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Applying the AFRAID and FRIGHT Clocks to Novel Preclinical Mouse Models of Polypharmacy

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Abstract

The Frailty Inferred Geriatric Health Timeline (FRIGHT) and Analysis of Frailty and Death (AFRAID) clocks were developed to predict biological age and lifespan, respectively, in mice. Their utility within the context of polypharmacy (≥5 medications), which is very common in older adults, is unknown. In male C57BL/6J(B6) mice administered chronic polypharmacy, monotherapy, and undergoing treatment cessation (deprescribing), we aimed to compare these clocks between treatment groups; investigate whether treatment affected correlation of these clocks with mortality; and explore factors that may explain variation in predictive performance. Treatment (control, polypharmacy, or monotherapy) commenced from age 12 months. At age 21 months, each treatment group was subdivided to continue treatment or have it deprescribed. Frailty index was assessed and informed calculation of the clocks. AFRAID, FRIGHT, frailty index, and mortality age did not differ between continued treatment groups and control. Compared to continued treatment, deprescribing some treatments had inconsistent negative impacts on some clocks and mortality. FRIGHT and frailty index, but not AFRAID, were associated with mortality. The bias and precision of AFRAID as a predictor of mortality varied between treatment groups. Effects of deprescribing some drugs on elements of the clocks, particularly on weight loss, contributed to bias. Overall, in this cohort, FRIGHT and AFRAID measures identified no treatment effects and limited deprescribing effects (unsurprising as very few effects on frailty or mortality), with variable prediction of mortality. These clocks have utility, but context is important. Future work should refine them for intervention studies to reduce bias from specific intervention effects.

Keywords: Biological age, Deprescribing, Lifespan, Mortality, Polypharmacy

It is important in studies of aging interventions to measure informative and clinically relevant outcomes. Biological age is emerging as an increasingly important outcome, which accounts for the heterogeneity in health status of people of the same chronological age and can be considered a measure of a person's overall health as they age (1–3). Biological age is also being increasingly measured in aging studies using preclinical models (4–8). Current translatable biological age measures in mice include the DNA methylation clocks, which predict age based on the methylation status of particular CpG genome sequences (DNA sequence where cytosine nucleotide is followed by a guanine nucleotide) (4), immune-based markers (5),

protein markers (6), and frailty indices (7,8). Frailty assessments are the least invasive and the cheapest of these measures (7).

Recently, the mouse clinical frailty index measures (7) were modeled using machine learning algorithms to provide the first frailty-based clocks to predict age and lifespan in mice (9). The Frailty Inferred Geriatric Health Timeline (FRIGHT) age is able to predict biological age within 1.3 months, and the Analysis of Frailty and Death (AFRAID) clock can predict lifespan within 1.7 months, even in mice of the same chronological age (9). These clocks were built in male C57BL/6J mice aged 21 months and older. They are proposed as tools to noninvasively measure biological age in longitudinal

aging studies (1), but their utility in cohorts of mice from diverse facilities, and with different interventions, has only been minimally explored. To date, these clocks have only been validated with 1 drug intervention, enalapril (an angiotensin converting enzyme inhibitor). The clocks predicted the known increase in healthspan (as assessed by the clinical frailty index score) but not lifespan (10,11). In addition, following dietary intervention of methionine restriction, these clocks also predicted the known increased healthspan and lifespan (12–14). These clocks also show that aerobic exercise increases lifespan in aged female mice and that sedentary male mice exhibit an increase in biological age (15). However, the utility of these clocks within the context of polypharmacy, the concurrent use of 5 or more medications, which is very common in older adults, is unknown.

We recently published a large study determining the effect of polypharmacy with increasing Drug Burden Index (DBI; measure of total exposure to sedatives and anticholinergics) on frailty and physical function in healthy aging mice (16). The DBI specifically measures an individual's total exposure to medications with anticholinergic and sedative effects (17). Both polypharmacy and increasing DBI are associated with poor outcomes, including frailty in older adults (18–22). We found that polypharmacy with increasing DBI in aging mice increased frailty and reduced function and activity, and that this was reversible after ceasing some drug regimens (deprescribing) in old age (16). Deprescribing is the process of withdrawal of medications that are currently considered inappropriate in an individual (23). While the study was not powered to detect differences in mortality, no significant differences were seen in survival between treatment groups on Kaplan–Meier analyses (15).

Here we apply the frailty-based FRIGHT and AFRAID clocks to the data from our polypharmacy cohort. Specifically, we aim to (1) describe the distribution of FRIGHT, AFRAID, and frailty index scores in this cohort; (2) compare these clocks between treatment groups; (3) investigate whether treatment affected the correlation of these clocks with mortality in our cohort; and (4) investigate factors that may explain variation in predictive performance of the clocks between intervention groups by examining prevalence of specific frailty items. We hypothesized, based on our published study (16), that chronic polypharmacy and monotherapies with drugs that had anticholinergic or sedative effects would increase frailty index and FRIGHT ages and decrease AFRAID scores, this may be reversed with deprescribing. We also hypothesized that these treatments may influence the correlation of the frailty measures with mortality.

