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Review article

The impact of short-lived controls on the interpretation of lifespan experiments and progress in geroscience – Through the lens of the "900-day rule"

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ABSTRACT

Although lifespan extension remains the gold standard for assessing interventions proposed to impact the biology of aging, there are important limitations to this approach. Our reanalysis of lifespan studies from multiple sources suggests that short lifespans in the control group exaggerate the relative efficacy of putative longevity interventions. Results may be exaggerated due to statistical effects (e.g. regression to the mean) or other factors. Moreover, due to the high cost and long timeframes of mouse studies, it is rare that a particular longevity intervention will be independently replicated by multiple groups. To facilitate identification of successful interventions, we propose an alternative approach particularly suitable for well-characterized inbred and HET3 mice. In our opinion, the level of confidence we can have in an intervention is proportional to the degree of lifespan extension above the strain- and species-specific upper limit of lifespan, which we can estimate from comparison to historical controls. In the absence of independent replication, a putative mouse longevity intervention should only be considered with high confidence when control median lifespans are close to 900 days or if the final lifespan of the treated group is considerably above 900 days. Using this "900-day rule" we identified several candidate interventions from the literature that merit follow-up studies.

1. Introduction

It has been argued that short-lived and metabolically unhealthy control animals can complicate the interpretation of mouse studies. In addition, mouse lifespan studies are often small, limited to one sex and fail to report potential confounding factors. Multiple authors have pointed out these problems and recommended steps to alleviate them (Spindler, 2012; Ladiges et al., 2009; Martin et al., 2010; Bischoff and Volynets, 2016).

Incorporating many of these suggestions for optimal mouse husbandry and avoiding pitfalls of other lifespan studies, the rigorous National Institute of Aging Interventions Testing Program (ITP) has become a gold-standard for mouse longevity studies (Nadon et al., 2017). In the ITP, studies are performed on both sexes, with large sample

Abbreviations: ITP, Interventions Testing Program; CR, caloric restriction; ILSXISS, recombinant inbred cross of ILS Inbred Long Sleep, ILS and ISS Inbred Short Sleep, ISS mice; FGF-21, fibroblast growth factor 21; GH, growth hormone.

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sizes and across three different centers to address idiosyncratic issues of mouse husbandry. Furthermore, the UM-HET3 mice used by the ITP are relatively long-lived compared to most inbred strains and genetically heterogenous, thereby reducing the likelihood that mice die of strain-specific pathologies, a factor that may confound lifespan data.

A majority of compounds tested by the ITP have not been previously published to extend lifespan in mice, thus we lack a "ground truth" for their expected effect size. Notably, however, the ITP has failed to replicate published lifespan extension for several compounds such as metformin (Strong et al., 2016), resveratrol (Strong et al., 2013) and nicotinamide riboside (Harrison et al., 2021), raising concerns about the robustness of published mouse longevity data.

Although differences in genetic background, age of treatment onset, husbandry, and dosing between the original study and the ITP cohorts may explain replication failures, another potential factor is methodological rigor. For example, many of the ITP-tested compounds that were supported by positive published data had already produced inconsistent results in earlier studies, e.g. aspirin (Hochschild, 1973), or only minimal lifespan extension (<5 %), e.g. nicotinamide variants (Zhang et al., 2016) and metformin (Martin-Montalvo et al., 2013). In other cases, compounds were predominantly tested in short-lived and/or unhealthy controls, e.g. resveratrol (Baur et al., 2006) and curcumin (Kitani et al., 2004). Avoiding the above-mentioned experimental shortcomings already at the study conception stage could reduce the amount of time and money spent on failed replication efforts and follow-up studies, thereby improving replicability of mouse research and accelerating progress towards truly geroprotective compounds.

In this manuscript, we reanalyze data from caloric restriction (CR) studies performed in multiple species, the ITP and other large mouse lifespan studies with a particular focus on control lifespan as one potential explanation for inflated effect sizes and lack of replicability. As a solution, we emphasize the importance of long-lived controls in mouse studies which should reach a median lifespan of around 900 ±50 days, or the comparison to appropriate historical controls, and we term this the "900-day rule". Finally, applying this new rule, we compare reported interventions to uncover the most promising candidates for follow-up studies.

2. Results

2.1. Short-lived strains within a species respond more favorably to lifespan-extending interventions

In a meta-analysis of metformin studies (Parish and Swindell, 2022), the lifespan benefit of the drug was largest in studies with the shortest-lived controls (see "The metformin case-study" in Supplementary results; Figs. S1-2). This important example led us to a more thorough investigation since the inverse relationship between control lifespan and the effects of metformin could be confounded by the differences in mouse strain, drug dose or husbandry conditions between

