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The impact of diet-protein content in telomarase regulation

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Abstract: Higher telomere length (TL) is associated with longevity, while telomeres shortening are related with some non-communicable diseases (NCDs) such as cancer or cardiovascular disease, and with higher risk of mortality. Telomerase RNA component (TERC) and peroxiredoxin-1 (PRDX1) are involved in the regulation of Telomerase, an enzyme capable to extent TL. Diet could play a role in telomere shortening by regulation of cellular oxidative stress and by modulate the expression of certain genes involved in Telomerase Activity (TA) as presented before. This paper study how low-protein diet (LPD) and leucine deprivation (LEU(-)) in tandem with fibroblast growth factor 21 (FGF21) can affect the relative gene expression of *Terc* and *Prdx1* in mice. Murines with and without FGF21 were fed with LPD and LEU(-), with corresponding Control Diet (CD) group for each one. Relative liver mRNA levels of *Terc* and *Prdx1* were determined by RT-qPCR. Results suggested that diet-protein content and FGF21 could impact on *Terc* and *Prdx1* expression by modulating oxidative stress of the cell. LPD synergized with FGF21 to increase *Prdx1* mRNA levels (p=0.00096), but inconsistency and the contradictions of the findings did not allow to suggest accurate conclusions and for that reason further investigations with better designs are needed.

Keywords: Telomerase RNA component; peroxiredoxin-1; fibroblast growth factor 21; low-protein diet; leucine deprivation; lifespan

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1. Introduction

The ends of linear chromosomes are comprised for repetitive noncoding DNA sequences called telomeres, that protects genetic material in these vulnerable regions [1]. These repetitive sequences, TTAGGG in mammals, are bonded to a protein complex known as shelterin or telosome.

This protein complex is formed by six different proteins: protection of telomeres 1 (POT1), telomere repeat-binding factor 1 (TRF1), telomere repeat-binding factor 2 (TRF2), TRF1-interacting nuclear factor 2 (TIN2), adrenocortical dysplasia protein homologue 1 (TPP1) and repressor activator protein 1 (RAP1), also called telomeric repeat-binding factor 2-interacting protein 1 [2].

Owing to the so-called "end of replication problem" (Figure 1), telomeres shorten with every cell division [3].

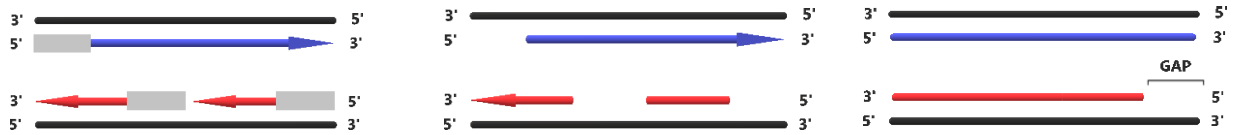

Figure 1. DNA polymerase is an enzyme capable to catalyze the synthesis of new DNA molecules from parental DNA strands (black). Due to the antiparallel structure of DNA jointly with the incapacity of DNA polymerase to synthesize in 3′-5′ direction, this enzyme synthesizes new DNA strands in opposite directions: leading strand (blue) and lagging strand (red). DNA polymerase needs a 3′-OH group to add the first nucletide, this is the function of the primer (grey), a short strand of RNA that provides a 3′OH group. Due to the lack of 3′OH in the 5′ ends of the linear DNA molecules, primer can't be replaced for DNA, therfore a GAP is produced.

Advanced telomere shortening cause unestable cromosomes forms that compromise cell division capacity leading to cellular senescence. At this point, cell is unable to keep their functionality and mutations rates rise, thus increasing the cell death by apoptosis. Even tough, this not ocurr in cancer cells, where genes that regulate the programmed cell death are suppressed [4]. The shorteness of the telomeres have been associated with a higher incidence of non-communicable diseases (NCDs) and age-related diseases, such as cancer [5-7] or cardiovascular diseases [8-10] two of the main causes of death in developed countries. In a recent paper, Muñoz-Lorente et al. [11], proved that mice with hyper-long telomeres are protected from cancer, show longer lifespans and an increased longevity.

