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High dietary protein and fat contents exacerbate hepatic senescence and SASP in mice

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Dietary choices have a profound impact on the aging process. In addition to the total amount of energy intake, macronutrient composition influences both health and lifespan. However, the exact mechanisms by which dietary macronutrients influence onset and progression of age-associated features remain poorly understood. Cellular senescence is a state of stable growth arrest characterized by the secretion of numerous bioactive molecules with pro-inflammatory properties. Accumulation of senescent cells is considered one of the basic mechanisms of aging and an important contributor to chronic inflammation and tissue degeneration. Whether dietary macronutrients affect the accumulation and the phenotype of senescent cells with age is still unknown. Here, we show that feeding on diets with varying ratios of dietary macronutrients for 3 months has a significant effect on different senescence-associated markers in the mouse liver. High protein intake is associated with higher expression levels of the two classical senescence-associated growth arrest genes, p21 and p16. Furthermore, the expression of many pro-inflammatory secretory markers was increased in diets enriched in protein and further enhanced by increases in fat content. These results provide preliminary evidence that dietary macronutrients have a significant influence on senescence markers and merit further investigation.

Introduction

Global eating habits are shifting toward hyper-caloric diets at the expense of diets enriched in nutrient-dense foods. Poor eating habits have profound effects on health, and constitute a primary, but potentially preventable, risk factor for several noncommunicable chronic diseases [1]. One of the major risk factors for

Abbreviations
CDK, cyclin-dependent kinase; CR, calorie restriction; DDR, DNA damage response; GLM, generalized linear modeling; LMNB1, Lamin B1; mTOR, mechanistic target of rapamycin; SASP, senescence associated secretory phenotype.

a plethora of chronic diseases is increased age [2]. Importantly, the impact of nutrition on the modulation of the aging process and age-associated dysfunctions is becoming more evident. In fact, it is long established that calorie restriction (CR), with the maintenance of adequate nutrient intake, is one of the most efficient interventions for extending lifespan and healthspan [3]. CR diets were demonstrated to retard and even prevent the onset of many age-related diseases in various organisms, ranging from yeast and invertebrates to nonhuman primates, and possibly humans [1,4,5]. In contrast, increased adiposity due to excess energy storage is a risk factor for many age-related diseases and correlates with premature mortality [6,7].

Besides the total amount of calorie intake, the proportion of dietary macronutrients can also influence the aging process. In support of this, mounting evidence suggests that a reduction, but not elimination, of dietary protein intake has a positive impact on longevity and metabolic health [8,9].

Aging tissues are typically enriched in senescent cells—cells that lose their proliferative capacity and develop a heterogeneous secretory phenotype (the Senescence-Associated Secretory Phenotype or SASP) enriched in pro-inflammatory factors [10]. Accumulation of senescent cells contributes to chronic low-grade and age-associated inflammation (also known as inflammaging) and promotes many age-related diseases [11]. In support of this, elimination of senescent cells from old tissues is sufficient to reduce inflammaging and alleviate onset and progression of age-associated conditions [12–14]. Therefore, eliminating senescent cells using senolytics or dampening the deleterious effect of SASP through the usage of senomorphics, are potential approaches for enhancing healthy longevity [15].

An alternative strategy to interfere with the detrimental function of senescent cells is to prevent their accumulation. Studies have shown that CR reduces senescence markers in mice and humans, potentially by preventing and/or repairing cellular damage that can lead to senescence induction [16,17]. However, the effect of different dietary macronutrient composition during *ad libitum* feeding on senescence has not yet been studied. Therefore, we investigated the expression level of various markers of senescence and SASP in the mouse liver in response to short-term exposure (3 months) to a series of diets with different macronutrients composition. In relation to lifespan, 3 months in the mouse is equivalent to about 9 years in a human.

