.001
BMI (kg/m^2)	32.11 (3.40)	−0.09 (0.81)	33.07 (3.58)	−2.05 (1.14) ¥	<0.001
Waist circumference (cm)	104.14 (10.39)	0.03 (3.24)	107.33 (8.44)	−7.94 (4.69) ¥	<0.001
Biochemical parameters					
Total cholesterol (mM)	217.63 (38.56)	−5.00 (40.81)	202.00 (34.93)	−5.68 (26.19)	0.937
LDL cholesterol (mM)	126.56 (33.16)	0.41 (41.12)	116.23 (20.41)	−1.23 (19.18)	0.853
HDL cholesterol (mmol/L)	51.82 (12.21)	−1.61 (6.10)	46.77 (13.14)	3.32 (5.47)	0.001
Triglycerides (mmol/L)	224.79 (167.78)	−20.76 (105.93)	208.10 (107.73)	−51.55 (76.07) ¥	0.179
Glucose (mmol/L)	101.08 (14.59)	−3.13 (12.96)	97.58 (12.65)	−5.29 (8.47)	0.428
HbA1c (%)	5.86 (0.50)	−0.07 (0.36)	5.73 (0.37)	−0.11 (0.26)	0.586
Leisure-time PA (METs.min/d)	385.02 (383.64)	−17.83 (307.89)	302.65 (262.30)	329.53 (403.68) ¥	<0.001
17-point erMedDiet score	8.00 (2.73)	2.53 (2.78) ¥	6.90 (2.55)	7.45 (2.72) ¥	<0.001
Telomere Length	3.14 (0.51)	0.65 (0.83) ¥	3.11 (0.80)	0.67 (0.80) ¥	0.922
8-OHdG plasma levels	2.84 (0.65)	0.01 (0.56)	2.95 (0.83)	0.12 (0.81)	0.528

Data are shown as means (SD) for continuous variables or number (%) for categorical variables for baseline characteristics. ¥ means statistical significance within a group. p values for differences between groups by ANOVA or chi-squared test, as appropriate. Telomere length and 8-OHdG plasma levels data were transformed applying log2. Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; BMI, body mass index; erMedDiet, energy-restricted Mediterranean Diet; HbA1c, glycated haemoglobin; PA, physical activity.

Significant differences in changes between groups were observed in different parameters. Compared with the control group, a higher decrease in total body weight ($p \leq 0.001$), BMI ($p \leq 0.001$) and waist circumference ($p \leq 0.001$), and a higher increase in HDL-cholesterol ($p = 0.001$) concentrations, and leisure-time physical activity ($p \leq 0.001$) was shown in those participants of the intervention group. No significant differences in changes between groups were found in relation to total and LDL-cholesterol, triglycerides, glucose, HbA1c, telomere length, or 8-OHdG plasma levels. In addition, both groups showed a greater improvement in the 17-point erMedDiet score, which was also significantly different between the two intervention groups.

3.4. Telomere Length and 8-OHdG Plasma Levels

Changes in telomere length and 8-OHdG plasma levels by intervention group, time, and by the interaction between intervention group and time (effect of the intervention) are shown in Table 4. In the case of telomere length, the effect of the intervention was not statistically significant, in both the unadjusted (β coefficient of -0.142 (95% CI: −0.453 to 0.169) and multivariable-adjusted (β of −0.310 (95% CI: −0.650 to 0.025) models. Moreover, no significant β coefficient was found, neither for the intervention group (0.014 (95% CI: −0.206 to 0.234) in the unadjusted model nor the multivariable model (0.070 (95% CI: −0.150 to 0.289). Interestingly, a significant β was found for time towards increasing values through the follow-up. In the multivariable-adjusted model, the β coefficient was 0.700 (95% CI: 0.477 to 0.922). No significant changes in 8-OHdG plasma levels between intervention groups were observed.

Table 4. One-year changes in telomere length and 8-OHdG plasma levels according to the intervention group, time, and its interaction (effect of the intervention).

	β for Intervention Group [1]	β for Time [2]	β for Intervention Group [1] x Time [2]
Telomere length			
Unadjusted model	0.014 (−0.206, 0.234)	0.740 (0.529, 0.951)	−0.142 (−0.453, 0.169)
Multivariable model	0.070 (−0.150, 0.289)	0.700 (0.477, 0.922)	−0.310 (−0.650, 0.025)
8-OHdG plasma levels			
Unadjusted model	0.122 (−0.212, 0.456)	−0.018 (−0.248, 0.213)	0.139 (−0.200, 0.478)
Multivariable model	0.041 (−0.172, 0.254)	−0.054 (−0.270, 0.163)	0.089 (−0.237, 0.415)

We used linear mixed models (with unstructured correlation matrix and robust variance estimators) to conduct these analyses. β coefficients (95% confidence intervals) are reported. [1] Intervention group versus control group; [2] visit at one-year follow-up versus at baseline. We adjusted for the following variables: intervention group, time, sex, age, BMI, education, total energy intake, 17-point erMedDiet score, physical activity, smoking, and alcohol intake. Additionally adjusted by baseline telomere length or 8-OHdG plasma levels values, as appropriate.

4. Discussion

In this PREDIMED-Plus sub-study, we observed greater adherence to the specific recommendations (higher increase in the MedDiet adherence and leisure-time physical activity), and greater metabolic benefits (higher weight loss and increase in HDL concentrations) in participants in the intervention group following an energy-restricted MedDiet and physical activity recommendations, than in those in the control group in which we advised to follow an unrestricted caloric MedDiet with no advice on weight loss. Despite these differences between groups, we found a favourable change in telomere length in both groups during the follow-up without significant differences observed between intervention groups. In addition, no significant changes in oxidative 8-OHdG plasma levels were shown throughout the follow-up in any of the intervention groups.

The lack of differences between intervention groups in relation to telomere length can be explained because both interventions have produced beneficial effects on MedDiet adherence. In fact, not enough significant differences between intervention groups have been established in terms of metabolic benefits, and it has been reflected in the absence of differences in LDL-cholesterol, blood pressure, glucose concentrations, glycated haemoglobin, and 8-OHdG plasma levels between groups. The increase in MedDiet adherence in both intervention groups could be responsible for this favourable change in telomere length within groups observed in our trial, although the mechanisms implicated are largely unknown.

Several studies have shown how healthy dietary patterns may potentially impact telomeres maintenance. Our results are in line with a recent systematic review and meta-analysis of cross-sectional studies pooling data from different cohorts and performed by our group, in which we demonstrated that an increase in MedDiet adherence is associated with longer telomeres [16]. Although in a unique prospective cohort study analysing the association between MedDiet adherence and telomere lengthening, no significant association was found for the entire population including adult men and women, this association was significant in the case of women for whom MedDiet adherence was associated with faster telomere shortening after 10 y of follow-up, even though the effect estimate was small and thus clinically insignificant [17].

The results of our study differ from those reported in the PREDIMED-Navarra study evaluating the effect of a MedDiet intervention supplemented with either virgin olive oil or nuts, compared with a low-fat diet, on the risk of telomere shortening after 5 years of follow-up in a population of high cardiovascular disease [18]. Notably, in the PREDIMED-Navarra, subjects allocated to the MedDiet supplemented with extra virgin oil group showed no beneficial effect in telomere erosion in comparison with the control low-fat

group, whereas a detrimental effect in telomere shortening was observed in the MedDiet supplemented with the nuts group.

These contradictory results can be explained by differences in the population studied, the interventions used, but also because in the PREDIMED-Navarra study, telomere length changes were measured by qPCR and here, by hybridisation using a flow cytometer.

In a recent systematic review, it has been reported that high consumption of healthy foods typical in the traditional MedDiet pattern (vegetables, fruits, nuts, wine, and coffee, foods rich in antioxidants and other phytochemicals) was consistently associated with telomere lengthening, while high consumption of meat and processed meat and sweetened beverages may have the opposite effect [15]. In fact, in our study, we observed a tendency toward an increase in the consumption of different MedDiet foods such as vegetables, fruits, legumes, nuts, virgin olive oil, and whole-grain cereals, and a decrease in the consumption of meat and sweetened beverages in both intervention groups. While these changes were significant in some cases in the intervention group or in the control group, in the case of nuts, this increase was significant in both groups. This increase in nuts consumption can partly explain our results, as these results are in line with our previous short-term RCT showing that the consumption of 57 g/day of pistachios for 4 months had potential effects in the prevention of telomere shortening in prediabetic subjects [28].

The mechanisms by which traditional Mediterranean food may protect from telomere shortening are probably multiple and largely unknown. Some plant-based key foods of the MedDiet are especially rich in antioxidants and anti-inflammatory compounds and thus have been implicated in telomere maintenance by different mechanisms (reviewed by [29]). In fact, the consumption of these plant-based foods has consistently demonstrated beneficial effects on several anti-inflammatory markers and in reducing oxidative stress and cardiovascular risk factors (reviewed by [30]).

García-Calzón et al. demonstrated in children and adolescents that higher dietary total antioxidant capacity of the diet and lower refined white bread consumption (both typical of the MedDiet) were associated with longer telomeres [31]. High adherence to MedDiet was also associated with longer leukocyte telomeres, whereas a negative correlation was found between telomere length and inflammation parameters in elderly subjects from Italy [32]. In the aforementioned RCT published by our group, when participants were following the pistachio-enriched diet, they showed a tendency to decrease levels of DNA oxidation in parallel to a lower decrease in telomere length [28].

In our study, we did not measure peripheral or tissue-specific biochemical markers of inflammation and oxidation in order to understand if these mechanisms are responsible for telomere lengthening observed in both intervention groups. However, we measured 8-OHdG levels in plasma as an indirect measure of oxidative stress in the body. MedDiet has been demonstrated to decrease levels of 8-OHdG in DNA from peripheral blood leukocytes [33]. Although in our study both intervention groups adhered to the MedDiet, we did not observe significant effects of the interventions in this biomarker for either of the intervention groups. However, we cannot discard that the PREDIMED-Plus MedDiet interventions could have had beneficial effects on inflammation and oxidative stress when measured by other biomarkers.

Despite the fact that in the present study, we did not determine hormone or telomerase activity levels, other potential mechanisms by which the MedDiet and its components may have beneficial effects on telomere maintenance could be related to its capacity to regulate the integrity of the hypothalamic–pituitary–adrenal axis and the production of cortisol, leading to a reduction in stress levels (reviewed by [34]) through the downregulation of the influence of cortisol on telomerase activity [35] and regulating DNA methylation changes. However, the interactions between genetic, epigenetic, environmental, and lifestyle factors make the whole framework even more complex, and future studies evaluating these and other mechanisms are needed to support the hypothesis to better understand the effect of MedDiet on telomere maintenance.

This study has some strengths and limitations that should be highlighted. Among the strengths, the absence of previous RCTs evaluating the association between telomere length and MedDiet defines this research as an original and novel study. We used a validated technique for telomere length measurement instead of the conventional single-plex qPCR, which allows higher specificity and precision [21]. Moreover, we employed LMMs that allowed the analysis of repeated measurements while adjusting for several time-varying confounders and for baseline values of telomere length and 8-OHdG plasma levels.

On the other hand, the results should be understood in the context of some limitations. First, this is a pilot sub-study with only 69 subjects, and that may have limited the statistical power. Furthermore, one year of follow-up could be insufficient to evaluate the real effects of the diet on telomere length and 8-OHdG plasma levels. Second, as our study population was composed of individuals with overweight/obesity and metabolic syndrome and did not include subjects with type 2 diabetes, our results may not be applied to other populations such as healthy or to individuals—male and female—with type 2 diabetes. Third, in our study, we did not include the measurement of parameters such as inflammatory-related or telomerase activity to understand better our findings. Fourth, as we did not test a control group without following MedDiet, we could not assure that the results we observed in relation to telomere attrition were only the results of the effect of adhering to MedDiet. Fifth, we measured 8-OHdG levels in plasma as an indirect measure of oxidative damage in the body but not DNA oxidation of cells that may have been a more accurate measure of oxidative stress. Finally, we measured telomere length in PBMCs and not in other cell groups or tissues, although it has been studied that the use of PBMCs for this purpose is an appropriate approach [8].

5. Conclusions

In conclusion, our results suggest that MedDiet, an antioxidant-rich dietary pattern, could have an important role in preventing telomere shortening after 1 year of intervention, whereas calorie restriction and exercise promotion do not provide this additional advantage. However, further studies should evaluate the effect of MedDiet on oxidative stress and telomere length with a larger sample size and longer follow-up to further support the research benefits of healthy dietary patterns for promoting health and longevity.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Comitè d'Ètica d'Investigació Clínica Hospital Universitari Sant Joan de reus (protocol code 13-07-25/7proj2; date of approval: 25 July 2013).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data are not publicly available outside of the core PREDIMED-Plus research group as neither participants' consent forms nor ethics approval included permission for open access. However, the researchers will follow a controlled data-sharing collaboration model since in the informed consent, participants agreed with a controlled collaboration with other investigators for research related to the project's aims. Therefore, investigators who are interested in this study can contact the PREDIMED Steering Committee by sending a request letter to jordi.salas@urv.cat. A data-sharing agreement indicating the characteristics of the collaboration and data management will be completed for the proposals that are approved by the Steering Committee.

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Chapter 3

Effect of a 3-year lifestyle intervention on telomere length in participants from PREDIMED-Plus: A randomized trial

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Overview of this publication

What is already known?

- Short TL has been associated with a higher risk of chronic diseases and disease-related mortality.
- Identifying lifestyle factors that could reduce accelerated telomere shortening is important for disease prevention.

What does this study add?

- After 3 years of follow-up, women in the intervention group experienced a positive change in TL compared to women in the control group. Women in the intervention group had 83% lower odds of telomere shortening than women in the control group.
- No differences were found between groups for men, nor for the whole cohort.

What are the main conclusions?

- An intensive lifestyle intervention based on a MedDiet, physical activity, and weight loss encouragement increased telomere length in older women with high cardiometabolic risk but not in men. Women following this intensive intervention also had a lower risk of telomere shortening.

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Original article

Effect of a 3-year lifestyle intervention on telomere length in participants from PREDIMED-Plus: A randomized trial

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SUMMARY

Background & aims: Short telomeres have been observed in chronic disease patients. Identifying environmental and lifestyle factors that could reduce telomere attrition is crucial for disease prevention. The aim of this work was to determine whether weight-loss induced by an energy-reduced Mediterranean diet (erMedDiet) and physical activity (PA) could modify telomere length (TL).

Methods: In 317 randomized non-smoker participants (mean age, 65.8 ± 4.98 years) with metabolic syndrome from two "Prevención con Dieta Mediterránea-Plus" (PREDIMED-Plus) trial centers, we evaluated MedDiet adherence, PA, anthropometric variables and TL at baseline and after a 3-year intervention using an intensive lifestyle program (IG) with an erMedDiet and PA or an unrestricted MedDiet without PA promotion (CG).

Results: Participants in the IG displayed greater 3-year weight reductions (−3.7 ± 4 kg, $P < 0.001$) compared to those in the CG. No differences in TL changes between groups were observed in the cohort as a whole. However, an interaction was observed between the intervention group and sex for TL changes ($p_{interaction} = 0.039$). Women in the IG showed an increase in TL after 3-y (+0.25 ± 0.9, relative units) compared to women in the CG (−0.07 ± 1.0) ($p_{ANCOVA} = 0.036$), whereas no differences between groups were observed in men. Women in the IG had a lower risk of telomere shortening after the intervention (OR = 0.17, 95%CI: 0.05–0.64, p = 0.008) compared to women in the CG.

Conclusions: A 3-year lifestyle intervention based on an erMedDiet and PA slowed telomere shortening in women but not in men.

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Abbreviations

CG	Control group
ErMedDiet	Energy-reduced Mediterranean Diet
IG	Intervention group
MedDiet	Mediterranean Diet
MMqPCR	Monochrome multiplex real-time quantitative PCR metho
PA	Physical activity
PREDIMED	Prevención con Dieta Mediterránea
RCT	Randomized controlled trial
ROS	Reactive oxygen species
SD	Standard deviations
SEM	Standard error of the mean
TL	Telomere length

1. Introduction

Telomeres are the structures responsible for maintaining genomic integrity, and changes in telomere length are linked to several aging processes [1]. Reduced telomere length (TL) has been observed in chronic disease patients and has been associated with an increased risk of disease-related mortality [2]. Therefore, identifying environmental and lifestyle factors that could reduce telomere attrition is crucial for disease prevention. In this regard, a systematic review and meta-analysis of cross-sectional studies has reported an association between Mediterranean Diet (MedDiet) adherence and longer telomeres [3]. Another recent meta-analysis evaluating the effect of lifestyle interventions on TL showed beneficial effects on telomere attrition after promoting physical activity (PA) and dietary counseling [4].