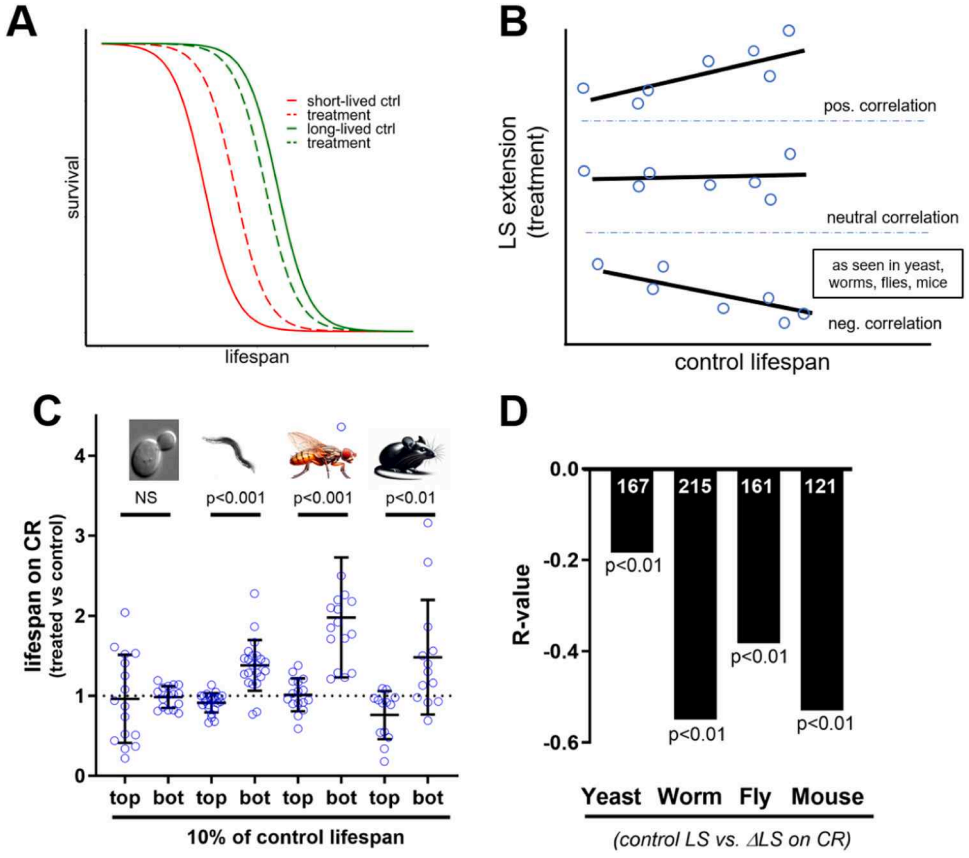

Fig. 1. Longer-lived strains within species respond less favorably to caloric restriction (CR). (A) Short-lived strains or species may benefit more from longevity treatments whereas long-lived strains or species benefit LS or even show lifespan shortening (schematic). (B) The three possible correlation patterns between control lifespan (LS) and the effect of treatments on LS extension: positive relation (top panel), neutral relation (mid) and negative relation (bottom, consistent with observed data). (C) LS extension under caloric restriction (CR) for the top 10 % longest-lived strains ("top") and the bottom 10 % shortest-lived strains ("bot") in each species (ratio of intervention to control). The longest-lived worm, fly and mouse strains show no LS extension under CR, whereas the shortest-lived strains do. This pattern is not evident in yeast. P-values based on Welch's T-test. (D) The correlation between control LS and LS extension under CR for different species shows a negative trend, where more negative values mean that long-lived strains within this species respond less favorably to CR. Sample sizes are indicated in a white font (number of cohorts). Data for yeast is from Schleit et al. (2013), for worms from worms (Snoek et al. 2019), for flies from Jin et al. (2020) and Wilson et al. (2020), and for mice from (Liao et al. 2010, Rikke et al. 2010, Unnikrishnan et al. 2021).

studies. Therefore, to mitigate this problem we searched the literature for studies that maintained consistent husbandry conditions and subjected cohorts with varying genetic backgrounds to a fixed drug or longevity treatment.

Such study designs are rare and none have been undertaken with lifespan extending drugs. Therefore, instead we re-analyzed the raw data from four large studies that imposed CR in yeast (Schleit et al., 2013), worm (Snoek et al., 2019), fly (Jin et al., 2020) and mouse strains (Liao et al., 2010, Rikke et al., 2010, Unnikrishnan et al., 2021) with differing lifespan. In all these studies differences in strain lifespan are primarily due to genetic determinants because the cohorts were kept under identical conditions in the same lab and subjected to the same degree of CR.

We hypothesized that longer-lived strains or cohorts would respond less well to lifespan-extending treatments (Fig. 1**A**) giving rise to a negative relationship between control lifespan and treatment effect (Fig. 1**B**) due to true biological effects or statistical effects (e.g. regression to the mean).

To explore the effects of strain lifespan, we plot the relative change in median lifespan with CR for the top 10 % longest-lived strains and the bottom 10 % shortest-lived strains. Indeed, in support of our hypothesis, CR was unable to extend the lifespan of the longest-lived strains across all four species examined (Fig. 1**C**) and we always observed a pronounced negative relationship between the lifespan of strains and lifespan extension with CR (Fig. 1**D**).

We show that this finding can be generalized and holds true for various datasets that will be discussed later on (Figs. 1–3, Table S1-**3**). We suggest that many longevity promoting interventions merely move the median lifespan closer to the strain-specific optimum and do not extend it further ("longevity-normalizing" effect, Fig. 1**A**). As we will argue, to consistently identify truly "longevity-extending" interventions, studies must be performed in the longest-lived strains possible under optimal conditions.

2.2. *Short-lived ILSXISS mouse strains respond better to caloric restriction*

In order to identify "longevity-extending" interventions, we need to understand why short-lived strains or cohorts show differential responses to interventions. For this we analyzed the mouse dataset from (Fig. 1) in more detail. In this experiment CR was imposed in 39 genetically different, inbred ILSXISS strains of two genders.

When we plot control lifespan against lifespan extension by CR a negative relationship is seen in both female (Fig. 2**A**) and male mice (Fig. 2**B**), which supports the idea that long-lived strains show less lifespan extension under CR.

An important unanswered question in the CR literature, however, is whether long-lived strains respond less favorably to CR due to statistical effects inherent to this comparison or biological effects. It has been argued that an apparent negative relationship between control lifespan and intervention lifespan may arise purely through regression to the mean effects (Garratt et al., 2017). Any time the control group in an experiment has a shorter lifespan than the true population mean, the treated group will likely be closer to the mean (thus longer-lived). For long-lived control groups the treated group will be likely shorter-lived. We tested this by resampling from the control population to generate both control and treated groups. Any negative relationship between control lifespan and lifespan extension based on these resampled values should be purely spurious and we would expect the regression line for the observed lifespans to be parallel to the resampled line. This resampling approach naturally also accounts for other statistical artifacts, e.g. a negative slope inherent to plotting X against (X-Y).

In the ILSXISS study, the observed regression line had a significantly more negative slope than the resampled line in female mice (Fig. 2**C**), with a trend in males (p=0.06, Fig. 2**D**). This suggests that long-lived

