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Supplementary information

Dietary restriction impacts health and lifespan of genetically diverse mice

In the format provided by the authors and unedited

Supplementary Tables

Supplementary Table 1: Pairwise comparison of diet effects on lifespan.
Significance (p-value) of all pairwise comparisons (1df) between diet groups from logrank tests. Overall (4df) significance p < 2.2e-16.

	1D	2D	20%	40%
AL	0.0433	2.65e-4	2.92e-8	1.39e-19
1D	--	0.0449	7.00e-5	2.47e-14
2D	--	--	0.0102	4.02e-10
20%	--	--	--	1.153e-5

Supplementary Table 2: Diet effects on median and maximum lifespan.
2a: Median and Maximum Lifespans by Diet

Diet	Deaths	Censored	Median (months)	Maximum (months)
AL	186	2	25.16	34.05
1D	185	3	28.12	36.15
2D	187	3	27.83	39.11
20%	186	3	29.74	41.64
40%	180	2	34.31	47.11

2b: Median lifespan pairwise non-parametric tests (overall p = 8.35e-7).

	1D	2D	20%	40%
AL	0.0548	0.0552	9.04e-4	1.35e-7
1D	--	0.0756	0.0161	2.54e-4
2D	--	--	0.0108	5.83e-4
20%	--	--	--	5.67e-3

2c: Maximum lifespan (90% survival) pairwise non-parametric tests (overall p = 2.91e-16).

	1D	2D	20%	40%
AL	0.0516	5.67e-4	3.21e-5	3.71e-9
1D	--	0.0168	5.89e-5	4.62e-10
2D	--	--	5.29e-3	1.88e-7
20%	--	--	--	2.02e-3

Supplementary Table 3: Fitting Gompertz models for DR effects on lifespan.
3a: Gompertz parameters.

Diet	Baseline Mortality Rate	Mortality Doubling Time	Percent Increase
AL	6.60	5.17	-
1D	5.03	5.05	-2.22
2D	5.80	5.71	10.6
20%	6.25	6.53	26.4
40%	5.22	7.39	41.0

3b: Gompertz slope pairwise tests (overall p = 7.28e-4).

	1D	2D	20%	40%
AL	0.823	0.316	0.0212	0.000797
1D	--	0.229	0.0132	0.000455
2D	--	--	0.193	0.0184
20%	--	--	--	0.292

Supplementary Table 4: Phenotyping schedule.
Filename: SupplementaryTable4.csv
Content: Phenotyping domains with age ranges**.**
Due to scheduling constraints, it was not possible to test all mice at precisely the same ages. In this table we record for each phenotyping event (indexed by Timepoint and Domain) the youngest (StartAge), median (MedAge), and oldest (EndAge) ages at which mice were tested. These data are presented in **Extended Data Figure 3a.**

Supplementary Table 5: Trait definitions.
Filename: SupplementaryTable5.csv
Content: Description of study data in FigShare file AllData.csv. Contains the following fields:
TraitName: three-part trait name used in R scripts.
ApplyRZ: true if rank normal scores transformation was applied for analysis.
UseForQTL: TRUE if used in QTL mapping.
UseForSurvTest: TRUE if tested for association with lifespan (**Extended Data Table 7**).
Timepoint: Categorical time of measurememnt, e.g., Y1, Y2, Y3.
Domain: type of assay used to generate the trait data.
Group: trait category BW/Health/Metabolic/Hematology/Immune.
Trait: trait name without timepoint or domain information.
BW.Var1: bodyweight trait used as covariate in lifespan association tests.
BW.Var2: additional body weight covariate for lifespan test.
Age.Var: age of the mouse at test.
Adjustment: variable(s) used to adjust data prior to analysis.
Description: description of trait including units and transformations.
Comments: additional information.

Supplementary Table 6: Longitudinal analysis.
Filename: SupplementaryTable6.csv
Content: Longitudinal analysis test statistics for diet, age and body weight effect on traits as described in **Methods**: *Longitudinal Trait Analysis.* File contains the following fields:
Domain: type of assay.
Trait: trait name.
Group: categorization of traits.
p_bw: p value for body weight association (adjusted for pll and diet).
p_pll: p value for pll association (adjusted for body weight and diet).
p_diet: p value for diet association (adjusted for pll and body weight).
p_diet.pll: p value for diet x pll interaction (adjusted for body weight).
p_age: p value for chronological age association (adjusted for body weight and diet).
p_diet.age: p value for diet x age interaction (adjusted for body weight).
q_bw, q_pll, q_diet, q_diet.pll, q_age, q_diet.age: false discovery rate adjusted significance for tests as above – Benjamini-Hochberg one-step method.