Therefore, the aim of the present research was to determine whether a lifestyle intervention consisting of an energy-reduced MedDiet (erMedDiet) together with PA promotion might play a role on the prevention of telomere shortening in older subjects with metabolic syndrome, compared to participants following an unrestricted caloric MedDiet without weight-loss encouragement.

2. Material and methods

2.1. Study design

The PREDIMED-Plus study is an ongoing 6-year parallel-group, multicenter randomized trial (RCT) involving 6874 participants recruited in 23 Spanish centers, aimed at assessing the effect of a lifestyle intervention on the primary prevention of cardiovascular disease. Eligible participants were women and men of 55–75 years old with no documented history of cardiovascular disease at enrolment, with overweight/obesity, and who had ≥3 components of metabolic syndrome. Participants were randomly assigned (1:1) to an intensive weight-loss lifestyle intervention with an erMedDiet and PA promotion or usual-care advice to follow an energy-unrestricted MedDiet (control). The PREDIMED-Plus protocol (Supporting information 1) has been detailed elsewhere [5,6] and was registered at (http://www.isrctn.com/ISRCTN89898870).

The current report aimed to evaluate the effects of the PREDIMED-Plus interventions on TL after a 3-year follow-up in participants from 2 of the 23 PREDIMED-Plus centers. This analysis represents a nested sub-study performed in the Reus and Pamplona centers including 317 non-smokers randomly selected with available DNA and information at baseline and after the 3-year intervention. The participant flowchart is shown in Fig. 1. The local institutional review board approved the study protocol. All participants provided written informed consent.

2.2. Telomere length determination

TL was analyzed in participants from the Reus and Pamplona centers based on our previous expertise in TL assessment in human samples [3,7–9]. These two centers were the only ones who offered to participate in this substudy. TL was measured in a subsample of 317 individuals at baseline and after 3 years of follow-up, due to limited resources.

Genomic DNA was isolated from frozen buffy coats using the Maxwell RSC Blood DNA kit (Promega, Madison WI, USA) with the Maxwell RSC Instrument. TL was measured by a monochrome multiplex real-time quantitative PCR method (MMqPCR) based on the Cawthon's method as previously described [7]. In a single reaction, the quantification of the relative copy numbers of telomeres (T) and a single copy gene (albumin; S) was performed in triplicates. TL is expressed as a ratio of these two parameters (T/S ratio).

2.3. Statistical analysis

A P-value <0.05 was considered statistically significant. R-Studio was used for all analyses. Means and standard deviations (SD) or percentages are shown for the description of baseline characteristics according to the intervention group. Analyses stratified by sex were performed as a pre-stated analysis, and therefore no other subgroup analyses were conducted. The primary outcome was TL change (3-year minus baseline values). First, to examine interactions between the intervention group and sex for TL changes, likelihood ratio tests were performed. Then, ANCOVA models were run to test the effect of the intervention groups on TL changes in women and men separately. Moreover, to estimate the risk for accelerated telomere shortening ($\Delta TL \leq$ percentile 20; $\Delta TL \leq$ p20) during the 3-year follow-up by intervention group, multivariable-adjusted logistic regression models were performed in analyses stratified by sex. All analyses were adjusted for potential confounders (see figure footnotes). The likelihood ratio test was used to examine interactions between the intervention group and sex for TL changes after the intervention in both the linear (ANCOVA) and the logistic models.

3. Results

A total of 317 participants (mean age, 65.8 ± 4.98 years; 47% women) with metabolic syndrome and a high prevalence of cardiovascular risk factors (62% obesity, 84% hypertension, 63% hypercholesterolemia, 28% diabetes) were included. No significant

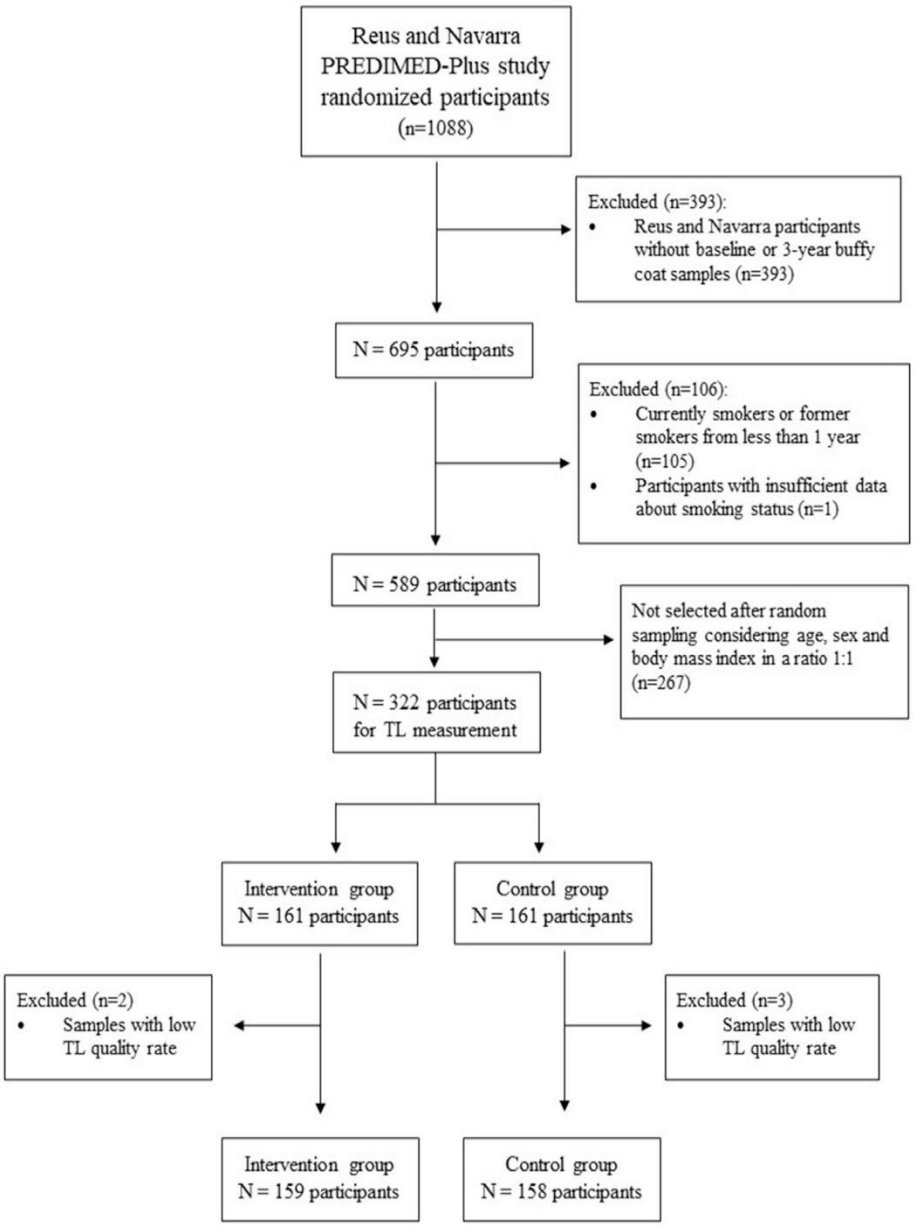

Fig. 1. Flowchart of participants included in the present analysis from the PREDIMED-Plus Study.

differences in the general characteristics between intervention groups at baseline were found, except for insulin treatment (Table 1 and Table S1). As expected, participants in the intensive lifestyle intervention (IG) increased their MedDiet adherence ($p < 0.001$) and PA ($p = 0.004$), achieving greater weight reductions (-3.7 kg $\pm$ 4.0; $p < 0.001$) compared to the control group (CG) participants after the 3-year intervention (Table S2). No differences were found between groups regarding therapy response after the intervention except from metformin, where an increase in the prevalence in the use of this drug was found in the CG compared to the IG (Table S3). No associations between changes in telomere length and changes in cardiovascular risk factors were found (Table S4).

No significant between-group differences in TL changes were found in the cohort as a whole. However, an interaction was observed between the intervention group and sex on TL changes ($p_{interaction} = 0.039$) (Fig. 2). Notably, after the 3-year intervention, a significant TL increase ($+0.25 \pm 0.9$, relative units) was observed in women in the IG *versus* the CG (-0.07 ± 1.0) in fully-adjusted models ($p = 0.036$). No between-group differences in TL were found in men (Fig. 2). Likewise, women in the IG showed an 83% lower risk (OR = 0.17, 95%CI: 0.05 to 0.64; $p = 0.008$) of having a greater telomere shortening rate than women in the CG (Table 2).

4. Discussion

In this PREDIMED-Plus sub-study we found a significant TL increase in women in the IG compared to those in the CG, after 3 years of intervention. Furthermore, women in the IG showed an 83% lower risk of telomere shortening compared to those in the CG. No between-group differences in TL were observed either in men or in the cohort as a whole.

Different RCTs have analyzed the effect of lifestyle interventions on TL with contradictory results. In the Finnish Diabetes Prevention study, no differences were found in the yearly TL change rate between the usual-care group and the IG, following a healthy diet and exercise [10]. Similarly, in a pilot PREDIMED-Plus sub-study

Table 1
Baseline characteristics of the subjects at randomization before the start of the study.

Characteristics	Control group (n = 158)	Intervention group (n = 159)	p-value
Age (years)	65.2 (4.9)	65.6 (5.1)	0.939
Women, n (%)	71 (44.9)	77 (48.4)	0.533
Weight (kg)	85.8 (12.3)	85.9 (14)	0.902
Waist circumference (cm)	106.5 (9.7)	105.9 (9.1)	0.624
BMI (kg/m^2)	32 (3.5)	32.2 (3.8)	0.633
Number of MetS components, n (%)			0.491
3	78 (49.4)	89 (56)	
4	56 (35.4)	48 (30.2)	
5	24 (15.2)	22 (13.8)	
Obesity (BMI≥30 kg/m^2), n (%)	98 (62)	99 (62.3)	0.965
Diabetes[b], n (%)	47 (29.8)	43 (27)	0.594
Cholesterol[a], n (%)	105 (66.5)	96 (60.4)	0.528
Hypertension, n (%)	129 (81.7)	137 (86.2)	0.274
Depression, n (%)	30 (19)	35 (22)	0.505
Smokers, n (%)			0.956
Former	77 (48.7)	77 (48.43)	
Never	81 (51.3)	82 (51.6)	
Educational level, n (%)			0.143
Higher degree or similar	12 (7.6)	13 (8.2)	
University school technician	23 (14.6)	11 (6.9)	
Secondary school	47 (29.8)	58 (36.5)	
Primary school	76 (48.1)	77 (48.4)	
Medication use, n (%)			
Lipid-Lowering drugs			
Statin	79 (50)	70 (44)	0.287
Other lipid-lowering drugs	9 (5.7)	10 (6.3)	0.824
Hypotensive drugs			
Renin direct inhibitor	0	1 (0.6)	0.918
Angiotensin receptor blocker	37 (23.4)	46 (28.9)	0.264
Angiotensin converting enzyme inhibitor	72 (45.6)	57 (35.9)	0.078
Thiazide drugs[c]	56 (35.4)	53 (33.3)	0.693
Antidiabetic drugs			
Insulin treatment	13 (8.2)	3 (1.9)	0.01
Metformin	35 (22.2)	35 (22)	0.976
Telomere length (T/S ratio)	1.10 (0.4)	1.17 (0.5)	0.177

Data are shown as means (SD) for continuous variables or number (%) for categorical variables.
P values for differences between groups by ANOVA or chi-squared test, as appropriate.
Abbreviations: BMI, body mass index; MetS, Metabolic syndrome.
[a] There were missing data for cholesterol in 2 participants (0.6%), one from each group.
[b] Diabetes was defined as previous diagnosis of diabetes or HbA1c ≥ 6.5% (48 mmol/mol), use of antidiabetic medication or having fasting glucose >126 mg/dL (7.0 mmol/L) in the screening visit plus fasting glucose >126 mg/dL (7.0 mmol/L) at baseline visit.
[c] Thiazide drugs include thiazides and thiazide-like diuretics.

performed with 69 non-diabetic participants, no differences in TL were found between intervention groups after 1 year of follow-up, whereas a significant 1-year TL increase in both intervention groups was observed [8]. After 5-year MedDiet interventions supplemented with virgin olive oil or nuts no beneficial effect on TL attrition compared to a low-fat diet was shown in 521 participants from the PREDIMED-Navarra study [9]. In that study, in women but not in men, cross–sectional associations between greater MedDiet adherence and longer telomeres were reported, showing that women had a lower risk of having short telomeres [9]. Finally, two RCTs aiming to evaluate the effect of exercise interventions on TL found a lengthening in the IG participants compared to the controls after 24 and 26-week interventions [11,12].

Studies performed only in women evaluating the effect of a lifestyle intervention have also reported inconsistent conclusions. In the Lifestyle Exercise And Nutrition study including breast cancer survivors, participants in the IG experienced a positive change in TL after 6 months, compared to the telomere shortening in the CG [13]. In contrast, 12-month TL changes were not different between control and intervention groups including different dietary and exercise approaches [14]. Another two RCTs in women found no differences between controls and exercise groups in TL after 6-month and 1-year interventions [15,16].

One possibility is that the discrepancies observed between these studies might be explained by differences in study design regarding the populations studied (men and women or only women, i.e. breast cancer survivors); type and length of the interventions (studies focused on the evaluation of changes in diet and/or physical exercise; the duration ranging from 6 months to 5 years); cell/tissue type and method used to measure TL determination. Further research is warranted to resolve these discrepancies, but this goes beyond the scope of our study.

Differences in TL between women and men have been previously reported [17,18]. A systematic review and meta-analysis of observational studies reported longer telomeres in women than in men [17]. Telomeres have a high sensitivity to oxidation processes [19], and levels of reactive oxygen species (ROS) in women are lower than in men to some extent because of the regulatory action of estrogen in the premenopausal stage [20,21], which might remain even during the postmenopausal period. On the other hand, the mechanisms underlying the observed response to the intervention could be partially explained by the influence of oxidative stress on telomere dynamics. Thus, the dietary pattern followed by the IG, based on the traditional MedDiet, has been proposed as an effective strategy to prevent telomere shortening due to its antioxidant ability [3]. Why this is only observed in women and not in

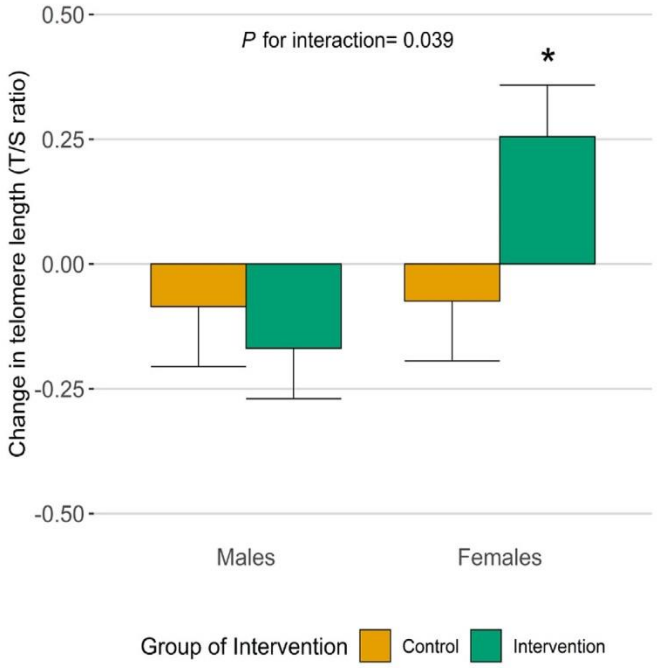

Fig. 2. Changes in Telomere length after 3 years of the PREDIMED-PLUS trial interventions in women and men separately. Interaction between the group of intervention and sex in determining 3-year changes in telomere length (*P* for interaction = 0.039). Mean and SEM changes in telomere length after 3 years of follow-up in women and men by intervention group. *$P = 0.036$ between intervention groups for women. All analyses are adjusted for the following confounding factors: age, BMI (in kg/m^2), baseline levels of TL (T/S ratio, relative units), physical activity (metabolic equivalent tasks in min/week), total energy intake (kcal/day), smoking status (never, former), adherence to MedDiet (17 point-score), diabetes status (yes/no), hypertensive status (yes/no), dyslipidemia status (yes/no). The likelihood-ratio test compares the goodness-of-fit of the following two models: 1) TL changes ~ group of intervention + sex + confounding factors and 2) TL changes ~ group of intervention + sex + confounding factors + sex * group of intervention. n = 315 (two individuals had missing data for dyslipidemia status).

men remains unknown. One explanation might be due to the age-dependent TL attrition observed mainly in men [18], which leave telomeres in women to be more prone to be modified by environmental factors. Furthermore, we cannot discard other sex-related potential mechanisms involved, as those attributed to lifestyle and behavioral factors differing in men and women [21]. More studies are needed to further clarify the mechanisms behind this sex-effect on telomere changes.