Telomerase is an enzyme capable to extend telomeric DNA sequences in order to reduce, stop or revert telomere shortening. This enzyme was discovered by E. Blackburn and C. Greider in 1985(12) whose were awarded in the 2009 with Nobel Prize in Physiology or Medicine, together with J.W. Szostak for abovementioned work and later investigations about telomeres and telomerase. Telomarase activity (TA) is high in germ cells, embryonic stem cells (ESCs) and tumor cells, but quite poor in somatic cells. With the activation of the telomerase, cancer cells preserve their telomeres after each cell division, and as a result they can divide relentlessly and become immortal [13]. The relationship between cancer and TA, and the association between low levels of TA and the activation of alternative tumor supression pathways [14], lead the scientific community to an area of controversy regarding TA and health.

It is well known that diet and health are closely and positively connected, although in many cases molecular mechanisms remain underlying the effects of diet over health remain unidentified. It has been described that diet could modulate telomere shortening through the regulation of cellular oxidative stress and of the expression of certain genes involved in telomeres homeostasis. Several human studies have linked diet and telomeres have been published. Galiè et al. [15], in a recent systematic review, collect the most important ones until today.

In one hand, healthy diets have been related with higher telomere length (TL). Mediterranean Diet (MD) has been the most studied [16-20], among others with a high diet quality indexes. Examples of those indexes are: Dietary Guideline Index (DGI) [21], Alternative Healthy Eating Index (AHEI) [22], Mediterranean Diet Index (MedDiet), Dietary Guidelines for Americans (DGA), Baltic Sea Diet Score (BSDS) or Prime Diet Quality Score (PDQS) [19,23,24]. Regarding food groups, fruits [25] and vegetables [26,27], have been the most clearly associated with TL, but also evidence for olive oil [28], whole grains [29], coffee [30,31] and nuts [32], have been described. Furthermore, ω-3 fatty acids [33,34], fiber [35], and some vitamins such as D [36,37], A [27] and E [38] could act in the same direction (Table 1).

On the other hand, some kind of diets can aggravate telomere shortening. Negative effects on telomere homeostasis have been described for pro-inflammatory diets [39,40] high consumption of red meat [41-43] ultra-processed food (UPF) [44], sugar-sweetened beverages(SSB) [45], alcohol [46,47] and for trans and saturated fat [48-50] (Table 2).

Table 1. Studies with positive association between diet and telomere length. +TA (positive association with TL); +TA (positive association with TA), -TL (negative association with TL).

Factor	Design	Population	Results	Year
MD	Cohort prospective (24 years)	Healthy woman (n= 4676)	+TL	2014
	Cross-sectional	Aged and healthy population (n=217)	+TA ; -TL	2015
	Cross-sectional	High risk of cardiovascular disease population (n= 520)	+TL	2016
	Randomized Controlled Trial (5years)	High risk of cardiovascular disease population (n= 520)	No associations	2016
	Cohort prospective (10 years)	Aged population (n= 1046)	-TL (in woman)	2019
	Cohort prospective (1 year)	Young and obese population (n=87)	+TL	2020
Quality Indexes	Cross-sectional	Middle-aged woman (n=5862)	No associations	2012
	Cross-sectional	Middle-aged population (n=4758)	+TL	2018
	Cross-sectional	Aged population (n=679)	No associations	2018
	Cohort prospective (10 years)	Middle-aged population (n=1046)	-TL (in woman)	2019
	Cross-sectional	Aged population (n=886)	+TL	2020
Fruits and vegetables	Case-control	Gastric cancer cases (n=300); healthy controls (n=416)	+TL	2009
	Cross-sectional	Healthy Italian adults (n=56)	+TL	2012
	Case-control	Pesticide-exposed cases (n=62); unexposed controls (n=124)	+TL	2016
Olive oil	Cross-sectional	Participants from CARDIOPREV study (n=1002)	+TL	2019
Whole grains	Cross-sectional	Woman from NHS (n=2284)	+TL	2010
Coffee	Randomized Controlled Trial (9 months)	Population with hepatitis C (n=40)	+TL	2013
	Cross-sectional	Woman from NHS (n=4780)	+TL	2016
	Cross-sectional	American population from NHANES (=5582)	+TL	2017
ω-3 fatty acids	Randomized Controlled Trial (6 months)	Aged population (n=33)	+TL	2014
	Randomized Controlled Trial	Young population (n=71)	+TL	2018
Fiber	Cross-sectional	American population from NHANES (n=5674)	+TL	2018
Vitamin D	Randomize Controlled Trial (12 weeks)	African American population (n=37)	+TL	2012
	Cross-sectional	Middle-aged woman (n=2160)	+TL	2018
Vitamin A	Cross-sectional	Healthy population 8n=56)	+TL	2012
Vitamin E	Cross-sectional	Japanese population (n=70)	+TL	2016

Table 2. Studies with negative association between diet and TL. –TL (negative association with TL).