Results

The transcription of the cyclin-dependent kinase inhibitors p16 and p21 increases with increased protein consumption

The liver is one of the major effectors of dietary interventions as it plays a central role in various biological activities related to nutrient metabolism. To study if diets with different macronutrient composition affected hepatic senescence markers, we measured the expression of the cyclin-dependent kinase (CDK) inhibitors p16 and p21, the two major senescence-associated cell cycle arrest genes [18]. Expression of p16 and p21 was evaluated in livers collected from mice fed diets with increasing protein content (10%, 15%, 25%, and 30% by energy) and with low (xx%) or high (yy%) amount of fat (Table S1). Data were analyzed using generalized linear modeling (GLM), using gene expression as the dependent variable and the level of dietary protein and fat as the independent variable (full analysis reported in Fig. S1). The balance of the diet is made up of carbohydrates, but it is not possible to enter all three macronutrients as predictors because of predictor colinearity. For both p16 and p21, there was a significant association between their expression and the level of dietary proteins in the diet (GLM, $P < 0.0005$, $P < 0.005$, respectively) (Fig. 1A and Table 1). In contrast, no significant association between p16 and p21 levels and amount of dietary fat was observed (GLM, $P \geq 0.05$) (Fig. 1A, Table 1 and Fig. S1). To further evaluate expression of senescence markers in low vs high protein dietary regimens, we measured the protein levels of p53, a major regulator of the senescence-associated growth arrest, and LaminB1 (LMNB1), a nuclear lamina protein which expression is normally lost in senescent cells [10]. In accordance to the results obtained at the transcriptomic level, the high protein diet showed an increase in p53 and a decrease in LMNB1 expression (Fig. 1B). Together, these data suggest that the level of hepatic senescence markers is proportional to the amount of dietary protein but independent of fat.

The transcription of proinflammatory SASP factors was dependent on both dietary protein and fat content

To evaluate the effect of different diets on SASP regulation, we measured transcriptional levels of some of the most common SASP factors (IL-6, IL-1α, IL-1β, TNF-α, CXCL-1, CXCL10, PAI-1, and CCL2) in the