The metabolic and molecular basis of action underlying the benefits of MedDiet on TL may be explained by the recognized potential antioxidant and anti-inflammatory properties associated to the combined consumption of high amounts of vegetables, fruits, nuts and seeds, legumes and virgin olive oil [9,22,23]. In fact, systematic reviews of cohort studies and RCTs concluded that a high adherence to the MedDiet dietary pattern is associated with telomere lengthening [3], whereas a high consumption of red meat and processed meat and sweetened beverages may have the opposite effect [24]. Energy restriction may also partially explain the beneficial effects on TL observed in the intervention group of our study, as it has been shown that energy restriction was associated with telomere lengthening [25] and improved health and lifespan in animal models and humans (reviewed in [26]).

It is worth mentioning that, in a meta-analysis, it has been demonstrated that patients with diabetes had shorter TL compared to healthy individuals [27]. Notably, elevated glucose and oxidative stress levels could interfere with telomerase activity, resulting in telomere shortening [28]. However, in our study, the prevalence of diabetes, baseline glycated hemoglobin levels (as a marker of glucose dysregulation) and changes in glycated hemoglobin levels did not differ between the intervention groups. Nevertheless, models have been adjusted by diabetes status to account for potential confounding.

Among the strengths of our sub-study, the PREDIMED-plus is a RCT which allowed us to explore long-term changes in TL. The technique used to measure TL (MMqPCR) quantifies telomere length and the single copy gene in one well, in a single reaction, thus reducing variability. Lastly, the models fitted were adjusted for several confounders. Among the limitations, our population included non-smoker participants with overweight/obesity and high cardiometabolic risk, thus making it difficult to generalize these results to other populations. Another limitation is that we did not have available the biological variables which might help to explain the underlying mechanisms behind the sex-differential effects on telomere changes in response to the intervention; thus, our results should be interpreted with caution. Nevertheless, the influence of sex on telomere biology is a topic that warrants further exploration.

Table 2
Risk for telomere shortening (ΔTL ≤20th percentile) after 3 years of follow-up by intervention group.

		OR for telomere shortening after 3 years of intervention								
		Crude			Model 1			Model 2		
		OR (95% CI)	p	p for interaction	OR (95% CI)	p	p for interaction	OR (95% CI)	p	p for interaction
All population (n = 317)	**Control**	1 (Ref.)		0.044	1 (Ref.)		0.009	1 (Ref.)		0.012
	Intervention	0.85 (0.49–1.47)	0.557		0.54 (0.26–1.13)	0.102		0.56 (0.26–1.22)	0.142	
Men (n = 169)	**Control**	1 (Ref.)			1 (Ref.)			1 (Ref.)		
	Intervention	1.47 (0.68–3.18)	0.333		1.43 (0.50–4.07)	0.506		1.35 (0.39–4.70)	0.637	
Women (n = 148)	**Control**	1 (Ref.)			1 (Ref.)			1 (Ref.)		
	Intervention	0.47 (0.21–1.05)	0.066		0.21 (0.07–0.66)	0.007		0.17 (0.05–0.64)	0.008	

Model 1: adjusted for sex, age and baseline TL.
Model 2: adjusted for sex, age, baseline TL, BMI (in kg/m2), physical activity (metabolic equivalent tasks in min/week), total energy intake (kcal/day), smoking status (never/former), adherence to MedDiet (17 point-score), diabetes status (yes/no), hypertensive status (yes/no), dyslipidemia status (yes/no). n = 315 (two individuals had missing data for dyslipidemia status).
P for interaction between the group of intervention and sex in determining the risk for telomere shortening after 3 years of intervention.

5. Conclusions

Our study suggests that an intensive lifestyle intervention based on a MedDiet, PA promotion and weight loss encouragement increased telomere length in older women with high cardiometabolic risk but not in men. Women following this intensive intervention also had a lower risk of telomere shortening. Whether the effect on TL is due to body weight loss, increased adherence to an energy-restricted MedDiet and/or increased in PA needs to be further addressed. Therefore, observational studies and clinical trials replicating these results and exploring possible implicated mechanisms are warranted.

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Authors' contributions

Conceptualization: AM, SG-C, SC and JS-S; methodology: AM, SG-C, SC and JS-S; software: MFdlP, CV-H and SG-C; validation: all authors; data analysis: MFdlP, CV-H and SG-C; statistical analysis and data interpretation: all authors; resources: all authors; writing—original draft preparation: AM, MFdlP, SC, CV-H, SG-C and JS-S; writing—review and editing: all authors; supervision: AM, MFdlP, SC, CV-H, SG-C and JS-S; project administration and coordination: MM-G and JS-S; funding acquisition: MM-G and JS-S. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

All authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.06.030.

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Supporting Information

More information on Methods:

1. PREDIMED-Plus protocol description

The PREDIMED-Plus study is an ongoing 6-year parallel-group, multicenter randomized clinical trial (RCT) involving 6874 participants recruited at 23 Spanish centers (Navarra (Epidemiology), Valencia (Genetics), Reus (Nutrition), Barcelona (Molecular biology), Alicante (Epidemiology), Balearic (Cardiology) , Navarra (Nutrition), Málaga (Nutrition), Córdoba (Internal medicine), Barcelona (Internal medicine), Granada (Epidemiology), Vitoria (Cardiology), Balearic (Physiology), Málaga (Endocrinology), Canary Islands (Epidemiology), León (Public Health), Sevilla (Primary care), Madrid (Endocrinology), Barcelona (Internal medicine), Barcelona (Endocrinology), Madrid (Nutrition), Jaén (Epidemiology), Madrid (Endocrinology)) from September 2013 until December 2016 and aimed at assessing the effect of a lifestyle intervention on the primary prevention of cardiovascular disease. Eligible participants were women (aged 60-75 years) and men (aged 55-75 years) with no documented previous history of cardiovascular disease at enrolment, but with overweight/obesity (BMI $\geq$27 and <40 kg/m^2) and having at least 3 components of the metabolic syndrome.

Each recruiting center randomly allocated candidates in a 1:1 ratio to either the control group (CG) or the intervention group (IG) with stratification by center, sex, and age (<65, 65–70, and >70 years) using a centrally controlled, computer-generated random number internet-based system. The randomization procedure was internet-based and blinded to all staff and to the principal investigators at each center.

Participants in the IG followed an energy-restricted MedDiet (erMEdDiet), accompanied by physical activity promotion and behavioral support to accomplish specific weight-loss objectives. The erMedDiet aimed at an energy reduction of 600 kcal/day (about 30% of estimated energy requirements) according to each participants' baseline metabolic rate and physical activity level (http://www.nap.edu/books/0309085373/html/), and with a macronutrient distribution of 40-45% carbohydrates, 35-40% fat and 20% protein. Dietary advice encouraged the consumption of typical and seasonal MedDiet foods and recommends refraining from foods characteristic of the Western dietary pattern. Participants were

encouraged to gradually increase their level of physical activity to at least 45 minutes per day after 6 months of intervention, and their progress was monitored. The physical activity program included aerobic activities, such as brisk walking or any equivalent activity of moderate intensity. Participants in the CG received recommendations to follow an energy-unrestricted traditional MedDiet and usual care administered by primary care physicians. No specific advice for increasing physical activity or losing weight was provided to participants in the control group.

Adherence to the erMedDiet was evaluated through a validated 17-point score [1]. Leisure-time physical activity was assessed using the validated REGICOR questionnaire (including questions in relation to the type of activity, frequency (number of days), and duration (min/day)) [2].

2. Biochemical determinations

Blood samples were collected in fasting conditions at baseline (before the intervention) and at three years of follow-up and frozen at -80ºC until analysis. Biochemical and TL measurements were taken in blinded conditions to the intervention group. The levels of total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), low-density lipoprotein cholesterol (LDL-cholesterol), triglycerides, serum glucose and HbA1c were measured using routine enzymatic methods.

3. Quality control for the determination of telomere length

The acceptable ranges of PCR efficiency for the single copy gene and telomere primers were 90 and 110%, respectively. For quality control, each sample was run in triplicate. In addition, a seven-point standard curve made from reference DNA samples was included for each plate using a 2-fold dilution series of DNA, ranging from 150 to 234 ng/mL. TL was expressed as a T/S ratio using the calibration curve (linearity agreement $R2 > 0.99$) to relative quantification. The intra-class correlation coefficient for TL was 0.793 (95% CI: 0.707, 0.857). In addition, as an extra quality control, baseline TL was correlated with chronological age (rho=-0.16, p=0.004) in all participants. The odds of having short telomeres was defined as TL below the 20th percentile as reported elsewhere [3–7].

Table S1. Baseline characteristics of the subjects at randomization before the start of the study, separately by sex

	Women			Men		
Characteristics	Control group (n=71)	Intervention group (n=77)	p-value	Control group (n=87)	Intervention group (n=82)	p-value
Age (years)	66.5 (3.8)	66.9 (4.2)	0.504	64.1 (5.4)	63.6 (5.4)	0.554
Weight (kg)	79.8 (10.9)	78.2 (11.4)	0.384	90.8 (11.2)	93.4 (12.1)	0.148
Waist circumference (cm)	102.9 (9.6)	101.5 (8.5)	0.374	109.4 (8.9)	110.1 (7.6)	0.594
BMI (kg/m²)	32.4 (3.6)	32.2 (3.9)	0.779	31.7 (3.4)	32.2 (3.7)	0.364
Number of MetS components, n (%)			0.699			0.050
3	38 (53.5)	36 (46.8)		40 (46)	53 (64.6)	
4	21 (29.6)	27 (35.1)		35 (40.2)	21 (25.6)	
5	12 (16.9)	14 (18.2)		12 (13.8)	8 (9.8)	
Obesity (BMI≥30 kg/m²), n (%)	48 (67.6)	49 (63.6)	0.612	50 (57.5)	50 (61)	0.643
Diabetes†, n (%)	21 (29.6)	21 (27.3)	0.756	26 (30)	22 (26.8)	0.660
Cholesterol*, n (%)	46 (64.8)	51 (66.2)	0.602	59 (67.8)	45 (54.9)	0.116
Hypertension, n (%)	54 (76.1)	65 (84.4)	0.201	75 (86.2)	72 (87.8)	0.758
Depression, n (%)	19 (26.8)	20 (25.9)	0.914	11 (12.6)	15 (18.3)	0.309
Smokers, n (%)			0.393			0.281
Former	20 (28.2)	17 (22.1)		57 (65.5)	60 (73.2)	
Never	51 (71.8)	60 (77.9)		30 (34.5)	22 (26.8)	
Educational level, n (%)			0.262			0.223
Higher degree or similar	6 (8.5)	4 (5.2)		6 (6.9)	9 (11)	
University school technician	8 (11.3)	5 (6.5)		15 (17.2)	6 (7.3)	
Secondary school	17 (23.9)	29 (37.7)		30 (34.5)	29 (35.4)	
Primary school	40 (56.3)	39 (50.7)		36 (41.4)	38 (46.3)	
Medication use, n (%)						
Lipid-Lowering drugs						
Statin	37 (52.1)	33 (42.9)	0.260	42 (48.3)	37 (45.1)	0.681
Other lipid-lowering drugs	4 (5.6)	5 (6.5)	0.827	5 (5.8)	5 (6.1)	0.923
Hypotensive drugs						
Renin direct inhibitor	0	0	-	0	1 (1.2)	0.302
Angiotensin receptor blocker	16 (22.5)	24 (31.2)	0.237	21 (24.1)	22 (26.8)	0.688
Angiotensin converting enzyme inhibitor	29 (40.9)	21 (27.3)	0.081	43 (49.4)	36 (43.9)	0.472
Thiazide drugs‡	23 (32.4)	29 (37.7)	0.502	33 (37.9)	24 (29.3)	0.234
Antidiabetic drugs						
Insulin treatment	6 (8.5)	2 (2.6)	0.116	7 (8.1)	1 (1.2)	0.037
Metformin	16 (22.5)	18 (23.4)	0.903	19 (21.8)	17 (20.7)	0.861
Telomere length (T/S ratio)	1.14 (0.4)	1.2 (0.4)	0.428	1.07 (0.4)	1.14 (0.5)	0.296

Data are shown as means (SD) for continuous variables or number (%) for categorical variables. *There were 2 missing data for cholesterol, one woman from intervention group and one man from the control group. †Diabetes was defined as previous diagnosis of diabetes or HbA1c ≥6.5% (48 mmol/mol), use of antidiabetic medication or having fasting glucose >126 mg/dL (7.0 mmol/L) in the screening visit plus fasting glucose >126 mg/dL (7.0 mmol/L) at baseline visit. ‡Thiazide drugs include thiazides and thiazide-like diuretics. *P* values for differences between groups by ANOVA or chi-squared test, as appropriate. Abbreviations: BMI, body mass index; MetS, Metabolic syndrome.

Table S2. Baseline and 3-year changes in anthropometric measurements and components of the intervention (physical activity and adherence to the MedDiet) by intervention groups

All population (n=317)

	Control group (n=158)		Intervention group (n=159)		
	Baseline	Change	Baseline	Change	P-value
Anthropometric measurements					
Body weight (kg)	85.8 (12.3)	-0.32 (4.0)	85.9 (14.0)	-3.7 (4.0) ¥	<0.001
BMI (kg/m²)	32 (3.5)	-0.04 (1.6)	32.2 (3.8)	-1.3 (1.5) ¥	<0.001
Waist circumference (cm)	106.5 (9.7)	0.3 (4.8)	105.9 (9.1)	-3.9 (5.6) ¥	<0.001
Leisure-time physical activity (METs-min/week)	2704 (2579.5)	26.8 (2239.3)	2666.3 (2393.4)	770.2 (2315.3) ¥	0.004
17-point erMedDiet score	8.9 (2.4)	2.1 (2.8) ¥	8.7 (2.6)	5 (3.1) ¥	<0.001
Biochemical parameters					
Total cholesterol* (mg/dL)	196.1 (40.6)	-10.8 (37) ¥	199.8 (34.6)	-8.6 (34) ¥	0.582
LDL cholesterol* (mg/dL)	121.1 (34.6)	-12 (31.4) ¥	121.3 (31.1)	-8.3 (28.4) ¥	0.304
HDL cholesterol* (mg/dL)	46.8 (10)	2 (7.4) ¥	48.3 (11.5)	2.8 (6.8) ¥	0.343
Triglycerides* (mg/dL)	154.1 (96.9)	-7.4 (105.9)	154.5 (78.4)	-13.2 (62.8) ¥	0.556
Glucose* (mg/dL)	111.6 (30.5)	1.4 (27.2)	109.7 (24.3)	-1.7 (22.1)	0.257
$HbA1_c$* (%)	6 (0.9)	0.1 (0.7) ¥	6 (0.7)	0.05 (0.5)	0.438

Women (n=148)

	Control group (n=71)		Intervention group (n=77)		
	Baseline	Change	Baseline	Change	P-value
Anthropometric measurements					
Body weight (kg)	79.8 (10.9)	0.006 (4.4)	78.2 (11.4)	-3.2 (3.4) ¥	<0.001
BMI (kg/m²)	32.4 (3.6)	0.05 (1.8)	32.2 (3.9)	-1.3 (1.4) ¥	<0.001
Waist circumference (cm)	102.9 (9.6)	0.1 (4.9)	101.5 (8.5)	-3.9 (5.2) ¥	<0.001
Leisure-time physical activity (METs-min/week)	2178.1 (2175.7)	-29.4 (1875.1)	1829.2 (1233.1)	700.9 (1829.5) ¥	0.018
17-point erMedDiet score	9.7 (2.4)	1.8 (3.2) ¥	8.3 (2.6)	4.5 (2.9) ¥	<0.001
Biochemical parameters					

Total cholesterol* (mg/dL)	202.5 (38.3)	-9.8 (34) ¥	208.5 (34.9)	-7.8 (36)	0.739
LDL cholesterol* (mg/dL)	126.8 (34.5)	-12.6 (28.6) ¥	128.5 (33.5)	-10.1 (30.9) ¥	0.628
HDL cholesterol* (mg/dL)	49.9 (9.5)	3.3 (7.9) ¥	51.8 (12.1)	2.7 (7.4) ¥	0.621
Triglycerides* (mg/dL)	145.2 (76.9)	-11 (61.3)	144 (57.6)	-4.4 (42.3)	0.449
Glucose* (mg/dL)	112.3 (32.3)	0.4 (25)	106.6 (21.5)	0.4 (20.5)	0.995
$HbA1_c$* (%)	6 (0.7)	0.1 (0.6)	6 (0.6)	0.01 (0.5)	0.873

Men (n=169)

	Control group (n=87)		Intervention group (n=82)		
	Baseline	Change	Baseline	Change	P-value
Anthropometric measurements					
Body weight (kg)	90.8 (11.2)	-0.6 (3.6)	93.4 (12.1)	-4.0 (4.4) ¥	<0.001
BMI (kg/m^2)	31.7 (3.4)	-0.1 (1.3)	32.2 (3.7)	-1.4 (1.5) ¥	<0.001
Waist circumference (cm)	109.4 (8.9)	0.4 (4.7)	110.1 (7.6)	-3.9 (5.9) ¥	<0.001
Leisure-time physical activity (METs-min/week)	3133.3 (2806.9)	72.6 (2507.5)	3452.3 (2907.8)	835.2 (2703.1) ¥	0.058
17-point erMedDiet score	8.3 (2.2)	2.4 (2.3) ¥	8 (2.5)	5.4 (3.2) ¥	<0.001
Biochemical parameters					
Total cholesterol* (mg/dL)	190.9 (41.9)	-11.6 (39.4) ¥	191.6 (32.4)	-9.3 (32.1) ¥	0.677
LDL cholesterol* (mg/dL)	116.4 (34.1)	-11.4 (33.6) ¥	114.1 (26.8)	-6.4 (25.5) ¥	0.311
HDL cholesterol* (mg/dL)	44.3 (9.7)	1 (6.8)	45 (9.9)	2.9 (6.1) ¥	0.060
Triglycerides* (mg/dL)	161.4 (110.5)	-4.5 (131.6)	164.4 (93)	-21.6 (76.8) ¥	0.314
Glucose* (mg/dL)	111 (29.1)	2.3 (28.9)	112.5 (26.3)	-3.7 (23.6)	0.142
$HbA1_c$* (%)	6.1 (1)	0.1 (0.7)	6 (0.7)	0.02 (0.4)	0.357

Data are shown as means (SD) for baseline characteristics. * Variables with missing data (6 participants for total cholesterol; 27 for LDL cholesterol; 6 for HDL cholesterol; 6 for triglycerides; 2 for glucose and 19 for HbA1c). ¥ means statistical significance within group. *P* values for differences between groups by ANOVA. Abbreviations: BMI, body mass index; erMedDiet, energy-restricted Mediterranean Diet; HbA1c, glycated haemoglobin; MET, metabolic equivalent of task.

