Universidad de Navarra

Facultad de Farmacia

TELOMERE LENGTH IN DIFFERENT SPANISH AGE GROUPS: ASSOCIATION WITH DIET, GENETICS AND ADIPOSITY TRAITS

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Memoria presentada por Dña. **Sonia García Calzón** para aspirar al grado de Doctor por la Universidad de Navarra.

Dña. Sonia García Calzón

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Ciencias de la Alimentación y Fisiología y autorizo su presentación ante el tribunal que lo ha de juzgar.

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Telomere length in different Spanish age groups: association with diet, genetics and adiposity traits

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¡Muchas gracias a todos!

A mis padres

LIST OF ABBREVIATIONS

ALT	Alternative lengthening of telomeres
BMI	Body mass index
bp	Base pairs
CAD	Coronary artery disease
CHO	Carbohydrates
CI	Confidence Interval
Ct	Threshold cycle
CV	Coefficient of variation
CVD	Cardiovascular disease
EVOO	Extra virgin olive oil
FFQ	Food Frequency Questionnaire
GENOI	Grupo de Estudio Navarro de la Obesidad Infantil
GWAS	Genome-wide association studies
HPC	Hematopoietic progenitor cells
HSC	Hematopoietic stem cells
MeDiet	Mediterranean Diet
MUFA	Monounsaturated fatty acids
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PREDIMED	Prevención con Dieta Mediterránea
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species

RT-PCR	Real-time PCR
SNPs	Single nucleotide polymorphisms
T2D	Type 2 Diabetes
TAC	Total antioxidant capacity
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TL	Telomere length
T/S ratio	Telomere to single copy-gene ratio
WC	Waist circumference
WHtR	Waist to height ratio

LIST OF PUBLICATIONS

I. **García-Calzón S**, Moleres A, Martínez-González MA, Martínez JA, Zalba G, Marti A. Dietary total antioxidant capacity is associated with leukocyte telomere length in a children and adolescent population. Clin Nutr. 2014; doi: 10.1016/j.clnu.2014.07.015.

II. **García-Calzón S**, Martínez-González MA, Razquin C, Corella D, Salas-Salvadó J, Martínez JA, Zalba G, Marti A. The Pro12Ala polymorphism of the *PPARγ2* gene interacts with a Mediterranean Diet to prevent telomere shortening in the PREDIMED-NAVARRA randomized trial. Circ Cardiovasc Genet. 2014; doi:10.1161/CIRCGENETICS.114.000635.

III. **García-Calzón S**, Moleres A, Marcos A, Campoy C, Moreno LA, Azcona-Sanjulián MC, Martínez-González MA, Martínez JA, Zalba G, Marti A. Telomere length as a biomarker for adiposity changes after a multidisciplinary intervention in overweight/obese adolescents: the EVASYON study. PLoS One. 2014; 24: e89828.

IV. **García-Calzón S**, Gea A, Razquin C, Corella D, Lamuela-Raventós RM, Martínez JA, Martínez-González MA, Zalba G, Marti A. Longitudinal association of telomere length and obesity indices in an intervention study with a Mediterranean diet: The PREDIMED-NAVARRA trial. Int J Obes (Lond). 2014; 38:177-182.

ABSTRACT

Telomeres are nucleoprotein structures that, together with shelterin proteins, protect the end of chromosomes maintaining genome stability. Telomere length (TL) has been proposed as a biomarker of biological aging processes because telomeres shorten within each cell division. But the fact that TL varies considerably among individuals of the same chronological age implies the need to investigate potential lifestyle and genetic aspects that influence TL. In addition, TL has also been considered a general risk factor for age-related chronic diseases, such as cancer, type 2 diabetes or cardiovascular disease. Obesity is a common risk factor for increased morbidity and mortality, contributing to the incidence of aging-related pathologies. Since obesity and shortened telomeres are associated with the increased risk of metabolic disorders, we wondered whether TL may be related to adiposity. In fact, some cross-sectional studies reported shortened telomeres in obese subjects, considering inflammation and oxidative stress as the possible connexion between them. However, little is known about the longitudinal association between TL and adiposity.

Therefore, the aims of this thesis were: 1) to assess the association between dietary factors, particularly dietary total antioxidant capacity (TAC), and TL in children and adolescents; 2) to evaluate the genetic variant Pro12Ala (rs1801282) of the *PPARγ2* gene on TL, as well as possible gene-diet interactions in subjects at high cardiovascular risk, and 3) to study the relationship between TL and changes in adiposity after a follow-up period in an adolescent and adult population.

In **Chapter 1**, the effect of macronutrients, food groups and dietary TAC on leukocyte TL was examined in 287 children and adolescents (6-18 years) from the GENOI study. This cross-sectional study found that higher dietary TAC intake and lower white bread consumption were associated with longer telomeres. In **Chapter 2**, high cardiovascular risk individuals carrying the Pro12Ala polymorphism of the *PPARγ2* gene displayed a lower rate of telomere shortening after a 5-year nutritional intervention in the frame of the PREDIMED-NAVARRA trial. Furthermore, a potent gene-diet interaction was observed since a higher adherence to a Mediterranean dietary pattern strengthens the prevention of telomere shortening among Ala carriers. In **Chapter 3,** the focus was analysing TL and adiposity changes in a subsample of 74

obese adolescents (12-16 years) from the EVASYON study, after a multidisciplinary weight loss intervention. A 2-month intensive treatment led to an increased leukocyte TL in 88% of the adolescents. Besides, higher baseline TL predicted a better weight loss response after the 2-month intensive period and also after a 6-month follow-up period, but only among boys, suggesting a modification effect by sex at puberty. In **Chapter 4**, leukocyte TL and adiposity parameters were evaluated after a long-term dietary intervention in 521 subjects (55-80 years) participating in the PREDIMED-NAVARRA trial. A decrease in obesity risk was linked to an increased TL after 5 years of a nutritional intervention. Moreover, those subjects presenting longer telomeres at baseline reported a better improvement of obesity traits after the 5-year period of dietary intervention.

In summary, the results presented in this thesis have suggested a potential role of an antioxidant rich diet as well as of the gene variant Pro12Ala in maintaining TL. Moreover, an inverse longitudinal relationship between TL and changes in adiposity in two different age groups, high cardiovascular risk adults and obese adolescents, was observed after lifestyle interventions. Notably, a higher baseline TL was associated with a better improvement in anthropometric parameters, proposing TL as a biomarker of adiposity. Further research is necessary to better understand TL homeostasis in metabolic diseases, and to identify potential factors that could modulate age-dependent telomere erosion.

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I. Introduction

The story of telomeres began in the 1930s with the observation that natural chromosome ends had special properties that protected them from end to end fusion (McClintock, 1939). But it was not until 1970s when Elizabeth Blackburn and Joseph Gall noticed that chromosome ends from *Tetrahymena thermophila* consisted of a simple sequence of hexameric TTAGGG repeats protecting the chromosomes from degradation (Blackburn and Gall, 1978). Afterwards, Blackburn and Jack Szostak made the incredible discovery that the presence of this sequence, named telomeric DNA repeats, was conserved throughout evolution and that a common mechanism might exist in eukaryotes for their maintenance (Szostak and Blackburn, 1982). Soon thereafter, in 1985, Blackburn and Carol Greider identified an enzymatic activity capable of extending telomeric sequences (Greider and Blackburn, 1985). All this magnificent research was recognized by the 2009 Nobel Prize to these brilliant researchers for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase.

1. Structure and function of telomeres

Telomeres (from the Greek *telos* for end and *meros* for part) are special structures at the ends of the chromosomes. Telomeric DNA is a type of noncoding DNA that ensures the stable inheritance of the genetic material by protecting chromosomes (Blackburn, 2010) (**Figure 1**).

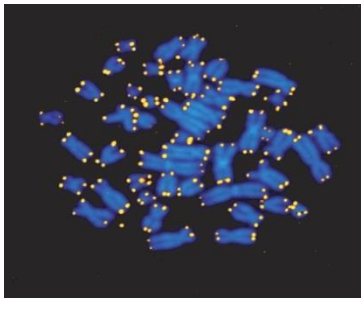

Figure 1. Telomeres capping the ends of chromosomes. Telomere repeats in a normal human lymphocyte are visualized in yellow using quantitative fluorescence in situ hybridization. Source: Aubert and Lansdorp, 2008.

Each species has a characteristic telomeric repeat common to protect the ends of chromosomes. In mammalian cells, telomere DNA contains double-stranded tandem repeats of TTAGGG followed by terminal 3′ G-rich single-stranded overhangs.

Specifically, in humans, telomeres are generally in the 4-14 kb length range (Lu *et al.*, 2013).

Due to the essential nature of telomeres in protecting the genome, a number of unique proteins have evolved to ensure that telomere length (TL) and structure are preserved. Therefore, telomere DNA is thought to adopt the t-loop structure which is proposed to mask the chromosome end from DNA damage. To generate the t-loop, the G-overhang is proposed to strand-invade into a region of the telomeric dsDNA to form a displacement-loop (D-loop). The t-loop is stabilized by shelterin complexes, which in mammals are composed of six protein subunits: TRF1, TRF2, TIN2, RAP1, TPP1, and POT1 (de Lange, 2005) (**Figure 2**).

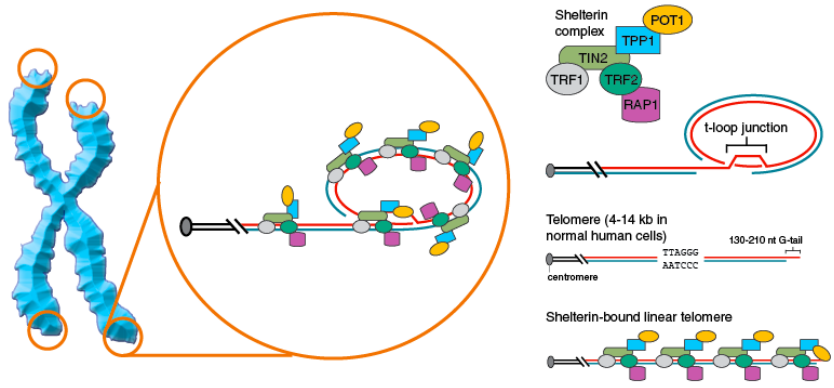

Figure 2. Structure of telomere: telomere forming a loop. Adapted from: Cesare and Reddel, 2010.

Telomeres provide a solution to the end-replication problem: the regular DNA replication machinery is unable to fully replicate the chromosomal ends; as a consequence information is lost with each cell division, eventually resulting in senescence and cell death (Nelson and Shippen, 2012). Highly proliferative cells, such as mammalian embryonic cells, solve this problem by expressing telomerase (Hiyama and Hiyama, 2007). The telomerase is a unique ribonucleoprotein complex that consists of the telomerase reverse transcriptase (TERT), and a telomerase RNA

component (TERC) that serves as the template for telomere extension during *de novo* addition of TTAGGG repeats into chromosome ends (Lu *et al.*, 2013) (**Figure 3**).

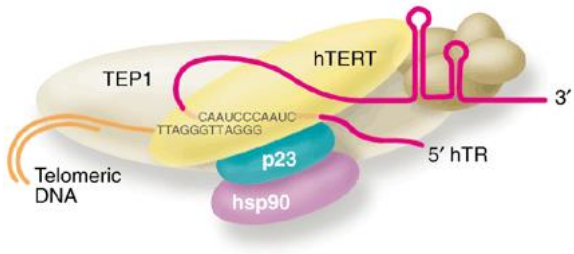

Figure 3. The human telomerase complex. The RNA component of telomerase contains a template region that directly binds to the G-rich overhang of telomeres at the ends of chromosomes. Adapted from: Shay and Wright, 1999.

2. Factors associated with telomere length

2.1. Socio-demographic factors

2.1.1. Age

Several studies have focused on TL as a molecular tool for estimating cellular aging (Saeed *et al.*, 2012; Muezzinler *et al.*, 2013; Zhang *et al.*, 2014). Thus, TL has been proposed as a good biomarker of biological aging.

In 1990, Harley *et al.* (Harley *et al.*, 1990) showed that the amount and length of telomeric DNA in human fibroblasts decreased during aging *in vitro* and possibly *in vivo*. For the first time, they showed that telomeres shorten with each cell division in somatic human cells, but not in immortal tumour cell cultures. This discovery led to the suggestion that telomeres might act as a mitotic clock (von Zglinicki, 2000). In fact, during each cell division, DNA loses telomeric repeats in somatic tissues with an estimated shortening rate of 25 base pairs (bp) per year, eventually causing replicative cell senescence as an ultimate DNA damage checkpoint (Muezzinler *et al.*, 2013). This shortening was thought to be due to the so-called end-replication problem, the inability of DNA polymerases to replicate the 3′-end of a linear DNA molecule to its very end (Olovnikov, 1971). At a certain critical short length, the cell does not divide further and enters senescence (Nitta *et al.*, 2011) (**Figure 4**).

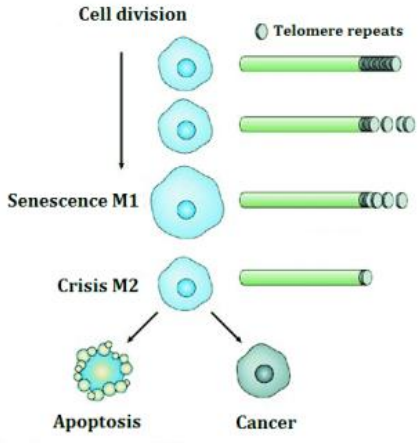

Figure 4. Telomeres as mitotic clocks. Adapted from: Shay and Wright, 2006.

The hypothesis of telomeres involvement in replicative senescence became more evident when it was demonstrated that ectopic expression of telomerase would lead to elongation of telomeres followed by immortalization of some human cell clones (Bodnar *et al.*, 1998). In most human somatic cells, except for stem cells and lymphocytes, telomerase activity is diminished after birth, so that TL shortens with each cell division (**Figure 5**). The longest telomeres are found in germline cells (mean TL about 16 kb), whereas mean TL in peripheral blood lymphocytes of older subjects or in senescent fibroblast cultures is about 5–6 kb (von Zglinicki, 2000). As stem cells, and especially progenitor cells have elongated proliferative capacity, these types of cells should have a mechanism that maintains TL through many cell divisions (Hiyama and Hiyama, 2007). Thus, these specialized cells avoid senescence by activating the telomerase complex (Hiyama and Hiyama, 2007).

Several theories explained that replicative senescence is driven by shortening and dysfunction of telomeres (Allsopp, 1996). In fact, age-dependent loss of telomeric DNA has been observed in both lymphocytes and neutrophils (Vaziri *et al.*, 1993), as well as in other tissues (Hiyama *et al.*, 1996). Therefore, TL may be the first genetic biomarker that has been correlated to cell replication and replicative cell senescence, both strong indicators of tissue aging in humans.

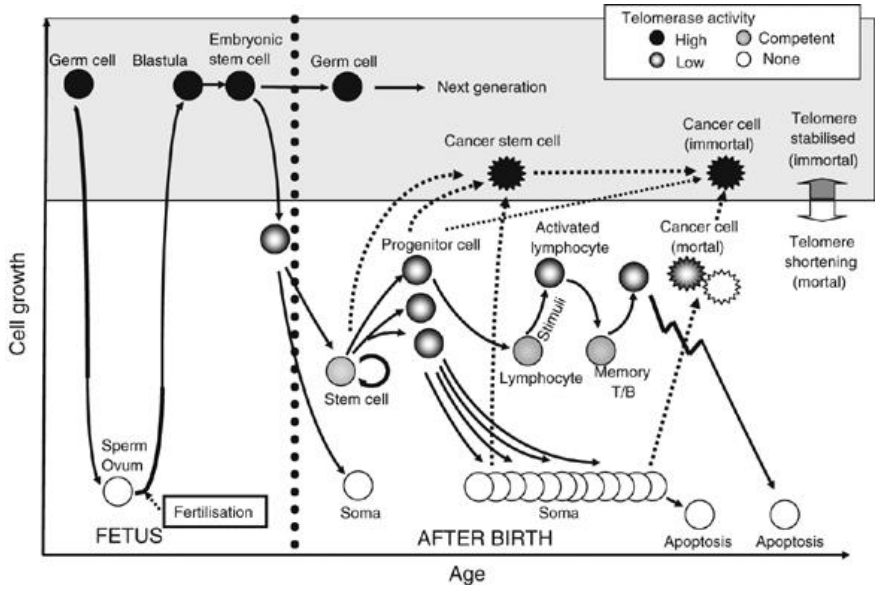

Figure 5. Telomere and telomerase dynamics in human stem cells. Germ cells have high levels of telomerase activity during rapid proliferation. In the developmental stage, telomerase activity gradually decreases and diminishes in most somatic cells after birth. In adult stem cells, the level of telomerase activity is low or undetectable, and upregulated in committed progenitor cells. Source: Hiyama and Hiyama, 2007.

A review on genetic markers for predicting biological age found that telomeres remain the most promising biomarker for age estimation since the mechanisms for telomere repeat attrition over time have been well documented (Saeed *et al.*, 2012). In agreement, a systematic review involving 124 cross-sectional and 5 longitudinal studies showed that the inverse relationship between age and leukocyte TL in adults was a very consistent observation in the literature (Muezzinler *et al.*, 2013). Interestingly, a recent study used the factor analysis method to build a biological age equation based on TL and chronological age, together with a number of indices, such as genetic index, renal function indices, cardiovascular function indices, brain function indices, and oxidative stress and inflammation indices (Zhang *et al.*, 2014). They suggested that TL and chronological age could be used as a new method to build a biological age equation.

In contrast, there are some studies reporting that TL is not a good biomarker of aging, but its association with cellular senescence remains unequivocal. Thus, a

review concluded that TL is implicated in cellular aging, but it is not a biomarker of aging in humans since it did not fully meet the criteria of the American Federation of Aging Research (Mather *et al.*, 2011). Briefly, these criteria include that a well-defined biomarker of aging must predict the rate of aging, being a good predictor of lifespan; it must monitor a basic process that underlies the aging process, not the effects of disease; it must be able to be tested repeatedly without harming the person; and it must be something that works in humans and in laboratory animals, such as mice (Johnson, 2006; Simm *et al.*, 2008). Similarly, Der *et al.* (Der *et al.*, 2012) published that TL did not satisfy the strict criteria for a biomarker of aging, but did add predictive power to that of chronological age. A meta-analysis, including 16 eligible studies comprising 10,157 individuals, reported that leukocyte TL behaved as a biomarker of somatic redundancy, the body's capacity to absorb damage, rather than biological age (Boonekamp *et al.*, 2013).

On the whole, evidence led to the fact that TL is a marker of biological aging, since we cannot ignore that average TL declines with age. This is further supported by research using the powerful twin model which clearly found that the co-twins with the shorter leukocyte TL were more likely to die first (Bakaysa *et al.*, 2007; Kimura *et al.*, 2008). In addition, telomeres are found to be shorter in adult men than women due to the faster telomere shortening rate in men, confirming the well-known sex difference in life expectancy (Bekaert *et al.*, 2007). These observations settle the proposition of leukocyte TL as a biomarker of human aging.

2.1.2. Sex

Cross-sectional and longitudinal studies reported that telomeres seemed to be longer in women than in men. A meta-analysis from 36 cohorts (36,230 participants) showed that on average females had longer telomeres than males, but this difference was not universally found in studies that did not use Southern blot methods (Gardner *et al.*, 2014). Several hypotheses have been postulated to explain this fact. It could be the action of the oestrogen that might stimulate telomerase or the circumstance that women produce fewer reactive oxygen species (ROS) than men presenting a lower accumulation of oxidative stress (Nawrot *et al.*, 2004). Furthermore, oestrogen

presents antioxidant properties that could help women in the metabolism of ROS (Nawrot *et al.*, 2004). In fact, telomere attrition rate was reported to be faster in men than women, leading to shorter telomeres (Bekaert *et al.*, 2007).

2.2. Genetic factors

2.2.1. Heritability and paternal age at conception

TL is strongly influenced by genetic factors with previous studies reporting heritability estimates ranging from 36 to 84% (Aviv, 2012). Studies in twins, siblings and families reported a greater paternal than maternal TL heritability (Nordfjall *et al.*, 2005; Njajou *et al.*, 2007; Nordfjall *et al.*, 2010), meanwhile Nawrot *et al*. (Nawrot *et al.*, 2004) observed an X-linked inheritance of leukocyte TL. Conversely, another study found no evidence of heritable effects in an elderly male twins' population (Huda *et al.*, 2007), probably because the samples were not randomized (Aviv, 2012). Interestingly, these previous works were clarified by a recent pivotal meta-analysis study considering 19,713 subjects, that revealed a high consistent heritability estimate for TL (70%) but supporting both paternal and maternal inheritance (Broer *et al.*, 2013). Unexpectedly, this latter meta-analysis showed a more robust evidence for a maternal inheritance component after adjustment for several environmental factors. Both maternal and paternal inheritability point out to the fact that TL implies the possibility of direct, non-genetic TL transmission through the germ cells, which it is considered to be an epigenetic-like mechanism (De Meyer *et al.*, 2014).

Several studies have also suggested that paternal age is an important determinant for TL, since an older paternal age at conception was associated with a longer leukocyte TL in the offspring (De Meyer *et al.*, 2007; Prescott *et al.*, 2012; Broer *et al.*, 2013). A possible explanation could be that there is a progressive age-dependent elongation of TL in the male germ-line due to the difference in telomerase activity between somatic cells and male germ-line cells (Aviv and Susser, 2013). This indicates that a subject's TL reflects the TL of his/her parents' specific germ cells, at least partially (De Meyer *et al.*, 2014). Nevertheless, more examination of the prominent sex-specific parental effects could shed new light on genetic determinants of TL.

2.2.2. Genetic variants

Genome-wide association studies (GWAS) have also reported genes and genetic variants that are known to be involved in telomere maintenance (Codd *et al.*, 2013; Lee *et al.*, 2013). Indeed, the impact of telomere pathway gene variants on TL has been studied, specifically, those single nucleotide polymorphisms (SNPs) encoding telomerase subunits. So far, common variants in the *TERC* and *TERT* locus were reported to be associated with TL and longevity in GWAS studies (Bojesen *et al.*, 2013; Codd *et al.*, 2013). A recent meta-analysis, enrolling 37,684 individuals, with replication of selected variants in additional 10,739 individuals, identified seven loci associated with mean leukocyte TL (Codd *et al.*, 2013) (**Figure 6**). Five of the loci contain candidate genes (*TERC, TERT, NAF1, OBFC1* and *RTEL1)* that are known to be involved in telomere biology. Of note, it has been identified strong evidence for novel genetic loci affecting variation in leukocyte TL on chromosomes 13q12, 18q22.2 and 3p14.1 in American Indians who suffer from high rates of diabetes and cardiovascular disease (CVD) (Zhu *et al.*, 2013).

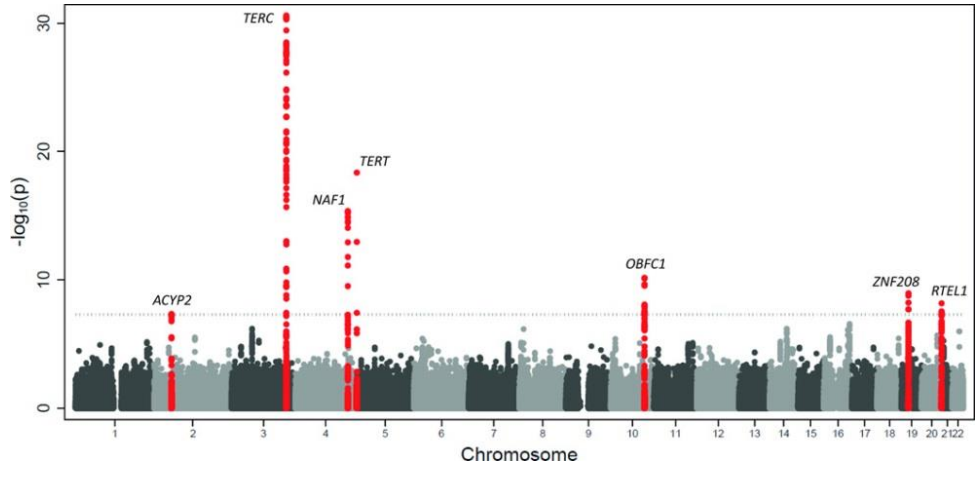

Figure 6. Signal intensity plot of genotype association with telomere length. Data is displayed as –log10P values against chromosomal location for the 2,362,330SNPs that were tested. The dotted line represents a genome-wide level of significance at *P*=5x10−8. The 7 loci that showed an association at this level are plotted in red. Source: Codd *et al.*, 2013.