Supplementary Table 7: Trait Association with Lifespan
Filename: SupplementaryTable7.csv
Content: Lifespan association test statistics as described in **Methods**: *Trait Association with Lifespan*. File contains the following fields:
TraitName: three-part trait name used in R scripts.
Timepoint: Categorical time of measurememnt, e.g., Y1, Y2, Y3.
Domain: type of assay.
Group: categorization of trait.
Trait: name of trait.
BW.Var: body weight covariate used in testing model.
coef: adjusted (partial) correlation of lifespan with trait.
p: p-value of trait-lifespan association adjusted for diet and body weight.
q: false discovery rate adjusted of above.
p_int: p-value significance of diet x lifespan interaction adjusted for body weight.
q_int: false discovery rate adjusted of above.
coef_AL: adjusted (partial) correlation of lifespan with trait, AL mice only.
p_AL: p-value of lifespan association adjusted for body weight, AL mice only.
q_AL: false discovery rate adjusted of above.
coef_1D: adjusted (partial) correlation of lifespan with trait, 1D IF mice only.
p_1D: p-value of lifespan association adjusted for body weight, 1D IF mice only
q_1D: false discovery rate adjusted of above.
coef_2D: adjusted (partial) correlation of lifespan with trait, 2D IF mice only.
p_2D: p-value of lifespan association adjusted for body weight, 2D IF mice only.
q_2D: false discovery rate adjusted of above.
coef_20: adjusted (partial) correlation of lifespan with trait, 20% CR mice only.
p_20: p-value of lifespan association adjusted for body weight, 20% CR mice only.
q_20: false discovery rate adjusted of above.
coef_40: adjusted (partial) correlation of lifespan with trait, 40% CR mice only.
p_40: p-value of lifespan association adjusted for body weight, 40% CR mice only.
q_40: false discovery rate adjusted of above.
N_AL, N_1D, N_2D, N_20, N_40: sample size used in testing broken down by diet.

Supplementary Table 8: Diet effects on lifespan stratified by 6-month body weight.
8a: Log rank pairwise tests light mice (overall p = 2.57e-08).

	1D	2D	20%	40%
AL	0.185	0.00546	2.95e-04	2.21e-08
1D	--	0.143	0.00978	1.78e-05
2D	--	--	0.113	0.0101
20%	--	--	--	0.0337

8b: Log rank pairwise tests Heavy mice (overall p < 1.20e-15).

	1D	2D	20%	40%
AL	0.238	0.0598	1.43e-05	3.68e-14
1D	--	0.264	0.00125	4.11e-11
2D	--	--	0.0201	1.32e-08
20%	--	--	--	4.50e-05

8c: Median and maximum lifespan by 6-month body weight

Diet	Median Light/Heavy	p value (7.63e-4)	Max (90%) Light/Heavy	p value (0.167)
AL	27.1 / 24.8	0.770	36.1 / 33.7	0.0112
1D	29.9 / 26.0	0.00141	36.8 / 34.6	0.125
2D	31.1 / 25.4	0.00383	39.3 / 38.6	0.471
20%	31.4 / 29.4	0.384	42.6 / 41.1	0.325
40%	33.4 / 34.4	0.657	46.2 / 47.5	0.802

Supplementary Table 9: Nomenclature for immune traits.
Filename: SupplementaryTable9.csv
Content: Flow cytometry trait names, gating parameters, common names
For immune traits (FLOW) reference in the paper, we provide information needed to define cell types. Note that a complete listing of FLOW traits is provided in **Extended Data Table 5**. This file contains the following fields:
Trait Name: name of trait as used in R scripts.
Parent gate: denominator cell type used to define proportions.
Identifying markers: markers used to identify cells (parent gate).
Activation – Negative: markers used to define negative activation states.
Activation-Positive: markers used to define positive activation states.
Common Name: name used in text and figures to describe the cell type.

