

1    **Title**

2    A Randomized, Controlled Clinical Trial Demonstrates Improved Cognitive Function in Senior  
3    Dogs Supplemented with a Senolytic and NAD+ Precursor Combination.

4

5    **Authors**

6    Katherine E. Simon<sup>1</sup>, Katharine Russell<sup>1,2</sup>, Alejandra Mondino<sup>1</sup>, Chin-Chieh Yang<sup>1</sup>, Beth C  
7    Case<sup>1</sup>, Zachary Anderson<sup>1</sup>, Christine Whitley<sup>1</sup>, Emily Griffith<sup>3</sup>, Margaret E. Gruen<sup>+1</sup>, Natasha J.  
8    Olby\*+<sup>1,4</sup>

9

10   <sup>1</sup>Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State  
11    University, Raleigh, North Carolina, United States of America

12   <sup>2</sup>Southeast Veterinary Neurology. Miami, Florida, United States of America

13   <sup>3</sup>Department of Statistics, College of Sciences, North Carolina State University,  
14    Raleigh, North Carolina, United States of America

15   <sup>4</sup>Comparative Medicine Institute, NC State University, Raleigh, North Carolina, United  
16    States of America

17

18   \*Corresponding Author: [njolby@ncsu.edu](mailto:njolby@ncsu.edu)

19   + Co-senior Authors

20

21

22

23

24 **Abstract**

25 Age-related decline in mobility and cognition are associated with cellular senescence and  
26 NAD<sup>+</sup> depletion in dogs and people. A combination of a novel NAD<sup>+</sup> precursor and senolytic,  
27 LY-D6/2 was examined in this randomized controlled trial. Seventy dogs were enrolled and  
28 allocated into placebo, low or full dose groups. Primary outcomes were change in cognitive  
29 impairment measured with the owner-reported Canine Cognitive Dysfunction Rating (CCDR)  
30 scale and change in activity measured with physical activity monitors. Fifty-nine dogs completed  
31 evaluations at the three-month primary endpoint, and 51 reached the six-month secondary  
32 endpoint. There was a significant difference in CCDR score across treatment groups from  
33 baseline to the primary endpoint ( $p=0.02$ ) with the largest decrease in the full dose group. There  
34 were no significant differences between groups in changes in measured activity. However, the  
35 proportion of dogs that improved in frailty and owner-reported activity levels and happiness was  
36 higher in the full dose group than other groups. Adverse events occurred equally across groups.  
37 All groups showed improvement in cognition, frailty, and activity suggesting placebo effect and  
38 benefits of trial participation. We conclude that LY-D6/2 significantly improves owner-assessed  
39 cognitive function and may have broader effects on frailty, activity and happiness as reported by  
40 owners.

41

42 **Key words**

43 Aging, Senolytic, NAD<sup>+</sup>; Canine Cognitive Dysfunction Syndrome; cognitive impairment;  
44 longevity

45

## 46 Introduction

47

48 Improved veterinary medical care and migration of dogs from a working role to family member  
49 have resulted in extension of the canine lifespan [1,2,3]. As a result, dogs, like humans,  
50 experience a wide range of age-related morbidities [4,5,6,7,8,9]. Indeed, inclusion of dogs in the  
51 family household with exposure to the same environmental contaminants and stressors, and  
52 adoption of the activity patterns and sometimes nutrition of their owners has resulted in a very  
53 similar array of these conditions occurring in both species [10,11].

54

55 Cognitive function and mobility have been proposed as key hallmarks of functional aging, and  
56 their age-associated attrition appears to be linked in both humans [12,13,14] and dogs [15,16].  
57 Normal age-related cognitive changes include alterations in sleep [17], memory [18,19],  
58 attention [20,21] and social interactions [22,23]. In a high proportion of people and dogs, these  
59 develop into Alzheimer's Disease [24] or Canine Cognitive Dysfunction Syndrome (CCDS)  
60 [25,26,27,28] respectively. Similarly, activity and mobility decrease with age in humans [29] and  
61 dogs [30] due to sarcopenia [31], decreased motivation [32] and common pathologies such as  
62 osteoarthritis [33]. These changes affect the patient's quality of life, and take a significant toll on  
63 the caregiver [34,35,36]. Supplements that can reduce cognitive decline and maintain mobility  
64 in aging dogs would have enormous impact on dogs and their caregivers, and translational  
65 relevance for aging people.