Table S3. Changes in medication use by intervention group after 3 years of intervention

All population (n=317)

	Control group (n=158)		Intervention group (n=159)		
	Increased use	Decreased use	Increased use	Decreased use	P-value
Lipid-Lowering drugs					
Statin	21 (13.3)	11 (7)	12 (7.6)	6 (3.8)	0.092
Other lipid-lowering drugs	5 (3.2)	0	5 (3.1)	5 (3.1)	0.080
Hypotensive drugs					
Renin direct inhibitor	0	0	0	0	-
Angiotensin receptor blocker	8 (5.1)	1 (0.6)	9 (5.7)	5 (3.1)	0.249
Angiotensin converting enzyme inhibitor	5 (3.2)	12 (7.6)	8 (5)	11 (6.9)	0.692
Thiazide drugs‡	16 (10.1)	4 (2.5)	16 (10.1)	6 (3.8)	0.819
Antidiabetic drugs					
Insulin treatment	0	2 (1.3)	1 (0.6)	0	0.222
Metformin	16 (10.1)	3 (1.9)	4 (2.5)	1 (0.6)	0.011

Women (n=148)

	Control group (n=71)		Intervention group (n=77)		
	Increased use	Decreased use	Increased use	Decreased use	P-value
Lipid-Lowering drugs					
Statin	8 (11.3)	7 (9.9)	6 (7.8)	3 (3.9)	0.246
Other lipid-lowering drugs	3 (4.2)	0	1 (1.3)	2 (2.6)	0.221
Hypotensive drugs					
Renin direct inhibitor	0	0	0	0	-
Angiotensin receptor blocker	5 (7)	1 (1.4)	3 (3.9)	3 (3.9)	0.467
Angiotensin converting enzyme inhibitor	3 (4.2)	5 (7)	3 (3.9)	5 (6.5)	0.985
Thiazide drugs‡	10 (14.1)	1 (1.4)	4 (5.2)	5 (6.5)	0.064
Antidiabetic drugs					
Insulin treatment	0	1 (1.4)	0	0	0.296
Metformin	7 (9.9)	1 (1.4)	2 (2.6)	1 (1.3)	0.181

Men (n=169)					
	Control group (n=87)		Intervention group (n=82)		
	Increased use	Decreased use	Increased use	Decreased use	P-value
Lipid-Lowering drugs					
Statin	13 (14.9)	4 (4.6)	6 (7.3)	3 (3.7)	0.267
Other lipid-lowering drugs	2 (2.3)	0	4 (4.9)	3 (3.7)	0.126
Hypotensive drugs					
Renin direct inhibitor	0	0	0	0	-
Angiotensin receptor blocker	3 (3.5)	0	6 (7.3)	2 (2.4)	0.175
Angiotensin converting enzyme inhibitor	2 (2.3)	7 (8.1)	5 (6.1)	6 (7.3)	0.462
Thiazide drugs‡	6 (6.9)	3 (3.5)	12 (14.6)	1 (1.2)	0.182
Antidiabetic drugs					
Insulin treatment	0	1 (1.2)	1 (1.2)	0	0.367
Metformin	9 (10.3)	2 (2.3)	2 (2.4)	0	0.040

Data are shown as number (%). ‡Thiazide drugs include thiazides and thiazide-like diuretics. *P* values for differences between groups by chi-squared test.

Table S4. Correlation analysis between changes in telomere length and changes in cardiovascular risk factors

All population (n=317)				
	Control group (n=158)		Intervention group (n=159)	
	Pearson correlation coefficient	P-value	Pearson correlation coefficient	P-value
Anthropometric measurements				
Body weight (kg)	0.0684	0.393	0.0353	0.659
BMI (kg/m²)	0.0838	0.295	0.0392	0.624
Waist circumference (cm)	0.0710	0.375	0.0287	0.719
Biochemical parameters				
Total cholesterol (mg/dL)	0.0778	0.336	0.0542	0.501
LDL cholesterol (mg/dL)	0.0693	0.408	0.0602	0.472
HDL cholesterol (mg/dL)	0.0259	0.749	-0.0252	0.754
Triglycerides (mg/dL)	0.0299	0.712	0.0186	0.817
Glucose (mg/dL)	0.0172	0.831	0.1116	0.163
$HbA1_c$ (%)	-0.0605	0.460	0.1086	0.190

Women (n=148)				
	Control group (n=71)		Intervention group (n=77)	
	Pearson correlation coefficient	P-value	Pearson correlation coefficient	P-value
Anthropometric measurements				
Body weight (kg)	0.1809	0.131	0.0297	0.797
BMI (kg/m²)	0.2029	0.089	0.0550	0.635
Waist circumference (cm)	0.1768	0.140	0.0602	0.603
Biochemical parameters				
Total cholesterol (mg/dL)	0.1412	0.247	0.0970	0.405
LDL cholesterol (mg/dL)	0.1091	0.383	0.1	0.393
HDL cholesterol (mg/dL)	-0.0007	0.995	0.0519	0.656
Triglycerides (mg/dL)	0.1566	0.199	0.0014	0.990

Glucose (mg/dL)	0.1780	0.138	0.0505	0.665
$HbA1_c$ (%)	0.0348	0.779	0.1498	0.216

Men (n=169)

	Control group (n=87)		Intervention group (n=82)	
	Pearson correlation coefficient	P-value	Pearson correlation coefficient	P-value
Anthropometric measurements				
Body weight (kg)	-0.0306	0.778	0.0239	0.831
BMI (kg/m^2)	-0.0384	0.724	0.0251	0.823
Waist circumference (cm)	-0.0254	0.816	0.0070	0.950
Biochemical parameters				
Total cholesterol (mg/dL)	0.0373	0.733	0.0130	0.909
LDL cholesterol (mg/dL)	0.0381	0.739	0.0375	0.758
HDL cholesterol (mg/dL)	0.0805	0.461	-0.0974	0.390
Triglycerides (mg/dL)	-0.0199	0.855	0.0054	0.962
Glucose (mg/dL)	-0.1045	0.338	0.1388	0.214
$HbA1_c$ (%)	-0.1301	0.238	0.0673	0.561

P-value for significance level of the correlation. Abbreviations: BMI, body mass index; HbA1c, glycated hemoglobin.

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Chapter 4

Sperm and leukocyte telomere length are related to sperm quality parameters in healthy men from the Led-Fertyl study.

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Overview of this publication

What is already known?

- Male factors account for almost half of the cases of couples affected by infertility and shorter sperm TL has been observed in men with impaired sperm parameters. However, evidence in men from the general population and accounting for lifestyle factors is very limited.

What does this study add?

- Longer leukocyte and sperm TL were associated with higher sperm concentration and sperm count in 200 volunteers of reproductive age.

What are the main conclusions?

- Sperm and leukocyte TL could be considered suitable candidates to be used as biomarkers of sperm quality, complementing the conventional semen analysis.

TITLE PAGE

Sperm and leukocyte telomere length are related to sperm quality parameters in healthy men from the Led-Fertyl study

Running title: Sperm and leukocyte telomere length and sperm parameters

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Abstract

Study question: Could sperm and leukocyte telomere length (TL) be associated with sperm quality parameters and reproductive health in men from the general population?

Summary answer: A positive association between sperm and leukocyte TL with sperm concentration and total count has been demonstrated.

What is known already: Male factors account for almost half of the cases of couples affected by infertility and shorter sperm TL has been observed in men with impaired sperm parameters. However, evidence in men from the general population is limited.

Study design, size, duration: A total of 200 volunteers of reproductive age were recruited between February 2021 and April 2023 to participate in the Led-Fertyl (Lifestyle and Environmental Determinants of Seminogram and Other Male Fertility-Related Parameters) cross-sectional study.

Participants/materials, setting, methods: Sperm and leukocyte TL were measured using quantitative Polymerase Chain Reaction (qPCR) in 168 and 194 participants, respectively. Sperm parameters, including concentration, total count, motility, vitality, and morphology, were analyzed using a computer-assisted sperm analysis (CASA) SCA® system according to the World Health Organization (WHO) 2010 guidelines. Multivariable regression models were performed to assess the associations between sperm and leukocyte TL, either in tertiles or as continuous variables, and sperm quality parameters adjusting for potential confounders.

Main results and the role of chance: Participants in tertiles 2 (T2) and 3 (T3) of sperm TL showed a higher sperm concentration (β: 1.09; 95% CI: 0.09 to 2.09 and β: 2.06; 95% CI: 1.04 to 3.09 for T2 and T3, respectively; p-trend<0.001), compared to those in the reference tertile (T1). Participants in the highest tertile of sperm TL showed higher total sperm count (β: 3.83; 95% CI: 2.08 to 5.58 for T3 Vs. T1; p-trend<0.001). Participants in the top tertile of leukocyte TL showed higher sperm concentration (β: 1.49; 95% CI: 0.44 to 2.54 for T3 Vs. T1; p-trend=0.004), and total count (β: 3.49; 95% CI: 1.62 to 5.35 for T3 Vs. T1; p-trend<0.001) compared with participants in T1. These results remained consistent when sperm and leukocyte TL were modelled as continuous variables.

Limitations, reasons for caution: The impossibility to establish a cause-effect relationship due to the cross-sectional study design. The sample size of the study cannot be considered large.

Wider implications of the findings: Sperm and leukocyte TL are associated with sperm quality parameters in the general population. Additional determinations and further studies with larger sample size are needed to clarify the mechanisms underlying these associations and to investigate beyond.

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Trial registration number: N/A

Key words

Telomere length

Sperm quality

Male infertility

Spermatozoa

Leukocyte

Reproductive age

Led-Fertyl study

What this means for patients?

The proportion of the global population who have experienced infertility during their life raised to 17.5% in 2020 and male factors are responsible for almost half of the cases of couples affected by infertility. In the recent years, the study of the mechanisms related to reproductive health has become essential due to the impact on mental health and quality of life.

In this sense, telomeres, the end of chromosomes that protect them, have been previously proposed as biomarkers of sperm quality and male infertility since short telomere length has been found in men with impaired sperm parameters. However, most of the published articles did not include men from the general population and instead used data from men attending fertility clinics.

To increase evidence in this field, we conducted a cross-sectional study including 200 healthy volunteer participants of reproductive age. We found positive associations between sperm and leukocyte telomere length and sperm concentration and total count, independently of the age, body mass index, and other potential confounding factors. Additional studies are needed to reproduce and elucidate the mechanisms underlying these associations.

Introduction

Total fertility rate, reported as the number of births per woman, has drastically decreased worldwide between the 1950s and 2022 (United Nations, 2022) and the estimated proportion of the population who have experienced infertility during their lifetime raised to 17.5% in 2020 (World Health Organization, 2021), with male factors accounting for almost half of the cases of couples affected by this condition (Agarwal et al., 2021). The causes of male infertility are multifactorial, with varicocele being the one of the most common, followed by endocrine, metabolic, and genetic disorders. These conditions, in association to environmental factors, may reduce semen quality (Tahmasbpour et al., 2014). Given the significant impact on the mental health and quality of life of the population, investigating the underlying mechanisms related to reproductive health is therefore essential (Lei et al., 2021).

The nucleoprotein complexes that protect and maintain genome integrity are known as telomeres (Blackburn, 1991). These structures, located at the end of eukaryotic chromosomes, consist of the repetition in tandem of the DNA sequence 5'-TTAGGG-3' and a specialized multiprotein complex, called Shelterin (De Lange, 2005). In somatic cells and under typical conditions, a small fragment of telomeric DNA is shortened after each cell division due to the end-replication problem (Shammas, 2011). In male germ cells, telomeres preserve and increase their length with age to ensure genome integrity across generations. Mature spermatozoa, in fact, showed longer telomere length (TL) when compared to somatic cells (Rocca et al., 2019).

Over the past few years, the relationship between TL and sperm quality has been investigated. Data from a recent systematic review and meta-analysis (SRMA) of case-control studies (Fernández de la Puente et al., 2024), showed that individuals diagnosed

with infertility had shorter sperm and leukocyte TL compared to fertile controls. Furthermore, it was observed that TL in spermatozoa was also shorter in men with poorer sperm concentration or total count, compared to men with normal sperm quality parameters (Fernández de la Puente et al., 2024). However, in that SRMA, the cross-sectional studies reviewed reported contradictory results. The majority of the articles included in this SRMA have been conducted in men from couples attending fertility clinics or included in assisted reproduction programs, but few studies were conducted in men from the general population. Additionally, these studies did not consider demographic characteristics or lifestyle factors that have been shown to modulate the relationship between TL and sperm quality, such as age, energy intake, smoking habits, physical activity (Gürel et al., 2024), body mass index (Raee et al., 2023; Moustakli et al., 2024), or nutrition (Galiè et al., 2020).

Therefore, the main aim of this study was to assess the associations between sperm and leukocyte TL and sperm quality outcomes in healthy volunteers of reproductive age from the Led-Fertyl study. The main hypothesis is that longer sperm and leukocyte TL are positively associated with better parameters evaluating sperm quality.

Materials and Methods

Study design

The Led-Fertyl (Lifestyle and Environmental Determinants of Seminogram and Other Male Fertility-Related Parameters) study is a cross-sectional study aimed to identify the associations between lifestyle and environmental factors and sperm quality, and to explore potential mechanisms elucidating these associations. The principal objective of the present analysis was to explore the potential associations between sperm and leukocyte TL and sperm quality parameters.

Study participants

A total of 200 volunteers were enrolled in the Led-Fertyl study from February 2021 to April 2023. The recruitment process was conducted through the dissemination of information at the Rovira i Virgili University and in various towns and villages across Catalonia (Spain). In addition, flyers and posters were exhibited in establishments, pharmacies, hospitals, primary healthcare centers and through social media and newspapers. The main inclusion criteria were men aged between 18 and 40 years with good health status and registered in the Spanish public health system. Exclusion criteria were: inability to follow scheduled intervention visits; institutionalization; history of intestinal resection or the presence of inflammatory bowel disease; history of major organ transplantation; known reproductive disease or vasectomy; documented history of cardiovascular disease; concurrent therapy with immunosuppressive drugs, cytotoxic agents, systemic corticosteroids or medications associated with sperm disorders; excessive weight loss (over 5kg in the last month); presence of cirrhosis or liver failure; endocrine diseases; immunodeficiency disorders, hepatitis B or C; alcoholism or drug abuse; active malignant cancer or a history of malignancy within the past 5 year; severe psychiatric disorder; any condition of severe comorbidity with less than 24 months of life expectancy, or any other condition that may interfere with compliance with the study protocol. Once enrolled in the study, participants were asked to complete online self-report questionnaires and attend a face-to-face visit at the Hospital Universitari Sant Joan de Reus (Reus, Tarragona, Spain) to collect biological samples. Therefore, blood and semen samples were collected the same day.