Supplementary Table 10: Network analysis details.
Filename: SupplementaryTable10.csv
Content: Trait clusters as in **Extended Data Figure 8a** and edge weights between traits and key metadata covariates in the glasso network. The definition of network weights is described in **Methods**. This file contains the following fields:
Trait Name: Name of trait as used in Python scripts.
Meta_DietGroup: Weight of network edge between trait and diet group.
Meta_Lifespan: Weight of network edge between trait and lifespan.
BW_BW: Weight of network edge between trait and body weight.
cluster: Trait cluster that a trait belongs to
Age (months): Approximate age of mice at time of collection

Supplementary Table 11: Path analysis of partial correlation network

Path	Score	
Diet → Lifespan	0.216	0.61
Diet → BWDelta → Lifespan	-0.038	0.108
Diet → CD4T → Lifespan	0.015	0.041
Diet → EE → Lean Mass → Lifespan	0.012	0.033
Diet → Lean Mass → Lifespan	0.011	0.032
Diet → RDW → Lifespan	-0.007	0.02
Diet → NLR → Lymphocytes → Lifespan	0.005	0.015
Diet → Eosinophils → Lifespan	0.004	0.01
Diet → CD4T → Lymphocytes → Lifespan	0.003	0.009
Diet → Food intake → Lean Mass → Lifespan	0.003	0.008
Diet → Glucose → Lean Mass → Lifespan	0.002	0.007

Supplementary Table 12: Genetic mapping results.
Filename: SupplementaryTable12.csv
Content: Summary of QTL peaks with LOD > 6 for all traits. The table contains the following fields:
Trait_Name: Three part trait name used in R scripts.
marker_id: marker names (GigaMUGA array) nearest to QTL peak.
chr, pos: chromosome and position (bp) of QTL peak.
lod: log-odds score at QTL peak.
A, B, C, D, E, F, G, H: estimated additive effects for each founder haplotype.

Supplementary Table 13: Diet effects on lifespan stratified by chromosome 18 QTL.
13a: All pairwise log-rank tests for mice without CAST allele.

	1D	2D	20%	40%
AL	0.0776	0.00161	2.08e-07	2.22e-15
1D	--	0.0759	0.000174	2.90e-11
2D	--	--	0.0113	7.46e-08
20%	--	--	--	0.000493

13b: All pairwise log-rank tests for mice with CAST allele.

	1D	2D	20%	40%
AL	0.326	0.439	0.171	2.97e-05
1D	--	0.983	0.472	0.00030
2D	--	--	0.610	0.00105
20%	--	--	--	0.00477

Supplementary Discussion

Using genetically diverse mice for longevity research

Most studies to date have examined metabolic responses to various forms of DR in male inbred mice, typically C57BL/6J, which have a propensity to develop diet-induced glucose and lipid dysregulation[14,66]. In our study, we used DO mice generated through randomized mating of eight inbred founder strains to obtain a genetically diverse outbred stock. Each DO animal is genetically unique and the cohorts in this study reflected the heterogeneity that is a hallmark of aging[67]. This approach circumvents the risk of confusing strain-specific idiosyncrasies with general properties of aging mice. Similar justifications have been proposed for UM-HET3 mice in aging studies[68]. UM-HET3 mice are the outbred progeny of a standardized 4-way cross; they capture only a fraction of the genetic variants present in DO mice and have fixed genotypes of Y and mitochondrial chromosomes.

One drawback of using DO mice is that some male mice can display aggressive behavior. This can result in the need to isolate or euthanize animals, which can bias study outcomes. Careful husbandry can minimize the incidence of fighting. While we acknowledge that our choice to include only female mice was a shortcoming, there is a high prevalence of male-only studies in preclinical research[69], and this study serves to expand our understanding of aging in female animals.

A notable feature of our study was the broad range of longitudinal phenotypes measured across the lifetime of the mice. This is important for two reasons: Firstly, we observed that across several traits, including body composition, association with lifespan varies with the proportion of life lived at time of measurement. Secondly, the effects of DR on health and lifespan are complex and pleiotropic, necessitating comprehensive phenotyping of multiple aspects of physiological health. In addition, some of the most important lifespan-associated traits, such as the ability to maintain body weight, could only be derived from longitudinal monitoring. Using a partial correlation network analysis, we demonstrated that a selected set of the measured physiological traits explains ~40% of the effects of DR on lifespan. As discussed in greater detail below, our analysis also indicated that DR affects multiple independent aspects of physiological health, and that such effects, even within groups of related physiological measures, can be both beneficial and detrimental.