66

67 Clinical manifestations of aging have their origin at a molecular level with 12 molecular  
68 hallmarks of aging now recognized [37]. The burgeoning field of geroscience has identified  
69 therapeutic targets to mitigate the aging process within these hallmarks and numerous clinical  
70 trials are now underway in people [38]. While the human anti-aging field is flourishing, far fewer  
71 studies target age-related decline in dogs. In this randomized, controlled, double-blinded clinical  
72 trial we evaluated a combination of supplements that target two important hallmarks of aging,  
73 cellular senescence and depletion of cellular nicotinamide adenine dinucleotide (NAD+)  
74 concentrations.

75

76 Senolytics have emerged as a promising anti-aging therapeutic strategy [39,40]. Cellular  
77 senescence increases with age and while it can be a protective mechanism, it can also cause  
78 an unwanted inflammatory response, the Senescence-Associated Secretory Phenotype (SASP)  
79 [37,41]. Senolytics include plant derived flavonoids such as quercetin and fisetin that act  
80 through inhibition of anti-apoptotic proteins (such as BCL-2 family proteins) [42]. Reducing  
81 senescence reduces age-related pathology *in vitro* and extends lifespan and a variety of  
82 different functional outcomes *in vivo* [43,44]. Senolytics are marketed widely as anti-aging  
83 supplements and human clinical trials evaluating senolytics in age-related diseases such as  
84 osteoarthritis, heart disease and Alzheimer's disease are underway [45], but clinical trials in  
85 dogs are lacking.

86

87 Supplementation with an NAD+ precursor takes a more broadly targeted approach to aging.  
88 NAD+ is involved in regulation of key biological processes, cellular signaling and electron  
89 transfer, but it declines with age [41,46,47], exacerbating age-related diseases [48,49].

90 Precursors such as nicotinamide mononucleotide have been shown to mitigate the effects of  
91 aging and age-related pathologies [50] and restoring levels of NAD+ improves cellular energy  
92 and metabolism, leading to improvements in health and lifespan [51,52]. Human clinical trials  
93 are ongoing [53], however there is only one canine study published to date, where muscle  
94 function improved following treatment with nicotinamide in dogs with Duchenne's Muscular  
95 Dystrophy [54]. Senescent cells secrete an NADase (CD38), causing depletion of NAD+. As a  
96 result, it has been suggested that combining NAD+ precursors with a senolytic amplifies NAD+  
97 anti-aging properties [55].  
98

99 This study evaluated the effects of a repeated monthly regimen of two consecutive days of the  
100 senolytic (LY-D6<sup>TM</sup>) and NAD precursor followed by daily NAD precursor (LY-D2<sup>TM</sup>) on cognition  
101 and activity in companion dogs. We hypothesized that supplementation with LY-D6/2  
102 combination would reduce cognitive and activity level decline in aging dogs. Companion dogs  
103 aged 10 years or older were randomized to receive either placebo or LY-D6/2 combination at  
104 two different doses (low and full) with primary outcomes of change in owner assessed cognition  
105 and collar mounted physical activity monitor (PAM) assessed activity levels after 3 months.  
106  
107

## 108 **Results**

110 Two hundred and forty-three surveys were completed, 91 dogs were brought in for screening  
111 appointments from which 70 dogs were enrolled and 67 completed baseline assessments  
112 (Figure 1). One dog was withdrawn due to pre-existing disease and 66 completed the one-  
113 month assessments. Two dogs died, one withdrew and one was excluded due to  
114 noncompliance, leaving 62 dogs to complete the three-month (primary endpoint) assessments.  
115 Four dogs were euthanized between month three and six and two withdrew due to mobility  
116 decline and neck pain. Fifty-six dogs completed all visits. Two dogs had active urinary tract  
117 infections (UTIs) at their six-month visit so their data were excluded from the six-month  
118 analyses. Data from three dogs were excluded from three- and six-month analyses due to  
119 treatment noncompliance (>20% missed doses).  
120

## 121 **Study Population**

123 Demographic details and baseline outcome measures of dogs at time of enrollment are  
124 provided in Table 1. The only significant difference between groups in baseline characteristics  
125 was age (p=0.02) with the full dose group being significantly older than the placebo or low dose  
126 groups. Owners were asked to maintain consistency in their pet's routine over the course of the  
127 trial, but the number of pets changed in 14 households (addition or euthanasia of a pet).  
128 Twenty-eight dogs had a change in medication; we compared the number of household and  
129 medication changes from baseline to primary endpoint (month three) and from primary endpoint  
130 to secondary endpoint (month six) across groups and found no significant differences between  
131 groups (Supplementary Table S1).  
132