Ethical approval

The study protocol was approved by the Institut d'Investigació Sanitària Pere Virgili Ethics Committee (Reference: CEIM: 181/2019) and the study was conducted according to the ethical standards laid down in the Declaration of Helsinki. All participants involved in the study signed a written informed consent.

Sperm quality parameters

Conventional complete semen quality analyses were performed after 3-7 days of sexual abstinence following the World Health Organization protocol (WHO 2010). An Olympus CX43phase-contrast microscope (Olympus Corporation, Tokyo, Japan) was used in conjunction with the Computer Aided Sperm Analysis (CASA) SCA® System version 6.5.0.67 (Microptic, Barcelona, Spain) to assess microscopic characteristics of spermatozoa. For each seminogram, the following parameters were analyzed: sperm concentration ($x10^6$/ml), sperm total count ($x10^6$/ejaculated), total motility (%), progressive and non-progressive motility (%), vitality (%), and normal morphology (%). Motility, vitality and morphology parameters were evaluated in 200 spermatozoa. Sperm vitality was assessed through the hypoosmotic swelling test (HOS test) and sperm morphology was evaluated using the Hemacolor (Merck KGaA, Darmstadt, Germany) staining protocol. Subsequently, aliquots of 15M/ml of sperm cells were frozen at -80ºC until used.

Sperm and leukocyte genomic DNA isolation

Frozen peripheral blood leukocytes and sperm samples were used to extract genomic DNA using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to manufacturer's recommendations. Quantity and quality of the isolated sperm and leukocyte DNA were analyzed using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). To evaluate somatic cell contamination on sperm samples, a DNA

methylation analysis was performed using the *DLK1* locus as quality control in a subsample of 60 participants. This region contains CpGs differentially methylated between somatic and sperm cells. In spermatozoa, these regions have low methylation levels compared to somatic cells.

Telomere length determination

TL in sperm and leukocytes was measured by quantitative real-time polymerase chain reaction (qPCR) following a protocol adapted from O'Callaghan (O'Callaghan and Fenech, 2011). The relative quantification of telomeres (T) was conducted using a single-copy gene (S, human 36B4) as a reference for each sample and results were expressed as telomere to single-copy gene ratio (T/S ratio). To complete a final volume of 20 µl per well, 9 µl of leukocyte or sperm DNA (5 ng/µl), 10 µl of SYBR Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 1 µl of primers (Merck KGaA, Darmstadt, Germany) were used. Samples were amplified in two PCRs: the first to determine the cycle threshold (Ct) value for telomere (T) amplification and the second to determine the Ct value for 36B4 single-copy gene using the corresponding primers. In addition, a six-point standard curve was included in each plate using 10-fold dilutions of known quantities of standards of telomere and 36B4 (linearity agreement $R^2 > 0.97$). The quantification of the relative copy numbers (T/S) was performed in triplicates using 96-well plates in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Primer and standard sequences for telomeres and 36B4 are detailed elsewhere (O'Callaghan and Fenech, 2011). Leukocyte and sperm DNA samples from the same participant were run in the same plate. Intra- and inter-assay coefficients of variability were calculated and when resulting above 10%, samples were reanalyzed.

Covariates

General socio-demographic (age, educational level), and lifestyle information (diet, physical activity, and smoking status) were collected by online questionnaires. Anthropometric measurements (weight, height and waist circumference) were recorded with the participants wearing light clothing and without shoes. Body Mass Index (BMI; kg/m2) was calculated by dividing weight (kg) by the square of the height (m). For physical activity information, participants completed the validated short version of the REGICOR (Registre Gironi del Cor) Physical Activity Questionnaire (Metabolic Equivalent of Task (MET) -min/week) (Molina et al., 2017). Dietitians used a validated food frequency questionnaire (FFQ) to estimate dietary food consumption (Fernández-Ballart et al., 2010). The responses for each food item were then converted into daily grams, using the standard portion size of each food. Total daily energy was then calculated using the e-Diet Base URV®.

Statistical analyses

We used the January 2024 Led-Fertyl database. The Kolmogorov–Smirnov test was used to assess normal distribution of the variables. Sperm and leukocyte TL, total sperm count, concentration, and normal morphology were cubic root-transformed to approach normality. For general characteristics and sperm-related parameters, mean and standard deviation (SD) or median [P25 – P75] for continuous variables and number (percentage, %) for categorical variables were reported. Differences across tertiles of sperm or leukocyte TL were analyzed by one-way analysis of variance (ANOVA) or Kruskal–Wallis test according to normal or skewed distributions, respectively. To compare categorical variables across TL tertiles, the Chi-square test was used.

In the present analysis, sperm and leukocyte TL, in continuous and in tertiles, were defined as the exposures, and sperm quality parameters as outcomes. Multivariable linear

regression models adjusted for several potential confounders were fitted to test the associations between sperm and leukocyte TL and sperm concentration, total count, total motility, progressive and non-progressive motility, vitality and normal morphology. Tertile 1, which corresponds to the shortest telomere length, was used as a reference. Model 1 was adjusted for age (years), BMI (kg/m^2) and sexual abstinence (days). Model 2 (fully adjusted model) was further adjusted for energy intake (kcal/day), physical activity (METs-min/week), smoking status (never, former, current) and educational level (high school or less, college or high education).

As a sensitivity analysis, participants with sperm concentration and/or total count below the reference lower limits according to the WHO 2010 guidelines (World Health Organization., 2010) were excluded from the analysis of the associations between sperm/leukocyte TL and sperm parameters.

A *P* value <0.05 was considered statistically significant and the Stata 14.2 software program (StataCorp LP, College Station, TX, USA) was used for all analyses.

Results

In **Figure 1** is depicted the complete flowchart of the study population included in the present analysis. From a total 200 participants initially included, 3 participants were excluded because of azoospermia. Sperm and leukocyte TL was measured in 168 and 194 participants, respectively.

General characteristics and sperm quality parameters of the 197 included Led-Fertyl participants are shown in **Table 1**. The median age of the participants was 28 years, and the mean BMI was 24.4 kg/m^2. Almost 69% of the volunteers were non-smokers and a 64% had highly educational level. In **Tables 2 and 3,** the same general characteristics according to tertiles of sperm and leukocyte TL are reported, respectively. Compared to

participants in the lowest sperm and leukocyte TL tertile, those in the top tertile had higher sperm concentrations and total count. The *DLK1* locus quality control confirmed that the set of subsamples analyzed were clear of somatic cell contamination (**Supplementary Figure 1**).

Table 4 and **Figure 2** summarize the multivariable-adjusted β coefficients (95% CI) of the linear regression analysis evaluating the cross-sectional associations between sperm TL and sperm quality parameters. Compared to participants in the reference tertile (T1) of sperm TL, those in tertiles 2 (T2) and 3 (T3) showed a higher sperm concentration (fully adjusted model, β: 1.09; 95% CI: 0.09 to 2.09 and β: 2.06; 95% CI: 1.04 to 3.09 for T2 and T3, respectively; p-trend < 0.001). Besides, participants in the highest tertile of sperm TL had a higher total sperm count (fully adjusted model, β: 3.83; 95% CI: 2.08 to 5.58 for T3 vs. T1; p-trend < 0.001) (**Table 4**). These positive associations with sperm concentration and total count were also found when sperm TL was modelled as a continuous variable (**Figure 2**). When sensitivity analyses were performed by excluding the 5 out of 168 participants with sperm concentration and/or total count below the reference lower limits, the observed associations remained significant.

Similar results were observed in relation to leukocyte TL. Participants in the top tertile of leukocyte TL showed higher sperm concentration (fully adjusted model, β: 1.49; 95% CI: 0.44 to 2.54 for T3 vs. T1; p-trend = 0.004), and total count (fully adjusted model, β: 3.49; 95% CI: 1.62 to 5.35 for T3 vs. T1; p-trend < 0.001) compared to participants in T1 (**Table 5**). Moreover, when leukocyte TL was expressed as a continuous variable in the analysis, a positive association between leukocyte TL and sperm concentration and sperm count was also identified (**Figure 3**). A total of 21 out of 194 participants had sperm concentration and/or total count below the reference lower limits. Upon exclusion of these participants, the results were consistent.

No associations were identified in the fully adjusted models between sperm or leukocyte TL and other sperm quality parameters considered (total, progressive and non-progressive motility, vitality and normal morphology), neither across TL tertiles (P for trends > 0.05), nor as continuous variable.

Discussion

In the present cross-sectional study, longer sperm and leukocyte TL were positively associated with higher sperm concentration and total count in a general population of healthy men of reproductive age, independently of the age, BMI and other potential confounding factors. The results were consistent regardless of whether TL was modelled as tertiles or as a continuous variable.

The study of the association between TL and sperm quality and male infertility is crucial since short telomeres have been associated with impaired sperm parameters. As reported in our recently published meta-analysis, shorter sperm TL was found in men with oligozoospermia or infertility, compared to fertile or normozoospermic individuals (Fernández de la Puente et al., 2024). In individuals with other altered sperm quality conditions such as teratozoospermia (i.e., over 96% of spermatozoa with abnormal morphology), TL in spermatozoa was also shown to be shorter compared to healthy donors (Fattahi et al., 2023). Consistent with our meta-analysis, other recent studies have shown that sperm and leukocyte TL were shorter in oligospermic men, compared to fertile men (Dhillon et al., 2024; Margiana et al., 2024). These results clearly indicate that sperm parameters are compromised when the mechanisms for maintaining or elongating TL are impaired. This situation has been shown to be accompanied by processes such as increased oxidative species' concentration and the expression of pro-apoptotic markers

that could partially lead reduced sperm quality and, consequently, male infertility (Margiana et al., 2024).

In the present analysis including healthy volunteer men of reproductive age, even after excluding participants who had a sperm concentration and total count below the reference limit, the positive associations found between sperm and leukocyte TL and sperm count and concentration remained consistent. Our results are in line with the positive correlations found between TL and the same seminogram parameters identified in recent research (Dhillon et al., 2024). Moreover, in that study, no correlations were reported between TL measured in both cell types and sperm total motility or morphology (Dhillon et al., 2024), which is also in line with our findings. These results suggest that the length of telomeres from spermatozoa and leukocyte cells could be used as a potential biomarker of sperm quality in terms of sperm concentration and total count. Further studies are warranted in the future to elucidate the relationship between TL and the remaining sperm parameters.

The evidence pertaining to the relationship between longer telomeres in spermatozoa and reproductive outcomes has yielded contradictory results. The positive association between sperm TL and percentage of fertilization rates found in one study (Berneau et al., 2020) was not replicated by other authors (Yang et al., 2015; Yuan et al., 2023), and the same occurred with embryological parameters (Yang et al., 2015; Berneau et al., 2020). In a meta-analysis, longer sperm TL was associated with higher rates of clinical pregnancy (Yuan et al., 2023). In addition, paternal age has been associated with longer leukocyte TL from the offspring (Kimura et al., 2008), suggesting a potential hereditary component. Longer telomeres at birth could contribute to healthier aging trajectories throughout life. However, the exact mechanisms and implications of sperm TL on fertilization and offspring inheritance are still an active area of research.

Telomeres, in conjunction with a complex of proteins, attach to the nuclear envelope forming a cluster during the prophase I step in meiosis. This process could facilitate homologous chromosomes alignment for recombination, which is a crucial stage in completing meiosis and ensuring the proper development of germ cells (reviewed in (Handel and Schimenti, 2010)). Whether telomeres themselves are involved in these meiotic processes is still unknown. Spermatocytes from idiopathic infertile men exhibit reduced meiotic crossover rates and an alteration in the association between the telomeres and the RNAs that maintain the integrity of the telomeric structure (Reig-Viader et al., 2014). Thus, failed recombination, in combination with an alteration of telomeric homeostasis, could be a significant factor in reducing sperm quality and, consequently, increasing the risk of infertility.

Based on previous evidence and the findings reported here, it seems that the physiological function of telomeres and the proteins responsible for maintaining their length and integrity are crucial for preventing segregation errors and ensuring proper spermatogenesis.

Strengths and limitations of the study

Considerable limitations should be taken into account in the interpretation of our findings. Firstly, the cross-sectional design of the study makes it impossible to establish a cause-effect relationship. Although it was a challenge to recruit 200 volunteers, the sample size cannot be considered large. The main strength of the Led-Fertyl study is the inclusion of a general population of reproductive age, consisting of young volunteers in good health state who did not attend fertility clinics. Secondly, the wide range of information collected on sociodemographic and lifestyle factors enabled us to control for several confounders in all analyses. However, it is important to note that residual confounding factors cannot

be completely ruled out. Additionally, semen samples were processed using a standardized protocol through the CASA SCA® system, thereby reducing any potential subjectivity.

Conclusion

In light of our findings, it can be concluded that sperm and leukocyte TL are associated with sperm parameters in terms of concentration and total count. Reduced TL may partially explain cases of impaired spermatogenesis. Nevertheless, additional determinations based on oxidative stress, protein activity and expression of telomere dynamic-related genes can supplement these findings and clarify the mechanisms underlying these associations. Further studies with a larger sample size are necessary to confirm our results and investigate beyond.

Authors' roles. M.F.d.l.P.: seminogram analysis, telomere length measurement, statistical analysis, data interpretation and manuscript writing and review; C.V.H.: dietary data collection, statistical analysis and manuscript review; A.S.H.: study conception and design, statistical analysis, data interpretation and manuscript writing and review; M.Á.M.: data collection and manuscript review; E.S.R.: data collection and manuscript review; S.C.: study conception and design, data interpretation and manuscript writing and review; D.T.O.: telomere length measurement and manuscript review; J.R.: telomere length measurement and manuscript review; N.B.: study conception and design and manuscript writing and review; J.S.S.: study conception and design, statistical analysis, data interpretation and manuscript writing and review. All authors revised the manuscript for important intellectual content and read and approved the final manuscript.

Data availability Statement. The data are not publicly available outside of the core Led-Fertyl research group, owing to data regulations and ethical considerations. This precaution has been taken to safeguard the consent of research participants, as their original agreement was limited to the utilization of their data by the initial research team. However, the researchers will follow a controlled data-sharing collaboration for research related to the project's aims. Therefore, investigators who are interested in the present study can contact the senior authors (nancy.babio@urv.cat, jordi.salas@urv.cat) by sending a request formal letter.

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418 **Conflicts of Interest.** All authors declare that they have no conflict of interests.

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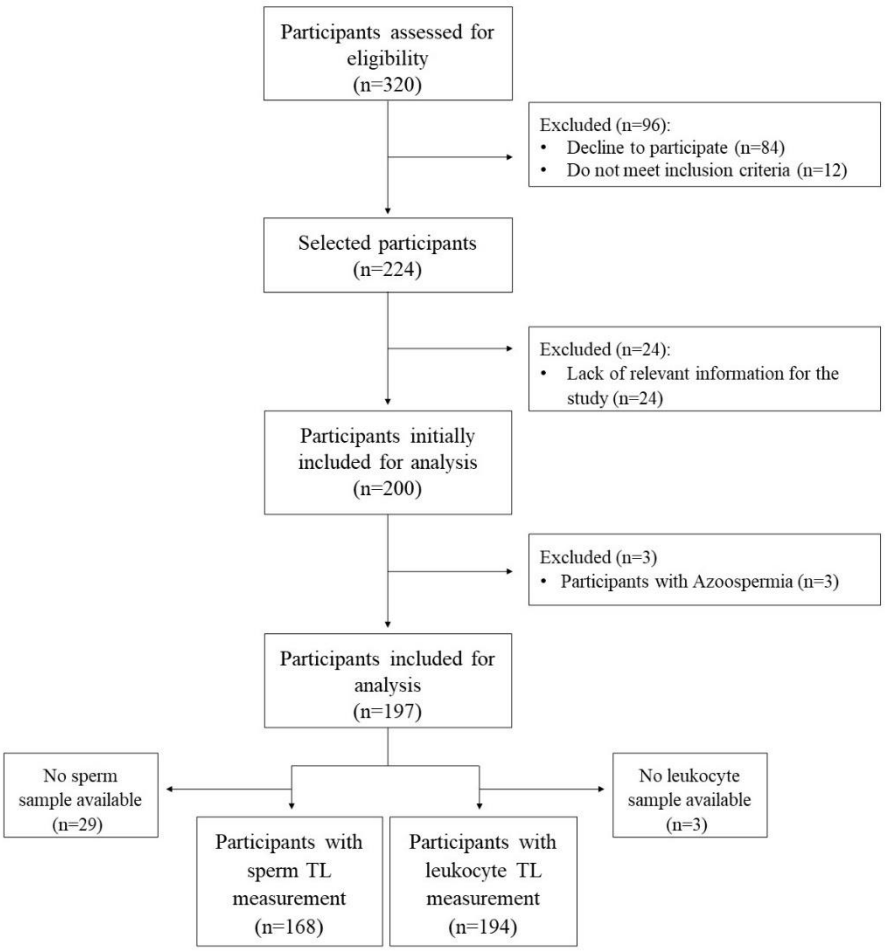
Figure 1. Flowchart of Led-Fertyl study participants included in the present analysis.