Factor	Design	Population	Results	Year
Diet Inflammatory Index	Cross-sectional	Population with high risk of cardiovascular disease (n=520)	-TL	2015
	Cohort prospective (10 years)	Population with high risk of cardiovascular disease (n=520)	-TL	2015
	Cross-sectional	Healthy middle-aged population (n=2509)	No associations	2018
Red meat	Cross-sectional	Population from MESA study (n=840)	-TL	2008
	Cross-sectional	Population from HSPS (n=2846)	-TL	2016
	Cross-sectional	Middle-aged population (n=300)	-TL	2018
UPF	Cross-sectional	Aged population from SUN project (n=886)	-TL	2020
SSB	Cross-sectional	Middle-aged population (n=5309)	-TL	2014
Alcohol	Cohort prospective (2 years)	Middle-aged population from Asklepios study (n=2509)	-TL	2007
	Case-control	Alcohol abuse cases (n=200); healthy controls (n=257)	-TL	2011
Trans and saturated fat	Cross-sectional	Aged population from HBCS study (n=1942)	-TL	2012
	Case-control	Diabetic premenopausal cases and healthy controls (n=4029)	-TL	2013
	Cross-sectional	Middle-aged population from NHANES study (n=5446)	-TL	2018

Among other diets, previous research of the group and of other authors show that leucine deprived diet (LEU(-)) [(51-53)], and low-protein diets (LPD, 5% protein) [54], improve insulin sensibility, promote weight loss and impact on lipid metabolism. Most of these effects has been associated with an increased expression/production of fibroblast growth factor 21 (FGF21) [55-58], a peptide hormone that play an important role for metabolic adaptations in response to nutritional inputs. FGF21 is increased in both LPD and LEU(-). Moreover, some of the metabolic changes in response to those diets has been also associated with longer telomeres and higher longevities [59]. For that reason, was plausible to think that FGF21 may impact on telomere homeostasis by modulating the expression of genes involved on it.

Based on the data mentioned in this introduction, the aim of this work is to study the impact of LDP and LEU(-) on gene expression involved in mice telomerase regulation, and in consequence on telomere homeostasis, and also how FGF21 takes part in this process. Nutritional interventions were previously conducted to the study and are described in order to contextualize this actual work

2. Results

Telomere homeostasis can be studied by analysing the relative expression of genes involved on it. Two of the main actors implied on this process are telomerase RNA component (TERC), that serves as a template for telomere replication by telomerase [60,61], and peroxiredoxin-1 (PRDX1), an antioxidant enyme crucial for TA [62].

2.1. LPD modulates the expression of Terc and Prdx1 in mice livers

In order to evaluate the impact of LPD and the role of FGF21 on the nutritional intervention effects over telomere homeostasis the mRNA levels of *Terc* and *Prdx1* were measured in locus of X-over P1 (LoxP) mice and in liver specific *Fgf21* knockout (L*Fgf21KO*) mice. LoxP mice fed with LPD (LoxP/LPD), showed a significant less expression of *Terc* than the control diet (CD) group (p=0.015). In contrast, even not statistically different, an opposite tendency for LPD was observed in L*Fgf21KO* animals. L*Fgf21KO* animals exhibited lower levels of TERC both in CD and in LPD versus LoxP/CD group, but no significant differences were observed between dietary groups in the absence of FGF21. Even so, the absence of FGF21 caused a significant decrease of *Terc* expression in LKO/CD group compared with LoxP under the same diet (p=0.006) (Figure 2).