## 133 **Primary Outcomes**

134

135 **CCDR:**

136 ***Primary Endpoint (3 months)***

137 All dogs entered the trial with mild to moderate cognitive impairment. The CCDR scores did not  
138 differ significantly between groups ( $p=0.28$ ) (Table 1). Over the first three months of the trial,  
139 CCDR score decreased (improved) in each treatment group. Individual frailty status at  
140 enrollment was significantly associated with change in CCDR and so was incorporated into a  
141 repeated measures model comparing CCDR score between groups at baseline, one and three  
142 months. There was a significant difference between treatment groups over the three-month  
143 period ( $p=0.02$ ), with the full dose group showing the largest decrease (improvement) in CCDR  
144 score (Figure 2).

145

146 Change in CCDR score was also categorized as failure (increase in score representing  
147 worsening cognition) or success (static or decreased score) and groups were compared using a  
148 chi-square analysis. In the full dose group 16/18 (88.9%) dogs were successes, compared with  
149 15/21 (71.34%) of low dose dogs and 12/20 (60%) of placebo dogs. However, these differences  
150 were not statistically significantly ( $p=0.11$ ).

151

152 ***Secondary Endpoint (6 months)***

153 We next assessed whether individuals maintained their CCDR scores to the final endpoint.  
154 Score differences from month three to month six were calculated and compared across groups.  
155 Median change in CCDR was static in all groups (Supplementary Table S2), with no significant  
156 difference across groups ( $p=0.44$ ).

157

158 **Activity Monitor**

159 Activity was initially analyzed using functional linear modeling (FLM) to allow patterns of change  
160 over the entire 24-hour period to be captured. At baseline, activity levels peaked around 6-10am  
161 and 4-9pm, reflecting times when owners interact with their dog. There was no significant  
162 difference between groups (Figure 3a,b). Weekend activity did differ between groups around  
163 12pm, reaching the global threshold for significance, with the full dose group showing higher  
164 activity (Figure 3c,d).

165

166 ***Primary Endpoint***

167 When change in activity level from baseline to month three was examined on weekdays, groups  
168 maintained similar levels of activity compared to baseline. Notably, all groups showed a small  
169 increase in morning and evening activity and decrease in afternoon activity with no significant  
170 difference between them (Figure 4a,b). Changes in weekend activity were more variable,  
171 reflecting the owners' less defined weekend schedules (Figure 4c). The placebo and full dose  
172 groups showed larger fluctuations than the low dose group, but the differences between groups  
173 did not reach global significance (Figure 4c,d).

174

175 ***Secondary endpoint***

176 Similarly, when evaluating the change in activity from months three to six, activity remained  
177 relatively unchanged on weekdays, apart from an increase in all groups in the afternoon to

178 evening, while the weekend activity fluctuated more widely (Figure 5a,c). There was no  
179 significant difference between groups on weekdays but at the weekend, groups differed  
180 significantly around 6am with the low dose groups showing a decrease in activity (Figure 5d).  
181

### 182 ***Cumulative Activity***

183 To incorporate covariates into the analysis, total cumulative activity for each dog was examined  
184 using repeated measures models. Weekday daytime activity was affected by age, and weekday  
185 and weekend daytime activity were affected by sex. Neither weekday nor weekend nights  
186 required correction for covariates. The repeated measures models for summated activity  
187 showed no significant difference over time (from baseline to primary endpoint) between groups  
188 during the day (weekday:  $p=0.95$ , weekend:  $p=0.71$ ) or during the night (weekday:  $p=0.31$ ,  
189 weekend:  $p=0.65$ ) (Supplementary Figure S3). When assessing the difference in cumulative  
190 activity between three and six months, we found no significant difference across either time  
191 period on weekdays (day:  $p=0.89$ , night:  $p=0.10$ ) or weekends (day:  $p=0.60$ , night:  
192  $p=0.83$ )(Supplementary Table S4).  
193