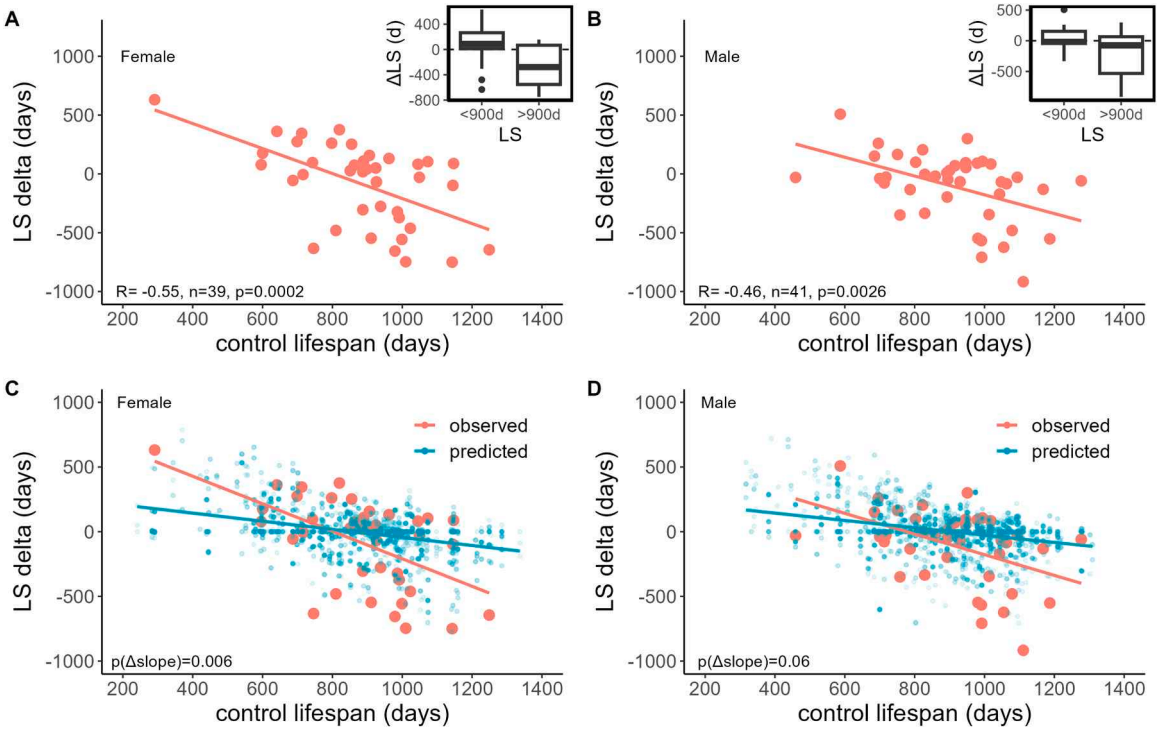

Fig. 2. Long-lived female and male ILSXISS strains respond less favorably to caloric restriction. Lifespan (LS) of female (A) and male (B) control mice from different strains on the X-axis (pink dots) plotted against the absolute change in lifespan with caloric restriction (CR) on the Y-axis when imposed in the respective strain (Δlifespan CR). Mouse cohorts with a lifespan of <900 days benefit from CR whereas mice with a lifespan of >900 days do not (see the insert). To test whether regression to the mean can explain exaggerated benefits in short-lived mice we resampled quasi-lifespan experiments from the control population. The resampled synthetic data (blue) is shown for female (C) and male (D) mice with the observed datapoints overlaid (pink). Figures based on data deposited in the Mouse Phenome Database which is comprised of a subset from Rikke et al. (2010) and Liao et al. (2010).

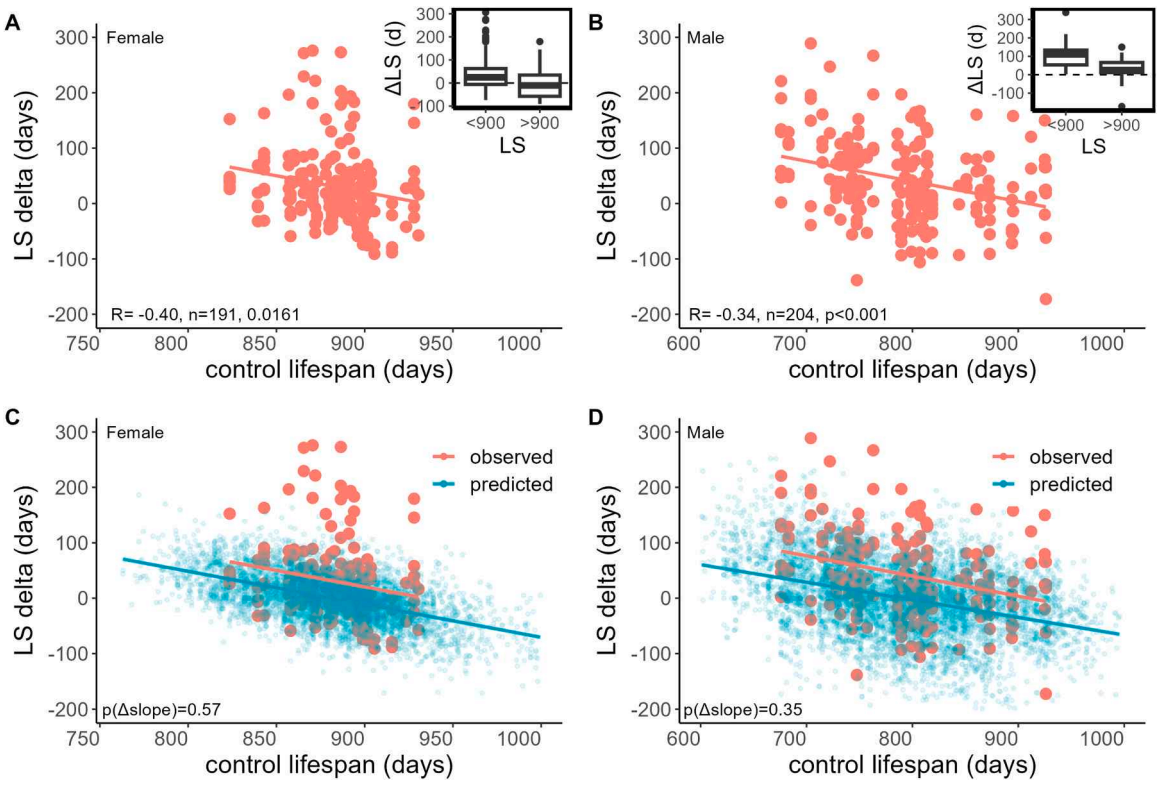

Fig. 3. Longer-lived cohorts of UM-HET3 mice show less pronounced lifespan extension in the interventions testing program (ITP). Lifespan (LS) of female (A) and male (B) control mice in the ITP study (pink dots) plotted against the change in lifespan with drug treatments on the Y-axis (Δlifespan in days). Each point corresponds to a unique combination of drug x gender x testing site. Mouse cohorts with a lifespan of <900 days benefit more from drug treatments than do mice with a lifespan of >900 days (see the insert). To test whether regression to the mean can explain exaggerated benefits in short-lived mice we resampled quasi-lifespan experiments from the control population. The resampled synthetic data (blue) is shown for female (C) and male (D) mice with the observed datapoints overlaid (pink). P-value in (A) and (B) based on a linear mixed effects model considering cohort year, test center and control lifespan.

strains responded less favorably to CR than expected based on regression to the mean effects, although other reasons could also attenuate the effects of CR in ILSXISS mice (Simons and Dobson, 2023).

Although in this paper we focus on mice, similar findings are seen in two invertebrate models, suggesting evolutionary conservation of this effect. In studies of calorie restricted flies (Fig. S3A**, B**) and worms (Fig. S3D**, E**), even though lifespan was extended, long-lived strains responded less favorably to CR than expected based on regression to the mean effects (Fig. S3C**, F**).

Finally, we also show that as expected regression to the mean effects are highly dependent on sample size and particularly pronounced in small studies (Fig. S4). This is important given the sample sizes in the ILSXISS study are around 5 mice per group. However, our analysis is able to account for this (Fig. 2**C, D;** Fig. S5**-7**) and other issues as discussed under "Data quality in the ILSXISS studies" and "Validation of resampling using synthetic data" in the supplementary.