Methods

Study Plan, Animal Testing, and Mortality

This is a retrospective analysis of data from our previously reported longitudinal study of chronic polypharmacy, monotherapy, and deprescribing in aging mice (16). In brief, in a longitudinal study, middle-aged (12 months) male C57BL/6J (B6) mice were randomly assigned into groups and administered control feed or feed and/or water containing polypharmacy or monotherapy with different DBI scores. The polypharmacy regimens consisted of polypharmacy zero DBI (DBI: 0; 20 mg/kg/day simvastatin, 350 mg/kg/day metoprolol, 10 mg/kg/day omeprazole, 100 mg/kg/day acetaminophen, and 5 mg/kg/day irbesartan), low DBI (DBI: 0.5; 20 mg/kg/day simvastatin, 350 mg/kg/day metoprolol, 10 mg/kg/day omeprazole, 100 mg/kg/day acetaminophen, and 10 mg/kg/day citalopram), and high DBI (DBI: 1.6; 20 mg/kg/day simvastatin, 350 mg/kg/day metoprolol, 27.2 mg/kg/day oxybutynin,

5 mg/kg/day oxycodone, and 15 mg/kg/day citalopram) and the monotherapy regimens consisted of individual medications that make up the high DBI polypharmacy regimen.

The medication regimens were chosen based on drug classes commonly used by older Australians, which are not routinely dose-adjusted in old age, have similar pharmacokinetics and pharmacodynamics between mice and humans, and were tolerated by mice in previous preclinical studies of chronic oral dosing. The DBI was used to identify medicines with anticholinergic and/or sedative effects. The 3 polypharmacy regimens have estimated DBI scores, that represent the range of exposure commonly observed in older adults. Zero, low (0–1) and high (≥1) DBI exposures are seen in 75%, 20%, and 5% of community-dwelling older Australian men (24), and 30%, 44%, and 26% of people living in residential aged care facilities (25).

At age 21 months, each treatment group was stratified by the mouse clinical frailty index (16) to either continue on treatment for life or to have treatment gradually withdrawn (deprescribed [DP]). Mouse clinical frailty index was assessed at 24 months by a single trained investigator (J.M.) who was blinded to the treatment group. Animal weights were monitored weekly. The date of death of each mouse was recorded to calculate the mortality age. In this cohort study (*n* = 342 total), the majority (*n* = 214, last 8 cohorts) of mice were euthanized for tissue analysis at 24 months of age, and a subset followed for mortality analysis (*n* = 128; the first 5 cohorts of mice).

Mortality was defined when mice were either found dead or were euthanized because they were deemed moribund by an experienced researcher or veterinarian. The details, including the cause of death if known, are listed in Supplementary Table 1. The criteria to determine whether mice were moribund were: severe lethargy, inability to eat or drink, rapid weight loss (>20%), persistent recumbence, severe gait and balance issues, dyspnea, ulcerated tumors, or prolapse that did not recover after veterinary treatment.

Study Inclusion Criteria

In the current study, all animals that had completed the clinical frailty index assessment at 24 months were included for the AFRAID clock, FRIGHT age, and frailty index analysis (total *n* = 274, *n* = 12–19 per group). This was a necessary inclusion criterion because the above clocks require data from the clinical frailty index assessment at 24 months.

To assess for treatment effects on mortality, mortality correlations with other assessments and to conduct precision and bias calculations, only animals that were assessed for frailty at 24 months and not euthanized for tissue collection were used (total *n* = 100, *n* = 5–7 per group, except citalopram DP *n* = 2). The number of animals in the follow-up cohort is provided in Supplementary Table 1. The citalopram DP group had a low number of animals due to randomization and 2 animals passing away prior to the 24 month mark. This follow-up cohort was used because it consisted of only animals with uncensored mortality results. To confirm that the follow-up cohort was representative of the whole cohort, the AFRAID clock, FRIGHT age, and frailty index were also assessed in this follow-up cohort (Supplementary Figure 1).

Frailty Assessment, AFRAID Clock, and FRIGHT Age

The mouse clinical frailty index was conducted as previously described on all mice at 24 months (7). In brief, 31 health-related deficits were examined and graded with increasing severity, with scores 0, 0.5, and 1. The frailty score was derived from the sum of

deficits divided by the total number of deficits (31). The AFRAID clock score and FRIGHT age were calculated as previously described (9). In brief, these clocks were calculated by applying random forest regression algorithms, trained using the mouse clinical frailty index items plus age and weight change (recent weight change: weight change 1 month before assessment; total weight change: weight change from 21 months) by Schultz et al. 2020 (9). We applied these models to the frailty index, age, and weight change data of our study at 24 months to measure either biological age (FRIGHT) or remaining lifespan (AFRAID) at 24 months. Previous research has shown that the items with the highest importance for FRIGHT age include breathing rate/depth, tail stiffening, kyphosis, total weight change and tremor, and those that are the most important for the AFRAID score include total weight change, age, tremor, distended abdomen, and recent weight change (9).

Data Analysis and Statistics

AFRAID clock scores, FRIGHT ages, frailty index scores, and lifespans were described and compared between groups using the Student's *t* test and one-way analysis of variance (ANOVA), with post hoc Tukey, for normally distributed data, or Mann–Whitney and Kruskal Wallis tests for non-normally distributed data. Levene's test was used to determine whether the data were normally distributed. The following comparisons were made; control versus all treatment groups, high DBI polypharmacy compared to other polypharmacy and monotherapy treatment groups and zero DBI verus low DBI polypharmacy groups. DP groups were compared to their corresponding continued treatment groups. All tests were 2 sided and a *p* value <.05 was considered statistically significant.