However, SNPs which do not contain candidate genes of telomere pathways have also been associated with leukocyte TL. These genes were related to several cancers and other diseases (Codd *et al.*, 2013; Lee *et al.*, 2013). In fact, findings suggested that TL regulation could be modified by gene variants in cancer susceptibility genes (Burke *et al.*, 2013). Regarding CVD, homozygocity for the D allele in the angiotensin converting enzyme gene was associated with shorter leukocyte TL and this polymorphism appears to strengthen the association of TL with increased cardiovascular risk (Fyhrquist *et al.*, 2013). On the contrary, in the Nurses' Health Study no association between TL and genetic predisposition (a genetic score including 68 gene variants) to higher body mass index (BMI) or type 2 diabetes (T2D) was found (Du *et al.*, 2013).

2.3. Environmental factors

Genetic factors are not sufficient to explain TL, and the impact of lifestyle factors needs to be evaluated. It is well-known that telomere attrition is likely to be a modifiable factor as there is substantial variability in the rate of telomere shortening that is independent of chronological age (Aviv *et al.*, 2009) (**Figure 7**).

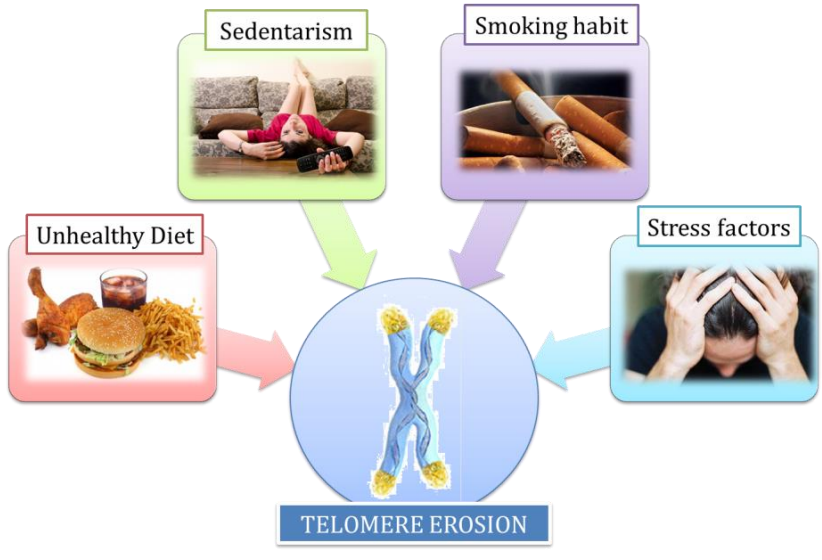

Figure 7. Environmental factors including behavioral and psychosocial aspects that can lead to telomere shortening.

There has been growing evidence that lifestyle factors may affect the health and lifespan of an individual by affecting TL. In this sense, Sun et al. (Sun *et al.*, 2012) observed that an adherence to a healthy lifestyle, defined by 5 modifiable factors (smoking, physical activity, adiposity, alcohol intake and diet), was associated with longer TL in U.S women. Similarly, another study found the same relationship in U.S men but defining the healthy lifestyle by 4 low-risk factors (low or no smoking, higher intake of fruit and vegetables, lower BMI and higher levels of physical activity) (Mirabello *et al.*, 2009). These factors are explained in detail below.

2.3.1. Behavioural factors

2.3.1.1. Diet

What we eat and how much we eat can significantly affect our telomeres, health, and longevity. Some studies described no association between energy intake and TL (Nettleton *et al.*, 2008; Cassidy *et al.*, 2010; Chan *et al.*, 2010) in middle-aged and elderly subjects, whereas other studies conducted in young adults showed an inverse relationship (Kark *et al.*, 2012). A possible explanation for this discrepancy might be that the attrition of TL is greatest in humans during the first years of life, when growth is most rapid (Sidorov *et al.*, 2009).

Since an *in vitro* study showed that vitamin C slows down telomere attrition in a human endothelial cell line (Furumoto *et al.*, 1998), several human studies have investigated the effect of dietary components on TL. A high consumption of fruit, vegetables (Diaz *et al.*, 2010; Tiainen *et al.*, 2012) or dietary fibre (Cassidy *et al.*, 2010) have been correlated with longer telomeres, whereas an increase intake in processed meat (Nettleton *et al.*, 2008) or alcohol (Strandberg *et al.*, 2012; Sun *et al.*, 2012) may lead to shortening the telomere. Interestingly, there are some articles in the literature reporting an inverse association between total fat or specific fatty acids, such as polyunsaturated fatty acids (PUFA) (Cassidy *et al.*, 2010; Kark *et al.*, 2012) and saturated fatty acids (Tiainen *et al.*, 2012; Song *et al.*, 2013), and TL. A small number of studies provide initial evidence that a higher intake in omega-3 fatty acids may be beneficial for slowing down the rate of telomere shortening (Farzaneh-Far *et al.*, 2010;

Kiecolt-Glaser *et al.*, 2013; O'Callaghan *et al.*, 2014). In relation to monounsaturated fatty acids (MUFA), one recent study showed no association with TL (Song *et al.*, 2013).

- *Mediterranean Diet*

The traditional Mediterranean diet (MeDiet) is the dietary pattern prevailing among the people of the olive tree-growing areas of the Mediterranean basin before the mid-1960s (Trichopoulou *et al.*, 2014). It is characterized by a high intake of fruit and nuts, vegetables, legumes, cereals, fish and seafood; a low intake of dairy products, meat and meat products; and a moderate ethanol intake mainly in the form of wine and during meals (Naska and Trichopoulou, 2014).

There is little information on the protective role of a Mediterranean dietary pattern on TL. The MeDiet has been widely considered as a model of healthy eating (Willett *et al.*, 1995). Numerous epidemiological studies have explored the health benefits of the MeDiet showing that individuals who adhere to this dietary pattern have healthier aging and a longer lifespan, including a better cardiovascular risk profile (Trichopoulou *et al.*, 2003; Buckland *et al.*, 2011; Esposito *et al.*, 2011; Estruch *et al.*, 2013). The MeDiet has a high content of total fat, as its most distinguishing feature, due to the high intake of olive oil and nuts. The beneficial effects of olive oil, especially extra virgin olive oil (EVOO), are linked to antioxidant, anti-inflammatory and anti-microbial activities (Martin-Pelaez *et al.*, 2013), and also its important cardio-protective effect (Martinez-Gonzalez and Sanchez-Villegas, 2004; Psaltopoulou *et al.*, 2004). In this sense, prospective studies have shown that the adherence to a typical MeDiet with a high intake in olive oil is associated with lower mortality and increased longevity (Trichopoulou and Dilis, 2007; Roman *et al.*, 2008).

The MeDiet pattern has been widely considered as a model of healthy eating and just three studies have shown its protective role on TL (Marin *et al.*, 2012; Boccardi *et al.*, 2013). Marin *et al*. (Marin *et al.*, 2012) reported that a 4-week intervention with a MeDiet (enriched in MUFA by virgin olive oil) prevents telomere shortening of endothelial cells in 20 elderly subjects. The work of Boccardi *et al*. (Boccardi *et al.*, 2013) suggested that a lower rate of telomere shortening and a higher peripheral blood mononuclear cells (PBMC) telomerase activity, might be involved in

lifespan and most importantly in health-span among subjects consuming traditional MeDiet. Similarly, Crous-Bou *et al.* (Crous-Bou et al., 2014) has just reported that a greater adherence to the MeDiet was associated with longer telomeres in 4676 women from nested case-control studies within the Nurses' Health Study. All these findings suggest the benefits of adherence to the MeDiet for promoting health and longevity.

- *Epigenetics Diet*

The epigenetics diet consists in the consumption of phytochemicals (sulphoranes, glucose restriction or green tea) that modulate epigenetic processes such as DNA methylation, histone modifications and non-coding RNA (Tollefsbol, 2014). Telomeres are under epigenetic control since epigenetic pathways play an important role in modulating *TERT* transcription (Liu *et al.*, 2004). In turn, alterations of DNA methylation in subtelomeric regions or of histone modifications in telomeric chromatin correlate with telomere-length deregulation (Blasco, 2007). This new concept of diet could modify telomerase activity creating a direct impact on aging (Tollefsbol, 2014).

2.3.1.2. Physical activity and sleep duration and quality

Several studies have looked into the effect of physical activity on leukocyte TL (**Table 1**).

Cherkas *et al.* (Cherkas *et al.*, 2008) evaluated the association between physical activity in leisure time and leukocyte TL in 2,401 white twin volunteers. Their finding suggested that a sedentary lifestyle had an effect on leukocyte TL and may have accelerated the aging process. Another study specified that moderate physical activity levels were responsible of the beneficial effect on leukocyte TL compared with high or low levels (Ludlow *et al.*, 2008). The Nurse's Health Study, involving 7,813 women, also found a positive association between moderate amounts of activity and leukocyte TL (Du *et al.*, 2012), in agreement with other findings in postmenopausal females (Kim *et al.*, 2012). Moreover, it has been observed that vigorous physical activity appears to protect leukocyte TL as well (Puterman *et al.*, 2010). Leukocyte TL of professional ultra-marathons runners was 11% higher, in comparison with healthy males (Denham *et al.*, 2013). This result suggested that ultra-endurance aerobic exercise attenuated

cellular aging, as another previous study had already reported (Werner *et al.*, 2009). In contrast, other studies reported no association between physical activity and leukocyte TL (Hovatta *et al.*, 2012; Mason *et al.*, 2013). Of particular interest is the metanalysis by Gardner *et al.* (Gardner *et al.*, 2013) showing little evidence for leukocyte TL to be a strong biomarker for physical performance at older ages.

Table 1. Studies investigating physical activity and leukocyte telomere length

Author	Type of activity	Study design; subjects (n)	Association
(Cherkas *et al.*, 2008)	Leisure time	Cross-sectional; Twins (2,401)	Positive
(Hovatta *et al.*, 2012)	Guidance to increase the levels	RCT; Glucose intolerance (190) and Control (188)	No
(Ludlow *et al.*, 2008)	Moderate levels (not high or low)	Cross-sectional; 50-70 yr (69)	Positive
(Du *et al.*, 2012)	Moderate levels	Cross-sectional; Women (7,813)	Positive
(Kim *et al.*, 2012)	Moderate levels	Cross-sectional; Postmenopausal women (44)	Positive
(Savela *et al.*, 2013)	Moderate levels (not high or low)	Cross-sectional ; Men (782)	Positive
(Mason *et al.*, 2013)	Moderate-to-vigorous aerobic activity.	RCT; Postmenopausal women (439)	No
(Puterman *et al.*, 2010)	Vigorous physical activity	Cross-sectional; Postmenopausal women (63)	Positive
(Werner *et al.*, 2009)	Long-term endurance training (athletes)	Cross-sectional; Young and old sedentary and athletes (25 per group)	Positive
(Denham *et al.*, 2013)	Ultra-endurance aerobic exercise (marathons)	Cross-sectional; Men (123)	Positive
(Gardner *et al.*, 2013)	Different types of activity	Meta-analysis; (4 studies)	No

RCT: Randomized Clinical trial.

Although it has been explained above that a sedentary behaviour is associated with shorter telomeres, sleep duration and quality could be essential for telomere

maintenance. Thus, it has been observed that sleep duration (>7 hours) and sleep quality may be modifiable behaviours associated with the attenuation of aging effects (Cribbet *et al.*, 2014). Other studies reported that just getting at least 7 hours of sleep at night may protect telomeres from damage, observing no association with sleep quality (Liang *et al.*, 2011; Jackowska *et al.*, 2012; Lee *et al.*, 2014). Another study carried out in 245 healthy women in midlife showed that a poorer sleep quality predicted shorter leukocyte TL independent of other covariates, but not association was found with sleep duration (Prather *et al.*, 2011). Moreover, telomere shortening has been observed in sleep apnea syndrome (Barcelo *et al.*, 2010; Lin and Li, 2011; Savolainen *et al.*, 2014).

2.3.1.3. Smoking

An unhealthy habit, such as tobacco, has been revealed to accelerate telomere attrition. Since Valdes *et al.* (Valdes *et al.*, 2005) showed that smoking a pack of cigarettes per day for 40 years corresponded to 7.4 years of aging in women, several studies have looked into the association of tobacco and leukocyte TL leading to inconsistent results (Morla *et al.*, 2006; McGrath *et al.*, 2007; O'Donnell *et al.*, 2008; Mirabello *et al.*, 2009; Cassidy *et al.*, 2010; Tiainen *et al.*, 2012). A recent work, regarding this association, measured leukocyte TL in 8,074 participants (Huzen *et al.*, 2014). The findings showed active smoking as a major independent factor in determining telomere attrition rate (approximately multiplied by 3 the loss of leukocyte TL per year). Noteworthy, it has been reported that high tobacco consumption is causally associated with increased all-cause mortality in a general population sample of 55,568 individuals, but not with shortened telomeres (Rode *et al.*, 2014). This latter study suggested that high cumulative tobacco consumption is associated with short telomeres observationally, but the genetic association remains unclear.

2.3.2. Psychosocial factors

2.3.2.1. Psychological stress

Chronic psychological traumatic stress has also been linked to shortened telomeres. Epel *et al.* (Epel *et al.*, 2004) provided evidence that psychological stress

was associated with higher oxidative stress, lower telomerase activity, and shorter TL in PBMC from healthy premenopausal women. Chronic stress is associated with altered T cell function and accelerated immune cell aging as suggested by excessive telomere loss in women (Damjanovic *et al.*, 2007; Parks *et al.*, 2009). Humphreys *et al.* (Humphreys *et al.*, 2012) showed that TL was significantly shorter in 61 formerly abused women compared to 41 controls, suggesting an association between partner violence, as an experience chronic stress, and cellular aging.

In relation to acute psychological stress, higher overnight urinary cortisol levels were associated with lower telomerase activity and shorter leukocyte TL in 62 healthy women (Epel *et al.*, 2006). An exposure to an acute stressor (brief laboratory psychological stressor) yielded to the activation of telomerase-mediated TL maintenance system (Epel *et al.*, 2010).

2.3.2.2. Prenatal maternal stress

Only two studies assessed the relationship between leukocyte TL and maternal stress during pregnancy. In the first study, offspring (average age 25 years) of mothers, who experienced a severe stressor in the index pregnancy, presented 178 bp less leukocyte TL than the offspring of the control group (Entringer *et al.*, 2011). Following their study from 2011, Entringer *et al.* (Entringer *et al.*, 2013) looked into leukocyte TL of cord blood PBMC of new born whose mother were exposed to specific stress during gestation. They reported that maternal psychological stress during pregnancy may exert a "programming" effect on telomere biology system, which is already apparent at birth.

2.3.2.3. Early life stress

Childhood is another time of apparent imprinting, where early adversity, such as exposure to trauma or neglect or low parental socioeconomic status predicts childhood or adulthood leukocyte TL (Needham *et al.*, 2012; Price *et al.*, 2013). The fact that childhood stress exposure would accelerate telomere erosion was recently tested in the first longitudinal study (Shalev *et al.*, 2013). The findings reported that children who experienced two or more kinds of violence exposure showed more telomere attrition than their counterparts, between the ages of 5 and 10 years.

Furthermore, childhood chronic or serious illness was reported to be the most significantly associated single event affecting TL at the adult age in a population-based epidemiological Health 2000 cohort (Kananen *et al.*, 2010). Similarly, multiple childhood adversities measured with the Childhood Trauma Questionnaire and a six-item tool were related to both increased interleukin-6 levels and shortened telomeres, compared with the absence of adversity, in a sample of 132 healthy older adults (Kiecolt-Glaser *et al.*, 2011).

2.3.2.4. Socioeconomic status and educational attainment

Several studies have documented a correlation between shortened telomeres and a lower socioeconomic status or education. A cross-sectional study including 1,542 men of the West of Scotland Coronary Prevention Study, found a relationship between leukocyte TL and employment status, among other socioeconomic factors (Batty *et al.*, 2009). Men who reported being out of work had significantly shorter leukocyte TL than those who were employed. Findings from The Multi-Ethnic Study of Atherosclerosis (MESA) suggested that socioeconomic status may contribute to rates of cellular aging, particularly in late life (Carroll *et al.*, 2013), whereas renting *vs.* owning a home was associated with shortened telomeres (Carroll *et al.*, 2013). However, Adam *et al.* (Adams *et al.*, 2007) showed no association between socioeconomic status and leukocyte TL in 318 individuals from a homogeneous birth cohort.

The role of education on telomere erosion is well described by others. The UK European Prospective Investigation Into Cancer–Norfolk Study (Surtees *et al.*, 2012) and another study enrolling participants of the Whitehall II epidemiological cohort (Steptoe *et al.*, 2011) found that lower educational attainment was associated with shortened leukocyte TL. However, the latter study did not find any association between household income or employment and leukocyte TL. Other cross-sectional studies reported that higher education appeared to have a protective association with telomere shortening independently of ethnic or racial differences (Adler *et al.*, 2013; Needham *et al.*, 2013). The only study involving children observed that children whose parents never attended college had telomeres shorter by 1,178 bp, which is roughly

equivalent to 6 years of additional aging, than those with at least one college-educated parent (Needham *et al.*, 2012).

3. Leukocyte telomere length dynamics: underlying mechanisms

During infancy and early childhood, leukocyte TL shortening is very rapid (>0.2 kb/year), whereas during adulthood the average rate is approximately 0.03 kb/year (Aviv, 2008). This could be accounted for the expansions of hematopoietic stem cells (HSC) and progenitor cells (HPC) in early life to accommodate age-related somatic growth and the concomitant increase in the peripheral leukocyte pool (Sidorov *et al.*, 2009). The slow rate of leukocyte TL shortening during adult life results principally from house-keeping proliferative activity of the HSC pool that sustains its own self-renewal, and replaces the loss from the HPC pool (Sidorov *et al.*, 2009). In fact, leukocyte TL dynamics mirror telomere dynamic in HSC, and therefore leukocyte TL is determined by TL of HSC at birth and its age-dependent shortening over life (Aviv, 2009). The process consists on a replication downstream from HSC to HPC, which ultimately increases leukocyte pool determining leukocyte TL (Sidorov *et al.*, 2009). However, leukocyte TL is also the result of the balance between telomere shortening and maintenance processes that can be induced by the activation of several mechanisms (**Figure 8**). Thus, leukocyte TL is heritable from TL of HSC at birth, but it is also modified by a host of environmental factors.

3.1. Telomere shortening regulation

Chronic oxidative stress and inflammation have been reported as the main underlying mechanisms responsible for telomere shortening (Aviv, 2008; Aviv, 2009; Epel, 2012). The rate of telomere erosion per cell division is not constant and may be a function of oxidative stress and inflammation processes.

Telomeres are highly sensitive to the detrimental action of hydroxyl radicals due to their high content of guanines (Kawanishi and Oikawa, 2004). Consequently, ROS produce single strand breaks which might cause DNA damage in telomeric DNA, that it is known to be deficient in the repair of these single-strand breaks (Kawanishi and Oikawa, 2004). Oxidative stress may influence damage in two ways: damage to

HSC that would increase their number of divisions to replenish the HSC pool, resulting in enhanced telomere erosion; or oxidative stress may also shorten biological life of leukocytes, increasing this way the demand on HPC and in last instance HSC to replicate (Aviv, 2008). Since ROS negatively influences TL, it is expected that an antioxidant treatment may be preventive in telomere shortening (Houben *et al.*, 2008). Several studies *in vitro* (Furumoto *et al.*, 1998; Kashino *et al.*, 2003) and *in vivo* (Tarry-Adkins *et al.*, 2006) support the model that antioxidants might reduce the rate of telomere shortening and therefore delay cellular senescence.

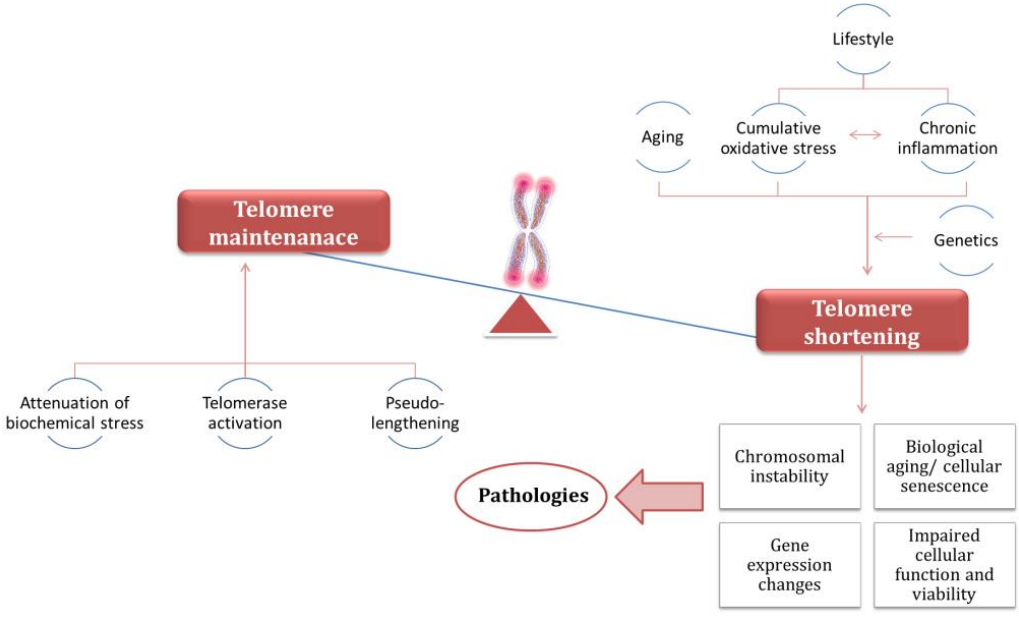

Figure 8. Mechanisms for telomere shortening or maintenance processes. Age-related telomere length shortening can be accelerated by factors that increase oxidative stress and/or cause chronic inflammation. Shortened telomere length may cause chromosomal instability and impaired cellular function, which may contribute to increased pathologies risks.

Inflammation could increase the rate of HSC replication to accommodate the increased demand for leukocytes due to their engagement in the inflammatory process (Aviv, 2008; Aviv, 2009). In addition, exposure to tumour necrosis factor-α also shorten TL through negative regulation of telomerase activity (Beyne-Rauzy *et al.*, 2005). Findings from a longitudinal study suggested that increased systemic inflammation was associated with decreased leukocyte TL after 2 years of follow-up

(Wong *et al.*, 2014). Other cross-sectional studies also observed that leukocyte TL is inversely associated with inflammatory markers (Bekaert *et al.*, 2007; Weischer *et al.*, 2012).

3.2. Telomere lengthening and maintenance regulation

First, an attenuation of biochemical stress through a decrease in oxidative stress and inflammation may have led to a decrease in the rate of telomere shortening and therefore it may induce a true reversal of telomere shortening (actual lengthening) (Epel, 2012).

Second, a reversal of telomeric aging also occurs when telomeres are elongated by telomerase (telomerase dependent elongation). Telomerase targets short telomeres causing them to lengthen more than long telomeres, in order to prevent critically short telomeres from shortening further (Hemann *et al.*, 2001). Few studies have observed that lowering psychological distress was associated with increases (from 30% to 43% increase) in telomerase activity of PBMC (Ornish *et al.*, 2008; Jacobs *et al.*, 2011; Daubenmier *et al.*, 2012; Lavretsky *et al.*, 2013). However, these studies have several limitations, specifically small sample size or lack of a control group. Nevertheless, one of the major proximal pathways for lengthening can be through telomerase activation.

And third, it has been suggested that cell redistribution toward more cells with longer TL or an influx of naïve cells into circulation could represent another mechanism that could explain telomere lengthening (Epel, 2012). Improvements in health behaviours have been reported to promote short term lengthening by increasing younger cells, called naïve T cells, which have longer telomeres than memory T cells and granulocytes (Kim *et al.*, 2012; Simpson *et al.*, 2012). Therefore, rather than telomere lengthening on a per cell basis, the increased TL seemed to be in part due to replenishment of cells in circulation (pseudo-lengthening). Homologous recombination between telomeric sequences, known as alternative lengthening of telomeres (ALT), could also maintain or elongate TL, particularly in cancer cells (Muntoni and Reddel, 2005).