### 194 ***Owner assessed Activity***

195 By contrast, when owners were asked to classify activity as static, reduced or increased at the  
196 primary endpoint, 8/18 (44%) owners in the full dose group reported static and 7/18 (39%)  
197 reported increased activity levels, compared with 13/21 (62%) static and 2/21 (10%) increased  
198 in the low dose group and 11/20 (55%) static and 4/20 (20%) increased in the placebo group  
199 (Supplementary Table S5). This difference between the groups was not significant ( $p=0.29$ ). A  
200 majority of dogs across all groups remained static at the final endpoint: 10/17 (59%) of the full  
201 dose group, 12/17 (71%) of the low dose group and 11/17 (65%) of the placebo group. The  
202 differences were not significant ( $p=0.64$ ).  
203

## 204 **Secondary Outcomes**

### 206 ***Primary Endpoint***

#### 207 ***Frailty Score***

208 The proportion of dogs that were frail (3 or more of 5 domains classed as impaired) and the  
209 number of impaired domains at study outset for each treatment group are provided in Table 1;  
210 groups were not significantly different. As expected with a senior pet population, there were  
211 changes in frailty over the course of the study which were categorized as success (static or  
212 improved) or failure (deteriorated) (Supplementary Table S5). The majority of dogs in full dose  
213 (13/18, 72.2%) and low dose (16/21, 76.2%) groups were classified as success after 3 months,  
214 as compared with 11/20 dogs (55%) in the placebo group; however groups did not differ  
215 significantly ( $p=0.32$ ).  
216

### 217 ***Cylinder Task (Inhibitory control)***

218 Inhibitory control is a test of executive function, similar to impulse control, which decreases with  
219 age in both humans and dogs [56,57]. The full dose group started with a lower mean baseline  
220 score (66.1%) than the low dose (78.79%) and placebo group (76.6%); this difference was not  
221 significant ( $p=0.57$ ) (Table 1). When assessing change from baseline to the primary endpoint,

222 inhibitory control required correction for changes to household and changes to medication.  
223 These were therefore incorporated into the repeated measures model, which showed all groups  
224 increasing in score (improving their performance in the task) (Figure 6a). However, all groups  
225 showed a similar trajectory of improvement and there was no significant difference over time  
226 across groups ( $p = 0.95$ ).

227

### 228 **Detour**

229 Detour is a further challenge to the cylinder task, requiring flexibility in problem solving, which  
230 becomes more challenging as dogs age [56]. Dogs had lower baseline scores on detour as  
231 compared to the cylinder task. Similar to the cylinder task, the full dose group started out with a  
232 lower baseline score (27.3%) compared to both the low dose (34.8%) and placebo group  
233 (34.6%); these scores again were not significantly different ( $p=0.52$ ) (Table 1). In the repeated  
234 measures analysis of change in score over time, detour required correction for fractional  
235 lifespan. The groups did not differ significantly across the 3 months ( $p=0.85$ ) (Figure 6b). We  
236 saw a small decrease in score (decline in performance) with the full dose group at the primary  
237 endpoint.

238

### 239 **Sustained Gaze**

240 Sustained gaze is a test of focus and attention, with aging dogs shown to decline in the time in  
241 which they can maintain gaze for a treat [21]. At enrollment, there was no significant difference  
242 in performance between groups ( $p=0.22$ ) (Table 1). No covariates reached significance to be  
243 included in the repeated measures analysis. While sustained gaze duration increased in all  
244 three groups at the primary endpoint, indicating an improvement, there was no significant  
245 difference between groups over time ( $p=0.59$ ) (Figure 6c).

246

### 247 **Off Leash Gait Speed**

248 Off leash gait speed is both a measure of physical ability and motivation (for a treat). This has  
249 been shown to decline with age in both dogs and humans [30]. There was no significant  
250 difference in off leash gait speeds across groups at enrollment ( $p=0.55$ ) (Table 1). Gait speed  
251 did not require correction for any covariates in the repeated measures analysis. All groups  
252 showed little change in speed, with no significant difference between treatment groups over time  
253 ( $p=0.59$ ) (Figure 6d).

254

### 255 **Secondary Endpoint**

256 Similar to the primary outcome measures, we assessed whether individuals were able to  
257 maintain their level of frailty, cognitive testing scores and gait speed from months three to six by  
258 comparing change in score across groups. We found the majority of dogs remained static  
259 (median change of approximately zero) across all groups from month three to month six  
260 (Supplementary Table S2). There was no significant difference between groups for frailty  
261 ( $p=0.80$ ), inhibitory control ( $p=0.33$ ), detour ( $p=0.28$ ), sustained gaze ( $p=0.33$ ) or off-leash gait  
262 speed ( $p=0.64$ ).