The correlations between frailty index scores, delta age (FRIGHT age—actual age), AFRAID scores, and actual lifespan were determined using Pearson's or Spearman's correlation analysis (based whether the data was normally distributed), for the whole cohort and each treatment intervention. All statistical analyses were conducted using SPSS (Version 24, IBM SPSS statistics, Chicago, IL). A *p* < .05 was considered statistically significant.

Predictive performance of the AFRAID score for actual mortality age was assessed in the whole cohort and in individual treatment and deprescribing groups. The predictive performance was assessed quantitatively represented by the mean error (ME) and root mean square error (RMSE), calculated according to the methods of Sheiner and Beal (26) with 95% confidence intervals. The ME is an estimate of bias in the prediction and the closer the value is to zero the less biased the model is. The RMSE is a measure of the precision of the model predictions and lower values indicate better fit.

To understand differences in the predictive performance between treatment groups, the distribution of the specific frailty index items between groups were generated for descriptive analysis. Mean scores for the 31-mouse clinical frailty index items plus recent and total weight loss were calculated for the whole cohort and in individual treatment and deprescribing groups.

Results

The Distribution of AFRAID Clock Score, FRIGHT Age, Frailty Index and Mortality in Aging Mice Exposed to Polypharmacy and Monotherapy and Deprescribing by Treatment Group

For each treatment group, the AFRAID clock score, FRIGHT age, and FI score for the whole cohort and mortality data for the

follow-up cohort are shown in Figure 1. Importantly, the whole cohort displayed similar results to the follow-up cohort (shown in Supplementary Figure 1). The results are summarized in Supplementary Table 2. The follow-up cohort displayed a mean lifespan of 31.1 months (range 26.8–34.1 months). Animals that died before the 24 month age point were not included in this study due to the inclusion criteria shown in Supplementary Table 3.

For the AFRAID score, compared to control, there was no statistically significant difference between any treatment groups (Figure 1A), indicating that no treatment group was predicted to live longer or shorter than the control group. Interestingly, mice in the zero DBI polypharmacy DP group had lower AFRAID clock scores than those in the zero DBI polypharmacy continued treatment group. This implies that for this treatment, deprescribing reduced predicted lifespan (Figure 1A).

In terms of FRIGHT age, which can be considered a measure of biological age, there was no difference between control and any treatment group (Figure 1B), indicating that no treatment changed biological age. The simvastatin DP group (Figure 1B) had a higher FRIGHT age compared to the group that continued simvastatin, which implies that the DP group had increased biological age.

For the mouse clinical frailty index, there was no statistically significant difference between control and any treatment (Figure 1C), indicating that treatment did not affect frailty at 24 months. However, in contrast to its effects increasing FRIGHT age, deprescribing simvastatin reduced the clinical frailty index score (Figure 1C).

For mortality age (Figure 1D) no difference was found between control mice and any drug treatment, implying that none of the polypharmacy or monotherapy drug regimens caused a change in lifespan. When mortality was compared between ongoing treatment and the corresponding group with that treatment DP, mice in the low DBI deprescribing group had reduced lifespan (Figure 1D).

The Effect of Treatment on the Correlation of the AFRAID Clock, FRIGHT Age, and Frailty Index with Mortality

To determine whether treatment affected the correlation of the AFRAID clock, FRIGHT age, and frailty index with mortality, we calculated the correlation between these measures for the whole follow-up cohort (which combined mice from all treatment groups [Figure 2]), and each individual treatment group (Supplementary Table 4).