2.3. Interventions tested in studies against short-lived controls are more likely to show lifespan benefits

To assess whether the above findings can be replicated outside of the context of CR we reanalyzed several, large meta-analytic datasets (Barardo et al., 2017; Garratt et al., 2017; Pedro de Magalhães et al., 2018; Swindell, 2012).

First, we reanalyzed lifespan data from a meta-analysis of CR studies by Swindell (2012), after excluding the ILSXISS data, to test whether studies with longer-lived controls showed smaller lifespan extension after CR. No significant correlation was seen in mice between control lifespan and lifespan extension (Fig. S8A), although there was a moderate significant negative correlation in rats (Fig. S8B). If this result were due to heterogenous study conditions across the many diverse datasets pooled by Swindell (2012) we would expect to see a significant correlation in a sufficiently large sub-study of this meta-analysis, and this was indeed the case (n=15; Fig. S9A**, B**).

Next, we reanalyzed mouse longevity interventions from the DrugAge database (Barardo et al., 2017). Although our data extraction strategy was different from the original publication, since we focused on absolute rather than relative lifespans, our results are nonetheless in good agreement with the reported data in DrugAge (Fig. S10). No significant negative correlation was observed between control lifespans and drug-induced lifespan extension (R= −0.09, n=147). However, as was the case for CR studies, the single largest dataset in DrugAge (n=22) revealed a strong negative correlation between control lifespan and treatment effect. Schroeder and Mitchener (1975) tested the impact of different metals on the longevity of Swiss mice across multiple experiments with varying control lifespans (Fig. S11A**, B**).

Two meta-analyses of genetic interventions also found evidence for an impact of control lifespan on the lifespan extension in various mutant mouse models. In our re-analysis of Garratt et al. (2017) we found that both IGF1/IRS mutants (Fig. S12A**, B**) and GH dwarfs (Fig. S12C**, D**) were less likely to show lifespan extension when the controls were long-lived. Similarly, the meta-analysis by Pedro de Magalhães et al. (2018) found that control lifespans significantly influenced the lifespan extending effects of genetic interventions (R= −0.55, n=33).

All in all, the strong negative relationship between control lifespan and treatment effect seen in large, controlled studies with multiple cohorts (Fig. 2**;** Fig. S9, S11) contrasts with a weaker relationship in meta-analyses. This suggests that between-study variability could mask the effects of control lifespan on experimental lifespan extension (Table S4).

2.4. In the ITP beneficial effects on lifespan are more likely in short-lived cohorts and less likely in long-lived cohorts

Since large heterogeneity in husbandry and interventions between experiments could mask the effect of control lifespan in meta-analyses, we searched for studies that tested different interventions under more comparable conditions. The only large study with consistent husbandry conditions that we identified was the ITP (Nadon et al., 2017).

The ITP dataset we analyze includes raw data for 68 drugs tested across 3 study sites. Since drugs are usually tested in both sexes, this yields 395 conditions in total, where a condition is defined as a particular combination of drug x gender x testing site.

Using the aggregated summary data, we again found a negative correlation between control lifespan and treatment effect in the ITP ($R=-0.21$, $p<0.05$, $n=132$; Fig. S13). This correlation becomes even more apparent when treating the results from each testing site as independent experiments ($R=-0.27$, $p<0.0001$, $n=395$; Fig. 3), which may be considered the more appropriate analysis due to relevant inter-site differences in lifespans.

Cohorts of longer-lived UM-HET3 mice showed less lifespan extension in response to various treatments whether lifespan extension was defined in absolute (Fig. S14A) or relative terms (Fig. S14B). Importantly, a significant negative correlation between control lifespan and treatment effect was seen in both females (Fig. 3**A**) and males (Fig. 3**B**), and across multiple testing sites, specifically the University of Texas Health Science Center for both sexes and the Jackson Laboratory for males (Table S5). However, our resampling analysis indicated that this effect was due to regression to the mean since the observed and the resampled regression line were almost parallel (Fig. 3**C, D**).

Arguably, the results in Fig. 3 may be an imprecise estimate of the true relationship because each treatment contributes only a few datapoints to the correlation. However, the ITP also provides a unique opportunity to address this issue. Since each drug was tested across three study sites with different control lifespan, we can perform a Spearman correlation analysis for every drug. We find a negative correlation between control lifespan and treatment effect in the pooled analysis for 51 out of 68 drugs tested (75 %, $p<0.0001$; p-value by permutation). Split by gender, we find a negative correlation between control lifespan and treatment effect for 52 out of 68 drugs in males (76 %, $p<0.0001$) and 40 out of 68 drugs in females (59 %, $p=0.053$).

2.5. The "900-day rule" defines a lifespan gold standard for mouse lifespan studies

In our earlier analysis we showed that studies in shorter-lived cohorts will produce exaggerated lifespan benefits, while the opposite is also true with long-lived cohorts producing underestimates. Although some of these findings can be explained by regression to the mean, conceivably long-lived animals may be biologically less likely to benefit from longevity interventions.

We propose that studies with long-lived mice are particularly valuable to aging researchers for two reasons. First, these mice might be a more faithful model for human physiology and longevity given the exceptionally long lifespans of humans compared to other animals (Buffenstein, 2009). Second, human lifespans in many countries are approaching a biological ceiling (survival curve rectangularization). We assume there is also a biological ceiling for the median lifespan of each strain (Fig. S15) which can be achieved with optimal mouse husbandry. Such mice maintained under favorable husbandry conditions would be a better model for human aging. Conversely, studies using shorter-lived mice would represent a systematic overestimate for lifespan extension (Fig. S16).

To define this lifespan ceiling, we first assembled normative median lifespans for the commonly used mouse strains in aging studies, C57BL/6 and UM-HET3.

Median lifespans for C57BL/6 mice were 801 days ($n=131$), with no overt sex (Fig. S17) or substrain differences (Fig. S18). The sample size-weighted median was lower at 740 days with a multimodal distribution displaying a large cluster of studies with lifespans around 800 days (Fig. S19). Median lifespans for UM-HET3 mice from the ITP were 883 days for females and 800 days for males (Fig. 4). Further details of the included studies are discussed in "Lifespans of commonly used mouse strains" in the Supplementary results.

Therefore, we propose the "900-day rule" for mouse lifespan experiments, which is easy to remember and sufficiently accurate to be useful to editors, reviewers, scientists and lay readers alike. Most healthy inbred or hybrid strains should have a median lifespan of close to 900 days (± 50 days). The appearance of a left-skewed, potentially bimodal, distribution of reported lifespans for both strains suggests that median lifespans pooled across all studies may be artificially decreased by this subset, which we presume to be mice kept under suboptimal husbandry conditions (Fig. S19, S20). Thus, it seems likely that at least C57BL/6 and UM-HET3 strains are well capable of lifespans around 900 days (Table S6-**7**). Furthermore, some labs with a reputation for good mouse husbandry consistently report lifespans above the historical average and closer in line with the 900-day rule (Fig. S21).