263

### 264 **Owner assessed happiness**

265 Owners were asked to categorize their dogs' level of happiness as the same, improved or  
266 deteriorated compared to the previous visit at three and six months. At three months, 10/18

267 (56%) owners with dogs in the full dose group reported static happiness, 8/18 (44%) improved  
268 and none reported a deterioration (Supplementary Table S5). While not significant ( $p=0.34$ ),  
269 this differs from the low dose and placebo groups in which 2/21 (10%) and 3/20 (15%) reported  
270 a deterioration. At six months, 6/17 (35%) owners with dogs in the full dose group and 8/17  
271 (47%) in the low dose group reported an increased level of happiness. Whereas only 4/17  
272 (24%) of owners in the placebo group reported an increase. These differences were not  
273 significant ( $p=0.42$ ).

274  
275 **Adverse Events**  
276

277 All dogs who received supplements/placebo were included in the adverse events analysis. At  
278 each visit, owners completed a checklist regarding possible adverse reactions (Supplementary  
279 Figure S6). There were 245 adverse events reported, ranging in VCOG grade: 1 ( $n=190$ ), 2  
280 ( $n=40$ ), 3 ( $n=7$ ), 4 ( $n=1$ ), 5 ( $n=6$ ). Events were only included if they were newly observable (not  
281 present at baseline) during the course of treatment. Of these reported events, 76 were in the  
282 placebo group, 102 in the low dose group and 67 in the full dose group (Supplementary Table  
283 S7). These events were evenly distributed across the groups.

284  
285 Most events observed were VCOG grade 1 ( $n=190$ ) or 2 ( $n=40$ ). Grade 1 events were  
286 considered minimally disruptive by the owner, required no intervention, and resolved quickly on  
287 their own. Grade 2 events moderately impacted the patient's daily life and usually necessitated  
288 outpatient veterinary care (antibiotics, pain management, minimally invasive procedure). There  
289 were seven severe (Grade 3) events that required medical intervention and had a significant  
290 effect on daily activities but were not immediately life threatening. These events were reported  
291 to us and medically managed by each patient's primary care veterinarian. None of these events  
292 could be related to LYD6/2. Only one event was graded as four (requiring urgent medical  
293 attention): this dog presented to their final visit with lethargy, weakness and ascites. Over the  
294 course of the trial, six dogs developed disease that ultimately led to euthanasia or death (Grade  
295 5); two in the placebo group, three in the low dose group and one in the full dose group. The  
296 reasons for these deaths included: severe respiratory disease, neoplasia, poor quality of life  
297 (severe cognitive dysfunction and incontinence) and surgical complications during a gastric  
298 dilation volvulus procedure.

299  
300 Adverse events occurred across a variety of systems for all groups (Supplementary Table S7  
301 and Figure 7). Only two adverse events occurred in the treatment groups that were not also  
302 seen in the placebo group (hypertension and an anaphylactic reaction). The hypertension was  
303 pre-existing and well controlled prior to the trial, but over the course of the trial, the dog  
304 developed worsening hypertension and was eventually euthanized due to poor quality of life.  
305 The anaphylactic reaction was in response to an antibiotic prescribed for a UTI which quickly  
306 resolved after discontinuation of the antibiotic. Therefore, it is unlikely that either of these events  
307 were a direct result of LY-D6/2.

308  
309 There were 284 lab work changes over the course of the trial (Supplementary Table S7) and of  
310 these, only two were graded as VCOG 2 and one was graded as VCOG 3. The rest were mild,

311 mostly incidental findings. The most common findings were anemia (n=21), lymphopenia (n=21)  
312 and hematuria (n=26). Any observed abnormalities were reported to the owner and pursued by  
313 the primary care veterinarian at their own discretion. Each group experienced a similar number  
314 of lab work abnormalities; placebo: 95, low dose group:106 and full dose group:87.

315

## 316 **Discussion**

317

318 This randomized controlled blinded trial is one of the first of its kind, evaluating an anti-aging  
319 supplement that targets two hallmarks of aging in senior dogs. This clinical trial used a  
320 pragmatic approach that included dogs with mild to moderate cognitive impairment who met  
321 age, weight and relatively broad health criteria. Dogs were followed to a primary endpoint at  
322 three months, representing nearly two human years, and a secondary endpoint at six months,  
323 representing approximately three and a half human years. There was a significant difference in  
324 change in CCDR score across treatment groups from baseline to the primary endpoint at three  
325 months, with the largest decrease in score in the group of dogs who received the full dose of  
326 LY-D6/2. There was no difference between groups in changes in activity level, gait speed or in-  
327 house cognitive testing. However, while not significant, a higher percentage of dogs in the  
328 placebo group showed a deterioration in frailty (45.0%) compared to both the low and full dose  
329 LY-D6/2 groups (23.8% and 27.8%, respectively). The LY-D6/2 supplements were well  
330 tolerated; while many health issues occurred in this group of old dogs over the six-month trial  
331 period, there was no difference in prevalence of adverse events between treatment groups and  
332 none could be attributed to LY-D6/2.