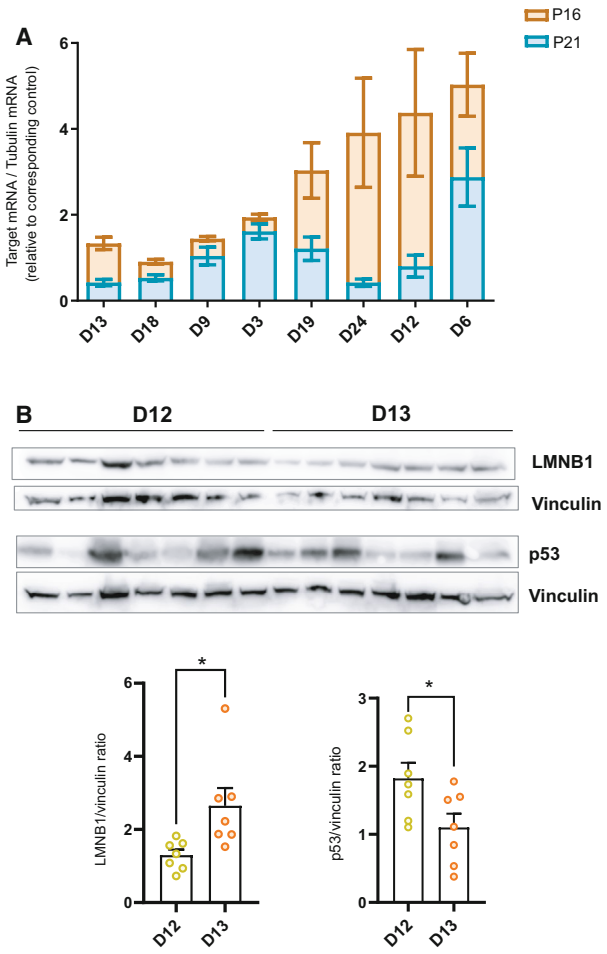

Fig. 1. Expression of senescence markers in liver of mice fed with different dietary macronutrients composition. Mice were fed diets with increasing protein content and with low or high amount of fat for 3 months (see Table S1 for details). (A) RNA was isolated from the liver and mRNA for the indicated genes quantified by qRT-PCR relative to tubulin. (B) Protein was extracted from the liver and p53 and LMNB1 (Lamin-B1) protein levels analyzed by Western Blot. Vinculin protein levels were used as internal controls. Data shown are the mean ± SEM (n = 7–12).

livers of mice fed the diets described in Table 1. As for p16 and p21, data were analyzed using generalized linear modeling (GLM), using gene expression as the dependent variable and the level of dietary proteins and fats as the independent variable (full analysis reported in Fig. S2). Again, the balance of the diet is made up of carbohydrates, but it is not possible to enter all three macronutrients as predictors because of predictor colinearity. Similar to the observations of p16 and p21, the levels of all the SASP factors analyzed, except for CXCL-10, were proportional to the protein content (Fig. 2 and Table 1). Importantly, increased fat intake further enhanced the expression of

Table 1. Significance of the Generalized Linear Modeling (GLM) analysis for all genes.

	P value	
Gene	Protein	Fat
P16	< 0.0005*	0.867
P21	0.003*	0.223
IL-6	< 0.0005*	0.001*
IL-1α	< 0.0005*	< 0.0005*
IL-1β	< 0.0005*	0.065
TNFα	0.001*	0.034*
CXCL1	0.001*	0.591
CXCL10	0.099	0.012*
PAI-1	< 0.0005*	0.016*
CCL2	< 0.0005*	< 0.0005*

*$P < 0.05$.

many SASP genes and resulted significantly associated with higher levels of IL-6, IL-1α, TNF-α, CXCL10, PAI-1, and CCL2 (Fig. 2 and Table 1). It is interesting to note that although increasing the amount of fat in the diet, while keeping the same amount of protein, showed a trend in increasing SASP expression, the effect was less noticeable when the protein content was low in the diet (Fig. 2 and Table 1). If we made the pairing for independent predictors protein and carbohydrate, rather than protein and fat, then we obtained significant effects for carbohydrate only for IL-1α, IL-6, and CCL-2 (in all cases increasing carbohydrates having a slightly negative effect on all expression of all three genes). These data suggest that high protein diets correlate with higher SASP levels, but also that SASP expression in high protein diets is further enhanced by fat content.

Discussion

Here, we show that modulating protein and fat content for a relatively short period of time can have profound effects on the accumulation of senescence-associated markers in the liver. In particular, we observe that the transcription and expression of different senescence markers is proportional to the protein content, whereas SASP expression is particularly exaggerated in mice fed high-protein and high-fat diets.

Previous studies have shown that protein/amino acid restriction or a decrease in protein-to-carbohydrate ratio have favorable effects on metabolic health and longevity [8,9,19–21]. Protein oxidation and DNA damage proportionally increase with dietary protein intake, and protein-restricted diets are associated to enhanced expression of endogenous antioxidants, and reduce hepatic tumorigenesis in rats. High-protein