Based on the 900-day rule we define treatments that extend the lifespan of short-lived cohorts as "longevity-normalizing", whereas those that work in long-lived cohorts are "longevity-extending". Importantly, without an appropriately long-lived control, it is impossible to attribute lifespan extension to effects on biological aging since the tested intervention could be simply offsetting idiosyncratic health issues. However, in the absence of a long-lived within-study control these values (Fig. 4**;** Table S7) can serve as a historical control. Interventions that result in median cohort lifespans well above 900 days in mice should be taken seriously independent of the within study controls (Fig. 4**C**). Conversely, even large lifespan increases against a short-lived background may be artefactual. As a corollary, the use of percentage increase in lifespan should be discouraged because it fails to capture, and indeed can often conceal, essential information about control lifespan.

To test whether the 900-day rule can successfully predict robust interventions, we asked whether interventions identified in DrugAge that passed the 900-day rule would be more likely to extend lifespan in the ITP than interventions that failed the rule. Although the available data for compounds found in both datasets is limited, NDGA and rapamycin were the only intervention that showed lifespan extension in long-lived DrugAge cohorts and these two were also successful interventions in the ITP (Table S8).

2.6. Re-ranking of interventions using meta-analysis, absolute lifespans and the 900-day rule

Using the 900-day rule we identified 19 interventional groups in the ITP that met our criteria in at least one cohort (Table S9). As expected, these included acarbose, rapamycin and 17-α-estradiol but also other compounds like glycine or captopril. In total, 10 unique compounds met the cut-offs. However, when data from all three cohorts was pooled, no interventions met our criteria consistently except rapamycin, and rapamycin combinations, in female mice (Table S10). This suggests that few compounds consistently increase lifespan across multiple cohorts of long-lived UM-HET3 mice. In a secondary analysis using a complementary approach, we also identified ACE inhibitors and rapamycin combinations as promising for further studies (Table S11**-12**, Fig. S22**-25**; see "Re-ranking of interventions in the ITP study" in Supplementary results).

Next, in our reanalysis of DrugAge we found 14 datasets comprising 12 different compounds that met the 900-day rule (Fig. 5**A**, Table S13). Interestingly, this set included three drugs that reduce heart rate, i.e. the two beta-blockers, metoprolol and nebivolol, and ivabradine.

Having shown that the 900-day rule can inform the interpretation of mouse lifespan studies using pharmacologic interventions, we extended