In short, larger longitudinal trials that examine telomerase and leukocyte TL are warranted to better understand how much telomeres can be lengthened. It is necessary to distinguish between true reversal of telomere shortening and replenishment of younger cells or ALT and whether these ways of lengthening slow clinical signs of aging.

4. Telomeres and associated pathologies

Biomarkers of aging are essential to predict mortality and aging-related diseases. In this sense, an important link between telomere deregulation and several age-related diseases, such as cancer and metabolic pathologies, have been reported. Here, a brief review of the roles of telomeres in human diseases, specifically in cancer, CVD, T2D and obesity is provided.

4.1. Telomeres and cancer

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (13% of all deaths) in 2008. This figure is increasing since 8.2 million people worldwide died from cancer in 2012. Lung, breast, colorectal, stomach, and prostate cancers cause the majority of cancer deaths. The majority of total new annual cases (60%) occur in Africa, Asia and Central and South America. Interestingly, it has been estimated that 30% of cancers could be prevented (WHO, 2014).

Telomerase is inactive in the majority of somatic cells. However, telomerase activity increases in most of immortalized cell lines and in 85-90% of human tumours (Taga *et al.*, 1999). In a study, in cultured cells (representing 18 human tissue types), it was found that a 98% had positive immortal cell telomerase activity, whereas it was negative in 100% of the populations of mortal cells. Similarly, positive telomerase activity was found in 90 of 101 biopsies (12 types of human tumours), but none activity in 50 normal somatic tissues (Gauthier *et al.*, 2001).

Many clinical studies have investigated the role of TL in the progression of different types of cancer. The majority of studies were retrospective studies leading to inconsistent findings. However, as listed in **Table 2**, there are some prospective

studies assessing the association between TL and cancer risks. These studies improve our understanding of TL as a possible biomarker of cancer progression and risk.

Table 2. Summary of prospective studies assessing the association between telomere length and different types of cancer

Type of cancer	Author	Number of individuals	Conclusion: is TL associated with the risk of cancer?
Lung	(Seow *et al.*, 2014)	847 cases and 847 controls	Yes
	(Lan *et al.*, 2013)	215 women cases and 215 controls	Yes
	(Shen *et al.*, 2011)	229 male cases and 229 controls	Yes
Breast	(De Vivo *et al.*, 2009)	1,122 cases and 1,142 controls	No
	(Pooley *et al.*, 2010)	199 cases and 413 controls	No
	(Kim *et al.*, 2011)	342 case and 735 controls	No
	(Duggan *et al.*, 2014)	478 cases	Yes
Colorectal	(Lee *et al.*, 2010)	357 women cases and 134 controls	No
	(Zee *et al.*, 2009)	191 male cases and 306 controls	No
	(Cui *et al.*, 2012)	441 women cases and 549 controls	Yes
	(Pooley *et al.*, 2010)	185 cases and 413 controls	No
Pancreas	(Campa *et al.*, 2014)	331 cases and 331 controls	No
	(Lynch *et al.*, 2013)	193 cases and 660 controls	Yes

Cancer cells present the ability to maintain TL, escaping senescence and termination of cell division, induced by critically shortened telomeres (Hanahan and Weinberg, 2011). The balance between telomere loss and telomere addition can maintain stable TL in cancer cells, over an extended period of proliferation. Consequently, long telomeres might be a marker of cancer risk. However, the opposite has also been hypothesized creating a paradox that several studies have already investigated (Hou *et al.*, 2012). In this sense, two recent meta-analyses have examined these two possibilities concluding that the presence of shortened telomeres may be a marker for susceptibility to human cancer (Ma *et al.*, 2011; Wentzensen *et al.*, 2011). Wentzensen *et al.* (Wentzensen *et al.*, 2011) observed in 27 studies that the strongest

evidence exists for bladder, esophageal, gastric, and renal cancers, obtaining a pooled OR of 1.96 (95% confidence intervals [CI]: 1.37-2.81, P = 0.0001) for the association of shortened TL and cancer. In addition, a more recent study, including 47,102 Danish participants, tested the hypothesis that shortened telomeres predicted a higher cancer risk (Weischer *et al.*, 2013). The latter work observed that short TL was associated with reduced survival after cancer but not with cancer risk. On the contrary, another review pointed out that either excessively short or long TL may both contribute to cancer development (Hou *et al.*, 2012). The possible explanation for the first hypothesis is that environmental factors may cause cancer, at least partially, via TL shortening mechanism (Hou *et al.*, 2012). Indeed, progressive telomere shortening might in turn favour the activation of telomere-elongation mechanisms, such as ALT and telomerase (Blasco, 2007). On the other hand, it is biologically plausible that cells with longer telomeres may favour a delayed cell senescence, hence, these cells could have more chances to develop chromosomal instability and acquire genetic abnormalities, being at higher risk of carcinogenic transformation (Mooi and Peeper, 2006; Hou *et al.*, 2012). Nevertheless, there is a need for longitudinal studies rather than retrospective to better understand the mechanisms implicated.

4.2. Telomeres and metabolic diseases

Since TL is considered as a marker of cellular and biological aging, several epidemiological studies have investigated the association between TL and aging-related diseases, with a particular focus on CVD and T2D. Obesity amplifies the risk of developing various age-related diseases, due to an increased body weight and accumulation of adiposity. Shorter telomeres have been observed in obese subjects and in individuals with CVD and T2D, suggesting the role of TL as a biomarker in metabolic diseases. This provides a better insight into the pathophysiology of these illnesses.

4.2.1. Telomeres and cardiovascular disease

CVD is the leading cause of death. The World Health Organization estimated that the 30% of all global death was attributed to CVD in 2008. Over 80% of CVD

deaths take place in low and middle-income countries. It is also expected that by 2030, over 23 million people will die from CVD each year (WHO, 2013).

Numerous *in vitro* and *in vivo* studies have looked into the possible association between TL and hypertension, atherosclerosis, stroke or coronary artery disease (CAD) (**Table 3**). Most of them found that shorter telomeres were associated with these cardiovascular pathologies. Moreover, some of them observed that shortened TL was related with a worse prognosis or a higher risk of presenting CVD.

Table 3. Summary of studies assessing the association between telomere length and cardiovascular disease

Author	Individuals or samples	Tissue	TL analysis	Association
HYPERTENSION				
(Jeanclos *et al.*, 2000)	40 Danish twins	Leukocytes	Southern blot	Yes
(Demissie *et al.*, 2006)	327 Caucasian Men	Leukocytes	Southern blot	Yes
(Benetos *et al.*, 2004)	163 Men	Leukocytes	Southern blot	Yes
(Fyhrquist *et al.*, 2011)	1,271	Leukocytes	Southern blot	No
(Jiang *et al.*, 2013)	450	Leukocytes	RT-PCR	Yes
(Insel *et al.*, 2012)	42	Leukocytes	RT-PCR	Yes
(Bhupatiraju *et al.*, 2012)	194 Indians	Leukocytes	RT-PCR	Yes
ATHEROSCLEROSIS AND CAD				
(Minamino *et al.*, 2002)	4 patients with heart disease	Vascular endothelial cells	N/A	Yes
(Ogami *et al.*, 2004)	11 patients with CAD and 22 without	Coronary endothelial cells	Slot blot	Yes
(Chang and Harley, 1995)	N/A	Human endothelial cell cultures	Southern blot	Yes
(Okuda *et al.*, 2000)	51 autopsies	Abdominal aorta	Southern blot	Yes
(Samani *et al.*, 2001)	10 patients with CAD and 20 controls	Leukocytes	Southern blot	Yes
(Brouilette *et al.*, 2007)	484 patients who developed CAD and 1,058 age-matched controls	Leukocytes	RT-PCR	Yes
(Farzaneh-Far *et al.*, 2008)	780 patients with CAD	Leukocytes	RT-PCR	Yes
(Zee *et al.*, 2009)	337patients who develop myocardial infarction and 337 matched controls	Leukocytes	RT-PCR	Yes

(Willeit *et al.*, 2010)	800	Leukocytes	RT-PCR	Yes *
(Perez-Rivera *et al.*, 2014)	203 men with acute coronary syndrome	Leukocytes	RT-PCR	Yes $
(Ye *et al.*, 2013)	1,917 (CAD incidence)	Leukocytes	RT-PCR	No
(Baragetti *et al.*, 2014)	768 (atherosclerosis incidence)	Leukocytes	RT-PCR	Yes
STROKE				
(Fyhrquist *et al.*, 2011)	1,271	Leukocytes	Southern blot	Yes
(Ding *et al.*, 2012)	1,309 stroke patients and 1,309 matched controls	Leukocytes	RT-PCR	Yes
(Zee *et al.*, 2010)	14,916 American men (stroke incidence)	Leukocytes	RT-PCR	No
(Schurks *et al.*, 2013)	504 women case-control pairs	Leukocytes	RT-PCR	No
(Jiang *et al.*, 2013)	150 stroke patients, 150 history stroke siblings and 150 healthy controls	Leukocytes	RT-PCR	Yes (but not causative)

NA: not applicable, TRF: terminal restriction fragment, RT-PCR: real-time PCR.
* TL was associated with advanced but not early atherosclerosis.
$ TL as good predictors of cardiovascular prognosis in men aged 50 to 75 years, but not in those > 75 years.

A recent meta-analysis involving 43,725 participants and 8,400 patients with CVD, integrating data from 24 prospective and retrospective studies, showed an inverse association between leukocyte TL and risk of coronary heart disease, independently of conventional vascular risk factors (Haycock *et al.*, 2014). However, the association with cerebrovascular disease was not clear. What is more, reduced leukocyte TL has been associated with all-cause mortality in patients with stable CAD (Farzaneh-Far *et al.*, 2008). On the other hand, a work carried out by Cui *et al.* (Cui *et al.*, 2014) observed a lack of causal relationship between leukocyte TL and coronary heart disease in a Chinese population. These controversial results suggest the need of more longitudinal studies in which changes in TL will be analysed to better understand the mechanisms implicated.

Some studies have mentioned possible mechanisms attributed to telomere shortening and risk of CVD, especially in atherosclerosis. Telomere shortening might contribute to atherosclerosis through various biological aging pathways, such as

cellular senescence. Indeed, the accumulation of senescent cells, a prominent feature of atherosclerotic plaques, reduces the regenerative potential of affected tissues and promotes apoptosis, which can further exacerbate inflammatory reactions and endothelial dysfunction (Minamino *et al.*, 2002; Matthews *et al.*, 2006). It has also been suggested that cellular senescence promotes the thinning of fibrous caps and the instability of atherosclerotic plaques by reducing the proliferative potential of vascular smooth muscle cells (Gorenne *et al.*, 2006). As demonstrated, a cumulative burden of oxidative stress and cardiovascular risk leads to the development of endothelial senescence and this is likely contributing to atherosclerosis. Potential therapeutic interventions in order to modify TL or telomerase activity can reverse some of the effects of cellular senescence, such as the loss of the proliferative potential of vascular smooth muscle cells and endothelial dysfunction (Minamino *et al.*, 2002; Gorenne *et al.*, 2006). Thus, anti-senescence therapy targeting the telomere complex is emerging as a new strategy in the treatment of atherosclerosis.

As a summary, it has been shown that telomere biology is altered in subjects with CVD. The assessment of TL could be as a good predictor of cardiovascular risk and mortality. Conversely, the results remained conflicting and the biological processes linking cardiovascular risk are still poorly understood.

4.2.2. Telomeres and type 2 Diabetes

T2D is a metabolic disorder resulting from impairments in insulin secretion and insulin action in target tissues (Yang *et al.*, 2011). T2D is the main comorbidity linked to obesity and its prevalence has also increased significantly in recent years (Chatzigeorgiou *et al.*, 2014). More than 360 million people have T2D in the world, and in 2030 it is projected that 552 million people will suffer this disease (Varemo *et al.*, 2013), becoming the 7th leading cause of death (WHO, 2013).

Several studies have examined the association between shortened TL and T2D. Case-control studies reported that shortened telomeres are presented in patients with T2D (Adaikalakoteswari *et al.*, 2005; Zee *et al.*, 2010; Xiao *et al.*, 2011; Harte *et al.*, 2012; Monickaraj *et al.*, 2012; Shen *et al.*, 2012). Interestingly, Ma *et al.* (Ma *et al.*, 2014) reported that T2D patients had significantly shorter TL than subjects without

T2D. But, treatment with sitagliptin gradually increased leukocyte TL in the T2D group. After 2 months, leukocyte TL was significantly elongated and it was almost equal to the leukocyte TL of healthy subjects. A plausible explanation of this result is that the sitagliptin improved glycaemic control, reduced oxidative stress, and slowed the rate of β-cell apoptosis minimizing the telomeric erosion (Ma *et al.*, 2014).

Notably, telomere shortening has already been seen even at the stage of impaired glucose tolerance (Adaikalakoteswari *et al.*, 2007). Another factor increasing telomere attrition is the duration of diabetes, since patients with a T2D for 10 years or more (since original diagnosis) presented shorter leukocyte TL than patients with less than a year of diagnosis (Murillo-Ortiz *et al.*, 2012).

With regard to analyse TL in other tissues, colonic epithelium in T2D patients did not differ significantly from control colonic epithelium in TL or oxidative DNA damage (Kejariwal *et al.*, 2008). However, Tamura *et al.* (Tamura *et al.*, 2014) reported that telomeres of β-cells were shortened in patients with T2D.

To identify the causal nature of association between TL and T2D, genetic variants have been evaluated. GWAS identified a new genetic variant (rs74019828) in the casein kinase II gene (*CSNK2A2*), that phosphorylates telomeric repeat binding factor 1 and plays an important role for regulation of TL homeostasis (Saxena *et al.*, 2014). Another study investigating the relationship between 11 telomere-pathway genes and incident of T2D in 22,715 Caucasian female participants, showed that genetic variation within the telomere-pathway may be useful predictor for T2D risk (Zee *et al.*, 2011). On the contrary, an analysis of 3,968 women participating in the Nurses' Health Study, reported that those genetically predisposed to T2D did not present shortened telomeres (Du *et al.*, 2013).

Longitudinal studies are other alternative approaches to make causal inference in observed reported associations. One example is the recent well-characterized longitudinal cohort of American Indians, participating in the Strong Heart Family Study, which examined leukocyte TL as a predictor of T2D (Zhao *et al.*, 2014). They reported that shortened leukocyte TL could increase the risk of diabetes development in American Indians after 5.5 years of follow-up. This is the first study indicating that TL may be in the causal pathway for the aetiology of T2D. On the other

hand, a previous study of You *et al.* (You *et al.*, 2012) in postmenopausal women concluded that there was no evidence for a causal relationship between short telomeres and diabetes. Similarly, the Diabetes Prevention Study showed that TL was not associated with the development of T2D after 4.5 years (Hovatta *et al.*, 2012). Overall, nine cohorts consisting of 5,759 cases and 6,518 controls were selected into a recent meta-analysis (Zhao *et al.*, 2013). Apparently, the results indicated that shortened TL was significantly associated with a higher risk of T2D (Odds Ratio [OR] = 1.291, $P<0.001$).

What are the underlying mechanisms for the connexion between shortened telomeres and T2D? Short telomeres may lead to premature β-cell senescence. Indeed, experimental evidence suggests that telomerase is important in maintaining glucose homeostasis in mice (Kuhlow *et al.*, 2010). Conversely, elevated blood glucose levels increase oxidative stress, potentially interfering with telomerase function and resulting in shortened telomeres (Serra *et al.*, 2000). There are several studies presenting similar findings (Sampson *et al.*, 2006; Ma *et al.*, 2013; Liu *et al.*, 2014). Moreover, it was reported an association of the *UCP2* functional promoter variant (−866G>A) and leukocyte TL, that implies a link between mitochondrial production of ROS and shortened TL in T2D (Salpea *et al.*, 2010).

4.2.3. Telomeres and obesity

Obesity is defined as an abnormal or excessive fat accumulation that presents a risk to health (WHO, 2014). Obesity is a multifactorial chronic disease characterized by the hypertrophy and hyperplasia of the adipose tissue, as a result of a positive balance between energy intake and energy expenditure (Chatzigeorgiou *et al.*, 2014).

The epidemic of obesity is prevalent worldwide and obesity is considered as the pandemic of XXI century (WHO, 2014). The rate has almost doubled since 1980, with half of this increase occurring between 2000 and 2008 (Scully, 2014). Recently, a systematic analysis for the global burden of disease reported that the number of overweight and obese individuals increased to 2.1 billion (36.9% men and 38.0% women) in 2013 (Ng *et al.*, 2014). The ENRICA study, conducted between 2008 and 2010 revealed that over 36% of Spanish adults have abdominal obesity (Gutierrez-

Fisac *et al.*, 2012) which is particularly alarming because of the associated comorbidities of this type of obesity (Hermsdorff *et al.*, 2011)

The prevalence of obesity in children and adolescents has increased at an alarming rate in the past two decades (WHO, 2012). In 2010, the IOTF estimated that over 200 million school-age children were overweight or obese. Recent published data revealed that rates of overweight in Spanish children are considered one of the highest in Europe (Lagerros and Rossner, 2011).

4.2.3.1. Obesity and aging

Obesity, in particular excess visceral adiposity, is associated with metabolic imbalances such as insulin resistance, hyperglycaemia, dyslipidaemia and hypertension (Esser *et al.*, 2014). In fact, these metabolic disorders increase the risk of development of T2D and CVD contributing to high rates of mortality and morbidity (Esser *et al.*, 2014). Then, what is the link between obesity and these detrimental health consequences?

In obesity, excess adiposity increases the production of a wide range of adipokines, including hormones, cytokines and immune factors that presented pro-inflammatory actions (Ouchi *et al.*, 2011). This dysregulation of production and secretion of adipokines contribute to a low-grade but chronic inflammation state. Moreover, adipose tissue in obese individuals is infiltrated by a large number of macrophages, and this recruitment is linked to systemic inflammation and insulin resistance (Weisberg *et al.*, 2003).

Obesity per se may induce systemic oxidative stress and this could be the underlying cause of dysregulation of adipokines and development of metabolic disorders (Furukawa *et al.*, 2004). Thus, this imbalance in body composition is now recognized as a state of increased oxidative stress and inflammation for the organism (Furukawa *et al.*, 2004). Therefore, these two mechanisms are responsible for increasing the risk of the development of obesity-related diseases.

However, obesity goes further since it has been reported that this disease not only increases the onset of metabolic imbalances, but also accelerates aging, through deterioration of the structure and function of organs and disturbance of homeostatic

pathways (Tzanetakou *et al.*, 2012). In fact, chronic inflammation and oxidative stress linked to obesity have been related to accelerated telomere shortening, which are considered biological markers for age.

4.2.3.2. Telomere length and obesity

Despite the fact that inflammation and oxidative stress play an important role in accelerated telomere attrition (Aviv, 2009), the relationship between TL and adiposity is not clear: most of the studies reported a significant association but a few did not observe any correlation (**Table 4)**.

Table 4. Summary of studies assessing the association between telomere length and obesity disease or adiposity status

Author	Study; Individuals (n)	Tissue	Association
(Valdes *et al.*, 2005)	Cross-sectional White women(1,122)	Leukocytes	Yes
(Zannolli *et al.*, 2008)	Cross-sectional; Children and Adults (76)	Leukocytes	Yes (only adults)
(Nordfjall *et al.*, 2008)	Cross-sectional; Adults (989)	Leukocytes	Yes (only women)
(Kim *et al.*, 2009)	Cross-sectional/Longitudinal Women (647)	Leukocytes	Yes
(Al-Attas *et al.*, 2010)	Cross-sectional; Middle-age arabs (193)	Leukocytes	Yes
(Moreno-Navarrete *et al.*, 2010)	Cross-sectional; Adults (72)	Subcutaneous adipose tissue	Yes
(Al-Attas *et al.*, 2010)	Cross-sectional; Children (148)	Leukocyte	Yes *(only boys)*
(MacEneaney *et al.*, 2010)	Cross-sectional; Adults (67)	Endothelial progenitor cells	No
(Diaz *et al.*, 2010)	Cross-sectional; Adults (317)	Leukocytes	No
(Zhu *et al.*, 2011)	Cross-sectional; Adolescents (667)	Leukocytes	No
(Lee *et al.*, 2011)	Cross-sectional; 8-80 years (309)	Leukocytes	Yes
(Monickaraj *et al.*, 2012)	Cross-sectional; Adults (59)	Subcutaneous and visceral adipose tissue	Yes

(Cui *et al.*, 2013)	Cross-sectional; Chinese Women (2,912)	Leukocytes	Yes
(Weischer *et al.*, 2014)	Cross-sectional/Longitudinal Danish adults (4,576)	Leukocytes	Yes*
(Buxton *et al.*, 2014)	Cross-sectional 31 years (5,598)	Leukocytes	Yes
(Chen *et al.*, 2014)	Cross-sectional; American Indians (3,256)	Leukocytes	Yes
(Rode *et al.*, 2014)	Cross-sectional; Danish adults (45,069)	Leukocytes	Yes
(el Bouazzaoui *et al.*, 2014)	Cross-sectional; Women (21)	Adipocytes	Yes
(Buxton *et al.*, 2011)	Case-control; Children (793)	Leukocytes	Yes
(Formichi *et al.*, 2014)	Case-control/Weight loss bariatric surgery (237)	Leukocytes	Yes
(Gardner *et al.*, 2005)	Longitudinal; Young adults (70)	Leukocytes	Yes
(Hovatta *et al.*, 2012)	Longitudinal; Adults (340)	Leukocytes	No
(Njajou *et al.*, 2012)	Longitudinal; Elderly (2,721)	Leukocytes	Yes
(O'Callaghan *et al.*, 2009)	Weight loss intervention; Men (12)	Midrectal biopsies	Yes
(Mason *et al.*, 2013)	Weight loss intervention; Postmenopausal women (439)	Leukocytes	No
(Muezzinler *et al.*, 2014)	Meta-analysis; (16 studies)	Leukocytes	Yes

*Only the cross-sectional association.
Individuals were either obese or overweight and in some studies there is a control group.

In 2005, Valdes *et al.* (Valdes *et al.*, 2005) reported for the first time that telomeres of obese women were 240 bp shorter than those of lean women. Similarly, a recent study involving Chinese women found an inverse association between leukocyte TL and body anthropometric indices (Cui *et al.*, 2013). Moreover, they reported that maintaining body weight within a normal range helps to maintain leukocyte TL. This is

in agreement with another study which indicated that leukocyte TL was associated with an obesity-phenotype but only in women (Nordfjall *et al.*, 2008).

The majority of cross-sectional studies have investigated men and women together. Higher total and abdominal adiposity was associated with shorter leukocyte TL in a cross-sectional sample of 309 white participants (52% females) from The Fels Longitudinal Study (Lee *et al.*, 2011). Other publications have also observed an inverse association between obesity and TL (Al-Attas *et al.*, 2010; Chen *et al.*, 2014; Rode *et al.*, 2014; Weischer *et al.*, 2014), but some did not (Diaz *et al.*, 2010; MacEneaney *et al.*, 2010). Interestingly, there are two large studies published in 2014 including 3,256 American Indians and 4,576 Danish adults. In the first one, obese participants had significantly shorter leukocyte TL than non-obese individuals (P=0.0002)(Chen *et al.*, 2014), while in the second one, increased body weight was associated with shorter leukocyte TL cross-sectionally ($P=7\times10^{-14}$), but not with leukocyte TL change after 10 years of follow-up (Weischer *et al.*, 2014).

Few studies have investigated the association of obesity and leukocyte TL in children and adolescents. A case-control study showed that obese children had a mean leukocyte TL that was 23.9% shorter than that of their non-obese counterparts (Buxton *et al.*, 2011). In agreement, Al-Attas *et al.* (Al-Attas *et al.*, 2010) observed that obese boys presented shorter leukocyte TL than the non-obese ones, but this result was not evident in girls. On the contrary, a study carried out in 53 Italian children found no difference in leukocyte TL between obese and non-obese participants (Zannolli *et al.*, 2008). There is just one study analysing leukocyte TL and adiposity parameters in adolescents (Zhu *et al.*, 2011). The data showed that adiposity and adipokines were not associated with leukocyte TL at this age.