Effects of body weight and composition on longevity

This study was the first to directly compare CR to IF (1 or 2 days fasting), and to report lifespan extension in response to 1D or 2D IF. Mice on CR diets displayed a reduced rate of aging that was proportional to the level of restriction and a corresponding extension of both median and maximal lifespan. IF mice showed extended median lifespan to a lesser degree than CR and did not significantly differ from AL mice in maximum lifespan or rate of aging[70].

We confirmed an inverse relationship between early body weight and lifespan that progressively decreased in magnitude and reversed later in life[33,71]. We propose that this reflects a shift in the relative importance of beneficial effects of smaller body size versus higher adiposity as animals

age. These observations agree with findings in humans, in which higher weight during childhood is associated with higher morbidity and mortality, and weight loss at older ages is associated with increased frailty and mortality risk[72,73]. Although the relationships between growth, body size, and longevity have been extensively studied, our study unveils several novel interactions between body weight and response to diets, including the lack of lifespan extension in response to IF among the heavier mice. The loss of lean mass seen in the CR groups but not in mice on IF underscores the potential undesirability of CR in humans if the effect translates across species. However, it is important to note that our measure of lean mass does not directly correspond to muscle mass. We observed that muscle strength and endurance, as measured by grip strength and spontaneous wheel running, were higher in the 40% group compared to mice in other diet groups[74].

We observed seemingly paradoxical effects of response to DR. Body weight and composition were associated with lifespan in the opposite direction from the intervention effects. DR drove weight loss and reduced adiposity, but mice that maintained more weight and higher adiposity on DR had longer lifespans. The association between lifespan and ability to maintain body weight was consistent across all diet groups. Collectively, these results argue against the notion that DR works simply by counteracting the negative effects of obesity.

Metabolic parameters, health, and longevity

Improved metabolic outcomes, including weight loss and reduced fasting glucose, represent some of the primary outcomes in human trials of CR, IF and other aging interventions[18-24,40,75-78]. While we saw similar changes in metabolic function in the mice, these were not associated with lifespan extension[56]. This raises a question about the interpretation of metabolic response in human trials[23]. The health benefits of reduced adiposity and glucose metabolism for humans are well established, but these may not be good indicators of changes in the rate of aging when analyzed in the context of DR. Intuitively, we may expect that weight loss in response to DR is a sign of the efficacy of the treatment. Rather, our study suggests that individuals who maintain weight on such a regimen may have better long-term outcomes. Specifically, our findings suggested that while DR-induced changes in metabolic traits can be beneficial for health, they may not necessarily translate into a significant extension of lifespan. This poses a challenge when determining the most appropriate primary endpoints for evaluating the effects of interventions in human aging trials. Researchers may need to consider other factors or endpoints when designing and evaluating interventions aimed at promoting healthy aging and longevity. This could include measures related to incidence of chronic disease and mortality and potentially other biological markers associated with functional abilities and resistance to stress.

Body temperature and lifespan

It has been suggested that lower body temperature (Tb) may play a role in the lifespan-extending effect of CR in mice[79], non-human primates[80], and humans[81]. Lowering Tb via genetic manipulation[82] or changes in ambient temperature[83] have been reported to promote lifespan independent of energy intake. On the contrary, a study examining the impact of CR on female mice from 28 strains, reported that strains experiencing the most significant drop in Tb were more susceptible to shortened lifespan as compared to those maintaining higher Tb under CR[84]. In this

study, we showed that Tb generally declines with age and in response to DR. Yet, mice that maintained higher Tb within diet groups had longer lifespan, which seems at odds with the notion that reduced Tb is a mediator of lifespan extension under CR.