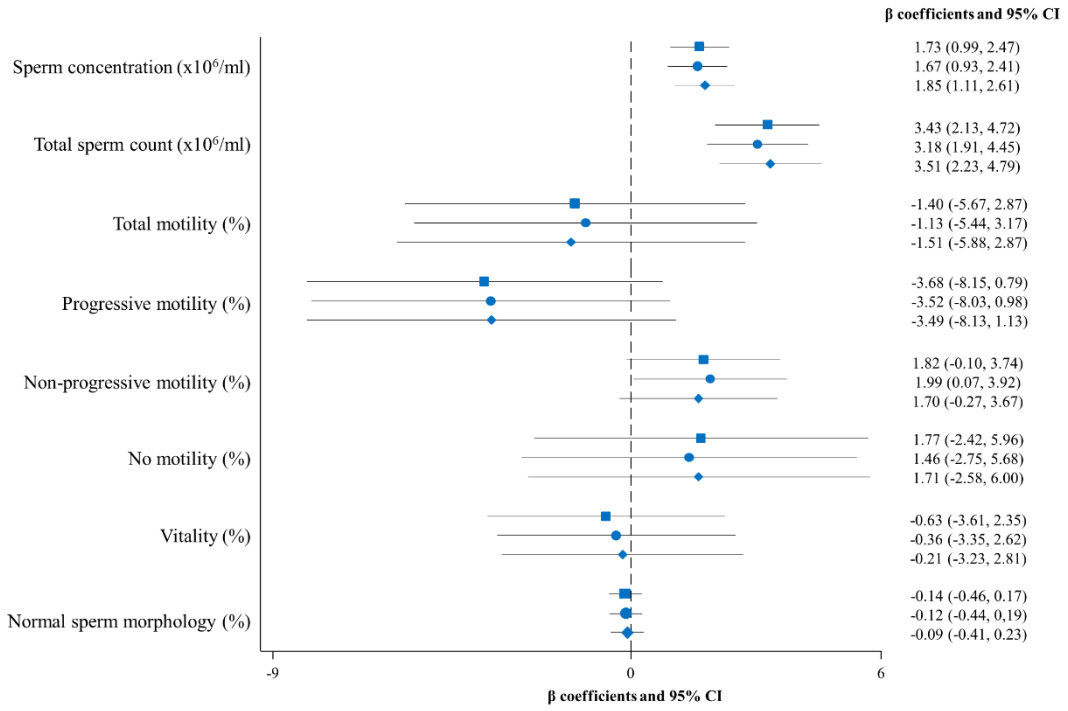


Figure 2. Multivariable-adjusted β coefficients and 95% CI of the associations between sperm telomere length as a continuous variable and sperm parameters. The crude model is represented with a squared. Model 1 is represented with a circle and was adjusted for age (years) and body mass index (kg/m^2) and abstinence (days). Model 2 is represented with a rhombus and was further adjusted for energy intake (kcal/day), physical activity (METs-min/week), smoking status (never, former, current), educational level (high school or less, college or high education). Sperm TL, total sperm count, concentration, and normal morphology were cubic root-transformed to approach normality.

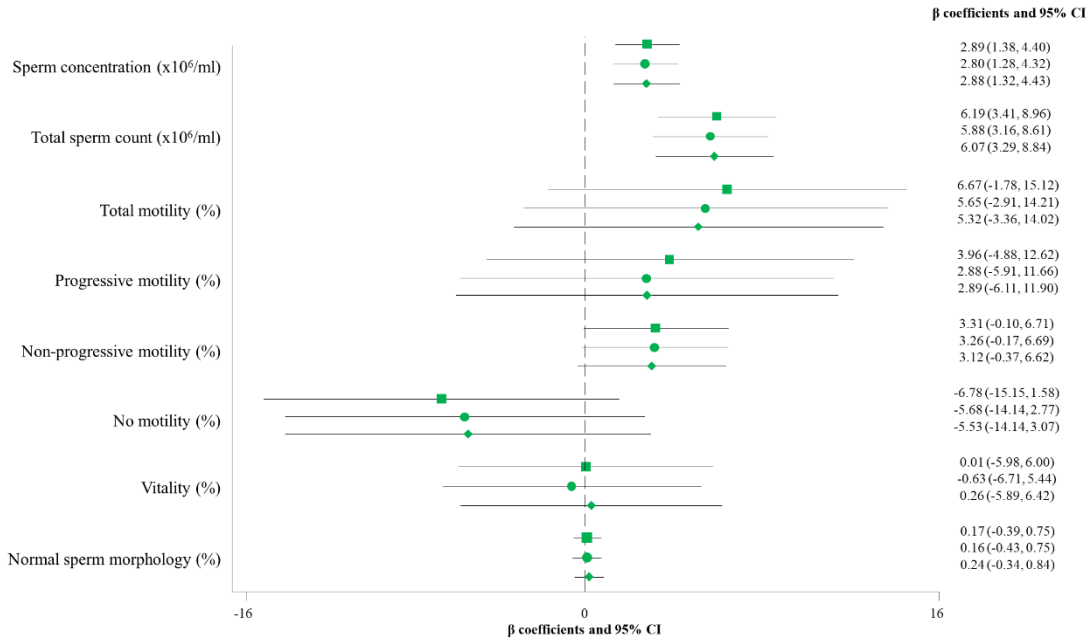


Figure 3. Multivariable-adjusted β coefficients and 95% CI of the associations between leukocyte telomere length as a continuous variable and sperm parameters. The crude model is represented with a squared. Model 1 is represented with a circle and was adjusted for age (years) and body mass index (kg/m^2) and abstinence (days). Model 2 is represented with a rhombus and was further adjusted for energy intake (kcal/day), physical activity (METs-min/week), smoking status (never, former, current), educational level (high school or less, college or high education). Leukocyte TL, total sperm count, concentration, and normal morphology were cubic root-transformed to approach normality.

Table 1. General characteristics of the study population

Characteristics	**n**	
Age (years)	197	28 [24 – 32]
Anthropometric measurements		
Weight (kg)	197	75.5 [70 – 81.7]
Waist circumference (cm)	197	81.9 [77.2 – 87.6]
BMI (kg/m²)	197	24.4 (3.2)
Energy intake (kcal/day)	197	2649.7 (635.4)
Leisure-time physical activity (METs-min/week)	197	3572.9 [1767.8 – 5307.7]
Educational level, n (%)	197	
High school or less		71 (36)
College or high education		129 (64)
Smoking status, n (%)	197	
Current		24 (12.2)
Former		25 (12.7)
Never		136 (69)
Not reported		12 (6.1)
Seminogram parameters		
Sexual abstinence (days)	197	4 [3 – 5]
Semen volume (ml)	197	3.5 [2.5 – 4.5]
Sperm concentration ($x10^6$/ml)	197	48.6 [29 – 84.6]
Total sperm count ($x10^6$)	197	168.4 [98.8 – 283.8]
Motility	197	
Total motility (%)		60.1 (16.4)
No motility (%)		39.6 (16.2)
Vitality (%)	196	81.5 [75.8 – 88.5]
Normal sperm morphology (%)	196	9.3 [5 – 15]
Sperm TL (T/S ratio)	168	3.2 [2.3 – 4.8]
Leukocyte TL (T/S ratio)	194	1.5 [1.1 – 2]

Data ere expressed as mean and standard deviation or median and [25^{th}-75^{th}] percentile for continuous variables and number and percentage for categorical variables. Abbreviations: BMI, body mass index; MET, Metabolic Equivalent of Task; TL, Telomere length.

Table 2. General characteristics of the study population according to tertiles of sperm telomere length

	Sperm telomere length (T/S ratio)			
	T1 (n=56)	**T2 (n=56)**	**T3 (n=56)**	**P-value**
Age (years)	27 [24 – 32]	29 [24.5 – 32]	28.5 [23 – 33]	0.553
Anthropometric measurements				
Weight (kg)	76.2 [71.5 – 80.1]	74.9 [68.8 – 80.3]	76.7 [68.8 – 81.6]	0.663
Waist circumference (cm)	81.9 [77.1 – 86]	81.6 [77.5 – 85.3]	81.5 [76.7 – 87.6]	0.933
BMI (kg/m²)	24.4 (3.3)	23.8 (2.7)	24.5 (2.8)	0.278
Energy intake (kcal/day)	2658.8 (724.5)	2606.2 (569.3)	2661.7 (593)	0.872
Leisure-time physical activity (METs-min/week)	3123.5 [1866.4 – 4631.7]	3779.5 [1841.5 – 5324]	3584.1 [1591.6 – 5489.5]	0.585
Educational level, n (%)				0.832
High school or less	21 (37.5)	19 (33.9)	18 (32.1)	
College or high education	35 (62.5)	37 (66.1)	38 (67.9)	
Smoking status, n (%)				0.040
Current	5 (8.9)	4 (7.1)	11 (19.6)	
Former	8 (14.3)	10 (17.9)	2 (3.6)	
Never	38 (67.9)	41 (73.2)	37 (66.1)	
Not reported	5 (8.9)	1 (1.8)	6 (10.7)	
Seminogram parameters				
Sexual abstinence (days)	4 [3 – 5]	4 [3 – 4.8]	4 [3 – 5]	0.559
Semen volume (ml)	3.8 [2.9 – 5]	3.6 [2.7 – 4.8]	3.5 [2.9 – 4.5]	0.874
Sperm concentration ($x10^6$/ml)	40.1 [28.8 – 66.1]	62.1 [35.9 – 91.8]	66.5 [47.9 – 109.3]	<0.001
Total sperm count ($x10^6$)	135.5 [102.8 – 226]	194 [127.8 – 303]	253.7 [171.9 - 361]	<0.001
Motility				
Total motility (%)	64 (13.1)	63.3 (16.4)	60.6 (15.9)	0.224
No motility (%)	35.9 (13.2)	35.9 (15.4)	39.4 (15.9)	0.352
Vitality (%)	81 [72.5 – 88.5]	83.8 [77 – 89.8]	80 [75.8 – 87]	0.167
Normal sperm morphology (%)	11.5 [6 – 19]	11 [5 – 15]	7.8 [4.8 – 14]	0.131
Leukocyte TL (T/S ratio)	1.2 [0.9 – 1.6]	1.6 [1.2 – 1.9]	2 [1.5 – 2.6]	<0.001

Data ere expressed as mean and standard deviation or median and [25^{th}-75^{th}] percentile for continuous variables and number and percentage for categorical variables. Abbreviations: BMI, body mass index; MET, Metabolic Equivalent of Task; TL, Telomere Length.

Table 3. General characteristics of the study population according to tertiles of leukocyte telomere length

	Leukocyte telomere length (T/S ratio)			
	T1	**T2**	**T3**	
	(n=65)	**(n=65)**	**(n=64)**	**P-value**
Age (years)	29 [24 – 33]	29 [24 – 31]	28 [23 – 33]	0.878
Anthropometric measurements				
Weight (kg)	76 [73 – 83.5]	75.2 [68.5 – 81.7]	75.8 [68.3 – 81.1]	0.251
Waist circumference (cm)	83 [78.3 – 89.4]	81 [77 – 84.5]	81.7 [76.8 – 87.3]	0.139
BMI (kg/m²)	25.2 (3.5)	23.9 (2.9)	24 (2.9)	0.298
Energy intake (kcal/day)	2566.8 (646.1)	2656.2 (621.3)	2699.3 (623.6)	0.477
Leisure-time physical activity (METs-min/week)	3729.1 [1965 – 5137.5]	3615.4 [1864.8 – 5757.6]	3128.7 [1521.7 – 5311.2]	0.509
Educational level, n (%)				0.244
High school or less	20 (30.8)	21 (32.3)	28 (43.8)	
College or high education	45 (69.2)	44 (67.3)	36 (56.2)	
Smoking status, n (%)				0.571
Current	7 (10.8)	7 (10.8)	9 (14.1)	
Former	11 (16.9)	9 (13.9)	5 (7.8)	
Never	43 (66.1)	47 (72.3)	44 (68.8)	
Not reported	4 (6.2)	2 (3.1)	6 (9.4)	
Seminogram parameters				
Sexual abstinence (days)	3 [3 – 5]	4 [3 – 5]	4 [3 – 5]	0.189
Semen volume (ml)	3.2 [2.3 – 4]	3.5 [2.7 – 5]	3.7 [2.8 – 4.5]	0.100
Sperm concentration ($x10^6$/ml)	42 [21.5 – 74.2]	43.5 [25.4 – 65.7]	63.7 [37.6 – 111.8]	0.001
Total sperm count ($x10^6$)	130.1 [64.3 – 230.1]	141.9 [83.8 – 232.7]	243.9 [137.9 – 350.7]	<0.001
Motility				
Total motility (%)	59.1 (17.5)	58.8 (16.5)	62.5 (15.3)	0.562
No motility (%)	40.8 (17.6)	40.9 (16.1)	37 (15.1)	0.486
Vitality (%)	80.8 [73 – 87.8]	85.5 [76 – 91]	80.8 [75.8 – 85.8]	0.112
Normal sperm morphology (%)	8 [4.5 – 15]	10 [5 – 16.5]	9.5 [5.5 – 14.5]	0.841
Sperm TL (T/S ratio)	2.2 [1.4 – 3.1]	3.2 [2.3 – 4.5]	4.2 [3.2 – 5.6]	<0.001

Data ere expressed as mean and standard deviation or median and [25^{th}-75^{th}] percentile for continuous variables and number and percentage for categorical variables. Abbreviations: BMI, body mass index; MET, Metabolic Equivalent of Task; TL, Telomere Length.

Table 4. Multivariable-adjusted β coefficients and 95% CI of sperm parameters across tertiles of sperm telomere length.

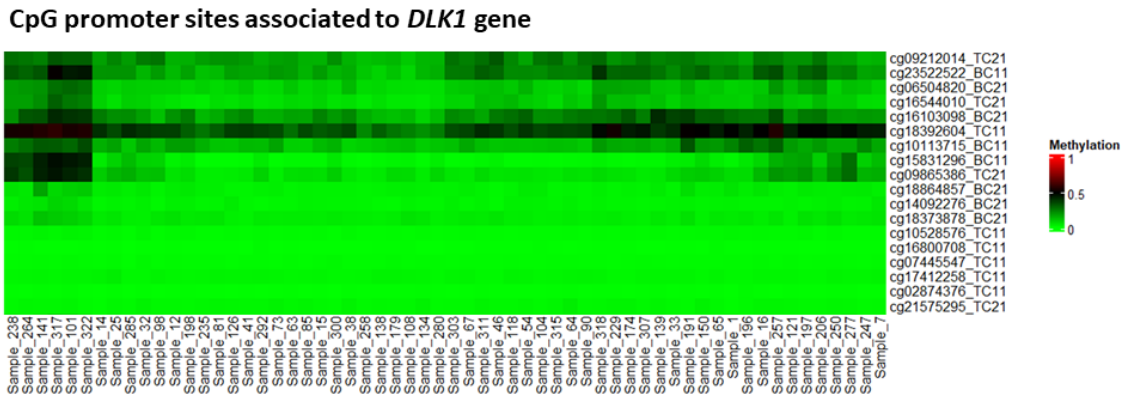
	Sperm telomere length (T/S ratio)			
	T1 (n=56)	**T2 (n=56)**	**T3 (n=56)**	**P-trend**
Sperm telomere length	[0.26 – 1.59]	[1.59 – 2.02]	[2.02 – 3.06]	
Sperm concentration (x10^6/ml)				
Crude model	0 (Ref.)	**1.11 (0.11, 2.12)**	**1.91 (0.91, 2.92)**	**<0.001**
Model 1	0 (Ref.)	**1.02 (0.01, 2.03)**	**1.85 (0.83, 2.86)**	**<0.001**
Model 2	0 (Ref.)	**1.09 (0.09, 2.09)**	**2.06 (1.04, 3.09)**	**<0.001**
Total sperm count (x10^6)				
Crude model	0 (Ref.)	1.35 (-0.41, 3.12)	**3.75 (1.98, 5.52)**	**<0.001**
Model 1	0 (Ref.)	1.11 (-0.61, 2.85)	**3.39 (1.65, 5.13)**	**<0.001**
Model 2	0 (Ref.)	1.12 (-0.58, 2.84)	**3.83 (2.08, 5.58)**	**<0.001**
Total motility (%)				
Crude model	0 (Ref.)	-0.67 (-6.33, 4.99)	-3.36 (-9.02, 2.30)	0.234
Model 1	0 (Ref.)	-0.63 (-6.34, 5.07)	-2.97 (-8.69, 2.75)	0.298
Model 2	0 (Ref.)	-0.07 (-5.77, 5.63)	-3.79 (-9.61, 2.00)	0.193
Progressive motility (%)				
Crude model	0 (Ref.)	-2.88 (-8.80, 3.02)	-6.25 (-12.16, -0.33)	**0.038**
Model 1	0 (Ref.)	-3.02 (-9.00, 2.94)	-5.92 (-11.91, 0.05)	0.052
Model 2	0 (Ref.)	-2.67 (-8.71, 3.36)	-6.06 (-12.21, 0.08)	0.052
Non-progressive motility (%)				
Crude model	0 (Ref.)	**3.18 (0.65, 5.72)**	**2.64 (0.11, 5.18)**	0.051
Model 1	0 (Ref.)	**3.35 (0.82, 5.89)**	**2.84 (0.30, 5.38)**	**0.037**
Model 2	0 (Ref.)	**3.48 (0.93, 6.03)**	2.31 (-0.28, 4.90)	0.091
No motility (%)				
Crude model	0 (Ref.)	-0.01 (-5.56, 5.55)	3.45 (-2.11, 9.01)	0.210
Model 1	0 (Ref.)	-0.04 (-5.63, 5.55)	2.98 (-2.61, 8.58)	0.281
Model 2	0 (Ref.)	-0.53 (-6.13, 5.05)	3.61 (-2.08, 9.30)	0.206
Vitality (%)				
Crude model	0 (Ref.)	2.36 (-1.55, 6.28)	-0.77 (-4.69, 3.14)	0.638
Model 1	0 (Ref.)	2.53 (-1.39, 6.46)	-0.39 (-4.33, 3.53)	0.778
Model 2	0 (Ref.)	2.24 (-1.66, 6.16)	-0.24 (-4.23, 3.74)	0.878
Normal sperm morphology (%)				
Crude model	0 (Ref.)	-0.18 (-0.60, 0.23)	**-0.46 (-0.87, -0.04)**	**0.030**
Model 1	0 (Ref.)	-0.18 (-0.6, 0.24)	**-0.43 (-0.64, -0.01)**	**0.045**
Model 2	0 (Ref.)	-0.24 (-0.66, 0.18)	-0.37 (-0.79, 0.05)	0.085