When analysing the possible link between obesity and TL measured in adipose tissue, three studies have come to the same conclusion. A work by Moreno-Navarrete *et al.* (Moreno-Navarrete *et al.,* 2010) reported that subcutaneous adipose tissue cells from obese subjects showed shortened TL. Moreover, the study also observed that BMI contributed independently to 16% of TL variance. Likewise, adipocyte hypertrophy appeared to be strongly associated with shortened telomeres in subcutaneous and visceral adipose tissue of obese and T2D patients (Monickaraj *et al.*, 2012). In addition,

Bouazzaoui *et al.* (el Bouazzaoui *et al.*, 2014) found that TL of adipocytes correlated negatively with waist and adipocyte size. These studies revealed that the association of shorter telomeres with obesity disease is not only happening in leukocytes but also in adipose tissue.

Several studies have assessed the association between BMI and TL to better understand whether telomere shortening is related to obesity. Several studies reported a negative association between BMI and leukocyte TL (Valdes *et al.*, 2005; Nordfjall *et al.*, 2008; Lee *et al.*, 2011; Cui *et al.*, 2013; Rode *et al.*, 2014), whereas others did not find a correlation with this parameter (Diaz *et al.*, 2010; Zhu *et al.*, 2011). Noteworthy, Rode *et al.* (Rode *et al.*, 2014) studied leukocyte TL in 45,069 individuals from the Copenhagen General Population Study, showing that leukocyte TL decreased in 7 bp per unit of increase in BMI. Furthermore, a recent meta-analysis provided a systematic review of studies on the relationship between BMI and leukocyte TL (Muezzinler *et al.*, 2014). For cross-sectional studies, the pooled estimates for correlation and regression coefficients were -0.057 (95% CI: -0.102 to -0.012) and -0.008 kBP·kg·m^{-2} (95% CI: -0.016 to 0.000), respectively, suggesting a biologically plausible inverse association between BMI and leukocyte TL in adults (Muezzinler *et al.*, 2014).

Not only BMI has been associated with leukocyte TL, but also other anthropometric parameters such as waist circumference (WC) (Nordfjall *et al.*, 2008; Al-Attas *et al.*, 2010; Cui *et al.*, 2013) or waist to height ratio (WHtR) (Cui *et al.*, 2013). Since these measurements are considered indices of central obesity, shorter telomeres may be also associated with abdominal obesity increasing the morbidity risk.

Two studies in the literature analysed changes in TL after a weight loss dietary intervention, leading to different conclusions. Increased TL was observed after 12 and 52 weeks of a calorie-restricted diet in midrectal biopsies of 12 obese men (O'Callaghan *et al.*, 2009). On the other hand, a 12-month randomized intervention trial of weight loss and/or exercise was not sufficient to yield significant changes in leukocyte TL in postmenopausal women (Mason *et al.*, 2013). In the same research line, a little number of publications assessed the association of TL and adiposity in a longitudinal way. Remarkably, Gardner *et al.* (Gardner *et al.*, 2005) observed that

weight gain was associated with accelerated telomere attrition in 70 young adults after 10 years of follow-up. In agreement, Kim et al. (Kim *et al.*, 2009) found an inverse association of TL and self-reported weight gain and cycling in 647 women. However, other studies reported no longitudinal relationship between TL and adiposity (Hovatta *et al.*, 2012; Weischer *et al.*, 2014).

A prospective study by Njajou *et al.* (Njajou *et al.*, 2012) has been the only work considering TL as a possible biomarker of changes in adiposity parameters after 7 years of follow-up. They showed leukocyte TL at baseline to be associated with positive change in BMI and percentage of body fat after the follow-up in an elderly population.

All these studies suggest that obesity could be link to shortened telomeres and therefore a hastened aging process. However, it is still unclear whether telomere shortening is a cause or a consequence of changes in adiposity.

5. Justification for the research

Telomeres are essential regulators in cellular aging. In this sense, TL serve as a biomarker of biological age since telomere shortening is considered one of the underlying molecular mechanisms related to cellular aging via senescence or apoptotic cell death activation (Sanders and Newman, 2013). Therefore, identifying potential factors that could modify telomere homeostasis will contribute to a better understanding of age-related diseases.

The impact of environmental factors, such as diet, sedentarism, smoking habit or stress, could have an interaction with the genetic background playing a key role in TL (Harari *et al.*, 2013). Since inflammation and oxidative stress are considered the principal mechanisms in telomere shortening (Epel, 2012), several studies have tested that anti-inflammatory and antioxidant nutrients can reduce erosion of telomeres (Paul, 2011). But so far, no information is available on the relationship between TL and dietary total antioxidant capacity (TAC), a good predictor of dietary and plasma antioxidant status (Wang *et al.*, 2012; Yang *et al.*, 2013).

The wide range of inter-individual variation in TL leads to consider genetics as an important factor in telomere regulation (Aviv, 2012). Interestingly, the polymorphism Pro12Ala of the *PPARγ2* gene has reported to be beneficial improving insulin resistance, lipid and glucose homeostasis (Gonzalez Sanchez *et al.*, 2002) and it seems to regulate inflammation and decrease resistance to oxidative stress (Yao *et al.*, 2005; Luo *et al.*, 2008). Moreover, Ala carrier subjects appeared to be protected against cardiovascular disease (Regieli *et al.*, 2009) and aging (Barbieri *et al.*, 2004). However, no studies have investigated the possible role of this genetic variant on telomere shortening.

It has been reported that TL could be associated with obesity and consequently with aging-related diseases. Nevertheless, it is still unclear whether shortened telomeres could be a risk factor for increased adiposity, making it hard to understand implicated biological pathways.

II. Hypotheses and Aims

1. Hypotheses

Based on the available evidence, on the one hand we proposed that a diet rich in antioxidants would be positively associated with leukocyte TL. On the other hand, focusing on the Pro12Ala polymorphism, we hypothesized that subjects carrying the Ala allele of the *PPARγ2* gene would have a slower rate of telomere attrition and we investigated potential gene-diet interactions.

In this study was also hypothesized that an improvement in adiposity after a dietary intervention could be associated with an increase in leukocyte TL during follow-up. Moreover, we hypothesized that a higher leukocyte TL at baseline could predict a better response to a lifestyle intervention.

2. Aims

The **general aim** of this work was to study the association between leukocyte TL and adiposity traits in different Spanish age groups after a lifestyle intervention, as well as evaluating the impact of dietary and genetic factors on telomere attrition.

The **specific aims** were:

1. To assess the cross-sectional effect of macronutrients, food groups and dietary TAC on leukocyte TL in children and adolescents (*chapter 1*).

2. To explore the influence of the rs1801282 polymorphism of the *PPARγ2* gene on telomere shortening after 5 years of a MeDiet intervention, as well as potential gene-diet interactions in subjects at high cardiovascular risk (*chapter 2*).

3. To investigate leukocyte TL variation after a 2-month weight loss intensive program in obese adolescents, and to evaluate the biomarker role of baseline TL for adiposity changes *(chapter 3).*

4. To study the longitudinal association between leukocyte TL and adiposity traits in high cardiovascular risk individuals after 5 years of a nutritional intervention with a MeDiet, and to assess the biomarker role of baseline TL for adiposity changes (c*hapter 4).*

III. Subjects and Methods

1. GENOI study

The GENOI study ("Grupo de Estudio Navarro de la Obesidad Infantil") is a case-control study designed to understand the role of lifestyle and genetics factors on obesity development in children and adolescents in the region of Navarra (Spain). Obese (cases) and non-obese (controls) subjects were enrolled when they attended Primary Care Centres or Hospitals for routine medical examinations or vaccinations. The study was approved by the Ethics Committee of the University of Navarra and all parents and subjects over 12 years old provided written informed consent in agreement with the Declaration of Helsinki.

1.1. Study population

The GENOI study recruited, during the period 2001-2003, 370 children and adolescents between 5 and 19 years old. Cases (n=185) were children or adolescents with a BMI above 97th percentile of the Spanish BMI reference data for age and sex (Sobradillo *et al.*, 2004). By contrast, controls (n=185) were healthy subjects with a BMI below the 97th percentile of the same reference data charts. Controls were individually matched to cases by sex and age (±6 months). The inclusion criteria for all the subjects were being residents in Navarra. Exclusion criteria included exposure to special dietary interventions, exposure to hormonal treatment, alcoholism or drugs, development of secondary obesity due to endocrinopathy and serious concurrent illness. In the present thesis, a subsample of 287 Spanish and adolescents (55% males, 6-18 years), including both cases and controls, was analysed.

1.2. Data collection

All data were collected in a personal interview conducted by trained staff in a medical and relaxed atmosphere, in the presence of an adult (parent or legal tutor).

1.2.1. Dietary assessment

A 132-item validated food frequency questionnaire (FFQ) for Spanish population was used to assess the dietary intake of participants (Martin-Moreno *et al.*,

1993)(**Appendix 1**). To date, no validated FFQ specifically for children are available. However, this FFQ has been also used in other studies concerning Spanish children (Rodriguez-Artalejo *et al.*, 2003; Ochoa *et al.*, 2007).

Interestingly, the dietary TAC was calculated from the FFQ by adding the TAC values from the ferric reducing antioxidant power assay of each food, as reported elsewhere (Halvorsen *et al.*, 2006). TAC was expressed in mmol/100 g food.

1.2.2. Physical activity

A physical frequency questionnaire, including 17 activities (sports and games) with ten response categories from "never" to "11 hours or more a week", was used to evaluate physical activity. This questionnaire has been previously validated in Spanish adults (Martinez-Gonzalez *et al.*, 2005). Furthermore, questions were asked on sedentary leisure activities, such as the daily time spent watching television, computer, lying, sitting, etc.

1.2.3. Anthropometric measurements

Trained researchers took the following anthropometric measurements: weight, height, WC and hip, skinfold thickness and arm circumference following standard procedures. The percentage of body fat was determined by bioelectrical impedance (BES 200Z Biological ohm meter, Tanita).

1.2.4. Blood samples

Blood samples were collected after an overnight fasting. Concerning genetic analyses, 10 mL of blood for DNA extraction were collected in EDTA. It was stored at -80°C for later analyses.

2. EVASYON study

The EVASYON study (Development, implementation and evaluation of the efficacy of a therapeutic programme for adolescents with overweight and obesity: integral education on nutrition and physical activity) is considered a national pilot study to be implemented as a method of treatment for obesity in adolescents into the Spanish Health Care Service.

Initiatives, as the EVASYON study, contributed to the establishment of the Spanish Strategy for Nutrition, Physical Activity and the Prevention of Obesity (NAOS Strategy) initiated in 2005 by the Ministry of Health and Consumer Affairs. It is an integral intervention study conducted in 5 hospitals of Spanish cities (Granada, Madrid, Pamplona, Santander and Zaragoza) with a multidisciplinary team consisting of paediatricians, endocrinologists, psychiatrists, psychologists, physical activity specialists and dieticians.

This project followed the ethical standards recognized by the Declaration of Helsinki and the EEC Good Clinical Practice recommendations (document 111/3976/88, July 1990), and current Spanish legislation regulating clinical research in humans (Royal Decree 561/1993 on clinical trials). The study was approved by the Ethics Committee of each hospital. The study was explained to the participants before starting, and the volunteers, parents or tutors signed an informed consent (Martinez-Gomez *et al.*, 2009)

The main aims of the EVASYON study were: 1) to develop a treatment programme including education on nutrition and physical activity, 2) to implement this programme for one year in Spanish adolescents with overweight and obesity and 3) to evaluate its efficacy. Further details of the EVASYON study are published elsewhere (Martinez-Gomez *et al.*, 2009; Marques *et al.*, 2012).

2.1. Study population

The EVASYON study was composed of 204 adolescents (12-16 years old) who presented either overweight or obesity according to Cole *et al.* criteria (Cole *et al.*,

2000). Other inclusion criteria were being brought up in Spain, not having a diagnosed disease associated with obesity and receiving no pharmacological treatment.

In order to obtain a large sample size, the intervention was also mentioned in the media (press, radio and local television) and community pharmacies, and talks and brochures were given in schools in Navarra. In this study, a subsample of 74 adolescents (49% males, 12-16 years) was included.

2.2. Intervention

The EVASYON study is a longitudinal multidisciplinary intervention implemented for approximately one-year follow-up. During that time, the study was divided into two phases: intensive calorie-restricted phase for the first 2 months (1st to 9th visits), and an extensive body weight follow-up period during the last 10 months (10th to 20th visits). In the intensive phase participants visited the hospitals weekly for 2 months. Paediatricians explained the patients several motivational strategies, life and time management strategies including physical activity recommendations or sleep time, nutritional advice, family involvement and psychological support. In the extensive intervention participants visited the hospital monthly during 10 months. In these monthly visits participants received nutritional education, as well as guidelines for the maintenance of weight loss after the intensive phase. The different phases of the intervention and all the visits are detailed in **Figure 9**. Moreover, the adolescents were treated in small groups of 8-10 subjects and individual session's strategies offered motivation to encourage adherence of adolescents to the program.

2.3. Data collection

In order to assess the efficacy of the treatment, nine measurement categories were assessed: 1) diet; 2) physical activity and health-related physical fitness; 3) psychological profile; 4) body composition; 5) haematological profile; 6) biochemistry and metabolic profiles; 7) mineral and vitamin profile; 8) immunological profile; 9) genetic profile. All the parameters in each measurement category, excluding genetic profile, were assessed at least at four points: baseline, at the end of the intensive

intervention, at mid-point of the overall intervention and at the end of the EVASYON treatment programme.

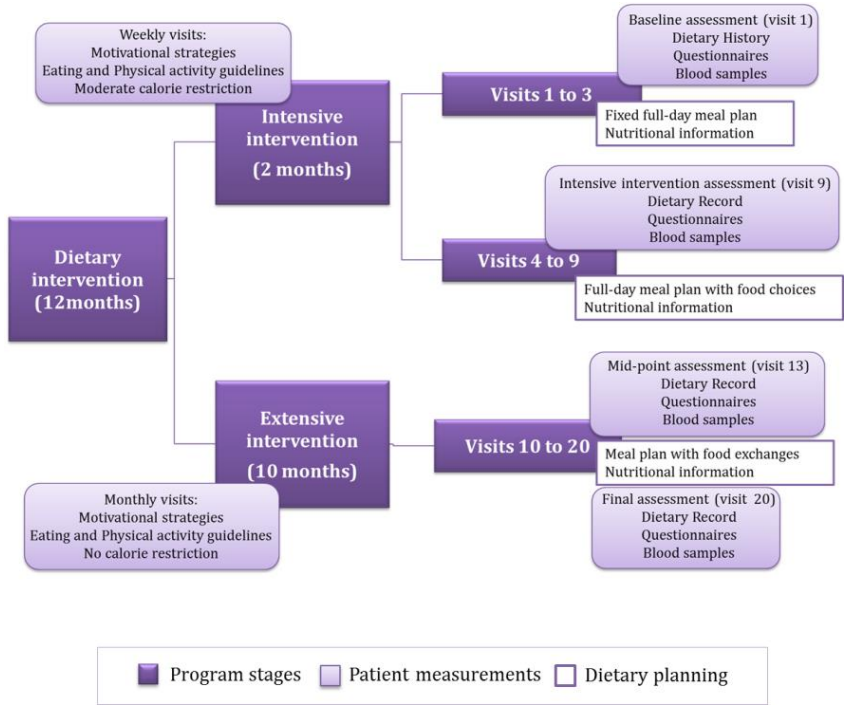

Figure 9. EVASYON study design: stages, questionnaires and intervention planning. Adapted from: Marques *et al.*, 2012.

2.3.1. Dietary assessment

Energy restriction was determined individually to each subject according to their basal metabolism (Schofield formula) and their BMI (Moreno *et al.*, 2006). Adolescents followed an energy-adjusted full-day menu during the first three weeks of intervention and full-day menus with food choices for the next seven weeks to complete the intensive period. In addition, subjects followed a full-day meal plan with exchanges during the extensive body-weight maintenance programme (**Figure 9).**

These meal plans were well-balanced distributed in macronutrients: 50% carbohydrates (CHO), 20% protein and 30% fat (Rusolillo *et al.*, 2003).

A semi-quantitative FFQ (**Appendix 1**), which was previously validated in Spain (Martin-Moreno *et al.*, 1993), and a 72-h dietary record were completed in order to evaluate compliance to the recommended diet, as well as changes in food intake habits during the intervention programme.

2.3.2. Physical activity

Each volunteer was given a personalized plan of physical activity, based on personal tastes during the intensive phase of the treatment. Everyone reached at least 5 weekly hours of moderate/vigorous physical exercise for at least 1 hour in a row. In the maintenance phase, the adolescents were advised to continue with the same habits acquired during those first 10 weeks of intervention.

Physical activity was assessed applying a combination of methods: accelerometers (Actigraph ™, LLC, Fort Walton Beach, FL, USA), adapted Spanish version from the Physical Activity Questionnaire for Adolescents (Martinez-Gomez *et al.*, 2009) and validated and standardized test batteries of fitness (Martinez-Gomez *et al.*, 2009).

2.3.3. Psychological evaluation

Three validated questionnaires for adolescent population were used during the EVASYON Study: Self-Concept Questionnaire (Quiles Marcos and Terol Cantero, 2009), Anorectic Behaviour Observation Scale (Vandereycken, 1992) and Eating Disorder Inventory (Schoemaker *et al.*, 1997).

2.3.4. Body composition

A complete set of measurements was performed and then repeated twice more. Weight and height were obtained by standardized procedures. Skinfolds thicknesses were measured on the left side of the body to the nearest 0.1 mm with a skinfold caliper (Caliper Holtain; Holtain Ltd., Walles, UK) at six different sites. Waist

and hip circumferences were measured with a flexible non-stretchable measuring tape (Type SECA 200). Fat mass percentage was calculated according the Slaughter formula (Slaughter *et al.*, 1988). In addition, BMI was converted into SDS according to the Spanish children and adolescents growth references (Moreno *et al.*, 2007).

2.3.5. Blood samples

Blood collection was carried out by experienced clinical staff after an overnight fasting. It was extracted from the antecubital vein (21.5 mL). Blood samples were divided into aliquots as follows: 1.5 mL in EDTA tube (for haematological study and immunophenotyping of peripheral blood cells), 10 mL in EDTA tube (for plasma extraction) and 10 mL in gel containing tube (for serum extraction). Within 1 hour of collection, blood was centrifuged and aliquots of plasma or serum were stored at -80°C.

3. PREDIMED study

PREDIMED is a long-term nutritional intervention study aimed to assess the efficacy of the MeDiet in the primary prevention of CVDs. The acronym PREDIMED comes from the Spanish words "Prevención con Dieta Mediterránea" that actually means prevention with MeDiet. It is a large randomized clinical trial including participants at high risk of CVD who were assigned to one of three different dietary groups of intervention. Thus, the main hypothesis was that two traditional MeDiets, one enriched with EVOO and another enriched with nuts, both high in total fat and unsaturated fat, would be superior to the usually recommended low-fat diet for the primary prevention of CVD. Other aspects of the design and methods of the PREDIMED study have been published elsewhere (Martinez-Gonzalez *et al.*, 2012).

The Institutional Review Board of Hospital Clinic (Barcelona) approved the study protocol on July 2002. The protocol was written in accordance to the Declaration

of Helsinki and registered at www.controlled-trials.com/ISRCTN35739639. The recruitment of participants was carried out between October 2003 and November 2008 in different Spanish primary care centres. This multicentre clinical trial involved 19 research centres located in Navarra, Valencia, Reus, Mallorca, Sevilla, Málaga, Vitoria, Las Palmas and Barcelona. Further details concerning the PREDIMED study are available at www.predimed.es.

3.1. Study population

Participants were men (55–80 years old) or women (60–80 years old) who were free of CVD at baseline but were at high cardiovascular risk. Inclusion criteria were to have either T2D or three or more major cardiovascular risk factors. The inclusion and exclusion criteria are detailed in **Table 5**.

Table 5. Inclusion and exclusion criteria

Inclusion criteria
- **T2D** - **Cardiovascular risk factors (≥3):** • Current smoking (>1 cigarette/day during the last month) • Hypertension (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or antihypertensive medication) • LDL cholesterol ≥160 mg/dl or lipid-lowering therapy • HDL cholesterol ≤40 mg/dl in men or ≤50 mg/dl in women • BMI ≥25 kg/m^2 • Family history of premature coronary heart disease
Exclusion criteria
- Previous history of CVD - Severe chronic illness, immunodeficiency or human immunodeficiency virus positive status - Illegal drug or alcohol misuse - BMI >40 kg/m^2 - History of allergy to olive oil or nuts or impossibility to follow a MeDiet, for religious reasons or due to the presence of disorders of chewing or swallowing - Low predicted likelihood of changing dietary habits according to the Prochaska and DiClemente stages of change model (Nigg *et al.*, 1999) - Participation in any drug trial or use of any investigational drug within the last year - Institutionalized patients for chronic care, those who lack autonomy, are unable to walk, lack a stable address, or are unable to attend visits in the primary health care centre every 3 months - Illiteracy. - Patients with an acute infection or inflammation (e.g., pneumonia) are allowed to participate in the study 3 months after the resolution of their condition

Finally, 7,447 subjects were included and specifically, the PREDIMED-NAVARRA recruitment centre recruited 1,055 participants that were randomly allocated to one of the three arms (**Figure 10**). In our study we included 521 subjects (55% women, 55-80 years) who had baseline and 5-year data and DNA samples available.

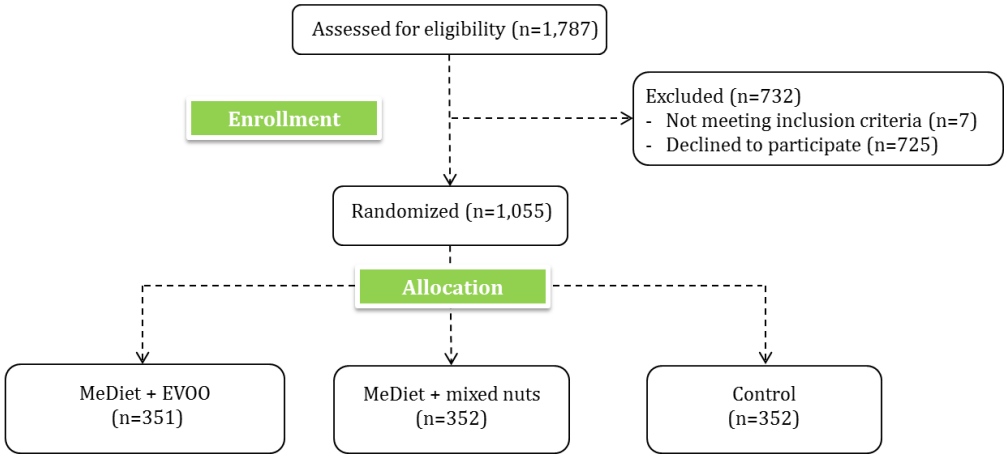

Figure 10. Flowchart of participants in the PREDIMED-NAVARRA.

3.2. Intervention

Study participants were randomized to three groups of intervention: MeDiet supplemented with EVOO, MeDiet supplemented with mixed nuts or a control group who were advised to follow a low-fat diet based on the American Heart Association's guidelines.

The two groups allocated MeDiets received intensive education to follow the MeDiet and supplemental foods at no cost. EVOO (1 L/week) was provided to the first group and 30 g/day of mixed nuts (15 g walnuts, 7.5 g hazelnuts and 7.5 g almonds) to the second group. Participants in the control group also received other gifts for their attendance to the formative sessions. The rationale for the free provision of these food items (EVOO and nuts) is that they may contribute to a higher compliance with the

overall MeDiet food pattern, as well as, the achievement of greater motivation of the participants.

The intervention consisted in quarterly individual interviews with the dietician and group visits that were repeated every 3 month (separated sessions are organized for each intervention group). Each visit included three steps: assessment, intervention, and future directions. Written material, cooking recipes, description of seasonal foods, shopping lists and weekly plan of meals were provided to the participants to ensure a better compliance to the dietary pattern. Promoting adherence and motivational education to the participants was vital through all the intervention.

3.3. Data collection

Participants attended different appointments: pre-screening evaluation, a screening visit, the randomization, a basal visit and finally the follow-up visits. Information was collected in each of the visits (**Table 6**).