AFRAID clock scores were not significantly correlated with lifespan for the whole follow-up cohort ($r = 0.04$, $p = .73$; Figure 2A) or any other treatment, except for oxycodone deprescribe ($r = 0.87$, $p < .05$; Supplementary Table 4). To explore this discrepancy between the findings of our study and the original study developing and validating the AFRAID clock (9), we investigated the predictive performance of the AFRAID clock for mortality across each of the treatment and deprescribing groups (Table 1) using precision and bias analysis. Based on the ME, the control and high DBI polypharmacy groups had the lowest bias in estimating mortality age (Table 1), but other groups, including zero and low DBI treatment, showed bias of more than 3 months. The ME for lifespan prediction across the whole cohort was 1.34 months (Table 1). The precision score (RMSE) for the whole cohort was 3.76 months, and the AFRAID clock model best fit the oxybutynin and high DBI polypharmacy treatment groups, and oxybutynin and low DBI polypharmacy DP groups, and was least precise for simvastatin and metoprolol deprescribing groups. These data suggest that, at least for some drug
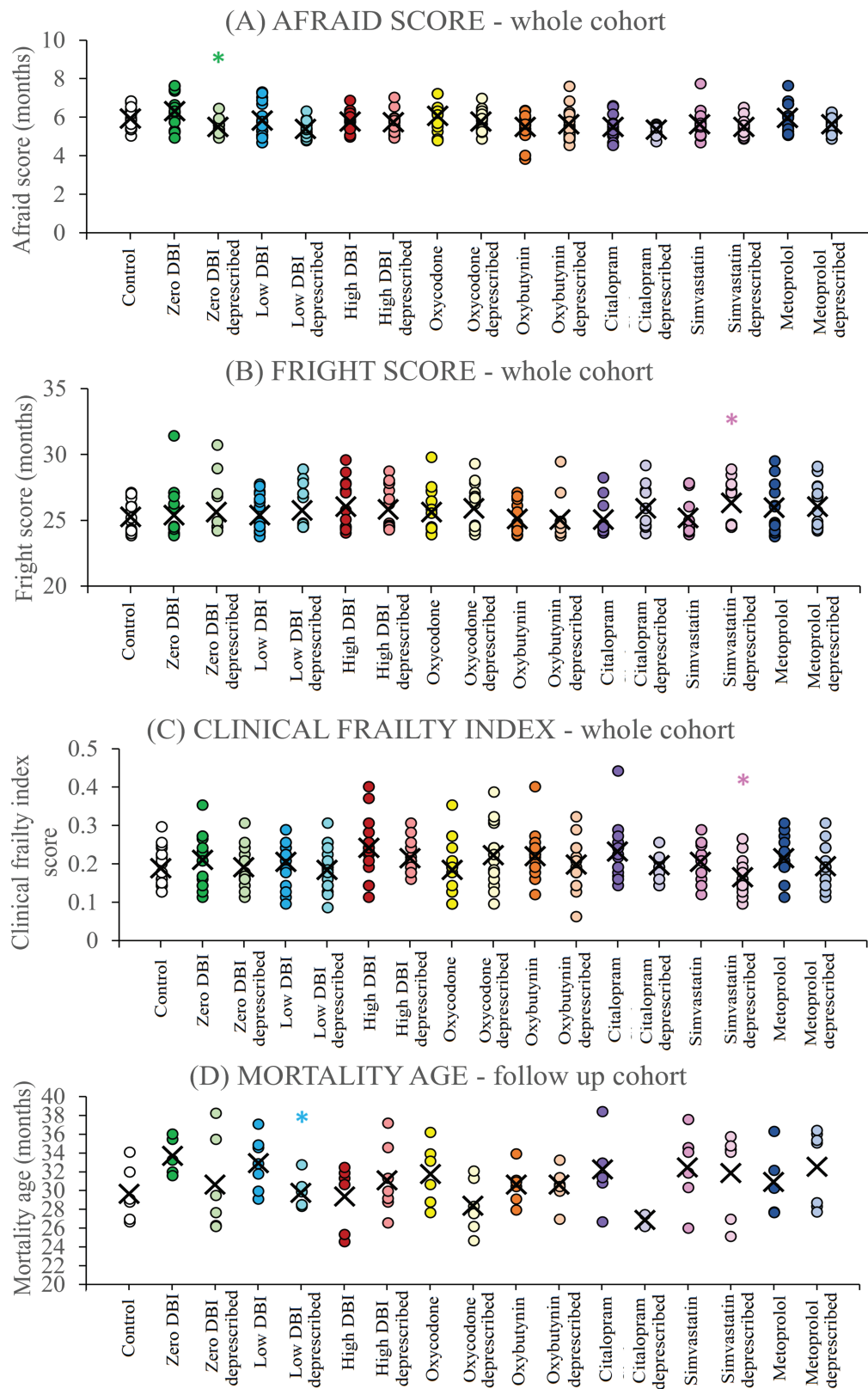
Figure 1. AFRAID score (A), FRIGHT score (B), and clinical frailty index (C) for C57BL/6J mice at 24 months of age (n = 12–19 per group). Mice were randomized from age 12 months to receive control feed or food/water with polypharmacy and monotherapy, which was continued lifelong or withdrawn (deprescribed [DP]) from 21 months. Mortality age (D) is shown for all mice that were not euthanised for tissue collection at 24 months (n = 5–7 per group, except citalopram DP n = 2 due to randomization and n = 2 found deceased before 24 months). Results are presented in dot plots where crosses represent the mean values. No statistically significant difference ($p <$ .05) was found comparing control with treatment, comparing high DBI polypharmacy with treatment and comparing zero DBI with low DBI polypharmacy. *indicates statistically significant difference ($p <$ .05) comparing treatment with their corresponding DP group. The legend is displayed below. DP groups are represented by a lighter color of the corresponding treatment group. For mortality age, no statistical analysis was conducted comparing continued citalopram treatment and DP because there were insufficient animals (deprescribe group n = 2). DBI = Drug Burden Index.   Full color version is available within the online issue.
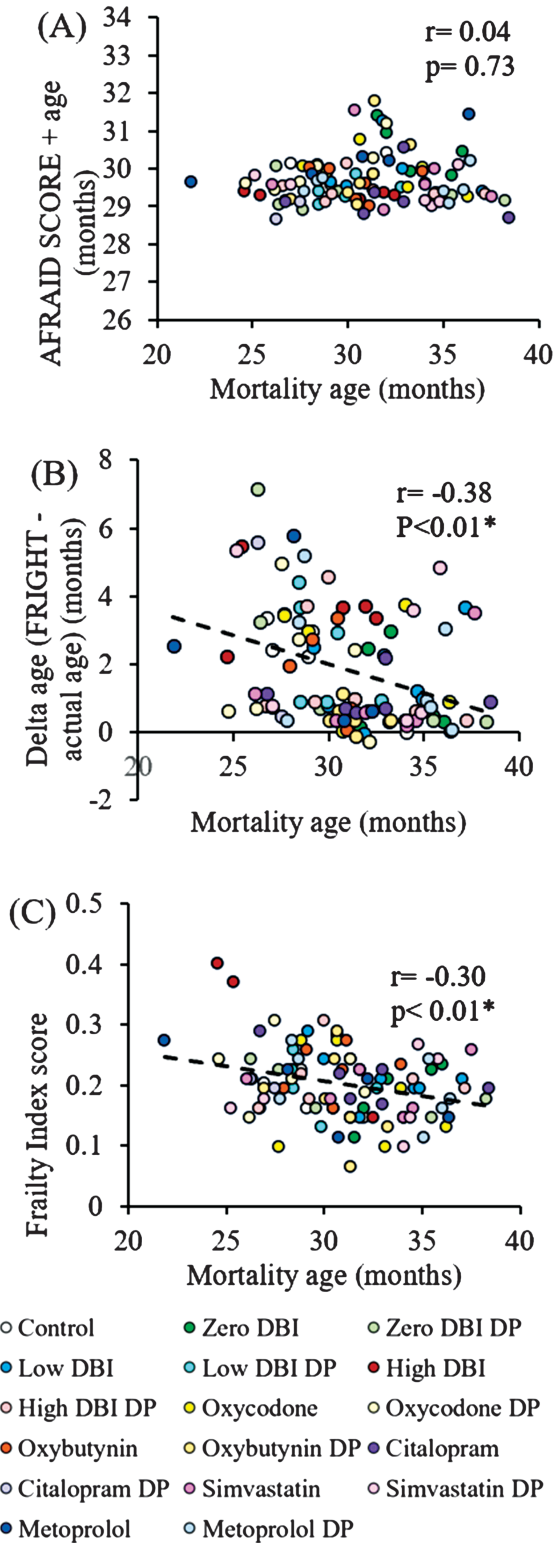
Figure 2. Correlations of AFRAID score (A), FRIGHT age (B), and clinical frailty index (C) with survival in male C57BL/6J mice (the whole follow-up cohort). Delta age (FRIGHT age—actual age) and frailty index score were correlated with mortality age for the whole follow-up cohort ($n = 100$). Pearson correlation coefficient values (r) and p values are shown on each of the graphs. *indicates a statistically significant correlation. Treatment groups are displayed in the legend. DBI = Drug Burden Index. Full color version is available within the online issue.