Model 1: adjusted for age (years) and body mass index (kg/m^2) and abstinence (days). Model 2: further adjusted for energy intake (kcal/day), physical activity (METs-min/week), smoking status (never, former, current), educational level (high school or less, college or high education). Sperm and leukocyte TL, total sperm count, concentration, and normal morphology were cubic root-transformed to approach normality.

Table 5. Multivariable-adjusted β coefficients and 95% CI of sperm parameters across tertiles of leukocyte telomere length.

	Leukocyte Telomere Length (T/S ratio)			
	T1 (n=65)	**T2 (n=65)**	**T3 (n=64)**	**P-trend**
Leukocyte telomere length	[0.64 – 1.10]	[1.10 –1.33]	[1.33 – 2.24]	
Sperm concentration (x10^6/ml)				
Crude model	0 (Ref.)	-0.36 (-1.39, 0.65)	**1.56 (0.51, 2.57)**	**0.002**
Model 1	0 (Ref.)	-0.41 (-1.43, 0.61)	**1.45 (0.42, 2.48)**	**0.004**
Model 2	0 (Ref.)	-0.35 (-1.33, 0.67)	**1.49 (0.44, 2.54)**	**0.004**
Total sperm count (x10^6)				
Crude model	0 (Ref.)	-0.40 (-2.25, 1.44)	**3.71 (1.85, 5.58)**	**<0.001**
Model 1	0 (Ref.)	-0.49 (-2.31, 1.33)	**3.39 (1.51, 5.22)**	**<0.001**
Model 2	0 (Ref.)	-0.43 (-2.18, 1.40)	**3.49 (1.62, 5.35)**	**<0.001**
Total motility (%)				
Crude model	0 (Ref.)	-0.34 (-6.04, 5.34)	3.39 (-2.32, 9.10)	0.224
Model 1	0 (Ref.)	-1.15 (-6.92, 4.61)	2.59 (-3.22, 8.38)	0.342
Model 2	0 (Ref.)	-0.95 (-6.67, 4.82)	2.32 (-3.64, 8.11)	0.423
Progressive motility (%)				
Crude model	0 (Ref.)	-1.69 (-7.51, 4.11)	2.27 (-3.56, 4.11)	0.405
Model 1	0 (Ref.)	-2.59 (-8.52, 3.30)	1.40 (-4.57, 7.34)	0.571
Model 2	0 (Ref.)	-2.37 (-8.16, 3.58)	1.33 (-4.72, 7.39)	0.609
Non-progressive motility (%)				
Crude model	0 (Ref.)	1.57 (-0.70, 3.90)	1.99 (-0.31, 4.29)	0.098
Model 1	0 (Ref.)	1.59 (-0.75, 3.87)	1.98 (-0.34, 4.31)	0.105
Model 2	0 (Ref.)	1.53 (-0.78, 3.86)	1.83 (-0.52, 4.20)	0.138
No motility (%)				
Crude model	0 (Ref.)	0.08 (-5.54, 5.72)	-3.49 (-9.45, 1.85)	0.171
Model 1	0 (Ref.)	0.97 (-4.77, 6.66)	-2.94 (-8.68, 2.78)	0.277
Model 2	0 (Ref.)	0.75 (-4.95, 6.46)	-2.69 (-8.51, 3.11)	0.333
Vitality (%)				
Crude model	0 (Ref.)	3.17 (-0.83, 7.18)	0.27 (-3.41, 4.29)	0.988
Model 1	0 (Ref.)	2.79 (-1.28, 6.86)	-0.13 (-4.22, 3.96)	0.843
Model 2	0 (Ref.)	2.82 (-1.26, 6.89)	0.44 (-3.69, 4.58)	0.919
Normal sperm morphology (%)				
Crude model	0 (Ref.)	0.09 (-0.21, 0.48)	0.10 (-0.28, 0.49)	0.597
Model 1	0 (Ref.)	0.07 (-0.32, 0.47)	0.09 (-0.30,0.49)	0.644
Model 2	0 (Ref.)	0.07 (-0.32,0.47)	0.15 (-0.25, 0.55)	0.465

Model 1: adjusted for age (years) and body mass index (kg/m^2) and abstinence (days). Model 2: further adjusted for energy intake (kcal/day), physical activity (METs-min/week), smoking status (never, former, current), educational level (high school or less, college or high education). Sperm and leukocyte TL, total sperm count, concentration, and normal morphology were cubic root-transformed to approach normality.



Supplementary Figure 1. Methylation analysis of the DLK1 locus for quality control of somatic cell contamination. CpG promoter sites associated to DLK1 gene are shown. These sites are differentially methylated between somatic and sperm cells. The methylation pattern showed no somatic cell contamination in the subsample analyzed.

VI. DISCUSSION

The present thesis was designed to study telomeres in two contexts. In the framework of the PREDIMED-Plus trial, this doctoral thesis aimed to provide evidence on the effect of an intensive lifestyle intervention on changes in TL measured in PBMCs and buffy coat. Secondly, it aimed to elucidate the potential role of TL in the context of male reproductive health by evaluating the associations between sperm and leukocyte TL and sperm quality in the context of the Led-Fertyl study.

Each chapter of this thesis includes a discussion section. However, a general discussion is provided to offer a summary of the findings, address additional points and, where available, present new evidence in the context of this dissertation.

1. General discussion

Effect of a lifestyle intervention on TL

A pilot study (**chapter 2**) was conducted to delve into the role of an intense lifestyle intervention on TL in an older Mediterranean population with overweight or obesity and MetS. After one year of follow-up, the 69 participants in both control and intervention groups experienced a positive change in TL, without an improvement in oxidative 8-OHdG plasma levels. No associations were observed between the intervention group or its interaction with time and changes in TL or 8-OHdG levels over 1 year.

Given that the common component of both PREDIMED-Plus groups was the MedDiet followed by the participants, it could be suggested a potential implication of this dietary pattern in the improvement of TL observed. As mentioned in the introduction section, several studies, including a meta-analysis of cross-sectional studies, have reported a beneficial association between healthy dietary patterns, with the MedDiet among them, and longer leukocyte TL[88,89,92]. In a recent analysis of the NHANES population, a higher adherence to a healthy plant-based diet was cross-sectionally associated with longer whole blood TL, whereas higher scores of an unhealthy plant-based diet were negatively associated with TL[197].

Although cross-sectional studies provide important evidence in relation to the relationship between diet and TL, RCTs incorporating MedDiet as a component of the intervention are needed to elucidate the effect of this dietary approach on telomere dynamics. However, the number of trials in this field is limited, and the results are somehow contrary to our findings since in the PREDIMED-Navarra group[198], the authors found no

differences in 5-year changes in leukocyte TL in the MedDiet enriched with extra virgin olive oil intervention group compared to the low-fat diet control group, whereas authors observed an accelerated telomere shortening in subjects following a MedDiet supplemented with nuts. To the best of our knowledge, no further RCTs have been conducted aimed at analyzing the effect of the MedDiet on TL. The discrepancies between the findings of this thesis and those of the PREDIMED-Navarra study may be attributed, at least in part, to differences in the characteristics of the study populations and the methodologies employed for the assessment of telomere length.

Even though the anticipated improvement in oxidative 8-OHdG plasma levels[199,200] was not observed, we should not dismiss the substantial antioxidant and anti-inflammatory potential of the phytochemical compounds in the MedDiet and their possible impact on telomere dynamics[201]. However, the mechanisms underlying the impact of this diet and its traditional food group consumption on TL need to be further explored. It has been observed that higher adherence to a MedDiet, compared with lower adherence scores, resulted in higher PBMCs telomerase activity, with MedDiet adherence positively associated with telomerase levels, as well as with longer TL[202]. In addition, the preliminary results from a 3-month interventional study aimed at improving lifestyle habits (e.g., diet, stress management, and behavioral support) showed an increase in telomerase activity measured in PBMCs[203]. Considering these findings, it could be hypothesized that the lengthening observed in this pilot study could be due to an activation of telomerase, although direct measurement of telomerase activity was not feasible in this study.

The second aim was to further investigate the potential effect of the PREDIMED-Plus intervention on TL, using a larger sample size and a longer follow-up period than in the previous pilot study. In **chapter 3**, a total of 317 non-smoking participants were included to evaluate the effect of the intervention on TL over a three-year follow-up period. It was observed that only women in the intervention group experienced an increase in TL after three years of lifestyle intervention, compared with women in the control group. Hence, it was observed that women in the intervention group had 83% lower odds of telomere shortening compared to women in the control group. In contrast, no significant changes in TL were observed in men, whose changes in TL did not differ between intervention groups, indicating a sex-specific response to the intervention.

The findings observed in women are in line with a comprehensive meta-analysis of interventional trials, which demonstrated the beneficial impact of the combination of physical activity and healthy diet intervention on leukocyte TL, compared to control groups[100]. The benefits of MedDiet adherence and exercise promotion have been demonstrated in other studies to yield similar results. Women in the exercise group who had higher adherence to this dietary pattern during pregnancy showed longer placental TL after birth relative to participants in the control group who had lower MedDiet adherence[204]. Although we included older adults, it is worth mentioning that in children with abdominal obesity following a moderate hypocaloric MedDiet, changes towards increasing physical activity (e.g., 30 minutes of light activity or moderate-to-vigorous exercise and 1000 daily step routine) were positively associated with increased TL after a 2-month intensive period[205].

However, our study reveals a sex-specific response, with women showing greater benefits from the intervention in comparison to men. Further research is required to explore the underlying biological mechanisms and to gain a deeper understanding of the reasons behind the more pronounced improvement observed in women. Cross-sectional evidence from PREDIMED-Navarra reported that in women, but not in men, greater MedDiet adherence was associated with longer telomeres, as well as with lower odds of having short telomeres[198]. PREDIMED-Plus-Valencia recently reported similar results only in women, but not in men, using a DNA methylation estimator of TL[206]. In addition, in cohort studies, it has been observed a slower TL attrition in women than in men[51–53]. This would be an advantage because it would leave telomeres in women to be more prone to be modified by environmental factors, in this case, by the PREDIMED-Plus intervention. Furthermore, we cannot discard other sex-related potential mechanisms involved, as those attributed to lifestyle and behavioral factors differing in men and women[207].

Associations between TL and sperm quality parameters

The systematic review and meta-analysis included in the introduction section demonstrated that individuals with impaired sperm quality parameters and infertile men had shorter sperm and leukocyte TL compared to fertile or normozoospermic participants (**chapter 1**). However, a significant limitation of the existing literature was identified, as the majority of the reviewed articles focused on men attending fertility clinics, with minimal or absent representation of the general population.

Therefore, taking advantage of the Led-Fertyl study, we addressed this gap by examining the cross-sectional associations between TL, measured in germ and somatic cells, and sperm quality parameters in 200 volunteer healthy men of reproductive age (**chapter 4**). The findings indicated that longer sperm and leukocyte TL were positively associated with higher sperm concentration and total count, independently of age, BMI, and other potential confounding factors.

Recent results have also reported shorter sperm and leukocyte TL in individuals with altered sperm quality[208–210]. These findings, in conjunction with the results included in the present thesis, suggest that sperm parameters are impaired when the mechanisms responsible for maintaining or elongating TL are compromised. This situation has been shown to be accompanied by processes such as increased oxidative species' concentration and the expression of pro-apoptotic markers, which could partially contribute to reduced sperm quality and, subsequently, male infertility[210].

Indeed, several studies have explored the implication of sperm TL on reproductive outcomes. However, the results were contradictory since the positive association between sperm TL and the percentage of fertilization rates found in one study [85] was not replicated by other authors[211,212], and the same occurred with embryological parameters [85,211]. A meta-analysis reported that longer sperm TL was associated with higher rates of clinical pregnancy[212].

Telomeres and associated protein complexes attach to the nuclear envelope, forming a cluster during the prophase I step in meiosis. This process could facilitate homologous chromosome alignment for recombination, which represents a crucial stage for meiosis and the proper development of germ cells[213]. Whether telomeres themselves are involved in these meiotic processes is still unknown. Spermatocytes from men with idiopathic infertility exhibited reduced meiotic crossover rates and an alteration in the association between the telomeres and the RNAs that maintain the integrity of the telomeric structure [214]. Thus, failed recombination, in combination with an alteration of telomeric homeostasis, could be a significant factor in reducing sperm quality and, consequently, increasing the risk of infertility. It appears that the physiological function of telomeres and the proteins responsible for maintaining their length and integrity are crucial for preventing segregation errors and ensuring proper spermatogenesis and sperm quality.

In summary, this thesis provides insights into the complex relationships between TL, lifestyle intervention, and reproductive quality as measured through sperm parameters. It highlights the necessity for further research to elucidate the mechanisms underlying these associations and to explore potential sex-specific responses and implications.

2. Strengths and limitations

The findings included in this doctoral thesis are subject to potential strengths and limitations that need to be highlighted and considered when interpreting the results.

In the context of the PREDIMED-Plus studies, the main strength is the RCT design that allowed us to explore changes in TL over one and three years of intervention, providing a high level of evidence of cause-effect. Besides, validated techniques were used to measure TL (Luminex technology and MMqPCR), thus ensuring specificity and precision. Lastly, thanks to the high number of variables and information collected for the PREDIMED-Plus trial, all models fitted were adjusted for several potential confounders. Among the limitations, our population included participants with overweight/obesity and high cardiometabolic risk, thus it is difficult to generalize these findings to other populations. A further limitation is that we did not have the availability of additional biological variables or parameters such as inflammatory-related or telomerase activity which might help to explain the underlying mechanisms behind the findings observed, specifically, the sex-differential effects on telomere changes in response to the intervention. Given these limitations, our results should be interpreted with caution.

In relation to the findings derived from the Led-Fertyl study, considerable limitations should be considered in the interpretation of the results. Firstly, the cross-sectional design of the study makes it impossible to establish a cause-effect relationship. Secondly, although it was a challenge to recruit 200 volunteers, the sample size cannot be considered large. The main strength of the Led-Fertyl study is the inclusion of a general population of reproductive age, consisting of young volunteers in good health state who did not attend fertility clinics. Secondly, the wide range of information collected on sociodemographic and lifestyle factors enabled us to control for several confounders in all analyses. However, it is important to note that residual confounding factors cannot be completely ruled out. Additionally, semen samples were processed using a standardized protocol through the CASA SCA® system, thereby reducing any potential subjectivity and increasing the reliability of the results.