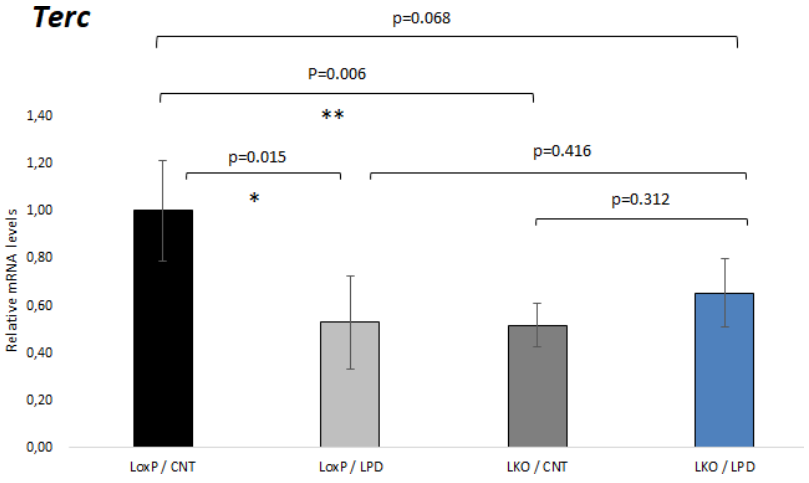

Figure 2. Relative mRNA levels of *Terc* in liver. LoxP/CNT (black bar), LoxP/LPD (light grey bar), LKO/CNT (dark grey bar) and LKO/LPD (blue bar). Error bars represent the mean ±SEM, *p<0.05, **p<0.01 (n=6-8/group).

Regarding *Prdx1*, its expression increased in mice fed with a LPD and this effect is at least in part dependent of the FGF21. In LoxP animals, LPD induced the expression of *Prdx1* (p=0.00096) (Figure 3) and this upregulation is partially blunted in LKO mice fed LPD where the *Prdx1* induction, even not statistically different (p=0.054), is lower than in LoxP/LPD mice. Even so, in the absence of FGF21, LPD exert an affect over the expression of Prdx1 as LKO/LPD mice showed a significant increase on the expression of *Prdx1*.

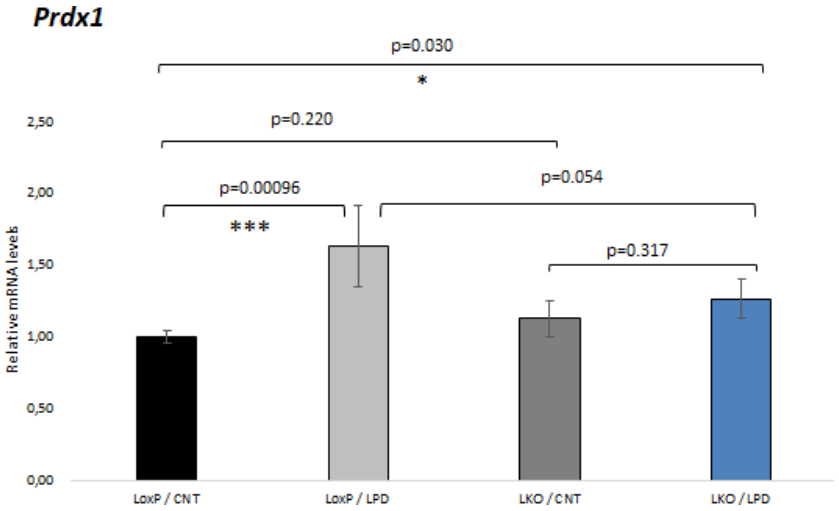

Figure 3. Relative mRNA levels of *Prdx1* in liver. LoxP/CNT (black bar), LoxP/LPD (light grey bar), LKO/CNT (dark grey bar) and LKO/LPD (blue bar). Error bars represent the mean ±SEM, *p<0.05, ***p<0.001 (n=6-8/group).

2.2. LEU(-) diet reduced the expression of Prdx1 in mice livers

To assess the impact of LEU(-) diet and the role of FGF21 on telomere homeostasis, mRNA levels of *Terc* and *Prdx1* were measured in wild-type (WT) mice and in total *Fgf21* knockout (*Fgf21KO*) mice fed with control diet or leucine-deprived diet. Figure 4 shows that not statistically differences on mRNA levels of *Terc* were observed of diet or genotype. A slightly tendency to increase *Terc* expression is observed in LEU(-) fed animals (Figure 4). It is remarkable that standard deviation in these groups and for the expression of this gene was too high, reason why these results may be difficult to interpret.