Table 1. Precision and Bias Calculated According to the Methods of Sheiner and Beal (26) for AFRAID Score with the Gold Standard, the Actual Mortality Age (months) for all Participants ($n = 100$) and Stratified by Intervention Groups

	n	ME (95% CI)	% ME (95% CI)	RMSE (95% CI)	R^2
Whole cohort	100	1.34 (0.64, 2.04)	3.09 (0.86, 5.31)	3.76 (0.05, 9.78)	0.00
Control	6	−0.07 (−3.00, 2.86)	−0.97 (−10.53, 8.60)	2.55 (0.44, 4.52)	0.09
Zero DBI	5	3.18 (0.06, 6.31)	9.13 (0.48, 17.78)	3.90 (0.21, 5.71)	0.49
Zero DBI DP	6	1.31 (−4.13, 6.75)	2.18 (−13.90, 18.27)	4.91 (0.18, 9.10)	0.04
Low DBI	7	3.16 (0.28, 6.05)	8.98 (0.99, 16.96)	4.28 (0.45, 7.70)	0.10
Low DBI DP	7	0.24 (−1.60, 2.08)	0.54 (−5.36, 6.44)	1.02 (0.53, 3.30)	0.00
High DBI	6	0.02 (−3.58, 3.62)	−1.17 (−14.47, 12.13))	1.98 (1.48, 4.76)	0.03
High DBI DP	7	1.67 (−1.75, 5.09)	4.3 (−5.72, 14.33)	2.70 (0.05, 7.85)	0.04
Oxycodone	6	1.87 (−1.90, 5.64)	4.98 (−6.26, 16.22)	2.67 (0.06, 7.04)	0.32
Oxycodone DP	6	−1.65 (−4.10, 0.80)	−6.56 (−15.94, 2.82)	1.91 (0.87, 4.90)	0.76
Oxybutynin	6	0.98 (−1.28, 3.24)	2.85 (−4.36, 10.05)	1.55 (0.86, 4.02)	0.03
Oxybutynin DP	7	0.69 (−0.77, 2.15)	2.01 (−2.89, 6.92)	1.14 (0.39, 2.65)	0.32
Citalopram	6	2.96 (−1.17, 7.09)	8.12 (−3.41, 19,64)	4.65 (2.04, 9.78)	0.01
Citalopram DP	2	−2.02 (−6.87, 2.82)	−7.57 (−27.78, 12.65)	2.06 (1.64, 2.41)	NA
Simvastatin	6	2.66 (−1.86, 7.17)	6.88 (−6.80, 20.56)	4.74 (1.14, 8.38)	0.06
Simvastatin DP	6	2.44 (−2.52, 7.40)	5.81 (−10.53, 22.14)	4.96 (2.56, 5.71)	0.10
Metoprolol	5	−0.42 (−6.37, 5.54)	−4.1 (−27.86, 19.67)	4.31 (0.44, 7.83)	0.77
Metoprolol DP	7	3.00 (−7.40, 6.74)	7.97 (−2.97, 18.91)	4.80 (1.02, 6.67)	0.00

Notes: CI = confidence interval; DBI = Drug Burden Index; DP = deprescribed; ME = mean error (months). A measure of bias; %ME = mean error (bias) expressed as a percentage of mortality age; NA = not analyzed because insufficient number of animals for analysis; RMSE = root mean square error (months). A measure of precision; R^2 = correlation coefficient.

treatment groups, there was significant bias and large variability in the precision of the mortality predictions by the AFRAID clock.