VII. CONCLUSIONS

The conclusions from the three original research articles presented in this doctoral thesis are shown in this section in relation to the considered objectives.

Objective 1: To evaluate the effect of an intensive lifestyle intervention based on an erMedDiet, physical activity promotion, and behavioral support on TL and 8-hydroxy-deoxyguanosine (8-OHdG) levels after one year of follow-up, compared to an ad-libitum MedDiet, in non-diabetic Mediterranean older adults who had overweight or obesity, MetS and were at high risk of CVD.

- Healthy lifestyle interventions resulted in telomere lengthening after one year of follow-up with non-significant changes in plasma levels of 8-OHdG over the intervention period.
- Calorie restriction and exercise promotion did not provide an additional advantage for TL or 8-OHdG levels.

Objective 2: To investigate whether a lifestyle intervention consisting of an erMedDiet together with physical activity promotion and behavioral support might play a role in the prevention of telomere shortening in a non-smoking Mediterranean older population with overweight or obesity, MetS, and at high cardiovascular risk, compared to participants following a traditional MedDiet without weight-loss encouragement, in the context of a RCT.

- Women following an intensive lifestyle intervention with an erMedDiet and physical activity experienced a TL increase after 3 years of follow-up and had lower odds of telomere shortening compared to women following only traditional MedDiet recommendations.
- The intensive lifestyle intervention did not show a beneficial effect on TL in men after three years, indicating a sex-specific response to the intervention.

Objective 3: To assess the associations between sperm and leukocyte TL and sperm quality outcomes in 200 healthy volunteers of reproductive age from the general population.

- Sperm and leukocyte TL were positively associated with total sperm count and sperm concentration, but not with other sperm quality parameters. Therefore, TL may be explored in the future as a candidate to be a potential biomarker of sperm quality or infertility.

VIII. GLOBAL AND FUTURE INSIGHTS

This doctoral thesis provides new evidence on the significance of TL research in two important contexts: its response to lifestyle intervention and its relationship with male reproductive impairment as determined by sperm parameters. These findings can be of value in the context of aging, lifestyle modulation of TL, and reproductive health in terms of male infertility.

Given the potential implications of the findings presented in this thesis, it is essential to consider several key points for future research in the field of telomere science:

- **Long-Term and Diverse Studies**: Further long-term longitudinal studies, encompassing diverse populations across different age groups and from other geographical regions are clearly required in order to gain a more comprehensive understanding of this area of research. In particular, studies conducted in children, ideally followed during different life stages, would provide valuable insights into telomere dynamics throughout the lifespan.
- **Randomized Controlled Trials (RCTs):** It would be advantageous to conduct RCTs that include a control group that does not adhere to a particular diet to gain a deeper comprehension of the long-term impact of an intervention, particularly one based on the MedDiet, on telomeres.
- **Mechanistic Studies:** In this line, given the incomplete understanding of the mechanisms underlying the findings included in this thesis, further studies are warranted, specially to explain the different responses to the intervention observed between women and men. It would be beneficial to incorporate further determinations in future studies in the context of telomeres, such as the measurement of inflammatory and oxidative markers, hormonal status, telomerase activity, and the quantification of gene expression or levels of proteins associated with telomere homeostasis, among others.
- **Reproductive Health Research:** In light of the global increase in couples affected by infertility, there is a social and medical concern to identify the causes and mechanisms related to this disease. It is therefore important to further explore the potential implications of telomeres on reproductive health, fertilization, and further clinical outcomes.
- **Interventional Studies for Sperm Quality**: With regard to this topic, it would be advantageous to conduct further research into the potential benefits of interventional studies aimed at improving sperm quality parameters. In particular,

an investigation into the role of telomeres in the context of these interventions could prove invaluable in advancing our understanding of reproductive health.

- **Standardization of TL Measurement:** It would be important for the scientific community to adopt standardized methods for the measurement of TL. Such a standardization would facilitate the comparison of results from different studies.
- **Mendelian Randomization Studies**: The increasing trend in conducting Mendelian randomization studies is an encouraging development in the context of telomere research. Further investigation into the associations between the onset of several diseases related to telomere dysfunction and insights into the genetic factors associated with TL would be helpful.

The objective of these insights and recommendations is to provide guidance for future research efforts and to facilitate an enhanced understanding of the role of telomeres in health and disease.

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X. APPENDICES

1. Scientific contributions

Publications derived from the present thesis

Fernández de la Puente M, Hernández-Alonso P, Canudas S, Marti A, Fitó M, Razquin C, Salas-Salvadó J. ***Modulation of Telomere Length by Mediterranean Diet, Caloric Restriction, and Exercise: Results from PREDIMED-Plus Study.*** Antioxidants (Basel). 2021 Oct 12;10(10):1596. doi: 10.3390/antiox10101596. PMID: 34679731; PMCID: PMC8533372.

Marti A*, **Fernández de la Puente M***, Canudas S, Zalba G, Razquin C, Valle-Hita C, Fitó M, Martínez-González MÁ, García-Calzón S, Salas-Salvadó J. ***Effect of a 3-year lifestyle intervention on telomere length in participants from PREDIMED-Plus: A randomized trial.*** Clin Nutr. 2023 Sep;42(9):1581-1587. doi: 10.1016/j.clnu.2023.06.030. Epub 2023 Jul 10. PMID: 37478811. (*co-first authorship).

Fernández de la Puente M*, Salas-Huetos A*, Valle-Hita C, Babio N, Murphy MM, Canudas S, Salas-Salvadó J. ***Is telomere length a biomarker of sperm quality***? ***A systematic review and meta-analysis of observational studies.*** Andrology. 2024 Feb;12(2):277-288. doi: 10.1111/andr.13482. Epub 2023 Jun 25. PMID: 37328426. (*co-first authorship).

Fernández de la Puente M, Valle-Hita C, Salas-Huetos A, Martínez MÁ, Sánchez-Resino E, Canudas S, Torres-Oteros D, Relat J, Babio N, Salas-Salvadó J. ***Sperm and leukocyte telomere length are related to sperm quality parameters in healthy men from the Led-Fertyl study.*** Hum Reprod Open. 2024.

Other publications

Tresserra-Rimbau A, Castro-Barquero S, Becerra-Tomás N, Babio N, Martínez-González MÁ, Corella D, Fitó M, Romaguera D, Vioque J, Alonso-Gomez AM, Wärnberg J, Martínez JA, Serra-Majem L, Estruch R, Tinahones FJ, Lapetra J, Pintó X, Tur JA, López-Miranda J, Cano-Ibáñez N, Delgado-Rodríguez M, Matía-Martín P, Daimiel L, Martín Sánchez V, Vidal J, Vázquez C, Ros E, Basterra FJ, **Fernández de la Puente M**, Asensio EM, Castañer O, Bullón-Vela V, Tojal-Sierra L, Gómez-Gracia E, Cases-Pérez E, Konieczna J, García-Ríos A, Casañas-Quintana T, Bernal-Lopez MR, Santos-Lozano JM, Esteve-Luque V, Bouzas C, Vázquez-Ruiz Z, Palau-Galindo A, Barragan R, López Grau M, Razquín C, Goicolea-Güemez L, Toledo E, Vergaz MV, Lamuela-Raventós RM, Salas-Salvadó J. ***Adopting a High-***

Polyphenolic Diet Is Associated with an Improved Glucose Profile: Prospective Analysis within the PREDIMED-Plus Trial. Antioxidants (Basel). 2022 Feb 4;11(2):316. doi: 10.3390/antiox11020316. PMID: 35204199; PMCID: PMC8868059.

Marhuenda-Muñoz M, Domínguez-López I, Langohr K, Tresserra-Rimbau A, Martínez González MÁ, Salas-Salvadó J, Corella D, Zomeño MD, Martínez JA, Alonso-Gómez AM, Wärnberg J, Vioque J, Romaguera D, López-Miranda J, Estruch R, Tinahones FJ, Lapetra J, Serra-Majem L, Bueno-Cavanillas A, Tur JA, Martín-Sánchez V, Pintó X, Delgado-Rodríguez M, Matía-Martín P, Vidal J, Vázquez C, Daimiel L, Ros E, Toledo E, **Fernández de la Puente M**, Barragán R, Fitó M, Tojal-Sierra L, Gómez-Gracia E, Zazo JM, Morey M, García-Ríos A, Casas R, Gómez-Pérez AM, Santos-Lozano JM, Vázquez-Ruiz Z, Atzeni A, Asensio EM, Gili-Riu MM, Bullon V, Moreno-Rodriguez A, Lecea O, Babio N, Peñas Lopez F, Gómez Melis G, Lamuela-Raventós RM. ***Circulating carotenoids are associated with favorable lipid and fatty acid profiles in an older population at high cardiovascular risk***. Front Nutr. 2022 Sep 29;9:967967. doi: 10.3389/fnut.2022.967967. PMID: 36245542; PMCID: PMC9557191.

Hernández-Alonso P, Boughanem H, Canudas S, Becerra-Tomás N, **Fernández de la Puente M**, Babio N, Macias-Gonzalez M, Salas-Salvadó J. ***Circulating vitamin D levels and colorectal cancer risk: A meta-analysis and systematic review of case-control and prospective cohort studies.*** Crit Rev Food Sci Nutr. 2023;63(1):1-17. doi: 10.1080/10408398.2021.1939649. Epub 2021 Jul 5. PMID: 34224246.

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Álvarez-Álvarez L, Rubín-García M, Vitelli-Storelli F, García S, Bouzas C, Martínez-González MÁ, Corella D, Salas-Salvadó J, Malcampo M, Martínez JA, Alonso-Gómez ÁM, Wärnberg J, Vioque J, Romaguera D, López-Miranda J, Estruch R, Tinahones FJ, Lapetra J, Serra-Majem L, Bueno-Cavanillas A, García Fernández C, Pintó X, Delgado-Rodríguez M, Matía-Martín P, Vidal J, Vázquez C, Daimiel L, Ros E, García-Arellano A, Martínez MÁ, Sorlí JV, Zomeño MD, García-Rios A, González-Palacios S, Monserrat-Mesquida M, Abete I, Colom Fernández A, Casas R, Cano Ibáñez N, Ugarriza L, Bernal-

López MR, Bes-Rastrollo M, Paz-Graniel I, Asensio EM, Fitó M, Arenas Larriva AP, Oncina-Cánovas A, Vázquez Z, **Fernández de la Puente M**, Pérez-Vega A, Tur JA, Martín-Sánchez V. ***Effect of a nutritional intervention based on an energy-reduced Mediterranean diet on environmental impact***. Sci Total Environ. 2024 Jun 10;928:172610. doi: 10.1016/j.scitotenv.2024.172610. Epub 2024 Apr 19. PMID: 38642762.

Fernández de la Puente M*, Marti A*, Canudas S, Zalba G, Razquin C, Boccardi V, Mecocci P, Babio N, Castañer-Niño O, Toledo E, Buil-Cosiales P, Salas-Salvadó J, García-Calzón S. ***Telomere length and 4-year changes in cognitive function in an older Mediterranean population at high risk of cardiovascular disease.*** (*co-first authorship). Age and Ageing. 2024 August.

2. Participation in national and international congresses and symposiums

Conference: 38th International Symposium on Diabetes and Nutrition, virtual event, 21-24 June 2021.
Authors: Fernández de la Puente M, Hernández-Alonso P, Canudas S, Salas-Salvadó J.
Title: Telomere length and DNA oxidation modulation beyond Mediterranean diet: Results from the PREDIMED-Plus study.
Format: Oral Communication.

Conference: VI Workshop of the Institute for Research in Nutrition and Food Security and the University of Barcelona (*Institut de Recerca en Nutrició i Seguretat Alimentària de la Universitat de Barcelona - INSA·UB*), Barcelona (Spain), 9 February 2022.
Authors: Fernández de la Puente M, Hernández-Alonso P, Canudas S, Marti A, Fitó M, Razquin C and Salas-Salvadó J.
Title: Modulation of Telomere Length by Mediterranean Diet, Caloric Restriction, and Exercise: Results from PREDIMED-Plus Study
Format: Oral Communication.

Conference: XVIII Biology of Reproduction Symposium, Barcelona (Spain), 24 March 2023.
Authors: Fernández de la Puente M, Hernández-Alonso P, Canudas S, Marti A, Fitó M, Razquin C and Salas-Salvadó J.

Title: Is telomere length a biomarker of semen quality? A systematic review and meta-analysis of observational studies.
Format: Oral Communication.

Conference: 40th International Symposium on Diabetes and Nutrition, Pula (Croatia), 15-18 June 2023.
Authors: Fernández de la Puente M, Marti A, Canudas S, Zalba G, Razquin C, Valle-Hita C, Fitó M, Martínez-González MÁ, García-Calzón S, Salas-Salvadó J.
Title: Effect of a 3-year lifestyle intervention on telomere length in an older Mediterranean population at cardiovascular risk.
Format: Oral Communication.

Conference: First PhD DAY of the Pere i Virgili Institute for Health Research and the Rovira i Virgili University *(Institut d'Investigació Sanitària Pere Virgili y Universitat Rovira i Virgili - IISPV·URV)*, Reus (Spain), 22 June 2023.
Authors: Fernández de la Puente M, Salas-Huetos A, Valle-Hita C, Babio N, Murphy MM, Canudas S, Salas-Salvadó J.
Title: Is telomere length a biomarker of sperm quality? A systematic review and meta-analysis of observational studies.
Format: Short Oral Communication.

Conference: Second PhD DAY of the Pere i Virgili Institute for Health Research and the Rovira i Virgili University *(Institut d'Investigació Sanitària Pere Virgili y Universitat Rovira i Virgili - IISPV·URV)*, Reus (Spain), 6 June 2024.
Authors: Fernández de la Puente M, Marti A, Canudas S, Zalba G, Razquin C, Boccardi V, Mecocci P, Babio N, Castañer-Niño O, Toledo E, Buil-Cosiales P, Salas-Salvadó J, García-Calzón S.
Title: Telomere length and 4-year changes in cognitive function in an older Mediterranean population at high risk of cardiovascular disease.
Format: Poster.

Conference: XV Symposium of the Pathophysiology of Obesity and Nutrition Consortium (*CIBER Fisiopatología de la Obesidad y Nutrición*), Zaragoza (Spain), 11 - 12 June 2024.

Authors: Fernández de la Puente M, Marti A, Canudas S, Zalba G, Razquin C, Boccardi V, Mecocci P, Babio N, Castañer-Niño O, Toledo E, Buil-Cosiales P, Salas-Salvadó J, García-Calzón S.
Title: Telomere length and 4-year changes in cognitive function in an older Mediterranean population at high risk of cardiovascular disease.
Format: Poster.

Conference: 41st International Symposium on Diabetes and Nutrition, Uppsala (Sweden), 15-18 June 2023.
Authors: Fernández de la Puente M, Marti A, Canudas S, Zalba G, Razquin C, Boccardi V, Mecocci P, Babio N, Castañer-Niño O, Toledo E, Buil-Cosiales P, Salas-Salvadó J, García-Calzón S.
Title: Telomere length and 4-year changes in cognitive function in an older Mediterranean population at high risk of cardiovascular disease.
Format: Short oral Communication.

3. Awards

Young Investigator Award to the best Oral Communication in the 38th International Symposium on Diabetes and Nutrition (June 2021).

First Award to the best Short Oral Communication in the first PhD DAY of the Pere i Virgili Institute for Health Research and the Rovira i Virgili University *(Institut d'Investigació Sanitària Pere Virgili y Universitat Rovira i Virgili - IISPV·URV)* (June 2023).

M. del Carmen de la Torre Boronat Award from the Catalan Association of Food Sciences (*Associació Catalana de Ciències de l'Alimentació)* for the best published Scientific Article – PhD student category (April 2024).

First Award to the best Poster in the second PhD DAY of the Pere i Virgili Institute for Health Research and the Rovira i Virgili University *(Institut d'Investigació Sanitària Pere Virgili y Universitat Rovira i Virgili - IISPV·URV)* (June 2024).

4. National and international mobilities

National mobilities

Length: three days (13, 14, 15 December 2021).

Institution: Faculty of Pharmacy and Nutrition, Department of Food Sciences and Physiology, University of Navarra.

Supervisor: Prof. Amelia Marti del Moral.

Objective: To learn the technique of telomere length analysis by polymerase chain reaction in PREDIMED-Plus samples.

Length: five days (15-19 May 2023).

Institution: Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona.

Supervisor: Dr. Marc Yeste Oliveras.

Objective: To analyze the DNA fragmentation of sperm samples from the Led-Fertyl study.

International mobility

Length: three months (September - December 2023).

Institution: Section of Gerontology and Geriatrics, Department of Medicine and Surgery, University of Perugia.

Supervisor: Prof. Patrizia Mecocci.

Objective: To analyze the longitudinal associations between telomere length and cognitive function in participants from PREDIMED-Plus.