Table 6. Measurement scheduled in the PREDIMED trial

Questionnaire/measurement	Basal	Year-1	Year-2	Year-3	Year-4	Year-5
1. Eligibility questionnaire	X					
2. General questionnaire*	X					
3. Food frequency questionnaire	X	X	X	X	X	X
4. 14-item score of MeDiet	X	X	X	X	X	X
5. Physical activity questionnaire	X	X	X	X	X	X
6. Follow-up questionnaire*		X	X	X	X	X
7. Blood samples	X	X	X	X	X	X

* Includes measurements of weight, height, waist circumference, blood pressure and ankle-brachial blood pressure index.

3.3.1. Socio-demographic information

A general questionnaire was answered at the beginning of the study collecting data about socio-demographic variables, such as age, sex, employment status or marital status, among others.

3.3.2. Clinical and lifestyle assessment

Information about medical conditions, new medical diagnosis of illnesses or the current use of medication (including doses) was collected at baseline and in the follow-up visits. Moreover, aspects about lifestyle, such as smoking habit, were also requested.

3.3.3. Anthropometric measurements

Professional nurses measured body weight, height, WC and blood pressure, following standardized protocols. Weight was measured using a calibrated balance beam scale with the subject barefoot and wearing light clothes. The nurse measured height using a wall-mounted calibrated stadiometer. WC was measured using an anthropometric measuring tape, at a horizontal plane. For blood pressure measurements, a validated semi-automatic sphygmomanometer (Omron HEM-705CP) was used.

3.3.4. Dietary assessment

To assess the dietary habits of the participants, a semi-quantitative FFQ with 137 items plus vitamin/minerals supplements was yearly filled in (**Appendix 1**). The frequency of food intake was requested according to 9 categories, from "Never or hardly ever" to "More than 6 times per day". This questionnaire was adapted from the Willet questionnaire and validated in Spain (Martin-Moreno *et al.*, 1993). In addition, its reproducibility has been re-evaluated in Spanish populations (de la Fuente-Arrillaga *et al.*, 2010; Fernandez-Ballart *et al.*, 2010).

The adherence to the MeDiet dietary pattern was assessed with the 14-item MeDiet assessment tool (**Appendix 2**). This questionnaire is less time-demanding, less expensive and requires less collaboration from participants than the usual full-length FFQ. Indeed, this 14-item tool is a key element in the intervention conducted in the PREDIMED trial and has been validated against the FFQ used in the study (Schroder *et al.*, 2011).

3.3.5. Physical activity evaluation

Physical activity was assessed through the validated Minnesota leisure time physical activity questionnaire for Spanish population (Elosua *et al.*, 1994; Elosua *et al.*, 2000).

3.3.6. Blood samples

Experienced nurses were directly responsible for collection, processing and storage of biological specimens. Overnight fasting venous blood samples were collected in EDTA added tubes at baseline and years 1, 3 and 5 of follow-up according to **Table 6**. After centrifugation of blood samples at 3,500 rpm for 15 minutes, the buffy coat was extracted and frozen at -80°C for future analysis.

4. Telomere length measurement: quantitative RT-PCR

4.1. DNA extraction

DNA was extracted from the buffy coat of the patients' blood samples which had been frozen at -80°C. It was performed using a commercial kit (Master Pure DNA purification kit for Blood cat) and in all cases the instructions of the manufacturer were followed.

In order to check the quality and the concentration of the DNA, a UV-visible spectrophotometer (NanoDrop Spectrophotometer ND-1000) was used. Once quantified, the samples were frozen at -20°C or -80°C. For the PCR TL analysis, the samples were diluted to 10 ng/μL using Tris 10 mM pH 8.5.

4.2. Quantitative real-time PCR

PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1990s (Mullis, 1990). PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only into a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. With this technique a

target sequence of DNA can be amplified a billion fold in several hours. The reaction has three main steps: DNA denaturation (95°C), primer annealing (50-60°C) and extension (72°C) (**Figure 11**).

The quantitative real-time PCR (RT-PCR) is a variant of the PCR used to simultaneously amplify and quantify absolutely or relatively the product of amplification of DNA. As conventional PCR, a DNA template, at least one pair of specific primers, dNTPs, reaction buffer suitable, and a thermostable DNA polymerase are used in RT-PCR. To this mixture a labelled substance with a fluorochrome is added for measuring fluorescence. The fluorochrome is excited, with the appropriate wavelength, to measure the rate of generation of one or more specific products. The amount of product is directly related to the fluorescence of the reporter dye. Such measurement is performed after each amplification cycle and that is why it is called RT-PCR (ie, immediate, simultaneous PCR). In our case, SYBR Green was the fluorochrome used, which binds the double stranded DNA generated, producing a fluorescence sign detected by the thermal cycler (**Figure 12**).

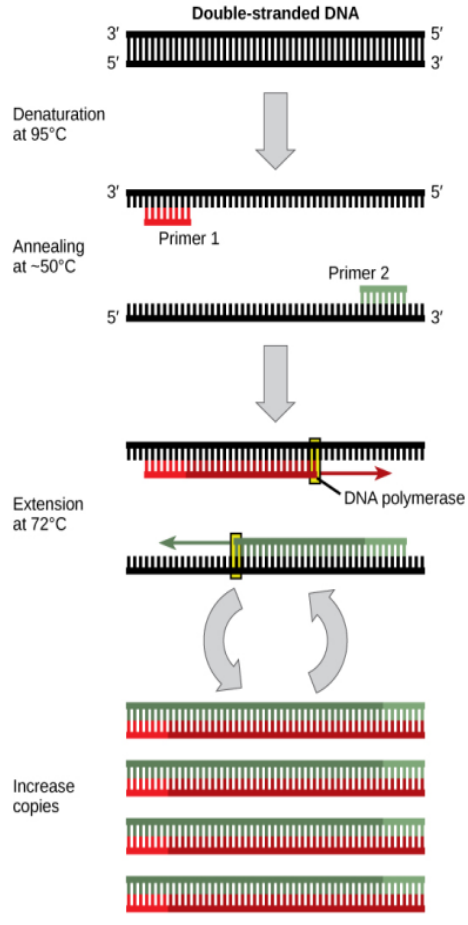

Figure 11. Schematic drawing of the PCR cycle. Source: OpenStax-College.

Estimation errors arising from variations in the quantification method can be the result of DNA integrity, enzyme efficiency and many other factors. For this reason a number of standardization systems have been developed. The most common are aimed at quantifying the specific gene being studied in relation to another gene called a normalizing gene (housekeeping gene), which is elected for its almost constant rate of expression. This enables to report a ratio for the expression of the genes of interest

divided by the expression of the selected normalizer, thereby allowing comparison of the former without actually knowing its absolute level of expression. The most crucial aspect of the process is that the reference gene must be stable (Pfaffl *et al.*, 2004).

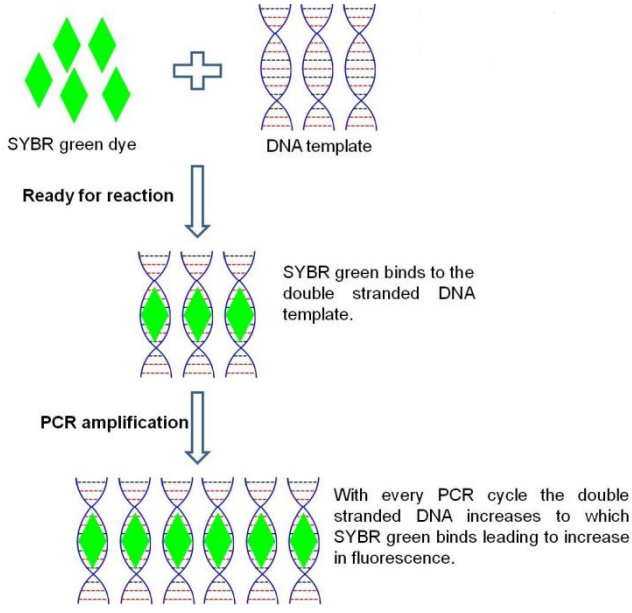

Figure 12. A quantitative RT-PCR assay using SYBR green dye. Adapted from: Ali and Ali, 2011.

4.3. Telomere length methodology process

TL was measured with a quantitative RT-PCR approach, according to the method of Cawthon (Cawthon, 2002), which uses a single-copy gene as a reference for each sample.

The primers were designed based on the sequence of interest. Therefore, a pair of Tel primers for the telomeres PCR and in order to correct for variations in the quantification of genomic DNA, a pair of single-copy gene 36B4 primers (codifies for a ribosomal protein RPLPO) were used. All primers were purchased from SIGMA-Aldrich, St.Louis, MO, USA, and their sequence and characteristics are described in **Table 7**.

Table 7. Characteristics of the primers designed for telomere quantification

Name	Size (bp)	Tm	Cf	Sequence
Tel 1	37	84.6	0.27 µM	5'GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT3'
Tel 2	39	77.7	0.9 µM	5'TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA3'
36B4u	23	67.5	0.4 µM	5'CCCATTCTATCATCAACGGGTACAA3'
36B4d	25	68.1	0.4 µM	5'CAGCAAGTGGGAAGGTGTAATCC3'

bp, base pairs; Tm, melting temperature; Cf, final concentration.

PCRs for telomere and single-copy gene expression were performed on white 384-well plates on an ABI-Applied Biosystems 7900 HT thermal cycler (Applied Biosystems, CA, USA). For quality control, all the samples were run in triplicates (EVASYON and GENOI study) or in duplicates (PREDIMED study) and checked for concordance between the values. In order to obtain a robust consistence, samples showing a high variation (more than 10%) were rerun and reanalysed. Samples from the PREDIMED and EVASYON study consisting in two samples of each patient (baseline and after intervention), were run in the same plate. Furthermore, the intra-assay and inter-assay coefficients of variation (CV) were calculated to control the variation of results within a data set obtained from one experiment and to ensure plate-to-plate consistency, respectively. In all cases the intra-assay coefficient was less than 3%, whereas the inter-assay was below 4%.

A calibration curve of the same DNA sample of reference (64 to 0.25 ng in 2-fold dilutions) was included as a standard in each plate to control the day-to-day variations. Standard curve with linearity $R^2 > 0.98$ was accepted. Moreover, a negative control was incorporated in each measurement.

The total reaction volume was 10 µL containing 10 ng of genomic DNA (1 µL), 5 µL of master mix and 2 µL of single-copy gene primers or 2.5 µL for Tel1 and 1.5 µL for Tel2. The commercial kit QuantiTect SYBR Green PCR (Qiagen, Valencia, CA, USA) was used as master mix. This master mix includes:

- *HotStarTaq DNA Polymerase* provides a stringent hot start, preventing the formation of nonspecific products. To activate the enzyme a previous step is needed for 15 min at 95°C.

- *QuantiTect SYBR Green PCR Buffer*, balanced combination of NH^{4+} and K^{+} ions that promotes specific primer annealing and enables high PCR specificity and sensitivity.
- *dNTP mix,* contains dUTP, enabling pretreatment with uracil-N-glycosylase (UNG) prior to starting PCR, which ensures that any contaminating PCR products do not affect subsequent PCR reactions.
- *SYBR Green I and ROX dyes,* the first one yields a strong fluorescent signal upon binding double-stranded DNA, whereas the second is used for normalization of fluorescent signals.

The amplification protocols for both PCRs are described in **Table 8.**

Table 8. PCR amplification protocol for telomeres and single-copy gene

Step	Time	Temperature
UNG pretreatment	2 min	50°C
PCR initial heat activation	10 min	95°C
3-step cycling		
Denaturation	15 s	95°C
Annealing	30 s	52-60°C*
Extension	2 min	72°C
Number of cycles	25-35†	

* 52°C for telomere expression and 60°C for 36B4 single-copy gene.

† 25 cycles for telomere expression and 35 cycles for 36B4 single-copy gene.

The analysis of the data was carried out with the informatics program of the thermal cycler (7900 HT Version 2.3 Sequence Detection Systems). There, the fluorescence increment in each cycle was registered and reflected in the kinetics of the reaction. Appearance of detectable fluorescence at an earlier cycle number indicates a greater amount of initial template substrate. The detectable amount of fluorescence, a signal significantly greater than background, is known as the threshold. The cycle, during which a reaction emits that threshold level of fluorescence, is known as the threshold cycle (Ct). Relative concentrations (arbitrary units) were obtained from the calibration curve, made with the standards, where the Ct and the logarithm of standard concentrations are represented (**Figure 13A and B**).

The identification of specific, amplified DNA fragments was checked using the analysis of their melting temperature (Tm). The DNA melting temperature is specific to the amplified fragment. Unlike conventional PCR, this method avoids the previous use of electrophoresis techniques to demonstrate the results of all the samples. This is because, despite being a kinetic technique, quantitative PCR is usually evaluated at a distinct end point. The technique therefore usually provides more rapid results and uses fewer reactants than electrophoresis (**Figure 13C**).

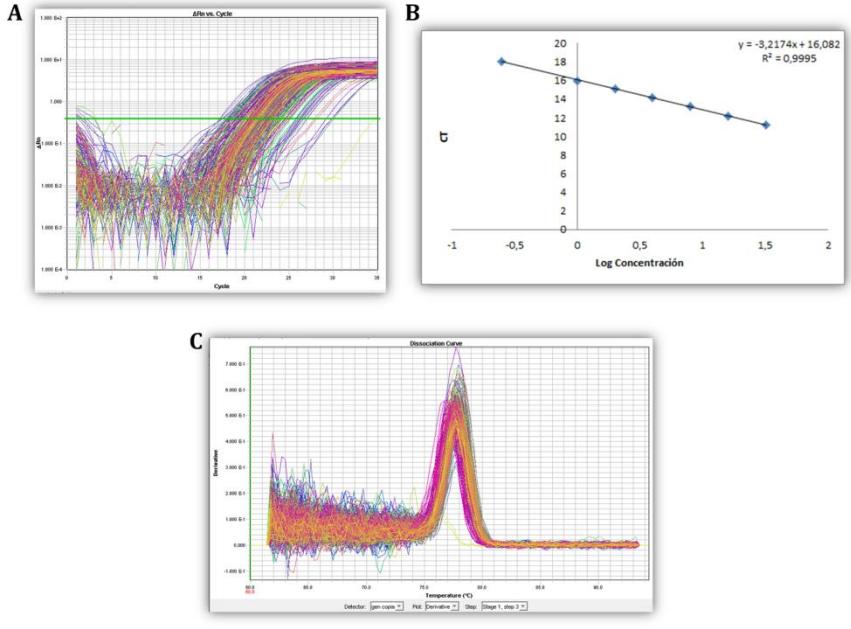

Figure 13. A: kinetics reaction, cycle *vs.* fluorescence. At the beginning of the reaction there is insufficient signal amplification, but as the number of cycles increase, an exponential increment in the concentration of the samples is produced, until the plateau phase is reached. **B:** calibration curve obtained from Ct values and the concentration logarithm of each sample. The calibration curve is calculated from a linear regression where a correlation coefficient (R^2) is obtained and its quality is defined when the value is greater than 0.98. **C:** Dissociation curve to check the specificity of the amplified DNA fragments. The procedure is based in the melting temperature characteristic of the amplicons.

The T/S ratio (telomere to single-copy gene) for each sample was obtained as a measure of relative TL and it was calculated as follows:

$$\Delta Ct = Ct\,(Tel) - Ct\,(36B4)$$

$$T/S\,ratio = 2^{(-\Delta Ct)}$$

The value regarding TL used in the different studies was the T/S ratio. TL was measured at two points regarding the PREDIMED study (basal and after 5 years) and the EVASYON study (basal and after 2 months), and in the GENOI study TL was assessed at one point due to its cross-sectional design.

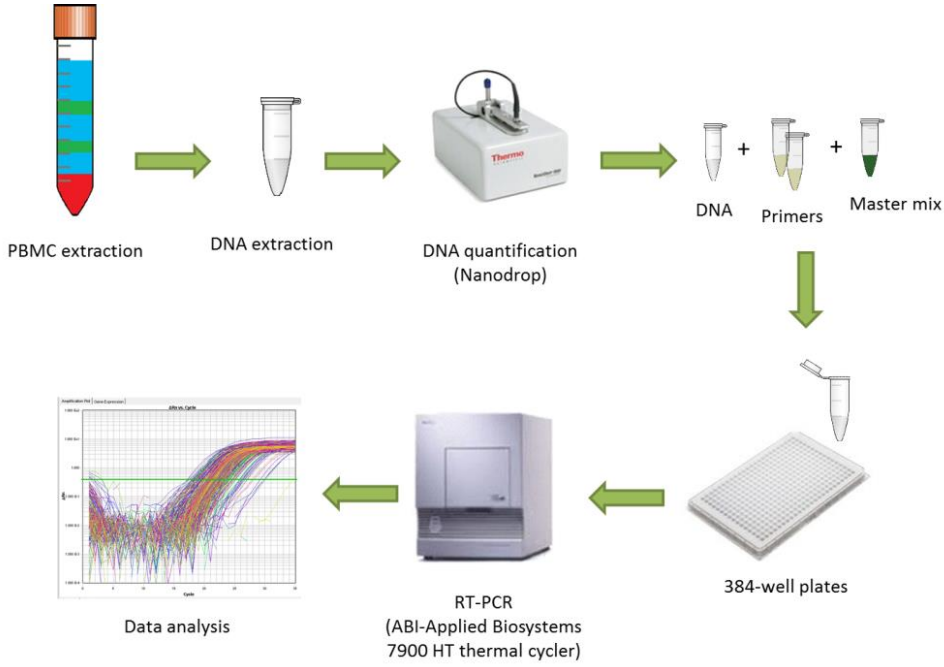

Figure 14. Flow diagram summary describing the methodology process of telomere length measurement.

5. Statistical analysis

T/S ratio was used as the relative measurement of TL. In the GENOI and EVASYON studies leukocyte TL was ln-transformed to achieve a better normal distribution conformation. Moreover, in chapter 4, TL was categorized into different groups (TL tertiles, TL gain and TL loss) to facilitate a better understanding of the results.

To analyse the cross-sectional association between the diet and TL, we performed multivariable linear regression models including age, sex, BMI-SDS and total energy intake as potential confounders. Foods were analysed as continuous variables and also stratified into quintiles for the assessment of leukocyte TL. Moreover, we carried out logistic regression models to establish the risk of having a very low TL (<10^{th} percentile) according to dietary TAC and white bread consumption.

For studying the relationship between *PPARγ2* genotype, telomere variation and changes in dietary components after a nutritional intervention, we used ANCOVA models after adjusting for potential confounders measured at baseline. Changes in dietary factors after the 5-year intervention programme were dichotomized based on individual's median levels, with the exception of the 14-item questionnaire that was divided into tertiles. Gene-diet interactions were tested for TL changes with the likelihood ratio test.

The longitudinal association between adiposity and leukocyte TL was evaluated in adolescents and in subjects at high cardiovascular risk after two different lifestyle interventions. In the first place, for the EVASYON study the analyses were carried out stratifying the adolescent population by sex. Changes in TL after a 2-month multidisciplinary intervention were assessed with a paired t-test. Pearson correlation coefficient was calculated between TL variation and baseline TL after adjustment for age and sex. Multivariable linear regression models were fit to analyse changes in anthropometric parameters according to leukocyte TL at baseline. Moreover, Benjamini-Hochberg multiple testing correction (Benjamini and Hochberg, 1995) was used to control de overall two-sided alpha level at 0.05.

In the second place, for the PREDIMED-NAVARRA study, the associations between changes in anthropometric markers and baseline and follow-up TL were examined performing Pearson's correlations analyses and multivariable linear regression models. In the multiple-adjusted models, Benjamini-Hochberg multiple testing correction was applied. Furthermore, we also carried out multivariable logistic regression models to estimate the odds of remaining obese after the intervention according to TL.

The statistical analyses were performed using STATA version 12.0 (StataCorp, College Station, TX, USA). A *P*-value <0.05 (two-tailed test) was considered statistically significant. These analyses are explained in more detailed in each corresponding chapter.

IV. Results

CHAPTER 1

Dietary total antioxidant capacity is associated with leukocyte telomere length in a children and adolescent population

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CHAPTER 2

The Pro12Ala polymorphism of the *PPARγ2* gene interacts with a Mediterranean Diet to prevent telomere shortening in the PREDIMED-NAVARRA randomized trial.

García-Calzón S 1, Martínez-González MA 2,3, Razquin C 2, Corella D 3,4, Salas-Salvadó J 3,5, Martínez JA 1, 3,6, Zalba G 7, Marti A 1,3.

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23/164 in Genetics & Heredity (Q1)

CHAPTER 3

Telomere length as a biomarker for adiposity changes after a multidisciplinary intervention in overweight/obese adolescents: the EVASYON study

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PLOS ONE

Telomere Length as a Biomarker for Adiposity Changes after a Multidisciplinary Intervention in Overweight/Obese Adolescents: The EVASYON Study

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Abstract

Context: Telomeres are biomarkers of biological aging. Shorter telomeres have been associated with increased adiposity in adults. However, this relationship remains unclear in children and adolescents.

Objective: To evaluate the association between telomere length (TL) and adiposity markers in overweight/obese adolescents after an intensive program. We hypothesize that greater TL at baseline would predict a better response to a weight loss treatment.

Design, Setting, Patients and Intervention: The EVASYON is a multidisciplinary treatment program for adolescents with overweight and obesity that is aimed at applying the intervention to all possibly involved areas of the individual, such as dietary habits, physical activity and cognitive and psychological profiles. Seventy-four participants (36 males, 38 females, 12–16 yr) were enrolled in the intervention program: 2 months of an energy-restricted diet and a follow-up period (6 months).

Main Outcome: TL was measured by quantitative real-time polymerase chain reaction at baseline and after 2 months; meanwhile, anthropometric variables were also assessed after 6 months of follow-up.

Results: TL lengthened in participants during the intensive period ($+1.9 \pm 1.0$, $p<0.001$) being greater in overweight/obese adolescents with the shortest telomeres at baseline ($r=-0.962$, $p<0.001$). Multivariable linear regression analysis showed that higher baseline TL significantly predicted a higher decrease in body weight ($B=-1.53$, $p=0.005$; $B=-2.25$, $p=0.047$) and in standard deviation score for body mass index (BMI-SDS) ($B=-0.22$, $p=0.010$; $B=-0.47$, $p=0.005$) after the intensive and extensive period treatment respectively, in boys.

Conclusion: Our study shows that a weight loss intervention is accompanied by a significant increase in TL in overweight/obese adolescents. Moreover, we suggest that initial longer TL could be a potential predictor for a better weight loss response.

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Introduction

Telomeres are tandem TTAGGG repeats of DNA that, together with associated protein factors, cap the ends of chromosomes and promote chromosome stability [1]. For individuals at any age, telomere length (TL) depends on, first, the initial setting of TL in the newborn, and second, the magnitude of telomere erosion from birth onwards [2]. Telomere attrition, in turn, depends on cell replication rate, cumulative exposure to agents that produce DNA damage (such as oxidative, inflammatory, endocrine and other forms of biological stress), and activity of the telomerase enzyme [3].

The prevalence of obesity is increasing rapidly worldwide, with a high impact on children and adolescents [4]. Therefore, treating obesity in young people is critical to prevent adult obesity-related complications [5]. It is well known that obesity is characterized as a state of chronic inflammation and heightened oxidative stress [6,7]. However, existing data on the relationship between obesity and TL in adults have yielded equivocal results; several studies reported an inverse association of TL with obesity [8–11], but others did not [12,13]. Differing outcomes have also been found in pediatric population. Zannolli *et al.* [14] found no difference in TL between obese and normal-weight Caucasian children, whereas Al-Attas *et al.* [15] and Buxton *et al.* [16] reported significantly shorter TL in obese children compared with the nonobese ones. There is just one study regarding the relationship between adiposity and TL in adolescents that showed no association, but it demonstrated that race and sex differences in TL have already emerged during adolescence [17].

To date, a limited number of studies have explored the association between TL and adiposity indices after a lifestyle intervention, suggesting that maintaining or losing weight can lead to preservation or lengthening of the TL [10,11,18,19]. Moreover, only two prospective studies considered TL as a biomarker for adiposity changes in an elderly population [9,11].