To determine whether treatment affected the relationship between FRIGHT age and mortality, we calculated a delta age for each mouse (FRIGHT age—actual age). This measure is considered a measure of biological age, where those with FRIGHT ages higher than their actual ages would be biologically older, and vice versa. Delta age was negatively correlated with lifespan for the whole cohort ($r = –0.38$, $p < .01$) and control ($r = –0.93$, $p < .01$; Figure 2B, Supplementary Table 4), where mice that were biologically younger lived longer than those that were biologically older.

Frailty index scores were negatively correlated with mortality for the whole cohort ($r = –0.30$, $p < .01$; Figure 2C) and high DBI treated animals ($r = –0.92$, $p < .01$; Supplementary Table 4), indicating that mice that were frailer had shorter lifespans. On the other hand, frailty index score was positively correlated with mortality for zero DBI treated animals ($r = 0.90$, $p < .05$; Supplementary Table 4), suggesting that, in this group, frailer mice had longer lifespans.

Effect of Treatment on Individual Deficits of the Frailty Index and Weight Change

In order to understand whether specific items of the mouse clinical frailty index and weight change were more common in specific treatment groups, which could explain the differences in precision and bias of the AFRAID clock score between treatment groups, mean values for each of the items for the whole cohort and each group were calculated (Figure 3, Supplementary Table 5). Items that were

commonly observed across all groups (displaying the darkest color on the heat map table) included loss of fur color, coat condition, distended abdomen, forelimb grip strength, vestibular disturbance, and mouse grimace scale. Items that showed variability across treatment groups included kyphosis, eye discharge, vision loss, body weight score, recent weight change, and total weight change. The groups that showed both low precision scores and high bias for the AFRAID clock (citalopram, simvastatin, low DBI, zero DBI polypharmacy treatment, and metoprolol deprescribing), had higher than average deficit scores for distended abdomen, grip strength, piloerection, and weight change. Tremor, cataracts, penile prolapse, malocclusions, nasal discharge, and diarrhea were not observed in any mouse across all groups.

Discussion

For the first time, the AFRAID clock and FRIGHT age were calculated in a cohort of aging mice treated chronically with therapeutic doses of drugs such as polypharmacy or monotherapy, either continued or DP in old age. No statistically significant differences were observed for either outcome between drug treatment groups. Deprescribing effects on these measures were seen, with deprescribing of zero DBI drug treatment resulting in decreased AFRAID score and deprescribing simvastatin increasing FRIGHT score but decreasing frailty index. The FRIGHT age and frailty index, but not AFRAID score, were associated with mortality in the whole follow-up cohort.

In this study, we found no significant difference between control mice and any of the drug treatment groups in AFRAID score,

Deficit category		Control	Zero DBI	Zero DBI DP	Low DBI	Low DBI DP	High DBI	High DBI DP	Oxycodone	Oxycodone DP	Oxybutynin	Oxybutynin DP	Citalopram	Citalopram DP	Simvastatin	Simvastatin DP	Metoprolol	Metoprolol DP
	Number of animals	22	15	14	19	18	15	16	17	16	17	16	14	13	16	16	14	16
Integument	Alopecia																	
	Loss of fur colour																	
	Coat condition																	
	Distended abdomen α																	
	Kyphosis *																	
	Tail stiffening *																	
	Gait disorders																	
	Tremor * α																	
	Forelimb grip strength																	
	Body condition score																	
Vestibulocochlear / Auditory	Vestibular disturbance																	
	Hearing loss																	
	Eye discharge/ swelling																	
	Microphthalmia																	
	Vision loss																	
Resipratory system:	Breathing rate/ depth *																	
Discomfort	Mouse Grimace scale																	
	Piloerection																	
Temperature and weight	Temperature score																	
	Body weight score																	
Weight loss	Recent weight change (%)α																	
	Total weight change (%) * α																	

Figure 3. Scores for the criteria that make up the clinical frailty index score, for control, polypharmacy with increasing DBI, monotherapy and deprescribing (DP) in aged male C57BL/6J/mice ($n = 20$–40 per treatment group) at 24 months. Data is displayed with heat map color coding, where greater scores or weight changes are darker in color. Only the criteria that are relevant to the AFRAID and FRIGHT clocks or showed changes following treatment were included in the table. Supplementary Table 5 shows all 31 criteria. * and α indicate the items with the highest importance for FRIGHT age and AFRAID score, respectively. DBI = Drug Burden Index. Full color version is available within the online issue.