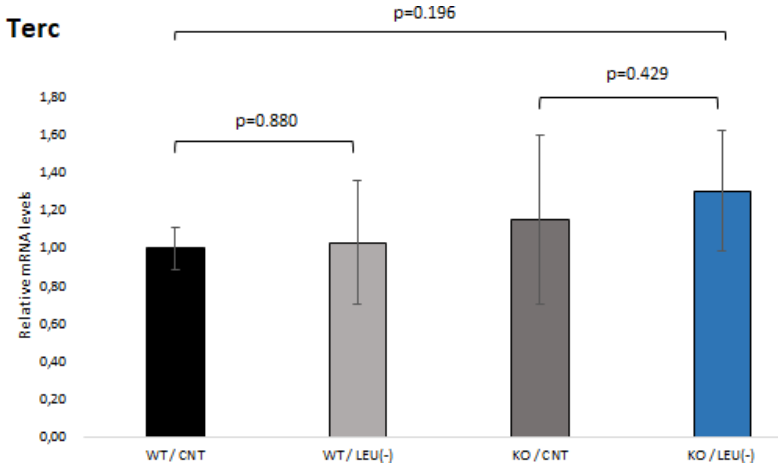

Figure 4. Relative mRNA levels of *Terc* in liver. WT/CNT (black bar), WT/LEU(-) (light grey bar), KO/CNT (dark grey bar) and KO/LEU(-) (blue bar). Error bars represent the mean ±SEM (n=6-8/group).

Regarding *Prdx1* expression, WT mice fed with LEU(-) diet shown a decrease on its mRNA levels (p=0.037). In *Fgf21KO* mice, the dietary intervention does not cause a significant reduction of the *Prdx1* RNA levels (Figure 5).

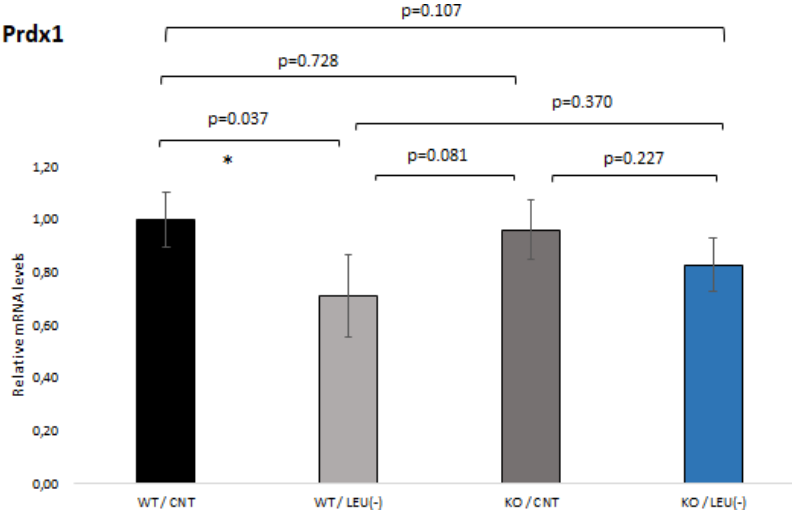

Figure 5. Relative mRNA levels of *Prdx1* in liver. WT/CNT (black bar), WT/LEU(-) (light grey bar), KO/CNT (dark grey bar) and KO/LEU(-) (blue bar). Error bars represent the mean ±SEM, *p<0.05 (n=6-8/group).

3. Discussion

LPD increases the expression of *Prdx1* (Figure 3). This effect can be explained through the capacity of LPD to increase oxidative stress in liver cells. PRDX1 is an antioxidant enzyme which is overexpressed in response to an increase of oxidative stress levels. It is known that LPD causes oxidative stress. Several studies have shown in rats that LPD decreases the levels of vitamin E [63] and glutathione [64], thus causing the raise of oxidative stress that has to be compensated by the upregulation of the *Prdx1* expression. In the same way, the hepatic downregulation of *Terc* mRNA levels may be also explained because the telomerase function decreases when oxidative stress of the cell is high. In the case of LKO mice fed a control show a reduction in the mRNA levels of *Terc* (Figure 2). This effect may be associated to an oxidative stress caused by the absence of FGF21 in the liver. It has been described that FGF21 protects against oxidative stress and inflammation in hepatocytes [65] and also has been observed their capacity to increase SOD activity, thus decreasing the oxidative stress of the cell [66]. The remaining question is the non-effect of LPD in LKO/LPD mice. The effects of LPD over *Terc* expression are blunted in KO mice.