To our knowledge, no prospective studies have assessed the relationship between TL and changes in adiposity traits in children or adolescents following a lifestyle educational program. Thus, the aim of this study was to assess the relationship between baseline TL and changes in anthropometric and obesity parameters after 2 and 6 months of a multidisciplinary intervention in overweight/obese adolescents. We hypothesize that greater TL at baseline would predict a better response to the multidisciplinary intervention, as has been previously observed in adult studies [11].

Subjects and Methods

Ethics Statement

Written consent to participate was requested from both parents and adolescents. The study protocols were performed in accordance with the ethical standards laid down in the 1961 Declaration of Helsinki (as revised in South Korea in 2008), following the European Economic Community (EEC) Good Clinical Practice guidelines (document 111/3976/88 of July 1990) and current Spanish law, which regulates clinical research in humans (Royal Decree 561/1993 regarding clinical trials). The study protocol was approved by the institutional review board and the Ethics Committee of each hospital that participated in this project (Madrid, Granada, Pamplona, Zaragoza, Santander) and by the Ethics Committee of the Spanish Council for Scientific Research (CSIC).

Study Population and Intervention

The study population included 204 overweight or obese adolescents within the EVASYON program; Development, Implementation, and Evaluation of the Efficacy of a Therapeutic Program for Adolescents with Overweight and Obesity: Integral Education on Nutrition and Physical Activity (EVASYON) study (http://www.estudioevasyon.com/). EVASYON study was carried out in 5 Spanish cities (Granada, Madrid, Pamplona, Santander, and Zaragoza) and it is a lifestyle education program supported by a multidisciplinary team of nutritionists, physical education specialists, psychologists, and pediatricians. The study was implemented in two stages: an intensive, energy-restricted period for the first 2 months, and an extensive body-weight follow-up period for the last 11 months.

In the present study, we present data from the intensive treatment period corresponding to the first 2 months (an energy-restricted phase) and 6 months regarding the extensive intervention, in a subsample of 74 participants (49% males) from whom

Table 1. Anthropometric measures of the overweight/obese adolescents at baseline and during the weight loss treatment, stratifying by sex.

	BOYS				GIRLS				
	Baseline	**2 months**	**6 months**	**P-value[1]**	**Baseline**	**2 months**	**6 months**	**P-value[1]**	**P-value[2]**
n	36				38				
Age	14.3 (0.9)				14.4 (1.1)				0.512
Weight (kg)	85.9 (15.2)	81.2 (14.4)	78.4 (13.6)	<0.001	84.3 (14.9)	80.6 (13.7)	81.1 (13.5)	<0.001	0.650
BMI-SDS	5.0 (1.6)	4.1 (1.7)	3.2 (1.6)	<0.001	4.6 (2.0)	3.9 (1.7)	3.9 (1.9)	<0.001	0.363
Body fat (%)	47.3 (8.5)	42.8 (8.1)	39.2 (8.4)	<0.001	44.1 (6.4)	40.1 (6.7)	40.3 (5.5)	<0.001	0.082
Waist circumference (cm)	101.9 (9.8)	98.5 (10.6)	92.0 (10.1)	<0.001	108.4 (12.0)	104.3 (10.6)	102.6 (12.4)	<0.001	0.014
Hip circumference (cm)	101.6 (22.8)	99.0 (20.8)	95.1 (19.6)	<0.001	85.9 (27.4)	81.5 (23.9)	81.8 (22.9)	0.133	0.011
Waist to hip ratio	1.08 (0.38)	1.06 (0.36)	1.03 (0.35)	0.087	1.39 (0.46)	1.39 (0.42)	1.36 (0.43)	0.695	0.002
Waist to height ratio	0.61 (0.05)	0.59 (0.06)	0.54 (0.05)	<0.001	0.67 (0.07)	0.64 (0.06)	0.63 (0.08)	<0.001	<0.001
Σ 6 skinfolds (mm)	182.9 (24.9)	166.5 (28.8)	150.2 (37.0)	<0.001	194.6 (27.6)	179.3 (28.3)	176.9 (31.5)	<0.001	0.113

The table shows means (SD). BMI-SDS: Standard Deviation Score for BMI.
[1]: p value in three different time points analyzed by repeated-measures ANOVA in subjects distributed by sex.
[2]: p value for the comparison at baseline between boys and girls.
doi:10.1371/journal.pone.0089828.t001

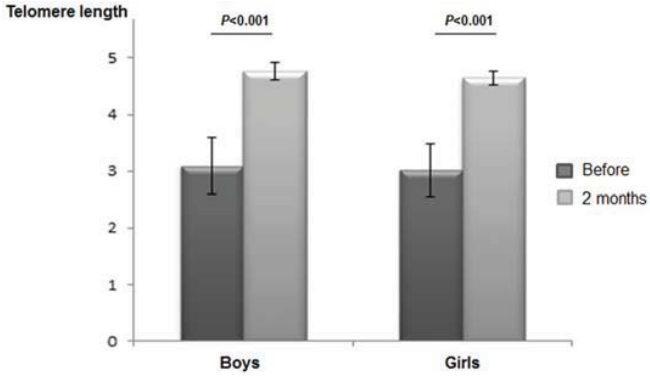

Figure 1. Telomere length distribution in boys and girls before and after the intensive lifestyle intervention. Significant differences were found after 2 months *vs.* before the multidisciplinary program.
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DNA samples were available. We could not do the analysis at 12 months of follow-up due to the observed drop-out rate (26%). 36 males (2.8% were overweight and 97.2% obese) and 38 females (5.3% overweight and 94.7% obese) were enrolled. The study included 12- to 16-year-old overweight or obese adolescents, according to Cole's criteria [20], who were brought up in Spain (inclusion criteria), had no diagnosed disease associated with obesity, were not receiving pharmacologic treatment and were not diagnosed of anorexia, bulimia or other eating disorders (exclusion criteria). Cole *et al.* [20] developed an internationally acceptable definition of child overweight and obesity, specifying the measurement, the reference population, and the age and sex specific cut off points. They obtained the reference population by averaging across a heterogeneous mix of surveys from different countries, with widely differing prevalence rates for obesity, whereas the appropriate cut off point was defined in body mass index (BMI) units in young adulthood and extrapolated to childhood, conserving the corresponding centile in each dataset.

Based on food intake questionnaires, a personalized diet (30% of energy (E) from fat, 15% E from proteins, and 55% E from carbohydrates) [21] was prescribed while the physical activity program was instructed to each adolescent. During the intensive program period, the adolescents attended weekly group sessions where they received nutritional education, dietary advice, physical activity recommendation, as well as psychological support. During the extensive body-weight maintenance period, adolescents attended monthly in person follow-up visits with the registered dietician. The participants and their families received group sessions on different aspects such as diet, physical activity, healthy habits and weight maintenance skills, how to engage in healthy weight control behaviors and relapse prevention. The description of the complete EVASYON study has been previously published elsewhere [22,23].

Weight and height were measured with an electronic scale (Type SECA 861; SECA, Hamburg, Germany) and with a telescopic height measuring instrument (Type SECA 225), respectively. BMI was calculated as weight (kg)/height^2 (m^2), then, individual BMI values were converted into SDS using age and specific cut-points according to the Spanish children and adolescent growth references [24]. Skinfolds were measured on the left side of the body to the nearest 0.1 mm with a skinfold caliper (Caliper Holtain; Holtain Ltd., Walles, UK) at triceps, biceps, subscapular, suprailiac, thigh, and calf. The waist and hip circumferences were measured with a flexible non-stretchable measuring tape (Type SECA 200). Fat mass percentage was calculated according to the Slaughter formula [25].

Telomere Length Assessment

TL was measured in genomic DNA extracted from human peripheral blood samples, using a real-time quantitative polymerase chain reaction (RT-PCR), as described by Cawthon [26]. Telomeres were measured at two points: at baseline and after 2 months of the intensive intervention period. Concentrations of telomere repeat copy number (T) and single-copy gene (Ribosomal Protein Large PO) copy number, (S) as a reference for each sample, were obtained with this method.

PCRs were performed on white 384-well plates on an ABI-Applied Biosystems 7900 HT thermal cycler (Applied Biosystems, Austin, TX, USA). The total reaction volume was 10 µL containing 10 ng of genomic DNA, and a quantiTect Syber Green PCR kit (Qiagen, Valencia, CA, USA) was used as master mix. The final telomere primer concentrations were as follows: for telomere amplification tel1, 675 nmol/L and for tel2, 1350 nmol/L; and for the amplification of the single copy gene RPLPO: hRPLPO1, 800 nmol/L; hRPLPO2, 800 nmol/L. The primer sequences (Sigma-Aldrich, St.Louis, MO, USA) were tel1 (5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGG-T-3′), tel2 (5′-TCCCGACTATCCCTATCCCTATCCCTATC-CCTATCCCTA-3′), hRPLPO1 (5′-CCCATTCTATCATCA-ACGGGTACAA -3′) and hRPLPO2 (5′-CAGCAAGTGG-GAAGGTGTAATCC -3′). This method normalizes T to S by taking the ratio (T/S ratio) for each sample. The T/S ratio was calculated as follows $[2^{CT(\text{telomeres})}/2^{CT(\text{single copy gene})}] = 2^{-\Delta CT}$, since the amount of the PCR product approximately doubles in each cycle of the PCR [26].

A calibration curve of the same DNA sample of reference (64–0.25 ng in 2-fold dilutions) was included for each measurement as a standard, to control the day-to-day variations. Standard curve with linearity $R^2>0.98$ was accepted. For quality control, all samples were run in triplicate and checked for concordance between triplicate values. Moreover the two DNA samples of each patient (at baseline and after 2 months of recruitment) were run in

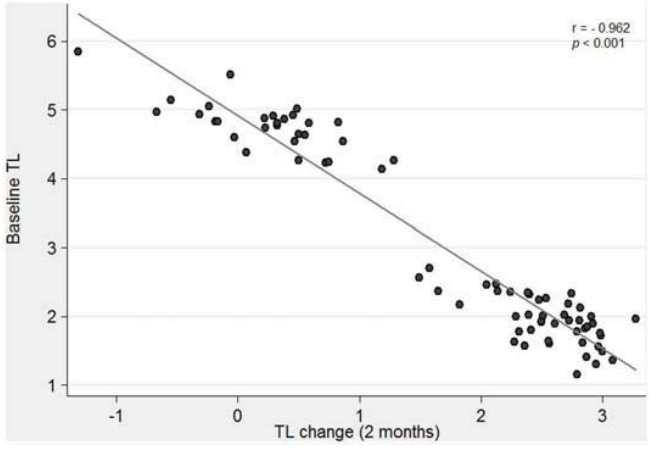

Figure 2. Telomere length change after the intensive intervention as a function of telomere length at baseline. The scatter plot shows the entire adolescent's sample (n = 74), each dot representing one individual. Relative telomere length is adjusted for age and sex.
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the same plate. In order to obtain a robust consistence, samples showing a high variation (more than 10%) were rerun and reanalyzed. The intra-assay coefficient of variation between triplicates was 1.6% and the inter-assay coefficient of variation between plates was 3.8%, which supports the power of this procedure.

Statistical Analysis

The sample size calculation led to conclude that at least 22 subjects were needed. This estimate was based on the following assumptions: a two-tailed alpha error of 5%, a power of 90%, and a mean (± standard deviation) difference of 50±50 in TL after the intervention. Our study involved 74 subjects from the EVASYON study who had DNA samples at baseline and after 2 months.

We compared the anthropometric measures of the adolescents at baseline and during the weight loss treatment, stratifying the sample by sex. We calculated means and SD for each variable and assessed the statistical significance of the differences among them with repeated-measures ANOVA. We used the unpaired t-test for comparing the parameters at baseline between boys and girls.

TL was ln-transformed to achieve a better normal distribution conformation. A paired t-test was performed to evaluate the increase in TL after 2 months of the intensive intervention period, dividing the participants by sex. In addition, we also calculated the Pearson correlation coefficient (r) between baseline TL and the change in TL after the intensive treatment period after adjusting for age and sex.

We fitted multivariable linear regression modeling to analyze changes in the anthropometric variables according to the increase of 1 SD in baseline TL. Comparisons were done separately for girls and boys and were adjusted for age, baseline BMI-SDS and for each basal anthropometric variable depending on the analysis. Moreover, we also assessed differences in the adiposity traits after 2 and 6 months in males, according to the median of baseline TL. B coefficients and 95% confidence intervals (CI) were calculated using those who had the shortest telomeres at baseline as the reference group and multiple testing correction (Benjamini-Hochberg) was used to control the overall two-sided alpha level at 0.05.

Statistical analyses were performed using STATA version 12.0 (StataCorp, College Station, TX, USA). The significance level for all the analyses was set at $\alpha = 0.05$ and all the tests were two-sided.

Results

Table 1 summarizes the general features of the 74 overweight/obese adolescents (mean age 14.3±1.0 yr, 49% males) at baseline and after 2 and 6 months of the weight loss program, dividing the population by sex. Sex differences were found at baseline regarding abdominal obesity ($p<0.050$). Both males and females did significantly lose body weight ($p<0.001$) after the intervention, which was accompanied by a significant improvement in the anthropometric measurements.

Average TL was ascertained in 74 subjects using RT-PCR. The analyses were carried out after ln-transformed of all T/S ratios. Interestingly, TL significantly increased after the intensive lifestyle intervention in 88% of the adolescents (+1.93 [1.69−2.18], $p<0.001$) (data not shown). In this sense, mean differences values (95% CI) were +1.66 (1.27−2.06) for boys and +1.64 (1.22−2.06) among girls (Figure 1). No differences in TL were found at baseline or during the 2 months of intensive intervention period in subjects according to sex.

Furthermore, the individual change in TL was inversely correlated with initial TL at a highly significant level after controlling for age and sex ($r = -0.962$, $p<0.001$; Figure 2), indicating that the lengthening rate was most pronounced in individuals with shorter telomeres at baseline. Due to this strong correlation, baseline TL was considered as a potential predictor factor for changes in adiposity in obese adolescents. Surprisingly, we did not observe a significant association between TL and participant's age ($r = -0.122$, $p = 0.301$; data not shown).

We fitted a multiple regression model to predict the changes in anthropometric and adiposity traits at follow-up according to sex (Table 2). In boys, during 2 months of the intensive treatment phase, higher baseline TL significantly predicted a greater

Table 2. Changes in anthropometric variables after 2 and 6 months of the weight loss program, according to the increase of 1 SD in baseline TL.

	Baseline TL							
	Boys (n = 36)				Girls (n = 38)			
	2 months		6 months		2 months		6 months	
	B (95% CI)	P-value	B (95% CI)	P-value	B (95% CI)	P-value	B (95% CI)	P-value
Δ Body Weight (kg)								
Unadjusted	−1.35	0.017	−2.08	0.086	0.44	0.487	0.27	0.767
	(−2.45 to −0.25)		(−4.48 to 0.31)		(−0.84 to 1.73)		(−1.59 to 2.13)	
Adjusted[1]	−1.53	0.005*	−2.25	0.047	0.84	0.149	0.71	0.406
	(−5.57 to −0.49)		(−4.48 to −0.03)		(−0.32 to 2.01)		−(1.01 to 2.43)	
Δ BMI-SDS								
Unadjusted	−0.20	0.014	−0.46	0.006	0.10	0.484	0.03	0.780
	(−0.35 to −0.04)		(−0.78 to −0.14)		(−0.18 to 0.38)		(−0.22 to 0.29)	
Adjusted[1]	−0.22	0.010*	−0.47	0.005*	0.20	0.102	0.09	0.443
	(−0.38 to −0.05)		(−0.79 to −0.15)		(−0.04 to 0.43)		(−0.15 to 0.34)	
Δ Body fat (%)								
Unadjusted	0.20	0.750	−0.45	0.686	0.99	0.246	0.98	0.252
	(−1.08 to 1.49)		(−2.68 to 1.79)		(−0.71 to 2.69)		(−0.72 to 2.68)	
Adjusted[1]	0.14	0.826	−0.75	0.482	1.15	0.152	0.95	0.146
	(−1.13 to 1.41)		(−2.91 to 1.41)		(−0.45 to 2.75)		(−0.35 to 2.25)	
Δ Waist circumference (cm)								
Unadjusted	−1.98	0.037	−1.85	0.257	0.74	0.577	2.04	0.191
	(−3.48 to −0.12)		(−5.12 to 1.41)		(−1.94 to 3.42)		(−1.06 to 5.14)	
Adjusted[1]	−1.47	0.065	−1.56	0.304	1.38	0.221	2.73	0.078
	(−3.04 to 0.09)		(−4.59 to 1.48)		(−0.87 to 3.62)		(−0.32 to 5.78)	
Δ Hip circumference (cm)								
Unadjusted	−0.72	0.505	0.50	0.725	3.86	0.125	4.70	0.067
	(−2.88 to 1.45)		(−2.38 to 3.38)		(−1.12 to 8.85)		(−0.35 to 9.74)	
Adjusted[1]	−1.83	0.065	−0.92	0.409	2.52	0.260	1.58	0.493
	(−3.78 to 0.12)		(−3.17 to 1.33)		(−1.96 to 6.99)		(−3.05 to 6.21)	
Δ Waist to hip								
Unadjusted	−0.01	0.461	−0.04	0.021	−0.05	0.103	−0.09	0.051
	(−0.05 to 0.02)		(−0.09 to −0.01)		(−0.11 to 0.01)		(−0.18 to 0.01)	
Adjusted[1]	0.01	0.914	−0.03	0.084	−0.04	0.209	−0.05	0.309
	(−0.03 to 0.04)		(−0.07 to 0.01)		(−0.09 to 0.02)		(−0.14 to 0.04)	
Δ Waist to height								
Unadjusted	−0.01	0.046	−0.01	0.245	0.005	0.499	0.01	0.220
	(−0.02 to −0.001)		(−0.03 to 0.01)		(−0.01 to 0.02)		(−0.01 to 0.03)	
Adjusted[1]	−0.01	0.078	−0.01	0.251	0.01	0.117	0.01	0.119
	(−0.02 to 0.001)		(−0.03 to 0.01)		(−0.01 to 0.02)		(−0.01 to 0.03)	
Δ Σ 6 skinfolds (mm)								
Unadjusted	−2.92	0.306	−4.08	0.347	4.04	0.300	7.37	0.220
	(−8.63 to 2.79)		(−12.77 to 4.61)		(−3.75 to 11.83)		(−0.71 to 15.46)	
Adjusted[1]	−2.18	0.440	−3.79	0.411	4.41	0.237	7.48	0.059
	(−7.87 to 3.51)		(−13.06 to 5.48)		(−3.04 to 11.87)		(−0.30 to 15.25)	

TL: telomere length, BMI-SDS: Standard Deviation Score for BMI, SD: Standard Deviation.
[1]Adjusted for age, basal BMI-SDS and the respective variable at baseline.
*P-value <0.05 after correcting for Benjamini–Hochberg multiple comparisons.
doi:10.1371/journal.pone.0089828.t002

Table 3. Multivariable-adjusted differences (95% confidence intervals) in the change of the anthropometric measures, by the median of baseline telomere length in boys.

	Baseline TL		
	<2.34	≥2.34	P-value
	(n = 18)	(n = 18)	
Change in obesity traits			
Body weight (kg)			
2 months	−2.85 (−4.21 to −1.49)	−6.54 (−7.89 to −5.18)	0.001*
6 months	−3.99 (−7.04 to −0.95)	−9.67 (−12.62 to −6.71)	0.014*
BMI-SDS			
2 months	−0.68 (−0.89 to −0.46)	−1.16 (−1.38 to −0.95)	0.004*
6 months	−1.06 (−1.51 to −0.62)	−2.12 (−2.55 to −1.69)	0.002*
Waist circumference (cm)			
2 months	−1.97 (−4.08 to 0.23)	−4.89 (−7.05 to −2.74)	0.067
6 months	−8.79 (−12.99 to 4.59)	−11.23 (−15.43 to −7.03)	0.429
Hip circumference (cm)			
2 months	−0.20 (−2.70 to 2.29)	−5.10 (−7.60 to −2.60)	0.012*
6 months	−5.56 (−8.57 to −2.54)	−7.51 (−10.53 to −4.50)	0.388
Waist to hip ratio			
2 months	−0.03 (−0.08 to 0.02)	−0.01 (−0.05 to 0.04)	0.450
6 months	−0.03 (−0.08 to 0.02)	−0.07(−0.12 to −0.02)	0.262
Waist to height ratio			
2 months	−0.01 (−0.03 to −0.001)	−0.03 (−0.05 to −0.02)	0.061
6 months	−0.06 (−0.09 to −0.04)	−0.08 (−0.10 to −0.06)	0.326
Σ 6 skinfolds (mm)			
2 months	−14.07 (−21.90 to−6.25)	−18.62 (−26.45 to −10.80)	0.429
6 months	−28.85 (−41.59 to −16.10)	−36.60 (−49.35 to −23.86)	0.408

Data is presented as B (95%CI). TL: telomere length, BMI-SDS: Standard Deviation Score for BMI.
Adjusted for age, basal BMI-SDS and the respective variable at baseline.
*P-value <0.05 after correcting for Benjamini–Hochberg multiple comparisons.
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reduction in body weight (B = −1.53, 95% CI: −5.57 to −0.49) and BMI-SDS (B = −0.22, 95% CI: −0.38 to −0.05) after adjusting for age and the respective variable at baseline. Moreover, a discernible trend was observed in waist circumference (B = −1.47, 95% CI:−3.04 to 0.09), hip circumference (B = −1.83, 95% CI:−3.78 to 0.12), and waist to height ratio (B = −0.01, 95% CI:−0.02 to 0.001). However, no differences were observed in girls. Interestingly, males with higher initial TL also presented a greater decrease in body weight (B = −2.25, 95% CI:−4.48 to −0.03) and BMI-SDS (B = −0.47, 95% CI:−0.79 to −0.15) after 6 months of the multidisciplinary intervention.

In addition, when we dichotomized the male sample at the median of baseline TL, those who presented longer telomeres displayed a greater reduction in body weight (p = 0.001), BMI-SDS (p = 0.004) and hip circumference (p = 0.012), and they showed a similar trend in waist circumference (p = 0.067), and waist to height ratio (p = 0.061) after the intensive lifestyle program (Table 3). Similarly, after 6 months of the intervention, the longer the initial TL, the higher the decrease in body weight (p = 0.014) and BMI-SDS (p = 0.002).

Discussion

In this study encompassing 74 overweight/obese adolescents, a significant increase in TL after 2 months of a multidisciplinary program was found. Interestingly, we observed a significant association between baseline TL and changes in adiposity traits in boys which remained significant after 6 months of follow-up. To our knowledge, this study assessed for the first time the relationship between TL and changes in obesity traits in an obese adolescent population.

We did not observe differences in TL according to age or sex, in agreement with studies in children and adolescent populations [15,16]. The narrow age range of our adolescent population (12–16 years) and the relatively small sample size did not favor this age-related phenomenon.

Interestingly, we showed that an integral intervention for weight loss may contribute to the prevention of telomere shortening. In adults, a 4-month supplementation with omega-3 did increase leukocyte TL [27]. In other cell types an increase in TL was observed after 1 or 3 months dietary intervention in adult subjects [18,28]. Two potential mechanisms could explain this telomere lengthening: a reduction in biochemical stress or a replenishment of younger cells with longer telomeres into circulation [29]. However, our findings need to be further explored in other populations to better understand this biological mechanism.

Besides, subjects who had the shortest telomeres at baseline presented the greatest increase in TL that is in concordance with other adult studies [30–32]. This finding might be due to TL maintenance machinery which is focused on protecting the

shortest telomeres [33–35]. It could be speculated that obese adolescents with longer telomeres at baseline had a lower rate of telomere lengthening, since the change in TL mainly depends on the initial TL, achieving a greater weight loss.

Few epidemiologic studies have investigated the association of TL and obesity traits in young population [14–17]. In adolescents a cross-sectional study did not observe association between TL and adiposity indices [17]. Obese children (French and Arab) showed shorter telomeres than their nonobese counterparts [15,16]. In Italian children no difference in TL between obese and nonobese children was found [14]. However, our findings give valuable information in a longitudinal way.

This study proposed TL as a biomarker for adiposity changes, as we showed that the longer the initial telomere, the greater the decrease in obesity parameters. In this regard, Njajou *et al.* [9] observed TL to be significantly associated with percentage of change in BMI and in body fat during a 7-year follow-up in the elderly. In the frame of the PREDIMED study, we recently reported that higher initial TL could predict a greater decrease in obesity anthropometric variables in an elderly population [11].