FRIGHT age, frailty index, or mean age at death (16). No difference was seen in the mean age of death between treatment groups, which is consistent with the survival findings from our previous publication (16). From available literature regarding the medications tested, only chronic metoprolol monotherapy has previously been documented to alter lifespan in preclinical models. In male B6C3F1 mice, chronic metoprolol extended lifespan by 10% (27). In our study, neither metoprolol alone nor all 3 of the polypharmacy regimens, which contain metoprolol, increased AFRAID scores or affected mortality. The differences in the 2 studies may be attributed to different strains of mice, larger dose of metoprolol, different base diet, and a larger sample size in the previously published study. Additionally, consistent with a previous lifespan study in genetically heterogeneous mice, we did not find a change in lifespan with chronic simvastatin treatment (28). While the effects of irbesartan (a component of the low DBI polypharmacy diet) on longevity in mice have not been studied, no effect was seen in a previous study of candesartan monotherapy on lifespan of male B6C3F1 mice, although in that study, the combination of simvastatin and ramipril appeared to extend lifespan (27), which is most analogous to the zero DBI polypharmacy that contained the related irbesartan and simvastatin. The effect of the other treatments on lifespan in mice has not been previously reported. Deprescribing had some effects on the measured outcomes compared to continuing each treatment. Specifically, predicted lifespan (AFRAID score) decreased with deprescribing in the zero DBI polypharmacy group, and mean age at death was decreased in the low DBI polypharmacy group. This is surprising because in our previous publication (16), we demonstrated that deprescribing zero DBI polypharmacy had no impact on functional outcomes (locomotor, gait speed, frailty, grip strength, daily activities, and motor coordination) (16). Interestingly, the zero DBI regimen contained irbesartan, which has a similar mechanism of action to enalapril, which has been shown to reduce frailty in longitudinal studies of mice (10). Additionally, in line with the effect of these medications, disruption of the angiotensin II type 1 receptor has been shown to increase life span and provide other beneficial effects (29). Our previous study showed that deprescribing low DBI polypharmacy causes functional improvements (16) but this is not consistent with the increased mortality seen here in the low DBI polypharmacy DP group. This paradox may reflect a lack of resilience to change in these old animals. The effects on biological age and lifespan of prescribing and deprescribing in healthy animals may be different to those in multimorbid people. Fortunately, strong evidence exists to support the safety of deprescribing in humans, with no evidence of increased mortality in randomized trials (30). However, there are potential harms. These include adverse drug withdrawal reactions and pharmacokinetic and pharmacodynamic changes (31), which may influence mortality. Compared to continued treatment, deprescribing simvastatin increased predicted biological age (FRIGHT score) but conversely decreased frailty index score. Interestingly, deprescribing simvastatin was associated with increasing kyphosis and total weight change, which are both highly important criterion items for the FRIGHT clock. Statins have been shown to affect bone turnover and bone density (32), which may explain the increase in kyphosis seen after deprescribing simvastatin.

In this study, we found that the AFRAID clock scores were not correlated with lifespan for the whole cohort but were in the group that had oxycodone treatment DP. The results for the whole cohort are not consistent with the study by Schultz et al. (2020) that established the AFRAID clock (9). The likely explanation for this finding is that different diets and treatments may differentially impact the key variables of the AFRAID algorithm and therefore increase variability and reduce predictive power in this cohort. Consistent with this idea, the bias and precision between the AFRAID score and actual mortality, varied between treatment groups. Bias in the overall cohort was 1.34 months, with the least bias for the control group (ME –0.07 months), the greatest overestimate for the zero DBI polypharmacy group (ME 3.18 months), and the greatest underestimate for the citalopram DP group (ME –2.02 months). A specific treatment or cohort effects on key items of the AFRAID score such as total weight loss, recent weight loss, distended abdomen, and tremor may explain the bias seen in the AFRAID score. Tremor, which is one of the most important items in the AFRAID score algorithm (8) was not observed across any groups in this study. Furthermore, the citalopram DP group (for which AFRAID scores were the most underestimated) had the highest scores of the cohort for total weight change and distended abdomen (Figure 3, Supplementary Table 5). Other treatment groups, including citalopram and simvastatin also had high levels of recent weight loss and overall deprescribing trended to increase distended abdomen (Figure 3, Supplementary Table 5). In a wild-type, cohort weight loss and distended abdomen would be detrimental (and thus contribute to decreased AFRAID score). but perhaps in the context of polypharmacy these changes reflect drug effects and are not predictors of lifespan. This suggests that care should be taken in using the AFRAID score for treatments with large effects on measures including weight loss and distended abdomen. Future studies could focus on building clocks with different frailty features in a variety of interventional cohorts in order to further increase the applicability of these tools. However, in the current study, the size of the overall ME remained smaller than the effect size on lifespan of previous interventions targeting aging such as metformin (33) and rapamycin (28), indicating the value in using AFRAID score as an outcome when studying the effects of interventions with significant effects on lifespan.

We did observe a negative correlation between delta FRIGHT age and mortality for the whole cohort and control-treated animals. This is consistent with the study by Schultz (8), suggesting that mice with lower delta FRIGHT age lived longer (biologically younger mice) and higher delta age lived shorter (biologically older mice). In addition, frailty index negatively correlated with mortality in the whole cohort as previously described by Rockwood et al. (34). Interestingly, high DBI treatment had a strong negative correlation, while zero DBI treatment caused a strong positive correlation, demonstrating how treatment can alter frailty and mortality relationships. These results indicate that these biological age clocks can be meaningfully applied to our mouse cohort, and in fact may provide more predictive information than the AFRAID clock, likely due to weight change being a less important factor in these models.