FGF21, that is overexpressed in LPD, increase fatty acid oxidation in liver. At the same time, fatty acid oxidation is associated with reactive oxygen species (ROS) [67]. Thus, FGF1 could increase oxidative stress and synergizes with LPD to decrease *Terc* levels (Figure 2) and increase *Prdx1* levels (Figure 3). Therfore,, the reduction of *Terc* mRNA levels in LKO mice, suggests that the absence of FGF21 can cause an increment of oxidative stress as well, supported by the bibliography above-mentioned [65,66].

LEU(-) also induces FGF21 expression in liver, therefore effects on *Terc* and *Prdx1* expression might be similar to LDP ones. In the case of *Terc* no effects of dietary intervention or genotype were observed. By contrast the effects of LEU(-) are opposite to the ones observe under LPD. Leucine deprivation causes a reduction in the expression of *Prdx1* through an unknown mechanism. It is obvious that metabolic stress by the dietary deficiency of an essential amino acid such leucine would not be the same that the reduc-

tion of the dietary protein content such as LPD. These differences may explain the differential impact of both nutritional interventions on *Prdx1*.

The lack of statistical difference in LEU(-) groups and their contradictory results comparing to LPD do not allow to propose new theories regarding LEU(-),LPD, FGF21 and telomere homeostasis. Further investigations, considering these results, are needed.

An important limitation of this work is that mRNA levels have been associated with function, without considering post-translational modifications that regulate functionality of proteins. For that reason, the quantification of the mRNA levels is only an approach to the final function. Additionally, TA is not only regulated by TERC and PRDX1, but there are also more genes involved in their activity such as TERT or shelterin genes presented before.

On the other hand, the animals used in this work are young (6-8 weeks-old). This is a big issue to detect changes on telomeres. These changes are more visible in aged subjects. Besides, the dietary intervention was too short. Changes in telomere homeostasis regulation caused by diet are detected after considerable time.

For further investigations it would interesting to analyze the expression of other genes involved in telomere homeostasis, quantify TA and also measure TL. Apart from that, long terms interventions and aged animals must be necessary.

3.1. Concluding remarks

The present work is the first study related with amino acid deficient diets, FGF21 and telomere homeostasis. The age of the animals and the duration of the nutritional intervention limit the reliable and robust results.

In spite of the results, this work is the first step on the road to investigate the molecular mechanisms through amino acid deprivation diets work. It has been shown on this paper that these diets can modify the expression of related telomere homeostasis genes.

Future investigations with better experimental designs and more specific analysis techniques are needed to discover if the positive metabolic effects of amino acid deprivation diets go together with an improvement in the telomere homeostasis.

4. Materials and Methods

4.1. Animals and diet intervention

4.1.1. LPD diet intervention

129S6/SvEvTac male mice were divided in two different genetic maps: L*Fgf21KO* and another group with Fgf21 flanked by two LoxP sites in liver. Animals were housed in a temperature-controlled room (22 ± 11ºC) on a 12h/12h light/dark cycle and were provided free access to commercial rodent chow and tap water prior to the experiments. For the feeding experiment, 8-week-old male mice were first fed with CD for 7 days and then L*Fgf21KO* and LoxP animals randomly assigned to CD or LPD group with free access to food and water for 7 days, resulting four experimental groups: LoxP mice under CD (LoxP/CNT), LoxP mice under LPD (LoxP/LPD), L*Fgf21KO* mice under CD (LKO/CNT) and L*Fgf21KO* mice under LPD (LKO/LPD).

The CD (Ref. D10001) and LPD (Ref. D12010401) were obtained from Research Diets, Inc. (USA). Both diets were isocaloric and had the following composition in mass percentage: 20% protein, 66% carbohydrates and 5% fat for the CD, and 5% protein, 81% carbohydrates and 5% fat for the LPD. After the nutritional intervention, animals were anesthetized by isoflurane inhalation. After euthanizing the animals, tissues were isolated immediately snap-frozen and stored at -80ºC.

For further information about genetic maps generation see the following paper published by our group [56].