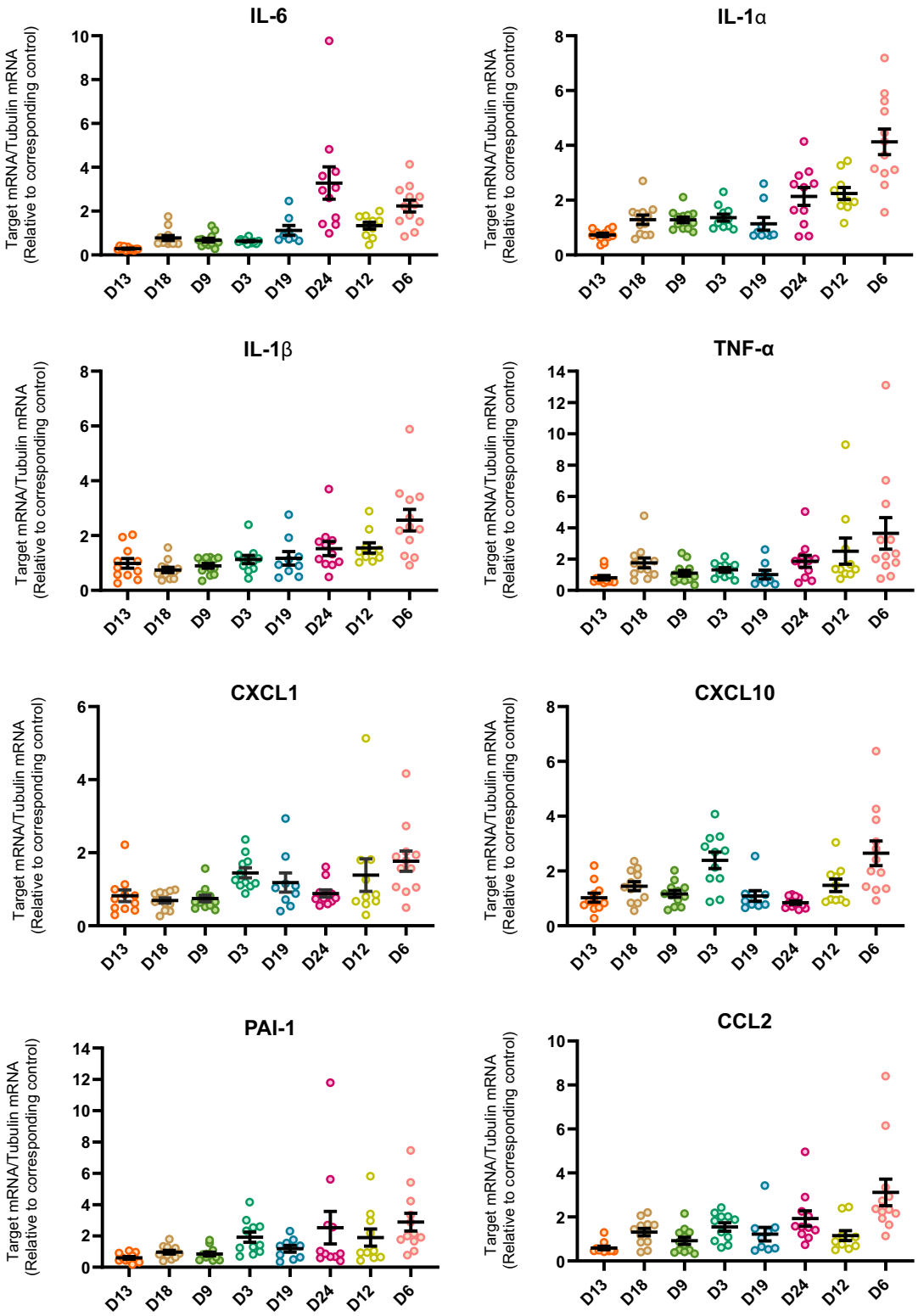

Fig. 2. Expression of senescence-associated proinflammatory genes in liver of mice fed with different dietary macronutrients composition. Mice were fed diets with increasing protein content and with low or high amount of fat (see Table S1 for details). After 3 months of feeding, liver was collected, RNA isolated, and mRNA for the indicated genes quantified by qRT-PCR relative to tubulin. Data shown are the mean ± SEM (n = 8–12).