The strengths of this study are that the cohort was held in a single facility for life, and the mouse clinical frailty index results were collected by a single trained investigator, who was blinded to the treatment group. There was no missing data, and the current study is powered to investigate treatment and deprescribing effects. The model is clinically relevant as old mice were investigated, and the medication regimens they were exposed to were comparable to those used by older adults, in terms of classes, doses, route, and duration of therapy and polypharmacy combinations (16). In addition, this model was designed to measure adverse effects from chronic medication use without the impact of disease and represents a clinical situation, whereby the patient is taking medication that they no longer need (harms out weight benefits), and deprescribing is used as an intervention (35). Frail older adults have a reduced ability

to cope with homeostatic changes caused by everyday or acute stressors (36). Prescriber inertia or fear of "rocking the boat" is a well-documented barrier to deprescribing (37). Therefore, this study provides an opportunity to explore the long-term clinical outcomes of deprescribing, which disrupts homeostasis and therefore could impact on the clocks and mortality, even without the effects of drugs on diseases.

A limitation of this study is that it is a secondary analysis of a larger cohort study, where a majority of the animals were euthanized for tissue analysis (16). This resulted in lower numbers of animals in the follow-up cohort. This particularly impacted the citalopram DP group, where 2 animals passed away before the age of 24 months, resulting in only 2 animals for analysis (Supplementary Table 2). Animals that died before the 24 month age frailty index time point were not captured in this study due to the study inclusion criteria (animals displayed in Supplementary Table 3).

Additionally, there may be some bias with the stratification for deprescribing at 21 months based on clinical frailty index rather than body weight, given the important contribution of body weight factors to the AFRAID and FRIGHT models. The current study's moribund criteria identified one control animal that was euthanized for having a rectal prolapse that did not recover after veterinary treatment. The exclusion of this animal did not change the observed statistical significance of any analyses. Additionally, for this study, we only assessed outcomes at 24 months, and future studies could explore the effect of polypharmacy on FRIGHT and AFRAID in younger and older mice.

Importantly, there were some discrepancies in the results of the current study and our previous polypharmacy publication (16) due to different selection criteria and different statistics applied to address the different aims of the 2 studies. In the current study, we saw decreased mortality age following low DBI polypharmacy deprescribing, whereas no change in survival was seen for this group in the previous study (16). The statistical method to investigate differences in mortality age in the current study did not adjust for cohort, which was included in the previous survival analysis (time-to-event Cox model). In addition, not all animals from the longitudinal study were included. The current study only included mice that were assessed for frailty at age 24 months and not those that were deceased prior to 24 months. Furthermore, although high DBI polypharmacy and citalopram monotherapy treatments were found to elevate frailty in our previous study (16), this was not observed here. This is likely due to the cohort differences and the fact that the current statistics were limited to only the last 3 months of the 12 months of treatment (21–24 months), instead of incorporating a longitudinal model approach to account for the multiple time points from 12 to 24 months as in the previous study (16).

As previously described, the findings of this study may not be generalizable beyond the medications and animal strain and sex tested, in this case only male C57BL/6J (16). The mice in this study were also healthy and did not have multimorbidity common in patients of this age. Future studies are required to explore the interaction of polypharmacy treatment with multimorbidity.

Conclusion

In conclusion, AFRAID clock, FRIGHT age, and frailty index were applied to our cohort of aging mice, treated chronically with therapeutic doses of drugs as polypharmacy or monotherapy, either continued or DP in old age. We found that the selected chronic polypharmacy regimens and monotherapy treatments did not alter AFRAID clock and FRIGHT age, but deprescribing had variable effects on AFRAID score, FRIGHT age, frailty index, and mean age of death in some treatment groups. The FRIGHT age and frailty index, but not AFRAID clock, were associated with mortality in the whole cohort. Despite bias induced by specific treatment effects, the use of the combination of these 3 frailty-based scores may be useful to estimate the effects of interventions on mortality and health at 24 months, increasing the efficiency of lifespan studies. This study demonstrates that although the clocks do have utility, future studies are required to tailor them to use mouse models of polypharmacy, which is how medications are commonly taken by older people. Future applications to assess other aging interventions may also require adaptation to avoid bias from specific intervention effects.

Conflict of Interest

D.A.S. is a founder, equity owner, advisor to, director of, consultant to, investor in and/or inventor on patents licensed to Cohbar, Galilei, GlaxoSmithKline, EMD Millipore, Wellomics, Inside Tracker, Caudalie, Bayer Crop Science, Zymo Research, Immetas, and EdenRoc Sciences (and affiliates Arc-Bio, Dovetail Genomics, Claret Bioscience, and MetroBiotech, Liberty Biosecurity). Life Biosciences (and affiliates Selphagy, Senolytic Therapeutics, Iduna, Continuum Biosciences, and Jumpstart Fertility). D.A.S. sits on the board of directors of both companies. D.A.S. is an inventor on a patent application filed by Mayo Clinic and Harvard Medical School that has been licensed to Elysium Health. More information at https://genetics.med.harvard.edu/sinclair-test/people/sinclair-other.php. The other authors declare no conflict of interest.

Author Contributions

S.N.H. conceptualized and designed the work, supervised acquisition, analysis and interpretation of the data, and assisted with drafting and revising the manuscript. A.E.K. and J.M. made substantial contributions to the design, the acquisition, analysis and interpretation of data, and drafting and revising the manuscript. S.E.H. and D.A.S. made substantial contributions to the conception and design of the work, and to interpretation of the data. All authors revised the manuscript and have approved the submitted version.

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