333

334 The rationale of targeting two molecular mechanisms of aging was both to enhance the  
335 intervention strategy and to broaden the population of dogs that might benefit from the  
336 supplements. Both cellular senescence and depletion of cellular NAD<sup>+</sup> are well established  
337 molecular hallmarks of aging and manipulation of both events in either direction has been  
338 shown to either ameliorate or worsen the aging phenotype in an experimental setting [37]. The  
339 use of a variety of different NAD<sup>+</sup> precursors to increase cellular NAD<sup>+</sup> concentrations is one of  
340 the most popular anti-aging strategies available at this time [53]. While there are now multiple  
341 studies demonstrating the ability to increase blood cell concentrations of NAD<sup>+</sup> with oral  
342 supplements, and some evidence of a health benefit in middle aged and healthy old people  
343 [58,59,60], definitive evidence of an effect on the aging phenotype in people is still lacking [53]  
344 and the only data in dogs relates to a model of Duchenne's muscular dystrophy [54]. Cellular  
345 NAD<sup>+</sup> attrition interfaces with other mechanisms of aging and the possibility of a synergistic  
346 effect of NAD<sup>+</sup> precursors when combined with another therapy has been proposed [55]. There  
347 has been particular interest in the rather complex interplay between cellular senescence and  
348 NAD<sup>+</sup> depletion. It is clear that the SASP production by senescent cells is both metabolically  
349 taxing and reduces NAD<sup>+</sup> concentrations by increasing CD38 expression [61]. In this clinical  
350 trial we chose to maximize the potential benefit of the anti-aging intervention by combining a  
351 senolytic with an NAD<sup>+</sup> precursor.

352

353 Given the dearth of published placebo controlled randomized clinical trials examining anti-aging  
354 supplements in aged companion dogs, there is insufficient data available on parameters such

355 as anticipated death rates, frequency and impact of comorbidity development, and size of  
356 caregiver placebo effect. We powered this clinical trial around detection of change in owner-  
357 quantified change in cognitive function. We used preliminary longitudinal data on dogs with mild  
358 to moderate cognitive impairment and made the observation that the vast majority of such dogs  
359 decline over a six-month period. This is supported by other published studies [62] and  
360 prevention of this deterioration would represent a meaningful benefit to dogs and their owners.  
361 Thus, the clinical trial was designed to detect a 50% reduction in the proportion of dogs that  
362 would show a deterioration in owner reported cognitive status. A primary endpoint at three  
363 months was chosen because of concerns about case attrition due to development of conditions  
364 such as cancer in this elderly population of dogs.

365  
366 Clinical trials can be explanatory or pragmatic, with explanatory trials answering the question of  
367 whether an intervention is effective in a very specific patient population and pragmatic trials  
368 determining whether a therapy will be effective under normal conditions [63,64]. In this clinical  
369 trial, the study population was intentionally wide and the primary outcomes (owner reported  
370 cognitive status and activity detected by a collar mounted PAM) were straightforward to obtain  
371 and were directly relevant to a dog's day-to-day life. The inclusion of simple owner reported  
372 assessments of improvement or deterioration in activity levels and happiness allowed  
373 identification of meaningful changes by owners blinded to treatment group. The results of this  
374 pragmatic clinical trial can immediately inform recommendations by primary care veterinarians.