diets, in contrast, are associated with elevated levels of oxidative stress markers, including NADPH oxidase, hydroxynonenal, and malondialdehyde, and reduced levels of antioxidant enzymes, including superoxide dismutase, and glutathione peroxidase [22,23]. In accordance with the notion that oxidative stress causes persistent DNA damage and DNA damage responses (DDR) eventually leading to senescence [24], we observe a significant correlation between the expression of the CDK inhibitors p16 and p21 and increased protein content. Furthermore, an increase in p53 protein levels and a decrease in LMNB1 were detected when a high protein diet was compared to a low protein diet. In line with the expression of p16, we observed upregulation of various SASP factors in a similar protein-dependent manner, particularly when the diet was rich in fat, with IL-6 and IL-1α presenting the most significantly altered expression between the groups. Following DNA damage, IL-1α binds to the receptor IL-1R and triggers further activation of NF-kB, creating a feed-forward loop which induces the transcription of IL-6 and sustains the maturation of the pro-inflammatory SASP [25]. Importantly, a high-fat diet is associated with increased expression of various pro-inflammatory markers [26] and a consequence NF-kB hyperactivation [27]. In accordance, it has been shown that high-fat diets seem to accelerate accumulation of senescent cells with a pro-inflammatory and pro-disease phenotype [28–30].

Another potential molecular mechanism involved in senescence markers regulation could be the mTOR pathway. Due to its role in the regulation of cellular growth, mTOR has been implicated in the modulation of the geroconversion process of senescent cells [31]. In addition, mTOR is known to induce the SASP through various signaling pathways [32,33]. Since mTOR mediates key processes implicated in senescence, and that its activation is influenced by macronutrients, it is conceivable that imbalanced intake of macronutrients can promote cellular senescence and SASP via mTOR. Protein-restricted diets were shown to achieve similar outcomes to rapamycin-induced inhibition of mTOR for metabolic health and longevity [9]. In contrast, high fat diets were shown to activate mTOR in different tissues and eventually lead to senescence [34,35].

More research is needed to investigate the exact mechanisms behind the accumulation of senescence markers in certain dietary contexts, and also to understand what is the exact contribution of senescent cells to the elevated levels of pro-inflammatory factors in high-protein and high-fat diets. Additionally, this work was in mice, and extrapolation to humans must be made with caution. Nevertheless, this study suggests a potential negative impact of high dietary protein particularly when combined with high dietary fat, and that future studies using long-term interventions and in models where the senescence induction and phenotype can be inducibly modulated will help to define whether the anti-aging effects and metabolic benefits of certain diets might be associated with prevention of senescence.

Materials and methods

Mice

Experimental protocols were reviewed and approved by the IRB of the Institute of Genetics and developmental biology, Chinese Academy of Sciences, Beijing (approval numbers AP2014011 and AP2015004). All mice that were used in this study were C57BL/6 males purchased from Charles River and were acclimated to the animal house 2 weeks before starting the experiment. Mice were housed individually (in a specific pathogen free facility) and maintained in environmentally controlled conditions (temperature 22–24 °C, 12 : 12 LD cycle lights on at 0730 h). For housing temperature, we followed the suggestion that housing mice at standard room temperature (22–24 °C), when provided with bedding and nesting materials, may be a good representative of the situation in humans [36]. Mice were fed a low fat diet with 20% protein and 10% fat (RD 12450B Research diets limited) for 2 weeks as the baseline period. At age 12 weeks, mice were randomly allocated to different groups and were maintained on a range of diets that varied in their macronutrient composition for 12 weeks and were then euthanized at the age of 24 weeks, and visceral organs were extracted and preserved at −80 °C. For full experimental details see [19]. Macronutrient composition of diets (by energy) and their original codes can be found in Table S1. Diet composed of 25% protein, 8.3% fat, and 66.7% carbohydrates (D19) was used as reference.