4.1.2. LEU(-) diet intervention

B6N;129S5-Fgf21tm1Lex/Mmucd male mice were divided in two genetic maps: WT and Fgf21KO, obtained from the Mutant Mouse Regional Resource Center, were used in collaboration with Dr. Francesc Villarroya's laboratory group. Before the experiments, animals were housed in a temperature-controlled room (22 ± 11ºC) on a 12h/12h light/dark cycle and were provided free access to commercial rodent chow and tap water. Before diet intervention, 12-15 week old mice were first fed with CD for 7 days and then *Fgf21KO* and WT animals randomly assigned to CD or LEU(-) group with free access to food and water for 7 days, resulting four experimental groups: WT mice under CD (WT/CNT), WT mice under LEU(-) (WT/ LEU(-)), *Fgf21KO* mice under CD (KO/CNT) and *Fgf21KO* mice under LEU(-) (KO/ LEU(-)).

CD (Ref. A10021B) and LEU(-) (Ref. A05080202) were obtained from Research Diets, Inc. (New Brunswick, NJ), and both were isocaloric and compositionally the same in terms of carbohydrate and lipid components, and regarding amino acid content, LEU(-) were deficient in leucine. After the intervention, animals were anesthetized by isoflurane inhalation. After euthanizing the animals, tissues were isolated immediately snap-frozen and stored at -80ºC.

4.2. RNA extraction and RT-qPCR quantification

Total RNA was extracted from liver frozen tissues using a solution of phenol and guanidinium tiocianate (TRI Reagent® Solution; Ref.AM9738 Ambion Thermo Fisher Scientific, USA). Absorbance at 260 nanometers (A260) was determined to quantify total RNA after the extraction, also the ratio A260/A280 to verify the quality of the extraction. This step was followed by DNase I treatment (Thermo Scientific RapidOut DNA Removal Kit; Ref. K2981) in order to eliminate genomic DNA contamination. RT-qPCR was performed to mesure the relative mRNA levels: cDNA was synthesized from 1 microgram of total RNA by reverse transcriptase (Ref. 4368814) with random examers and using a thermocycler (iCycler BIO-RAD), and PCR amplification was conducted using SYBR Green (Ref. 4479242) and CFX96 thermocycler of BIO-RAD. Each mRNA sample was mesurated in duplicate with B2M and M36B4 as housekeeping genes. There primers used for the qPCR were: TERC (F-5′TCATTAGCTGTGGGTTCTGGT-3′;R-5′-TGGAGCTCCTGCGCTGACGTT-3′);PRDX1(F-5′AATGCAAAAATTGGGTATCCTGC-3′);R-5′-CGTGGGACACACAAAAGTAAAGT-3′);B2M(F-5′-CGTCGTGCTTGCCATTC AGA-3′;R-5′-AGGAAGTTGGGCTTCCCATTC-3′);M36B4(F-5′-TCGTTGGAGTGACATC GTCTT-3′; R-5′-TCTGCTCCCACAATGAAGCA-3′).

4.3. Statistics

Mean of the individual mRNA quantification of each experimental group with corresponding standard error (SEM) was used to statistical treatment. Two tailed Student's t-test was applied and p values lower than 0.05 was considered statistical significant.

Institutional Review Board Statement: The Animal Ethics Committee of the University of Barcelona approved these experiments (CEEA register: 48/15).

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Conflicts of Interest: The author has declared no conflict of interest.

Abbreviations

AHEI	Alternative Healthy Eating Index
CD	Control Diet
DGA	Dietary Guidelines for Americans
DGI	Dietary Guideline Index
ESCs	Embryonic Stem Cells
FGF21	Fibroblast Growth Factor 21
Fgf21KO	FGF21-null
LEU(-)	Leucine Deprivation
LFgf21KO	Liver FGF21 Knockout
LoxP	Locus of X-over P1
LPD	Low-Protein Diet
MedDie	Mediterranean Diet Index
MD	Mediterranean Diet
NCDs	Non-communicable Diseases
PDQS	Prime Diet Quality Score
POT1	Protection Of Telomeres 1
PRDX1	Peroxiredoxin-1
RAP1	Repressor Activator Protein 1
SEM	Standard Error of the Mean
SSB	Sugar-Sweetened Beverages
TA	Telomerase Activity
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transcriptase
TIN2	TRF1-Interacting Nuclear Factor 2
TL	Telomere Length
TRF1	Telomere Repeat-Binding Factor 1
TRF2	Telomere Repeat-Binding Factor 2
TPP1	Adrenocortical Dysplasia Protein Homologue
UPF	Ultra-processed food
WT	Wild-Type

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