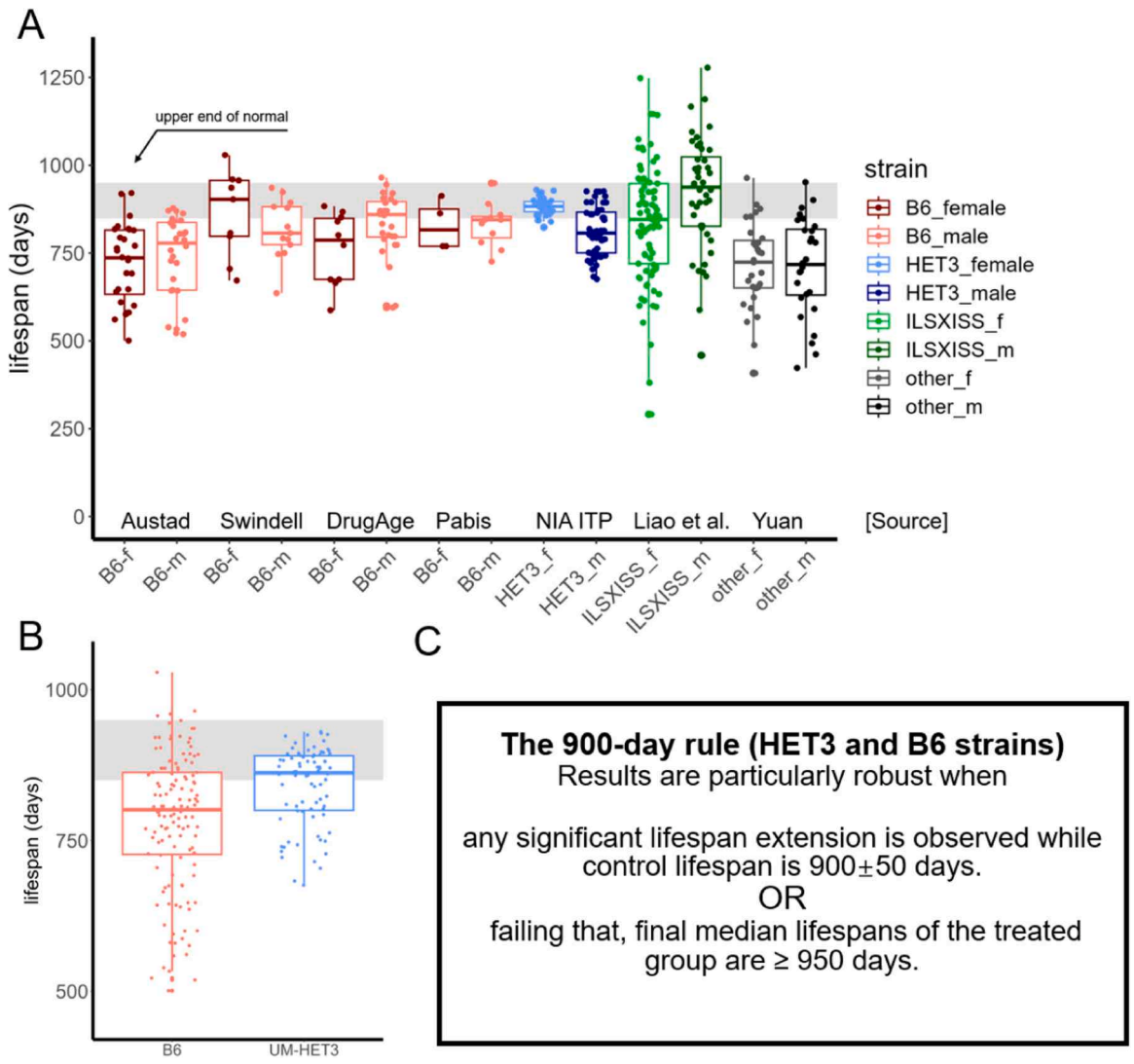

Fig. 4. Healthy inbred and hybrid mouse strains live close to 900 days. A) Under normal conditions, healthy control mice live close to 900 days. From left to right, lifespans for female (f) and male (m) C57BL/6 (B6) mouse cohorts from Austad (2011), Swindell (2012), DrugAge (Barardo et al. 2017) and from our own analysis. This is followed by lifespans for UM-HET3 (HET3) mouse cohorts tested by the ITP. Finally, for comparison we show data from the ILSXISS inbred panel (Liao et al. 2010, Rikke et al. 2010, Unnikrishnan et al. 2021) and from Yuan et al. (2012). Lifespans in the original datasets are either mean or median, depending on data-availability. The interval between 850 and 950 days is indicated with a shaded area. Boxplots show median ± 95 % CI. B) Pooling all the B6 and HET3 data from (A) it becomes clearer that 900 days represents the upper end of normal for these strains and few published cohorts using wildtype mice show median lifespans considerably above that value. The interval between 850 and 950 days is indicated with a shaded area. C) Based on these findings, the 900-day rule can be phrased in two ways. 1. It would be unusual to observe median lifespans considerably above 900 days in a mouse experiment, hence lifespan extension above 950 days - to allow for a buffer - compared to historical controls indicates that the given treatment shows robust lifespan extension, 2. If the controls are long-lived, i.e. 900±50 days, then any significant lifespan extension observed is more likely to be robust and not due to amelioration of premature death.

our analysis to genetic studies reported in GenAge (Tacutu et al., 2018). 24 out of 136 longevity genes also extended lifespan in studies with long-lived control mice (Table S14). These fell into four major categories: mTOR signalling, growth signalling, GH/IGF-1/Insulin-axis and diverse other pathways (e.g. telomerase, DNA repair or inflammation).

To narrow down the top genes we ranked the 24 candidates by the absolute lifespan of the intervention group and excluded interventions that led to lifespans of <950 days (Fig. 5**B**). The longest-lived animals were knock-outs in the growth hormone pathway (Ghrhr, Prop1, Pou1f1). Several other genes were also associated with exceptionally long lifespans in at least one studied cohort. This includes the overexpression of genes involved in DNA maintenance (Sirt6), telomere extension (Tert) and nutrient sensing (Fgf21) as well as the knock-out of Akt2, involved in growth signalling and glucose homeostasis. Out of these genetic interventions, FGF-21 overexpression appears to be the most robust since it extends lifespan in both sexes. The other interventions had sex dimorphic effects (Sirt6: male only) or were only tested in one sex (Tert, Akt2).

Based on our initial analysis of GenAge, we performed a literature search for confirmatory studies related to the top genes and pathways identified above. We searched for interventional studies using drugs or viral vectors specifically, because these approaches were not included in GenAge. Only two pathways were supported by such additional evidence, mTOR and telomerase. Somewhat surprisingly, studies targeting the GH/IGF-1 pathway pharmacologically have been less successful, with only one study showing lifespan extension in long-lived mice that was furthermore limited to females (Duran-Ortiz et al., 2021; Mao et al., 2018).

We identified studies of the mTOR inhibitor rapamycin based on a recent review (Selvarani et al., 2021) and for telomerase activation we searched the literature for published studies. Although the lifespans of most controls were short for both these interventions, comparison with