Furthermore, significant associations between TL and changes in adiposity indices were observed only in boys. The sex effect elicited in this study confirms the findings of Al-Attas *et al.* who observed a relationship between obesity and TL in boys aged 5–12 years [15]. Nevertheless, the possible explanation for our sex difference might be due to biological sex differences that may modulate this association. In fact, our participants were at a high growing rate where hormones, particularly in girls, could play an important role.

Some limitations should be acknowledged. There is a debate on whether observed telomere lengthening is real or an artifact caused by measurement errors. Thus, Steenstrup *et al.* [36] showed that the effect of measurement error can be reduced in longitudinal studies by presenting meticulous attention to potential measurement problems. Therefore, we have carefully controlled the experimental conditions to avoid potential errors: genomic DNA was processed following a standardized protocol to preserve its stability; and also the two DNA samples per patient (at baseline and after 2 months) were run in the same plate. Nevertheless, it should be recognised that our study consisted in a short intervention period leading to the possibility of individuals to be misclassified as TL gainers [36]. The small sample size and the progressive pubertal stages among participants are also weaknesses of this study. However, the fact that important statistical differences were found suggests that potential type-II errors were overcome. On the other hand, strengths of our study include: 1) the design allows reproducing real-time conditions with home-prepared foods in free-living individuals, as in usual clinical practice; 2) measurements in young subjects are not confounded by chronic obesity-related disorders.

In conclusion, our results suggest that a multidisciplinary intervention in obese adolescents did achieve not only weight loss, but an increase in TL. Moreover, we show that initial longer telomeres could predict a better weight loss response to a multidisciplinary intervention program in overweight/obese male adolescents. However, further larger longitudinal studies are warranted to confirm these results and better understand this complicated association, especially at a young age in order to prevent adult obesity-related complications.

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CHAPTER 4

Longitudinal association of telomere length and obesity indices in an intervention study with a Mediterranean diet: The PREDIMEDNAVARRA trial

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V. Discussion

Since TL has been proposed as the key to aging and cancer, several studies have investigated what aspects can slow down telomere loss associated with age. Genetics, lifestyle factors, weight-loss and nutritional interventions have been suggested to have an impact on TL. In this study, we have shed light into this matter looking into the association between leukocyte TL and diet, genetic variant or lifestyle interventions. The importance to study TL comes out to the fact that extending the telomere not only increases longevity but also allows you to have great health in the process.

In this research we reported that a higher dietary TAC and a lower consumption of refined cereals were cross-sectionally associated with longer telomeres in children and adolescents. This result suggests that antioxidants from the diet could potentially decrease telomere attrition. In addition, the Pro12Ala polymorphism of the *PPARγ2* gene prevented telomere shortening associated with age after a 5-year nutritional intervention in high cardiovascular risk subjects. Moreover, a gene-diet interaction was found showing that a high adherence to the MeDiet could slow down telomere shortening but only in those subjects carrying the Ala allele.

In addition, we suggested the possible role of leukocyte TL as a potential biomarker of adiposity, since the higher the telomere at baseline, the greater the improvement in adiposity traits after two nutritional interventions in adolescents and in subjects at high cardiovascular risk. Notably, we also found an inverse relationship between telomere shortening and changes in adiposity indices after 5 years in the frame of the PREDIMED-NAVARRA trial.

1. Effect of dietary factors on telomere length

Lifestyle factors may affect health and lifespan of individuals by affecting the rate of telomere attrition (Mirabello *et al.*, 2009; Sun *et al.*, 2012). It is shown that dietary components (Paul, 2011) and the overall healthy eating pattern (Tollefsbol, 2014) could have a potential influence on TL. Whereas a higher consumption of healthy food has been associated with longer telomeres (Cassidy *et al.*, 2010; Diaz *et al.*, 2010; Tiainen *et al.*, 2012), unhealthy dietary habits may have the opposite effect (Nettleton *et al.*, 2008; Strandberg *et al.*, 2012).

To improve our understanding of this matter, we assessed the association between food groups and leukocyte TL in a subsample of Spanish children and adolescents from the GENOI study. Given that various nutrients influence TL through oxidative stress mechanisms, we wondered whether the dietary TAC could have a beneficial role on TL. In fact, the dietary TAC is considered an integrated parameter assessing the overall capacity of all antioxidants present in food (Halvorsen *et al.*, 2006). Noteworthy, those subjects consuming a higher dietary TAC exhibited longer telomeres, suggesting a potential role of an antioxidant diet in maintaining the telomere. This is likely to be possible, since TL is highly sensitive to damage by oxidative stress due to their high guanine content (Houben *et al.*, 2008). Similarly, *in vitro* and *in vivo* studies have shown that the intake of vitamin C or E (antioxidant vitamins) was positively associated with longer telomeres (Furumoto *et al.*, 1998; Tanaka *et al.*, 2007; Xu *et al.*, 2009). Other antioxidant dietary components such as polyphenols, tea or curcumin have also resulted in increased TL (Thomas *et al.*, 2009; Chan *et al.*, 2010). However, to date there is no available research data on the association between dietary TAC and TL.

Moreover, a lower consumption of cereals, particularly white bread intake, was associated with longer telomeres in the frame of the GENOI study. There are no previous works examining this relationship, but in contrast the Multi-Ethnic Study of Atherosclerosis found no correlation between shorter telomeres and refined grains (includes white bread) among 840 white, black and Hispanic adults (Nettleton *et al.*, 2008). Our findings can be explained because the high glycaemic load of white bread (reference food in determining glycaemic index) could cause oxidative stress, inflammation and endothelial dysfunction (Botero *et al.*, 2009; Neuhouser *et al.*, 2012) and it is reasonable to speculate that this biochemical stress may affect TL. Indeed, hyperglycaemia generates oxidative stress that pushes normal endothelial cells to premature senescence (Hayashi *et al.*, 2006; Yokoi *et al.*, 2006) by telomere-related mechanisms (Matsui-Hirai *et al.*, 2011).

2. The Pro12Ala polymorphism of the *PPARγ2* gene and telomere lengthening

PPARγ is a member of the nuclear hormone receptor superfamily that activates adipocyte differentiation and fat metabolism through a complex program concerning gene expression (Tyagi *et al.*, 2011). This transcription factor is located at 3p25 region of the genome and it is expressed predominantly in adipose tissue (Pirat *et al.*, 2012). From this gene, three different RNAm are developed (γ1, γ2, γ3) resulting in two protein isoforms: *PPARγ1* (from RNAm 1 and 3) and *PPARγ2* (Elbrecht *et al.*, 1996).

The most studied variant of the *PPARγ* gene is a cytosine to guanine transversion (C/G) resulting in a proline 12 to an alanine substitution in exon B and so in *PPARγ2* protein (Pro12Ala; rs1801282) (Yen *et al.*, 1997). *In vitro* assays have shown that the Ala allele decreased binding affinity to the related promoter element, reducing the ability to transactivate responsive promoters (Deeb *et al.*, 1998). This fact predisposes people to lower levels of adipose tissue mass accumulation, which in turn may improve insulin sensitivity and lipid and glucose homeostasis (Gonzalez Sanchez *et al.*, 2002; Gouda *et al.*, 2010). It is well known that decreasing lipid adiposity not only reduces the release of inflammatory cytokines but also shows a greater resistance to oxidative stress, diminishing the burden of biochemical stress (Chae *et al.*, 2013). For instance, the presence of the Ala allele has been associated with lower levels of inflammation and oxidative stress (Yao *et al.*, 2005; Luo *et al.*, 2008). As TL is mainly affected by these two processes, we wanted to test whether Ala carrier subjects presented a lower rate of telomere attrition. Not surprisingly though, our study shows that Ala allele prevented telomere shortening associated with age after a 5-year nutritional intervention with a Mediterranean dietary pattern. In line with our findings, the Pro12Ala polymorphism was potentially associated with increase lifespan in humans carrying this mutation (Barbieri *et al.*, 2004). Furthermore, studies carried out in *PPARγ*+/− mice suggested that these rodents presented longer lifespans (Luo *et al.*, 2008; Heikkinen *et al.*, 2009). Since TL is considered a biomarker of age and can be

modulated by genetic variants, it could be speculated that *PPARγ2* could be a possible longevity candidate gene.

Another study showed that activation of *PPARγ2* can inhibit telomerase activity, elucidating a potential connexion between this gene and TL (Lehrke and Lazar, 2005). In other words, it could be hypothesized that Ala carrier subjects may present an increased telomerase activity and therefore lower telomere attrition, as the Pro12Ala gene variant results in a decrease of *PPARγ2* gene expression. Subsequently, the association of the polymorphism and TL could be mediated not only by oxidative stress or inflammation, but also by telomerase induction. More studies are needed to confirm the role of Pro12Ala on aging, but our study has contributed in longitudinal terms.

Given that individuals carrying the Ala allele are considerably protected against CVD (Rittig *et al.*, 2007; Regieli *et al.*, 2009) and shorter telomeres have been found in subjects with CVD (De Meyer *et al.*, 2011), it might be proposed that the Pro12Ala gene variant may protect against telomere erosion, as found in our population at high cardiovascular risk. Hence, TL could be the link for the association between the Pro12Ala polymorphism and a reduction in CVD risk, suggesting the biomarker role of TL in the prediction of CVD and other age-related diseases. In this context, shorter telomeres can contribute to CVD, and particularly to atherosclerosis, through various biological aging pathways, such as cellular senescence (Minamino *et al.*, 2002; Gorenne *et al.*, 2006; Matthews *et al.*, 2006).

Interestingly, this study also shows that the Mediterranean dietary pattern interacts with the Ala allele resulting in higher TL, displaying that the response of subjects might be different depending on the genotype. Nutrigenetics aims to understand how the genetic makeup of an individual coordinates their response to diet, and thus considers underlying genetic polymorphisms. It has the potential to provide a basis for personalized dietary recommendations based on the individual's genetic background in order to prevent common multifactorial disorders before their clinical manifestation (Ordovas and Mooser, 2004). Focusing on our study, an important gene-diet interaction has been identified for changes in leukocyte TL after a nutritional intervention. The adherence to a MeDiet and therefore, a higher

consumption of MUFA and PUFA at the expense of CHO intake, was found to be beneficial for attenuating telomere shortening in those subjects carrying the Ala allele. Accordingly, three studies have looked into the relationship of TL and adherence to a MeDiet, reporting its protective role on telomere erosion (Marin *et al.*, 2012; Boccardi *et al.*, 2013). But caution is needed when interpreting results since one had a cross-sectional nature (Boccardi *et al.*, 2013) and in the other one only 20 elderly subjects were included (Marin *et al.*, 2012). Nevertheless, the recent work by Crous-Bou *et al.* (Crous-Bou *et al.*, 2014) found a strong relationship between MeDiet and TL in a large sample of women (*P* for trend=0.004). It has been suggested that this positive effect of the MeDiet could be due to two biological processes (Epel, 2012; Boccardi *et al.*, 2013). On the one hand, specific nutrients of the MeDiet could stimulate telomerase activity in lymphocytes leading to telomere lengthening. On the other hand, the global effect of this dietary pattern reduces systemic oxidative stress and inflammation which have been proposed as the main causes of accelerated telomere shortening (Aviv, 2009). We have to keep in mind that our study showed a favourable effect of the MeDiet but considering the genetic background, in this case the Pro12Ala gene variant.

A higher increased TL among Ala carrier subjects was observed in the MeDiet groups supplemented with EVOO or nuts in comparison with the control group. This might be due to the fact that the two intervention groups were encourage to follow a MeDiet receiving intensive education and supplemental food at no cost, which ensures a higher compliance to the dietary pattern. Therefore, the Mediterranean dietary pattern on its own has a beneficial effect on the association between Pro12Ala gene variant and TL. This finding could contribute to optimize health through the personalization of diet providing a better view of the relationship between genetics and dietary factors in terms of aging.

3. Adiposity traits and telomere length

An inverse association between adiposity and TL have been observed in human studies. But, most of them had a cross-sectional design which does not allow causal inferences. There are two cross-sectional recent large works that should be particularly highlighted. The Strong Heart Family Study, enrolling 3,256 American

Indians (14-93 years old, 60% women), showed that leukocyte TL was inversely correlated with several obesity indices, including BMI, WC, percent of body fat, waist-to-hip and waist-to-height ratio in multivariate analyses (Chen *et al.*, 2014). In addition, obese participants had shorter leukocyte TL than non-obese individuals (age-adjusted $P<0.001$). The Copenhagen General Population Study, comprising 45,069 individuals (20-100 years), observed that leukocyte TL decreased by five bp per unit increase in BMI, after adjustment for C-reactive protein and other baseline characteristics of the participants (Rode *et al.*, 2014). Noteworthy, a recent meta-analysis compelling 14 cross-sectional studies also reported the inverse association between BMI and leukocyte TL in adults but without reaching statistical significance ($\beta = -0.008$, $P = 0.058$) (Muezzinler *et al.*, 2014).

There are a few studies looking into adiposity and TL in children and adolescents (Zannolli *et al.*, 2008; Al-Attas *et al.*, 2010; Buxton *et al.*, 2011; Zhu *et al.*, 2011). Just one study has been carried out in adolescents (n=667, aged 14-18 years) reporting no cross-sectional association between adiposity and TL (Zhu *et al.*, 2011). Alternatively, a case-control study, enrolling 793 French children (2-17 years), observed shorter telomeres in obese children than in the non-obese counterparts (Buxton *et al.*, 2011). In the same way, Al-Attas *et al.* (Al-Attas *et al.*, 2010) showed shorter leukocyte TL in 19 obese boys, but not in 33 obese girls (5-12 years). Likewise, the present research work found an inverse longitudinal association between TL and changes in adiposity traits in boys aged 12-16 years and not in girls. Interestingly, our work gives valuable information since we observed that a weight loss multidisciplinary intervention may slower telomere attrition in obese adolescents in a longitudinal way.

Little is known about the longitudinal association between adiposity and TL. Although some studies have reported no association between changes in adiposity and TL after a follow-up period (Hovatta *et al.*, 2012; Mason *et al.*, 2013; Weischer *et al.*, 2014), others did observe an inverse relationship. Hence, Gardner *et al.* (Gardner *et al.*, 2005) showed that weight gain was associated with accelerated telomere attrition in 70 young adults (mean age 25 years) after 10 years of follow-up. Similarly, Kim *et al.* (Kim *et al.*, 2009) reported that adult weight gain and frequent weight cycling were inversely associated with TL in 647 women (35 to 74 years). In the same way, weight

loss by 10.6 kg after 1 year of a calorie-restricted diet was associated with increased TL in rectal mucosa among 12 obese men (O'Callaghan *et al.*, 2009). However, these previous studies have often been performed with small samples, considered self-reported body weight data, measured TL only at one time or covered different age ranges, which yielded inconsistent results. For this reason there is an urgent need of longitudinal large studies investigating telomere dynamics with respect to changes in adiposity markers.

Our research was performed to clarify the longitudinal relationship between TL and anthropometric parameters. As previously shown, we did find an inverse association between TL and changes in adiposity markers. Regarding the EVASYON study, we found an increased leukocyte TL after a 2-month weight loss intensive program. Similarly, the PREDIMED study showed that a decrease in obesity risk was linked to higher leukocyte TL after 5 years of nutritional intervention. Moreover, both studies revealed that a higher baseline leukocyte TL was associated with a better improvement in anthropometric parameters, proposing TL as a biomarker of adiposity. In line with our results, there is a 7 year follow-up study considering TL as a predictor of changes in adiposity parameters reporting that baseline TL was negatively associated with changes in BMI and percentage of total body fat in 2,721 elderly subjects (Njajou *et al.*, 2012). Likewise, shorter baseline TL assessed in 2,848 participants (18–65 years) was significantly associated with unfavourable scores of most metabolic syndrome components at 2 and 6-year follow-ups (Revesz *et al.*, 2014).

According to our findings in two different aged populations, shortened telomeres might predict unfavourable adiposity changes. Therefore, it should be suggested that leukocyte TL may well serve as a genetic biomarker for obesity traits, although more longitudinal studies in large cohorts need to be performed to clarify this issue. In view of this, several underlying processes have been proposed to support this association.

Inflammation and oxidative stress have been suggested as the fundamental mechanisms for the relationship between adiposity and shorter telomeres (Aviv, 2009). It is likely to come to the conclusion that the low-grade chronic inflammation or oxidative stress linked to excess weight could play an important role in telomere

shortening. In fact, telomeres are highly sensitive to the detrimental action of hydroxyl radicals which might cause DNA damage in TL with each replication of the HSCs. Moreover, inflammation could increase the rate of HSCs replication to accommodate the increased demand for leukocytes due to their engagement in the inflammatory process (Aviv, 2009). However, the opposite hypothesis can also be true since this study showed that shortened telomeres could lead to higher body adiposity. For instance, it has been reported that cells presenting short telomeres could activate senescence processes leading to a release of inflammatory cytokines, inducing insulin resistance and defective HDL cholesterol (Fyhrquist *et al.*, 2013). Another mechanism implicated could be that telomere erosion activates p53 pathway causing impaired mitochondrial function and increased ROS content and also less fatty acid oxidation and glucose utilization (Sahin *et al.*, 2011). Hence, TL may be a biomarker for metabolic alterations and therefore it should be highlighted the possible role of TL in the onset of obesity. However, further research is required to deeper evaluate the biological mechanisms for this association and whether telomere attrition could become a possible target in nutritional interventions to prevent the progression of metabolic diseases.

4. Leukocyte telomere length dynamics: how reversible is telomere attrition?

The EVASYON study has shown an increase in leukocyte TL after 2 months of an intensive intervention in overweight and obese adolescents, accompanied by a decrease in adiposity. Moreover, the PREDIMED-NAVARRA study reported that 60% of participants after 5 years of follow-up decreased their TL meanwhile 40% of them increased it. Then, age-dependent telomere shortening could be somehow reversible. Two main mechanisms could be involved in these changes in leukocyte TL.

First of all, as previously explained, an attenuation of biochemical stress through a decrease in oxidative stress and inflammation may have led to a decrease in the rate of telomere shortening (Epel, 2012). Thus, decreasing adiposity or improving dietary habits could reduce the level of chronic inflammation and oxidative stress, and therefore it may induce a true reversal of telomere shortening (actual lengthening).

Alternatively, this telomere lengthening could also be possible through increasing telomerase activity (Epel, 2012). In fact, small-scale intervention studies have reported an increase in telomerase activity (from 18 to 43%) in blood cells in response to lifestyle modifications (Ornish *et al.*, 2008; Daubenmier *et al.*, 2012). Unfortunately, we did not measure telomerase activity changes in any of the studies.

Having said this, it is also suggested that cell redistribution toward more cells with longer TL or an influx of naïve cells into circulation could represent another mechanism that could explain telomere lengthening (Epel, 2012). In fact, leukocyte TL is comprised of the average TL across immune cell subpopulations present in the studied sample. In addition, improvements in health behaviours have been reported to promote short term lengthening by increasing younger cells, called naïve T cells, which have longer telomeres than memory T cells and granulocytes (Kim *et al.*, 2012; Simpson *et al.*, 2012). Therefore, rather than telomere lengthening on a per cell basis, the increased TL seemed to be in part due to replenishment of cells in circulation (pseudo-lengthening). Homologous recombination between telomeric sequences, known as ALT, could also maintain or elongate TL, as reported in cancer cells (Muntoni and Reddel, 2005). But little is known about the relevance of this mechanism in normal aging cells

Specifically the EVASYON study could exhibit a higher possibility of individuals to be misclassified as TL gainers, because it consisted in a short intervention period (Steenstrup *et al.*, 2013). Short follow-up periods result in some cases to leukocyte TL lengthening due to a mathematical artefact related to the measurement error rather than a biological result (Steenstrup *et al.*, 2013). In the same way, other studies have also reported changes in TL after short-time lifestyle interventions. In adults, Kiecolt-Glaser *et al.* (Kiecolt-Glaser *et al.*, 2013) did observe leukocyte telomere lengthening, after a 4-month supplementation with omega-3 in a randomized clinical trial. Moreover, other studies reported an increased TL in several cell types after 1 or 3 months dietary intervention in adult subjects (O'Callaghan *et al.*, 2009; Marin *et al.*, 2012).

Remarkably, the EVASYON study revealed that leukocyte telomere attrition rate was highly correlated with initial TL which is in agreement with other studies

(Aviv *et al.*, 2009; Nordfjall *et al.*, 2009; Hovatta *et al.*, 2012). We suggest that individuals with longer telomeres at baseline (a "healthier" status) had a lower rate of telomere lengthening, since the change in TL mainly depends on the initial TL. Although this finding was not reported in the PREDIMED-NAVARRA trial, additional statistical analyses actually confirmed that subjects who had the shortest telomeres at baseline presented the greater increase in TL after 5 years of follow-up, after adjusting for sex and age (r= -0.779, $P< 0.001$). This phenomenon can be explained because telomerase act preferentially on short telomeres, indicating that TL maintenance is focused on protecting the shortest TL (Samper *et al.*, 2001; Teixeira *et al.*, 2004). This preferential elongation of short telomeres by telomerase could be due to the fact that short telomeres lead changes in epigenetic status of telomeric and subtelomeric chromatin (Blasco, 2007). However, we could not rule out that this dependency of leukocyte TL attrition on baseline leukocyte TL might be accounted for the regression to the mean, a known statistical artefact (Verhulst *et al.*, 2013). To elucidate the underlying causes, more and larger longitudinal studies investigating telomere dynamics need to be conducted.

5. Strengths and limitations

This research is particularly robust by the design of the PREDIMED study and also the EVASYON study as both studies give a prospective nature which enabled us to measure TL at two points. Moreover, the reproduction of real-time conditions with home-prepared food (as in usual clinical practice) and the high compliance of the participants to the intervention make our research potent. The randomized design with a long follow-up period in the context of a MeDiet and the large number of subjects are other strengths of the PREDIMED study. The studies carried out in children and adolescent populations have the advantage that they are not affected by deleterious lifestyle practices. It should be highlighted that multiple-adjusted models were fitted in all the data to minimise small differences among individuals and potential confounders, which is much more informative than simply obtaining correlation coefficients. On the other hand some limitations in the present research should be declared.

First, TL technique could lead to errors in measurements. For this reason, we have carefully looked after the experimental conditions to avoid potential errors (Steenstrup *et al.*, 2013): genomic DNA was processed following a standardized protocol to preserve its stability; the two DNA samples, where appropriate, were run on the same plate; the samples were run either in duplicates or triplicates for quality control; CV were calculated; a calibration curve and a negative control were included in all the plates. In addition, in a small subset of individuals, we had previously checked that the quantification of telomeres by PCR (T/S ratio) did perfectly correlate with telomeric restriction fragment length determined by Southern blot analysis. Although, RT-PCR technique can yield to errors due to its high variability, it provides the advantage of using smaller amounts of DNA, thereby making it amenable for large numbers of people, and only measuring the canonical region (strictly TTAGGG repeats) and not the subtelomeric region up in comparison to Southern blot analysis (Aviv *et al.*, 2011; Elbers *et al.*, 2014). In fact, we partly solve the variability of the RT-PCR analysis obtaining a lower CV in all studies, supporting then the strength of the present methodology.

Second, the extent of inter-individual variation of leukocyte TL at a given chronological age should be stated as a weakness (Shiels, 2010; Gingell-Littlejohn *et al.*, 2013). Leukocyte TL dynamics not only depends of age-dependent leukocyte telomere shortening, but also leukocyte TL at birth which can vary on average 4-6 kb among newborns (Okuda *et al.*, 2002). Despite this last issue is difficult to control, we were able to ensure the age-dependence of relative TL in adults from 55-80 years within the PREDIMED-NAVARRA trial, either at baseline (rho= -0.104, *P*= 0.018) and after 5 years of intervention (rho= -0.124, *P*= 0.004). Surprisingly, we found no association between leukocyte TL and age in children and adolescents. It could be explained by the apparent plateau in telomeric repeats between age 4 and young adulthood (~25 yr) and also the small sample size should be mentioned (Frenck *et al.*, 1998). These data suggest that the loss of telomeric repeats is a dynamic process that is differentially regulated in children and adults.