375  
376 In dogs, cognitive decline is strongly age-associated, with prevalence estimates of mild  
377 cognitive impairment in 28% of 11-12 year olds, and up to 68% of 16 year olds [65,66]. The  
378 odds of developing canine CCDS increase by 52% with each additional year of age [67]. There  
379 are several different validated owner questionnaires to quantify canine cognitive decline and the  
380 development of CCDS. The initial power analysis and patient recruitment was performed using  
381 CADES because this scale identifies mild cognitive changes through an option to choose an  
382 event frequency of q6 months. However, owner scores of cognition were solicited at baseline,  
383 one, three and six months and so the CCDR was chosen as a more reliable means of sampling  
384 cognitive status repeatedly within six months. Using this measure, only 2/18 dogs (11.1%) in the  
385 full dose group showed a deterioration in score compared with 6/21 (28.6%) in the low dose  
386 group and 8/20 (40%) in the placebo group. Moreover, the repeated measures analysis, using a  
387 model that accounted for frailty status at study start, showed a significant effect of  
388 supplementation with the full dose group showing the largest decline in score (clinical  
389 improvement). This effect was not maintained through six months with CCDR scores plateauing  
390 or increasing slightly in all three groups. Given this time period represents nearly 3.5 years of  
391 human life, this is perhaps not surprising.

392  
393 Owners of 60% of dogs in the placebo group documented an improvement in cognition over a  
394 three-month period suggesting that there was a sizable and long-lasting caregiver placebo  
395 effect [68]. This caregiver placebo effect could be the result of optimism on the part of the  
396 owner; in addition, many owners participating in a longitudinal study of aging with our research  
397 group report that the interactions their dogs have when they visit the research site improve their  
398 attitude and engagement. Thus, this trial might be capturing the effect of increased social

399 interaction and problem solving in elderly dogs through study participation. This is supported by  
400 studies in purpose bred dogs in which behavioral enrichment was associated with preservation  
401 of learning [69,70], through elevation of brain levels of BDNF expression [71].  
402  
403 The results of the secondary cognitive tests of attention and executive control performed at the  
404 research site did not differ between groups, with most groups showing a slight improvement.  
405 These cognitive tests have not been used in a longitudinal clinical trial previously, and it is  
406 possible that these dogs learned how to perform the tests better with each visit. In addition, we  
407 might again be capturing the positive effect of trial participation.  
408  
409 Assessing activity levels in senior and geriatric dogs is challenging because they tend to lead  
410 quite sedentary lives and, as for all companion dogs, their activity is strongly influenced by their  
411 owner's activity [72,73]. We used collar mounted activity monitors to collect data over a two-  
412 week period at each evaluation point, and evaluated weekdays and weekends separately to  
413 allow for owner schedules differing over the weekend [74]. The baseline data showed the typical  
414 peaks in activity when the household rises in the morning and comes home from a day of work  
415 during the week. We first performed an FLM analysis as this captures and smooths data  
416 allowing a per-minute comparison between groups over a 24-hour period. Given that multiple  
417 comparisons are made when performing an analysis in this way, only group differences that  
418 reach the threshold of global significance are compelling, while those that reach pointwise  
419 significance highlight areas of interest. However, this analysis did not reveal any pattern of  
420 consistent change between groups. It was interesting to note that morning and evening activity  
421 on weekdays did increase in all three groups, again suggesting either placebo or a true  
422 beneficial effect of trial participation. Given these elderly dogs are not very active, but were  
423 reported to demonstrate small bursts of energy by owners, we also compared the sum of the  
424 total daytime and nighttime activity counts for each dog between groups. Unlike the FLM, which  
425 cannot be performed with covariates, this allowed us to build a model taking covariates into  
426 account, but no differences were noted between groups. Finally, we compared the owner  
427 reports of activity changes and more full dose group owners noted an improvement than the  
428 other groups. This raises the question of how best to capture activity changes in elderly dogs.  
429  
430 Frailty is a well described syndrome in aging people but descriptions of frailty are few in dogs  
431 [75,76,77]. We have developed a rapid screening tool for frailty that combines owner responses  
432 to questions around the five key frailty domains, and assessment of body and muscle condition  
433 score by the research group [78]. While there was no significant difference between groups in  
434 percentage of frail individuals at study start, approximately 35% of the placebo group were frail  
435 compared with 48% in both treatment groups. One would anticipate that aged dogs would  
436 become frailer over time, but in our study, the majority of dogs in all three groups improved in  
437 their frailty status over the first three months with a lower percentage of dogs in the treatment  
438 groups deteriorating than in the placebo group. Currently there is a dearth of data following  
439 frailty in elderly dogs longitudinally and so it is difficult to comment on whether this is unusual,  
440 but once again, it does suggest either a placebo effect, or a positive effect of trial participation.  
441