RNA isolation and cDNA synthesis

Frozen liver tissues were pulverized manually using liquid nitrogen-cooled pestle and mortar crusher, and approximately 0.5 g of homogenized tissue powder was collected for analysis for each sample. RNA was extracted from the obtained homogenized tissue powder using Bioline Isolate II Kit (London, UK, BIO-52073). The concentration and purity of the extracted RNA were measured using a NanoDrop spectrophotometer. Purity was assessed using the A260/A280 ratio. The purity of all of the obtained RNA samples was between 1.9 and 2.1. Per sample, 800 ng of RNA was reverse transcribed into cDNA using Applied Biosystems cDNA Reverse Transcription Kit (Waltham, MA,

USA, 4368814). The final product was diluted using RNase-free water and stored at −20 °C.

Real time QPCR

The generated cDNA was used as a template for the subsequent RT-qPCR. The RT-qPCR was performed to assess the transcription of prominent senescence-associated markers and various SASP constituents using Bioline SENSIFast Probe kit (BIO-86005) coupled with Roche Life Science Universal Probe Library. All of the conducted procedures were performed following the manufacturers' protocols. The relative gene expression analysis was conducted using Livak method. The relative abundance of genes was calculated based on the values of the reference diet. Target mRNA levels were determined following normalization to Tubulin gene. For each diet, 8–12 biological samples were analyzed; and for each gene, two technical replicates were made, and their average was used for analysis. The primers used in this study are detailed in Appendix 1.

Western blot

Liver tissues were lysed in cold RIPA buffer (Abcam, Cambridge, UK, ab156034) supplemented with proteinase and phosphatase inhibitors, and protein concentration was measured with Precision Red Advanced Protein Assay (ADV02-A, Cytoskeleton, Denver, CO, USA). Thirty micrograms of protein lysate were loaded on a SDS-PAGE gel and then blotted onto a 0.2 µm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA, 162-0112). Membranes were incubated with the following primary antibodies for 18 h at 4 °C: rabbit anti-Lamin B1 (Abcam, ab16048), mouse anti-p53 (SCBT, Dallas, TX, USA, sc-126), mouse anti-vinculin (Sigma-Aldrich, St. Louis, MO, USA, V9131). Secondary antibody incubation was performed for 1 h at RT with either Goat-anti-mouse-HRP (Dako, Glostrup, Denmark, P044701) or Goat-anti-rabbit-HRP (Dako, P044801). The signal detection was performed with ECL Prime Western blotting detection reagent (GE Healthcare, Chicago, IL, USA, RPN2236) in an ImageQuant LAS 4000 imager (GE Healthcare). Quantification of bands intensity was performed using IMAGEJ Software (NIH and University of Wisconsin, USA) and normalized to vinculin values.

Statistical analysis

All data were analyzed using generalized linear modeling (GLM), using gene expression as the dependent variable and the level (%) of dietary protein and fat as the independent variables, with the protein*fat interaction. We set the significance level at 0.05 and applied a Bonferroni correction to account for multiple testing. Hence, since we studied 10 genes, the P value had to be < 0.005 to reach the significance criterion. The full analyses are reported in Figs S1 and S2.

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Conflict of interest

M.D is founder, shareholder and advisor for Cleara Biotech. Clear Biotech was neither involved nor funded the study.

Author contributions

Conceptualization: JN, JRS, and MD; Data curation: JN, JRS, and MD; Formal analysis: JN, JRS, and MD; Funding acquisition: JRS and MD; Investigation: JN, DY, AA, M V-E, LW SH, YW, JT, CN; Methodology: JN, JRS, and MD; Project administration: JRS and MD; Supervision: JRS and MD; Writing—original draft: JN, JRS, and MD; Writing—review & editing: JN, JRS, and MD.

Peer review

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Data accessibility

The data that support the findings of this study are available from the corresponding authors (m.demaria @umcg.nl; j.speakman@abdn.co.uk) upon reasonable request.

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Supporting information

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

Fig. S1. General linear model p16 v protein and fat.
Fig. S2. General linear model IL-6 v protein and fat.
Table S1. Diets name and composition.
Appendix 1. List of primers and UPL probes used for Real time-qPCR.