Third, another key issue is whether leukocyte TL can be generalized to other tissues. Notably, Daniali *et al.* (Daniali *et al.*, 2013) recently shown that age-dependent

TL shortening is similar in proliferative (blood and skin) and minimally proliferative tissues (muscle and fat) of 87 adults (aged 19-77 years), regardless of their replicative activity. So consequently, stem cell division might be similar in these four somatic tissues since leukocyte TL dynamics reflect stem cell kinetics.

Fourth, the lack of telomerase activity and inflammation or oxidative stress markers could not confirm the mechanisms involved behind the observed associations between TL and adiposity or dietary and genetic factors, and neither how changes in TL could be generated. Therefore, in the present study we could only speculate about the underlying biological processes.

Fifth, the use of self-reported questionnaires in the dietary assessment may involve a possibility of measurement error leading to misclassifications of exposure. However, the FFQ and the 14-item score of MeDiet were previously validated and used in other studies (Martin-Moreno *et al.*, 1993; Ochoa *et al.*, 2007; de la Fuente-Arrillaga *et al.*, 2010; Schroder *et al.*, 2011). Furthermore, a registered dietician helped to collect the dietary information through individual sessions.

Finally, several weaknesses should be acknowledged in the two studies carried out in children and adolescents. The small sample size, particularly in the EVASYON study, and the progressive pubertal stages among participants should be stated as limitations. The lack of large samples could lead to increase the risk of type II errors (failing to detect real differences), mostly if several adjustments are performed (Smith, 2012). For this reason, confounders were limited in the analyses to discover new facts meaning the acceptance of more type I errors (asserting something that is absent) in order to avoid type II errors (Cohen *et al.*, 2013). The fact that important statistical differences were found suggests that potential type II errors were overcome. Another limitation is the cross-sectional nature of the GENOI study that limits the potential to discern causative relationships.

6. Summary and future perspectives

Altogether the results presented in this thesis clearly show that dietary and genetic factors could modulate telomere shortening. Notably, an antioxidant rich diet

and the Pro12Ala polymorphism in the *PPARγ2* gene, as well as the adherence to a MeDiet among Ala carrier subjects, could result in longer telomeres. Furthermore, our research work also contributes to better understand the role of TL homeostasis in adiposity. Hence, an inverse longitudinal association between TL and adiposity traits was observed in adults and adolescents after lifestyle interventions. Notably, we propose the assessment of leukocyte TL as a potential biomarker for changes in adiposity (**Figure 15**). However, the causal relationship behind these associations is not yet fully elucidated due to a great deal of controversy within the available literature. Indeed, it is not clear whether TL is a good genetic biomarker for adiposity or if telomere shortening could influence disease processes. These questions need to be answered in longitudinal large-scale epidemiological studies in different aged-populations. Moreover, the discrepancies regarding the most appropriate TL methodology make necessary to develop better assays that enable researchers to quantify leukocyte TL more precisely and accurately.

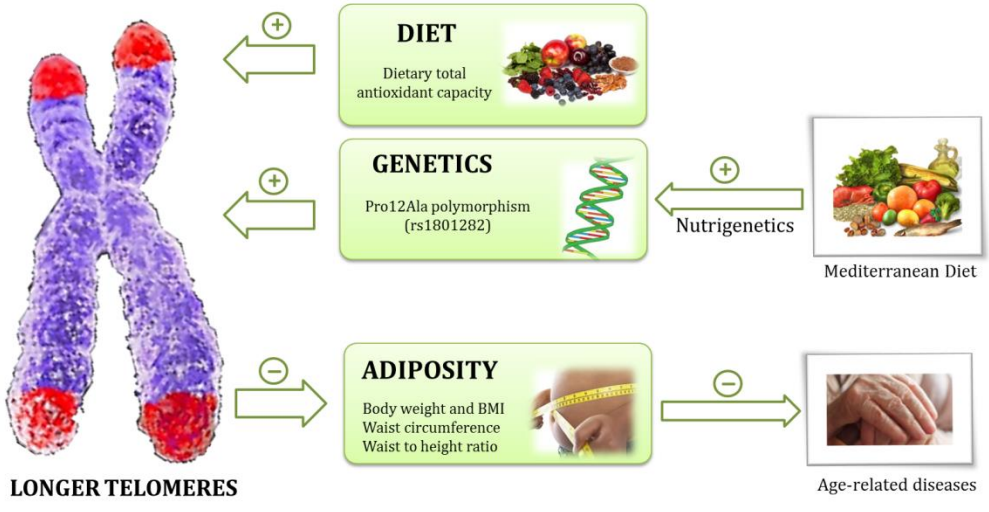

Figure 15. Summary depicting the main findings presented in the thesis.

The wide range inter-individual variation and the multitude of confounding variables, make TL maintenance as a complex process. Therefore, identifying how dietary and genetic factors modify TL could lead to understand potential molecular pathways. In this research we did find that dietary TAC, an indicator of antioxidant status of the diet, could have beneficial effects on TL. Moreover, a higher adherence to the MeDiet slows down telomere attrition but only in subjects carrying the Ala allele. The healthy properties of MeDiet and the fact of being a recommended diet worldwide make crucial to identify the mechanisms that link the MeDiet with TL. A very interesting possibility of continuing this research would be to test whether components of the MeDiet, for example EVOO or nuts, or perhaps specific nutrients such as antioxidant phenolic compounds or unsaturated fats, could be responsible for reverting telomeric aging. In this context, understanding the key molecular pathways involved, first *in vitro* and later on *in vivo*, could shed light on this matter.

Another point of interest to continue this line of research would be to detect potential gene variants that will condition TL, already since the birth. GWAS studies have emerged as powerful tools for identifying SNPs for complex diseases. What is more interesting is analysing the effects of the lifestyle habits depending on the genetic background. For this reason, nutrigenetics appear to be a good option to further investigate variations in the rate of telomere attrition and therefore provide personalized nutrition for preventing and treating age-related diseases.

VI. Conclusions

1. Higher dietary TAC intake and lower white bread consumption were associated with longer telomeres in a children and adolescent population, which suggests the potential capacity of antioxidant rich diets in maintaining TL.

2. The Pro12Ala polymorphism in the *PPARγ2* gene prevented telomere shortening associated with age after a 5-year nutritional intervention in high cardiovascular risk subjects.

3. The Mediterranean dietary pattern, as well as the ratio of unsaturated fat to CHO, modulated TL associated with the Pro12Ala gene variant. A higher adherence to a MeDiet (particularly in EVOO and nuts groups) reinforced the prevention of telomere shortening in Ala carrier subjects.

4. In obese adolescents, a 2-month weight loss intensive program did significantly increase leukocyte TL, reporting that an integral intervention for losing weight may contribute to the prevention of telomere shortening.

5. An inverse longitudinal association between leukocyte TL and obesity parameters was observed in adults at high cardiovascular risk after 5 years of a nutritional intervention. Interestingly, a decrease in obesity risk was linked to higher TL after the follow-up period.

6. A higher baseline TL predicted a greater decrease in adiposity parameters in two intervention studies enrolling high cardiovascular risk adults and obese male adolescents, suggesting the biomarker role of leukocyte TL for changes in adiposity.

7. The individual change in TL was inversely correlated with initial TL after controlling for age and sex in the EVASYON and PREDIMED-NAVARRA studies, indicating that the lengthening rate was most pronounced in individuals with shorter telomeres at baseline.

8. The age-dependent leukocyte telomere shortening was found in adult subjects (55-80 years), but not in the children and adolescent populations (6-18 years). This lack of correlation elucidates that leukocyte TL dynamics is differentially regulated among young and adult subjects.

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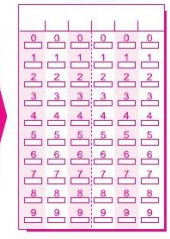
VIII. Appendices

Appendix 1. Food Frequency Questionnaire

Página 5

NÚMERO



En este cuadro debes repetir en las 6 casillas superiores el número que figura en el cuadro de la 1ª página y a continuación marcarlo igual que lo has hecho anteriormente.

ENCUESTA DIETÉTICA

Por favor, marca una única opción para cada alimento.

Para cada alimento, marca el recuadro que indica la frecuencia de consumo **por término medio** durante el **año pasado**. Se trata de tener en cuenta también la variación verano/invierno. Por ejemplo si tomas helados 4 veces/semana sólo durante los 3 meses de verano, el uso promedio al año es 1/semana	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
I LÁCTEOS									
Leche entera (1 taza, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Leche semidesnatada (1 taza, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Leche desnatada (1 taza, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Leche condensada (1 cucharada)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Nata o crema de leche (1/2 taza)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Batidos de leche (1 vaso, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Yogurt entero (1, 125 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Yogurt descremado (1, 125 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Petit suisse (1, 100 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Requesón o cuajada (1/2 taza)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Queso en porciones o cremoso (1, porción)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Otros quesos: curados, semicurados (Manchego, Bola, Emmental...) (50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Queso blanco o fresco (Burgos, cabra...) (50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Natillas, flan, puding (1 taza, 200 cc.)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Helados (uno)	☐	☐	☐	☐	☐	☐	☐	☐	☐

(Dobla por esta linea)

Por favor, marca una única opción para cada alimento.

Un plato o ración de 100-150 gr, excepto cuando se indica otra cosa	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
II HUEVOS, CARNES, PESCADOS									
Huevos de gallina (uno)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pollo o pavo CON piel	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pollo o pavo SIN piel	☐	☐	☐	☐	☐	☐	☐	☐	☐
Carne de ternera o vaca	☐	☐	☐	☐	☐	☐	☐	☐	☐
Carne de cerdo	☐	☐	☐	☐	☐	☐	☐	☐	☐
Carne de cordero	☐	☐	☐	☐	☐	☐	☐	☐	☐
Conejo o liebre	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hígado	☐	☐	☐	☐	☐	☐	☐	☐	☐
Otras vísceras (sesos, corazón, mollejas)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Jamón serrano o paletilla	☐	☐	☐	☐	☐	☐	☐	☐	☐
Jamón York, jamón cocido (1 loncha)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Embutidos (chorizo, salchichón, mortadela, 50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salchichas (50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Patés, foie-gras (25 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Morcilla (50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hamburguesa (unidad)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sobrasada (50 gr) / albóndigas (3 unidades)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tocino, bacon, panceta (50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pescado blanco: pescadilla, merluza, besugo, mero, lenguado (1 plato, pieza o ración)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pescado azul: sardinas, atún, bonito, caballa, salmón (1 plato, pieza o ración)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Bacalao	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pescados salados y/o ahumados: arenques, salmón	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ostras, almejas, mejillones, etc. (6 unidades)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Gambas, langostinos, cigalas, etc.	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pulpo, calamares, chipirones, jibia...	☐	☐	☐	☐	☐	☐	☐	☐	☐

Por favor, marca una única opción para cada alimento.

III VERDURAS Y HORTALIZAS — Un plato o ración de 250 grs, excepto cuando se indica	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
Acelgas, espinacas	☐	☐	☐	☐	☐	☐	☐	☐	☐
Col, coliflor, brócoles	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lechuga, endibias, escarola	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tomate crudo (1, 150 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Zanahoria, calabaza	☐	☐	☐	☐	☐	☐	☐	☐	☐
Judías verdes	☐	☐	☐	☐	☐	☐	☐	☐	☐
Berenjenas, calabacines, pepinos	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pimientos	☐	☐	☐	☐	☐	☐	☐	☐	☐
Espárragos	☐	☐	☐	☐	☐	☐	☐	☐	☐
Gazpacho andaluz	☐	☐	☐	☐	☐	☐	☐	☐	☐
Otras verduras (borraja, cardo...)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Patatas fritas (caseras, bolsa, 1 ración, 150 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Patatas asadas o cocidas (1 ración, 150 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Por favor, marca una única opción para cada alimento.

IV FRUTAS — Una pieza o ración	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
Naranja, pomelo (una), o mandarina (dos)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Plátano	☐	☐	☐	☐	☐	☐	☐	☐	☐
Manzana, pera	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fresas/fresones (6 unidades, plato postre)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Melocotón, albaricoque, nectarina	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cerezas, picotas, ciruelas (1 plato de postre)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Higos, brevas	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sandía (1 tajada, 200-250 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Melón (1 tajada, 200-250 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Uvas (un racimo, un plato postre)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Frutas en almíbar (2 unidades)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Frutas en su jugo (2 unidades)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Dátiles, higos secos, pasas, ciruelas-pasas (150 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Almendras, cacahuetes, avellanas, nueces (50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Aceitunas (10 unidades)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Aguacates	☐	☐	☐	☐	☐	☐	☐	☐	☐
Mangos, papaya	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kiwi	☐	☐	☐	☐	☐	☐	☐	☐	☐

¿Cuántos días a la semana tomas fruta como postre? 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐

Por favor, marca una única opción para cada alimento.

V LEGUMBRES Y CEREALES — Un plato o ración de 60 gr en seco	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
Lentejas	☐	☐	☐	☐	☐	☐	☐	☐	☐
Garbanzos	☐	☐	☐	☐	☐	☐	☐	☐	☐
Alubias (pintas, blancas o negras)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Guisantes	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pan blanco (3 rodajas, 60 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pan negro integral (3 rodajas, 60 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cereales desayuno (30 gr en seco)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Arroz blanco (60 gr en seco)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pasta: fideos, macarrones, espaguetis (60 gr en seco)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pizza (1 ración, 200 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐

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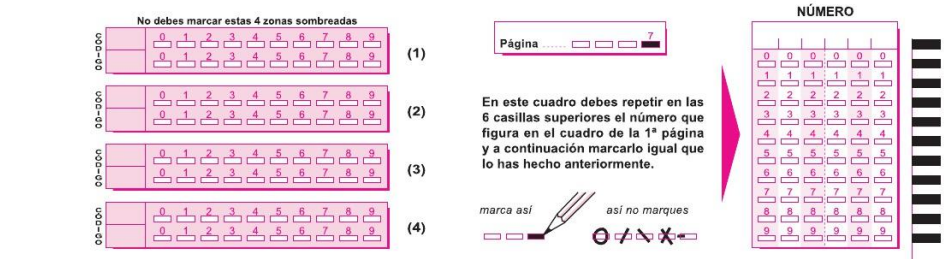

Por favor, marca una única opción para cada alimento.

Una cucharada o porción individual *Para untar, mojar en el pan, para aliñar, o para ensaladas, utilizas en total:*	CONSUMO MEDIO DURANTE EL AÑO PASADO NUNCA O CASI NUNCA	AL MES 1 - 3	A LA SEMANA 1	A LA SEMANA 2 - 4	A LA SEMANA 5 - 6	AL DÍA 1	AL DÍA 2 - 3	AL DÍA 4 - 6	AL DÍA 6 +
VI ACEITES Y GRASAS									
Mantequilla (porción individual)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Margarina (porción individual)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Aceite de oliva (una cucharada)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Aceite de girasol	☐	☐	☐	☐	☐	☐	☐	☐	☐
Aceite de maíz	☐	☐	☐	☐	☐	☐	☐	☐	☐
Manteca de cerdo	☐	☐	☐	☐	☐	☐	☐	☐	☐
Otros: (1)	☐	☐	☐	☐	☐	☐	☐	☐	☐
¿CON QUÉ FRECUENCIA CONSUMES?:									
Alimentos fritos en casa	☐	☐	☐	☐	☐	☐	☐	☐	☐
Alimentos fritos fuera de casa	☐	☐	☐	☐	☐	☐	☐	☐	☐

EN TU CASA, PARA FREIR SE UTILIZA:

Aceite de oliva ☐ Mantequilla ☐
Aceite de girasol ☐ Margarina ☐
Aceite de maíz ☐ Otros ☐ (2)

Marca de aceite de oliva que usas habitualmente (3)

(Dobla por esta línea)

Por favor, marca una única opción para cada alimento.

	CONSUMO MEDIO DURANTE EL AÑO PASADO NUNCA O CASI NUNCA	AL MES 1 - 3	A LA SEMANA 1	A LA SEMANA 2 - 4	A LA SEMANA 5 - 6	AL DÍA 1	AL DÍA 2 - 3	AL DÍA 4 - 6	AL DÍA 6 +
VII BOLLERÍA Y PASTELERÍA									
Galletas tipo María (4-6 unidades, 50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Galletas con chocolate (4-6 unidades, 50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Magdalenas comerciales (1-2 unidades)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Donuts (uno)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ensaimada, croissant u otra bollería **industrial comercial**... (uno, 50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Bollería, repostería **casera**	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pasteles (uno, 50 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Churros, porras y similares (ración, 100 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Chocolates y bombones (30 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Turrón (1/8 de barra)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Pastas de té, mantecados, mazapán (ración, 90 gr)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Por favor, marca una única opción para cada alimento.

VIII BEBIDAS									
Vaso de vino tinto	☐	☐	☐	☐	☐	☐	☐	☐	☐
Vaso de otro tipo de vino	☐	☐	☐	☐	☐	☐	☐	☐	☐
Vaso de vino en las comidas	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cerveza (1 jarra, 330 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Licores, destilados: whisky, ginebra, coñac, anís... (1 copa, 50 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Bebidas carbonatadas con azúcar: Coca-Cola, naranjadas... (1 botellín, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Idem, pero bajas en calorías, Bebidas light... (1 botellín, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Zumo de naranja natural (1 vaso, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Zumos naturales de otras frutas (1 vaso, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Zumos de frutas o de verduras en botella o enlatados (200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Café descafeinado (1 taza, 50 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Café (1 taza, 50 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Agua (del grifo) (1 vaso, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Agua embotellada (1 vaso, 200 cc)	☐	☐	☐	☐	☐	☐	☐	☐	☐

Marca de agua embotellada que bebes más habitualmente (4)

Por favor, marca una única opción para cada alimento.

	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
IX MISCELÁNEA									
Croquetas, buñuelos, empanadillas	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sopas y cremas de sobre	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salsa de tomate frito, ketchup (1 cucharadita)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Mayonesa (1 cucharadita)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Picante: tabasco, pimienta	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sal (una pizca)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Azúcar (1 cucharadita)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sacarina	☐	☐	☐	☐	☐	☐	☐	☐	☐
Mermeladas (1 cucharadita)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Miel	☐	☐	☐	☐	☐	☐	☐	☐	☐
Otros alimentos de frecuente consumo: (1)	☐	☐	☐	☐	☐	☐	☐	☐	☐
(2)	☐	☐	☐	☐	☐	☐	☐	☐	☐
¿Con qué frecuencia haces comidas fuera de casa?	☐	☐	☐	☐	☐	☐	☐	☐	☐

(1) No debes marcar esta zona sombreada — CODIGO: 0 1 2 3 4 5 6 7 8 9 / 0 1 2 3 4 5 6 7 8 9

(2) No debes marcar esta zona sombreada — CODIGO: 0 1 2 3 4 5 6 7 8 9 / 0 1 2 3 4 5 6 7 8 9

¿Tomaste vitaminas y/o minerales (incluyendo calcio) habitualmente durante el año pasado?

☐ No ☐ Sí

Si las tomaste, por favor indica la marca:

Marcas de los suplementos de vitaminas o minerales	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6 +
(1)	☐	☐	☐	☐	☐	☐	☐	☐	☐
(2)	☐	☐	☐	☐	☐	☐	☐	☐	☐
(3)	☐	☐	☐	☐	☐	☐	☐	☐	☐

(1) No debes marcar esta zona sombreada — CODIGO: 0 1 2 3 4 5 6 7 8 9 / 0 1 2 3 4 5 6 7 8 9

(2) No debes marcar esta zona sombreada: 0 1 2 3 4 5 6 7 8 9 / 0 1 2 3 4 5 6 7 8 9

(3) No debes marcar esta zona sombreada: 0 1 2 3 4 5 6 7 8 9 / 0 1 2 3 4 5 6 7 8 9

Habitualmente, ¿qué haces con la grasa de la carne?	1 La como ☐	2 Se la quito ☐

	SÍ	NO		SÍ	NO
¿Procuras tomar mucha fibra?	☐	☐	¿Evitas el consumo de mantequilla?	☐	☐
¿Procuras tomar mucha fruta?	☐	☐	¿Procuras reducir el consumo de grasa?	☐	☐
¿Procuras tomar mucha verdura?	☐	☐	¿Procuras reducir el consumo de carne?	☐	☐
¿Procuras tomar mucho pescado?	☐	☐	¿Limitas la sal en las comidas?	☐	☐
¿Sueles comer entre comidas (picotear)?	☐	☐	¿Le añades azúcar a algunas bebidas?	☐	☐
¿Sigues una dieta especial?	☐	☐	¿Procuras reducir el consumo de dulces?	☐	☐

Si has contestado SÍ, señala el tipo de dieta: .....

(3) No debes marcar esta zona sombreada: 0 1 2 3 4 5 6 7 8 9 / 0 1 2 3 4 5 6 7 8 9

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¿Dispones de correo electrónico (e-mail)? ☐ No ☐ Sí,

¿Dispones de acceso a Internet? ☐ No ☐ Sí

Appendix 2. 14-item Mediterranean assessment tool

ESTUDIO PREDIMED

Cumplimiento de la dieta

Identificador del participante:

Nodo	C.Salud	Médico	Paciente	Visita

Nodo: anotar el número de nodo correspondiente.
01. Andalucía - Málaga / 02. Andalucía - Sevilla - S.Pablo / 03. Andalucía - Sevilla - V.Rocío / 04. Baleares / 05. Cataluña - Barcelona norte / 06. Cataluña - Barcelona Sur / 07. Cataluña - Reus - Tarragona / 08. Madrid Norte / 09. Madrid Sur / 10. Navarra / 11. País Vasco / 12. Valencia

C.Salud: anotar el número del centro de salud correspondiente.

Médico: anotar el número del médico correspondiente.

Paciente: anotar el número del paciente correspondiente.

Visita: anotar el número de visita correspondiente.
00. Inclusión - exclusión / 01. Visita Inicial / 02. Visita 3 meses / 03. Visita 1 año / 04. Visita 2 años / 05. Visita 3años

Fecha del examen

__ / __ / 200__
Dia Mes Año

1. ¿Usa usted el aceite de oliva como principal grasa para cocinar?	Sí = 1 punto	☐
2. ¿Cuanto aceite de oliva consume en total al día (incluyendo el usado para freir, comidas fuera de casa, ensaladas, etc.)?	4 o más cucharadas = 1 punto	☐
3. ¿Cuantas raciones de verdura u hortalizas consume al día? (las guarniciones o acompañamientos = 1/2 ración) 1 ración = 200g.	2 o más (al menos una de ellas en ensalada o crudas) = 1 punto	☐
4. ¿Cuantas piezas de fruta (incluyendo zumo natural) consume al día?	3 o más al dia = 1 punto	☐
5. ¿Cuantas raciones de carnes rojas, hamburguesas, salchichas o embutidos consume al día? (ración: 100 - 150 g)	menos de 1 al dia = 1 punto	☐
6. ¿Cuantas raciones de mantequilla, margarina o nata consume al día? (porción individual: 12 g)	menos de 1 al dia = 1 punto	☐
7. ¿Cuantas bebidas carbonatadas y/o azucaradas (refrescos, colas, tónicas, bitter) consume al día?	menos de 1 al dia = 1 punto	☐
8. ¿Bebe usted vino? ¿Cuánto consume a la semana?	7 o más vasos a la semana = 1 punto	☐
9. ¿Cuantas raciones de legumbres consume a la semana? (1 plato o ración de 150 g)	3 o más a la semana = 1 punto	☐
10. ¿Cuantas raciones de pescado-mariscos consume a la semana? (1 plato pieza o ración: 100 - 150 de pescado o 4-5 piezas o 200 g de marisco)	3 o más a la semana = 1 punto	☐
11. ¿Cuantas veces consume reposteria comercial (no casera) como galletas, flanes, dulce o pasteles a la semana?	menos de 2 a la semana = 1 punto	☐
12. ¿Cuantas veces consume frutos secos a la semana? (ración 30 g)	3 o más a la semana = 1 punto	☐
13. ¿Consume usted preferentemente carne de pollo, pavo o conejo en vez de ternera, cerdo, hamburguesas o salchichas? (carne de pollo: 1 pieza o ración de 100 - 150 g)	Sí = 1 punto	☐
14. ¿Cuantas veces a la semana consume los vegetales cocinados, la pasta, arroz u otros platos aderezados con salsa de tomate, ajo, cebolla o puerro elaborada a fuego lento con aceite de oliva (sofrito)?	2 o más a la semana = 1 punto	☐