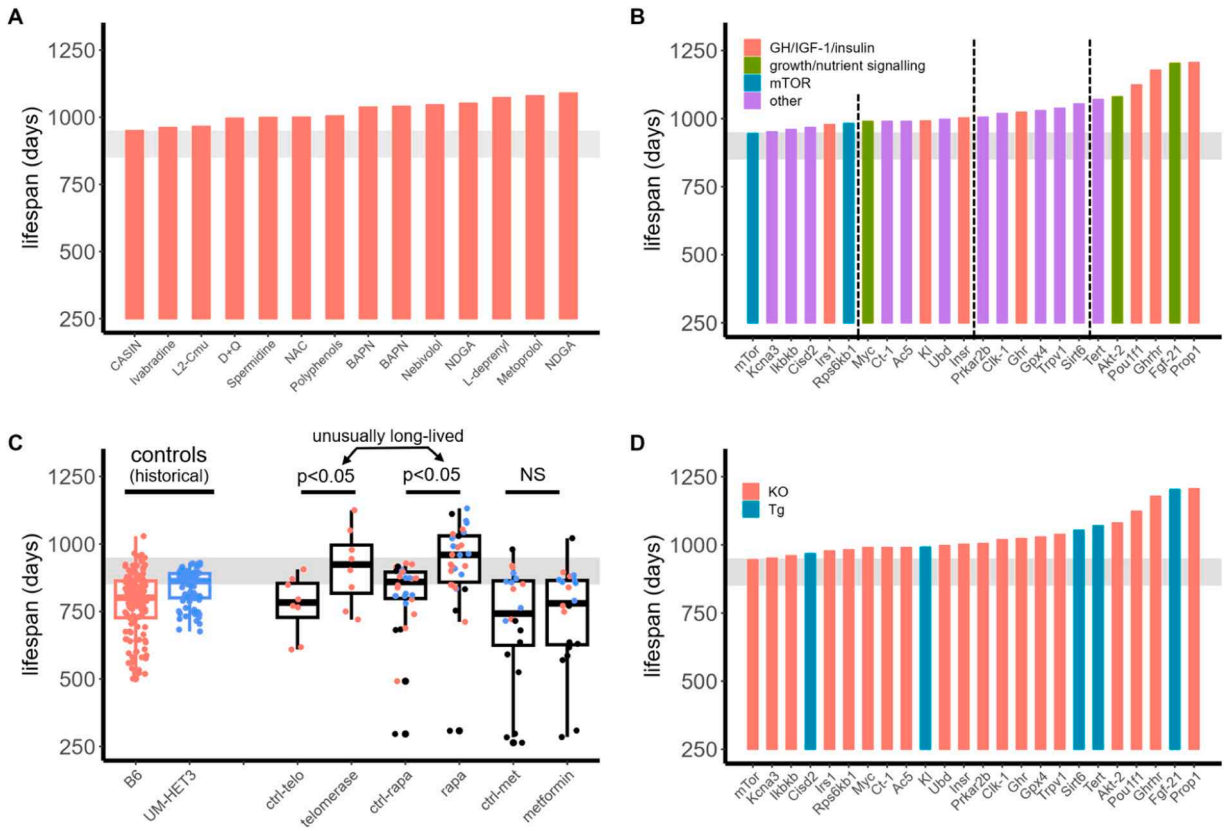

Fig. 5. Certain drugs and genetic interventions extend mouse lifespan compared to historical controls. For this figure any intervention producing a final median lifespan of ≥ 950 days was considered to pass the 900-day rule. A) 14 different cohorts with 12 unique compounds from DrugAge (Barardo et al. 2017) pass the 900-day rule. Abbreviations: D+Q = dasatinib + quercetin, NDGA = nordihydroguaiaretic acid, NAC = N-acetyl-L-cysteine, BAPN = beta-aminopropionitrile fumarate, CASIN = Cdc42 inhibitor, L2-Cmu = IGF-1R mAb. B) 23 different genetic interventions reported in GenAge (Tacutu et al. 2018) pass the 900-day rule. Although the mTOR hypomorphic strain failed the 900-day rule by a small margin (treated LS of 945 days) it was included as the 24th intervention due to prior plausibility. C) Mice treated with rapamycin (rapa) or subjected to telomerase activation live longer than most historical controls. From left to right, lifespans for C57BL/6 (B6) and UM-HET3 mouse cohorts of both sexes (n=131 and 78, respectively, based on the data in Fig. 4) used as historical controls. Followed by data from telomerase induced cohorts (n=8 per group), rapamycin treated cohorts (n=30, data from Selvarani et al. 2021), and metformin treated cohorts (n=20, Parish and Swindell, 2022) with the respective control (ctrl) and treated arm. The telomerase data includes studies using viral vectors and transgenic mice. The interval between 850 and 950 days is indicated with a shaded area. Boxplots show median ± 95 % CI. P-values based on paired T-test. D) The majority of interventions that robustly extend lifespan in GenAge are gene knock-outs (KO), whereas only few transgenic (Tg) mouse models were reported to extend lifespan.

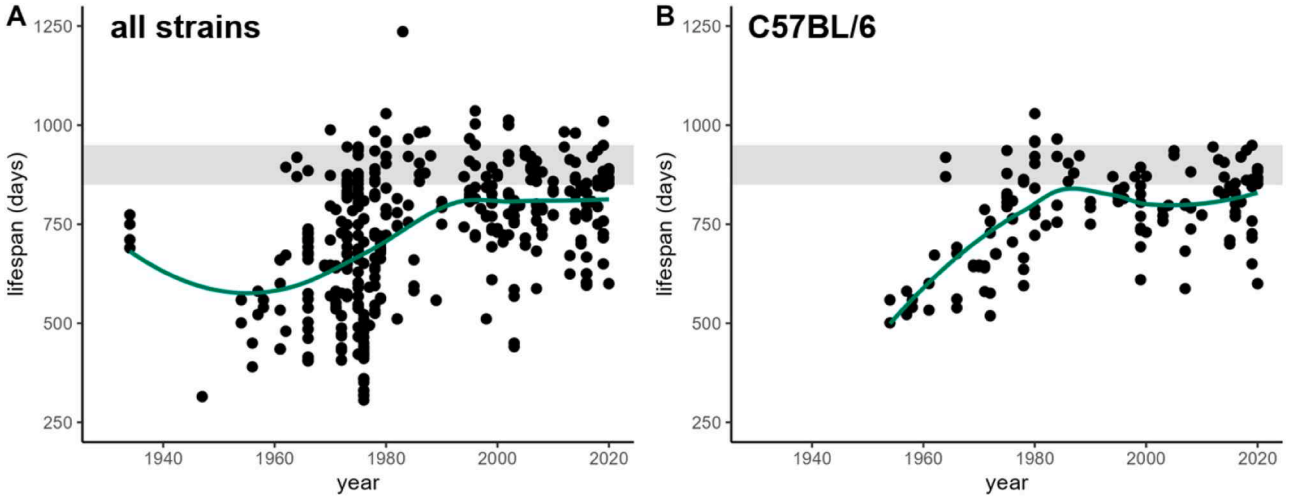

Fig. 6. Experimental mouse lifespans improved over time. Reported lifespans in mouse studies improved during the second half of the 20th century. The same trend is seen in an analysis including all mouse strains (A; n=428) and in an analysis limited to studies using C57BL/6 mice (B; n=129). Each datapoint represents the control lifespan in a study or a cohort within a study. Green trend line generated by locally estimated scatterplot smoothing (LOESS) method. The interval between 850 and 950 days is indicated with a shaded area. Data from Austad (2011), Swindell (2012), Barardo et al. (2017) and this manuscript.

historical controls enabled us to assess their longevity extending properties (Fig. 5**C**). Since a recent meta-analysis reported that metformin fails to extend the lifespan of mice, we used this dataset as a negative control (Parish and Swindell, 2022). We applied the 900-day rule to compare metformin, rapamycin and telomerase activation. 3 out of 9 telomerase studies passed our criteria (33 %), 16 out of 30 rapamycin studies (53 %) also passed whereas only 1 out of 20 metformin (5 %) studies did (Table S15). Other canonical interventions like CR and dwarfism are also correctly identified as robust by the 900-day rule, highlighting the usefulness of this heuristic (see "Pituitary dwarfism and the 900-day rule" in Supplementary results and Fig. S26, S27). Finally, comparative analysis of absolute lifespans reveals that drugs do not fully capture the lifespan benefits conveyed by genetic mutations (Fig. 5**A, C** vs Fig. 5**B, D**).

2.7. Control lifespans over the years – a need for further improvement

Looking at the historical development of mouse lifespan studies, we find that the late 70 s and early 80 s saw a marked improvement in lifespan (Fig. 6**A**). This is more likely due to improved husbandry rather than a shift towards the use of longer-lived strains since the same trend was observed when we limited our analysis to the popular C57BL/6 strain only (Fig. 6**B**). After this period of marked improvement, lifespan plateaued around 800 days. This increase in lifespan is consistent with a convergence towards a strain-specific optimum. However, we suggest that further improvements in husbandry and mouse lifespan would enable more robust identification of lifespan-extending compounds and interventions. See "Recommendations for lifespan studies" in Supplementary results for further discussion.

3. Discussion

Although it is often recommended that mouse studies should utilize healthy and long-lived animals, the impact of variation in the lifespan of control animals on experimental outcomes has not been rigorously explored so far. In this work we show that short-lived controls are prevalent in lifespan studies leading to exaggerated effect sizes of interventions which could affect the replicability of these studies.

To evaluate and improve confidence in longevity-extending interventions we propose a 900-day rule for mouse longevity studies. True slowing of aging can only be confidently measured against the backdrop of long-lived controls with median lifespans around 900 days (± 50 days), which is the upper end of a healthy normal lifespan. If a study fails the 900-day rule, i.e. an intervention extends the lifespan of a short-lived cohort, we cannot make any claims about aging with confidence except that the tested intervention allowed the animals to reach a lifespan closer to the natural lifespan of a healthy cohort (hence the term longevity-normalizing). In such a case the results must be interpreted with caution, the study repeated, or the data compared to appropriate historical controls that meet the 900-day rule.

We suggest three explanations for a longevity-normalizing effect. First, the intervention has no biological effect and the results are due to regression to the mean or other biases. Second, the intervention does not affect aging but instead improves the health of animals maintained under sub-optimal conditions, with a genetic predisposition toward short lifespan, or experiencing a diseased state. Third, the intervention did slow aging, but the effects were overwhelmed by unmeasured factors that lowered the lifespan of both the control and treatment group.