Immune and hematologic traits and lifespan

In contrast to the health and metabolic traits, immune and hematologic traits revealed strong associations with lifespan. The complete blood count is a routinely used medical diagnostic tool. Decreased hemoglobin levels and elevated RDW in aging humans is a strong predictor of all-cause mortality, even in the absence of anemia or other age-related diseases[85,86]. In our DO mice, these traits showed strong positive (Hgb, Hct, NumRBC) and negative (RDW, HDW) associations with lifespan. Ironically, 2D IF and 40% CR extend lifespan while driving important lifespan predictors like RDW in the wrong direction, again pointing to contradictory effects of DR. In other words, DR extended lifespan overall despite having a negative effect on lifespan specifically via pathways associated with RDW.

Previous studies have reported significant association between RDW and all-cause mortality in humans[53,54]. Despite significant genetic correlation between RDW and lifespan in UK Biobank, none of the nearly 400 genetic loci associated with RDW colocalize with significant lifespan associations[87,88]. In addition, none of these loci are concordant with the mouse chromosome 18 locus reported here.

The response of immune cell populations to DR was complex yet clearly important as an indicator of efficacy of DR. Our findings revealed that DR exacerbated the age-related decline in mature $CD11b^{+}$ NK cells and reduced the level of circulating inflammatory monocytes in a calorie titratable manner. A recent study indicated that DR modifies trafficking of immune cells between the circulation and tissue sites[89]. Thus, the observed changes in immune cell subsets impacted by diet may have been reflective of differences in tissue residence induced by acute effects of fasting and timing of blood sampling. Nonetheless, after adjusting for diet, changes in circulating immune cell subsets retained their predictive value for longevity.

NK cells are contributors to the innate immune response in that they respond immediately to viral and pathogen challenge in a non-antigen specific context. In some disease settings, mice undergoing caloric restriction have poor outcomes and this may be due to reduced mature NK cell subsets[90-93]. The result in the DO mice suggests that while the NK cell subsets may be exhibiting a less mature phenotype with DR, within the NK cell subsets defined by CD11c and CD11b expression there is an overall shift in frequency to CD62L positive cells with all dietary interventions and this shift is associated with longer lifespan. Since CD62L expression may enhance trafficking of NK cells to sites of viral infection[94], a relative increase in CD62L by the less mature NK cell subsets may offset potential negative effects of CR on innate NK cell mediated pathogen-directed responses. In general, maintenance of immune cells in a naïve or less activated state correlated well with longevity after adjusting for the effects of dietary interventions.

Tumors

While tumor incidence is difficult to evaluate using non-invasive exams, we did see that DR had an impact on easily observable palpable masses and distended abdomen. Other studies have shown that DR can impact both tumor incidence and lifespan extension[8,95,96]. The association between longevity and immune cell subsets could be at least in part attributed to an effect on tumor development. B cell lymphoma and the associated increase in B cells is commonly observed in mice and probable cause of death[97]. It is notable that the age-related increase in circulating B cells observed in this study was entirely inhibited only by 40% CR. We confirmed that early body weight is associated with a reduced incidence of tumors later in life. It seems plausible that this reduction in tumor incidence was partially responsible for the effects of early body weight on lifespan in mice. We also note that 2D fasting had a greater protective effect on tumor development than the 20% CR intervention. This suggests that caloric intake alone was not the direct cause of tumor control. Our study was not designed to identify the specific neoplastic lesions commonly observed in mice. Therefore, we may have not identified animals carrying less apparent yet lethal cancers. However, we speculate that the general trend in weight loss observed late-in-life may reflect the presence of an adipose tissue wasting syndrome associated with cancer[98].

Predictors of Lifespan

The top predictors of lifespan in this study broadly belonged to two categories: body weight/composition and immune/hematological parameters. The single strongest predictor of lifespan in this study was body weight retention in response to stress. We propose that the ability to maintain body weight during periods of stress is an indicator of resilience. More broadly, DR itself is a stressor and the more resilient animals within an intervention group are those that are better able to maintain body weight. Other top predictors of lifespan were a high proportion of lymphoid cells (particularly those characterized by naïve/antigen-inexperienced immune cell subsets), a low red cell distribution width, and maintained adiposity late in life. This implies that while some consequences of DR, such as the reduction of myeloid skew with age, contribute to its effect on longevity, the ability to withstand certain other impacts of DR, such as those on weight and adiposity, as well as on erythrocyte properties, is beneficial for long-term survival. Importantly, several of these parameters could only be derived from longitudinal monitoring, as performed in this study, highlighting the importance of continuous phenotyping in identifying regulators of DR effects on health and longevity.

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