We recognize that no experiment can guarantee, no matter how good the conditions, that the control lifespan will reach close to 900 days. The ITP, for instance, does not always achieve this goal in males. We provide additional "Recommendations for lifespan studies" in the Supplementary discussion (e.g. sufficient sample size per group, prevention of early life mortality from infections, etc). Furthermore, it is likely that many people are aging in non-optimal conditions such that so called longevity-normalizing interventions may have real benefits.

Metformin may be an example of a longevity-normalizing drug, because it works in short-lived mice but not in long-lived mice as shown by application of the 900-day rule. Nevertheless, the drug is associated with numerous health benefits in humans (Kulkarni et al., 2020) and we find evidence of synergistic lifespan benefits between rapamycin and metformin in mice.

Our analytical approach produces several other novel insights. We find that many compounds reported to extend mouse lifespan fail to extend lifespan in the ITP upon attempted replication, and this is more often true for compounds that did not pass the 900-day rule. By applying the 900-day rule and comparison with historical controls we were able to identify several promising interventions for further study, e.g. ACE inhibitors, telomerase activation, FGF-21 or rapamycin combinations. Therefore, the use of historical controls is highly recommended especially when the within-study control fails to reach the expected lifespan.

More generally, our approach provides an opportunity to address what is widely appreciated as a "reproducibility problem" in the field. There have been several notable examples where high-profile publications have initially claimed lifespan extension resulting from an intervention only to have subsequent studies fail to reproduce those claims (Harrison et al., 2021; Strong et al., 2016). This is particularly problematic in the context of mouse longevity studies, because attempts at replication take several years and require large amounts of resources. Additionally, the intense media and public interest in "anti-aging" regimens means that such reports are often widely disseminated to the general public, often accompanied by direct marketing of products to consumers. Hence, there is an urgent need for clear guidelines to confidently identify lifespan extending compounds.

4. Summary and limitations

Although the robustness of a mouse lifespan study should be proportional to the lifespan of the controls across the whole range of values, we nevertheless see certain advantages in the 900-day rule for practical purposes (Table S17**,** see also "Benefits of the 900-day rule" in Supplementary results).

Specifically, the advantages of a simple, binary rule are ease of use and ease of adoption. These often outweigh the disadvantages like lowered sensitivity and false-negatives. One example where this choice was made by convention would be the famous p-value cut-off α=0.05. Such rules should not discourage subject experts from a more thorough exploration of the raw data (e.g. consideration of maximum lifespans), while opening the field to a wider number of scientists and audiences. Importantly, even studies failing the 900-day rule make an important contribution to the literature and should be published.

Finally, there remain uncertainties in our estimation of optimal lifespan or regression to the mean from literature data due to the heterogeneity of reported studies.

5. Methods

5.1. Data collection and pre-processing

We collected median lifespans from the literature when possible, or mean lifespans when only these were provided by the authors. If neither was provided, we determined median LS from survival curves. Measures of maximum lifespan or mortality doubling time were not considered due to higher statistical uncertainty associated with these. When up-to-date data was not available, as was the case for recent studies of CR and telomerase activation, we performed a systematic literature search to identify studies and extend existing datasets.

All datasets used in this manuscript are summarized in Table S1-**2**. Correlation analysis was performed on the level of individual studies or cohorts, not individual animals. We removed datapoints deemed to be of low quality (e.g. no adequate information on strain and sex given). We further cleaned up some datasets as needed, e.g. removing duplicates, or

entries with missing references. Furthermore, we excluded the ITP and rapamycin data from DrugAge, which we analyze in more detail elsewhere. For GenAge, whenever multiple cohorts were reported in a paper, we chose the cohort with the highest lifespan for our analysis.

5.2. Literature search

Articles were retrieved by searching pubmed or google scholar. For our systematic review of CR studies we used the following terms: ("2011"[Date - Publication]: "3000"[Date - Publication]) (mice OR mouse OR murine OR mus) ("caloric restriction" OR "calorie restriction" OR "dietary restriction") and for our review of telomerase studies: telomerase AND (mice OR mouse OR mus OR murine) (telomerase OR TERT OR mTERT OR hTERT OR telomere OR telomeres) AND lifespan.

5.3. Analysis, linear regression and outlier removal

We performed Pearson correlation in this study, although the results were comparable using Spearman correlation (Table S3). To minimize denominator bias, we plot control lifespan against absolute change in lifespan rather than relative change ($lifespan^{treated}/lifespan^{control}$), although data is comparable for both (Table S1). For the ITP dataset, we calculated a p-value using the lmerTest package in R to construct a linear mixed effects model with a random term accounting for cohort year and test center. Corrected R-values for Fig. 2 are reported in the supplementary as the worst case of leave-one-out analysis. The code used to generate the main figures can be found on github under pabisk/mouse_longevity.

Analysis of sex and drug effects in the Interventions Testing Program

Raw data was obtained from the study authors. For the comparison of sex dimorphic effects only treatments that were tested in both sexes were included and the sex-specific survival advantage was calculated as absolute lifespan extension$^{male\text{-}female}$. To obtain results unbiased by multiple testing of one and the same drug, we randomly chose a lifespan study within each drug class for our analysis.

5.4. Resampling to model regression to the mean

Whenever the control group is longer-lived than the true population mean by chance, the treatment group will be on average closer to the mean and thus shorter-lived. The inverse will apply to short-lived controls giving rise to a negative correlation between control group lifespan and lifespan extension of the treated group (regression to the mean). To compare the observed lifespan data with theoretical data, we performed a bootstrap analysis. For each experiment, we resampled from the respective control population of each subcohort with replacement and group sizes matching the actual experiment. This means resampling takes place on the level of strains for the ILSXISS dataset and on the level of study site for each year of the ITP. The effect of regression to the mean is then estimated by comparing the slope of the resampled regression line with the slope of the observed regression line. To this end, we calculated a z-score for the difference between the slopes and used these to compute a two-tailed p-value. Please see "Validation of resampling using synthetic data" in the Supplementary for further information.

5.5. Defining lifespan gold standards

An idealized "healthy lifespan" of a mouse is defined as the longest median lifespan that a cohort of lean animals can achieve without slowing the rate of aging per se. Although this quantity is not knowable, we can estimate it by studying historical lifespan data. The median lifespan of a healthy cohort asymptotically converges towards a strain-specific lifespan optimum. Indeed, improvements in general health and husbandry lead to rectangularization of the survival curves and convergence towards this optimum in both mouse experiments (Hayflick, 1977) and human populations (Myers and Manton, 1984; Yashin et al., 2012).

Declaration of Competing Interest

None

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.arr.2024.102512.

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