442 This study has some weaknesses, first, this was a pragmatic clinical trial with no attempt to  
443 target dogs who exhibited higher levels of senescence or lower levels of NAD; a more targeted  
444 trial might be better able to capture changes due to alterations in these systems. Further,  
445 biomarkers of senescence and NAD+ levels were not measured. Future studies may benefit  
446 from incorporating these biological markers as outcomes. Our inclusion of cognitive tests was  
447 designed to capture changes in performance measures (as compared to owner-rated changes  
448 in signs) however these tests have not been used longitudinally, and while correlations have  
449 been seen between some of these measures and owner-questionnaires [79], their  
450 responsiveness as outcome measures was unknown. Dogs may learn to perform these tasks  
451 with practice, and additional longitudinal data will be needed to understand how performance on  
452 these tasks changes with time. Finally, the full dose group was significantly older than the other  
453 groups, although fractional lifespan did not differ. Cognitive decline is associated with age rather  
454 than fractional lifespan, and so this could have influenced the outcomes negatively for this  
455 group. However, age was examined in the univariate analysis of CCDR change and was not  
456 associated with the outcome.

457  
458 We conclude that LY-D6/2 can be used safely to mitigate cognitive decline in senior dogs and  
459 might have broader effects on dog health manifesting as improved happiness and reduced  
460 frailty. This trial highlights the viability of targeting hallmarks of aging to impact the health of  
461 aging dogs. The pragmatic design means the results are immediately applicable to an aging dog  
462 population and are potentially relevant to people. Clinical trial participation appears to be  
463 beneficial in an aging dog population.

464

## 465 **Methods**

466

467 This blinded, randomized, controlled (RCT) clinical trial was conducted and reported according  
468 to the CONSORT and ARRIVE Guidelines with the approval of the North Carolina State  
469 University Institutional Animal Care and Use Committee under protocol # 21-376-O. All  
470 procedures were performed in accordance with these approved protocols and institutional  
471 guidelines. Owners of the dogs who participated in these studies reviewed and signed an  
472 informed consent form. IRB approval was not sought because all collected data pertained to  
473 dogs and as such the work was categorized as “Non-Human Subject Research”.

474

## 475 **Study design**

476 This 3-arm RCT was designed to evaluate the effect of LY-D6/2<sup>TM</sup> on cognitive function and  
477 activity in aged dogs over a six-month period. The primary endpoint of the study was three  
478 months (representing approximately 1.75 human years) and the secondary endpoint was six  
479 months (representing approximately 3.5 human years). The primary outcomes were change in  
480 owner assessed cognitive function (via Canine Cognitive Dysfunction Rating (CCDR) scale) [80]  
481 and activity level (via a collar mounted physical activity monitor (PAM)) [81]. Secondary  
482 outcomes included changes in frailty phenotype, attention (sustained gaze), inhibitory control  
483 (cylinder task), cognitive flexibility (detour task) and off-leash gait speed. Preliminary data from  
484 six mild to moderately cognitively affected dogs (based on owner assessment using the Canine  
485 Dementia Scale (CADES) [62] were used to perform the power analysis. Five of these six dogs

486 showed a deterioration in owner reported cognitive function over a six-month period. It was  
487 determined that 20 dogs per group would detect a 50% reduction in the number of dogs who  
488 show cognitive deterioration with a power of 80%. Ten additional dogs were included in order to  
489 account for attrition. Dogs were randomized in blocks of 9 (3 per group) by the NC State  
490 pharmacy using a random number generator. Investigators and owners were blinded to  
491 treatment identity until data analysis was complete. The timeline of participation is provided in  
492 Supplementary Figure S8.

493

#### 494 **Inclusion and exclusion criteria**

495 In order to participate, dogs had to be greater than or equal to 10 years of age, weigh more than  
496 8kg, and be able to walk independently with sufficient hearing and vision to be able to perform  
497 cognitive tests. They had to be treat-motivated, willing to engage with the investigators, and  
498 show signs of mild to moderate cognitive impairment (based on CADES score). Owners had to  
499 be able to complete online questionnaires, administer the supplements, keep an activity monitor  
500 on their dogs for two-week periods and attend scheduled appointments.

501 Dogs with comorbidities likely to significantly adversely affect health over the course of the  
502 clinical trial were excluded. Examples include metastatic neoplasia, hyperadrenocorticism,  
503 diabetes mellitus, congestive heart failure, and refractory epilepsy. Aggressive dogs were  
504 excluded as they could not safely perform the cognitive tests. Dogs with evidence of an active  
505 urinary tract infection (clinical signs, bacteriuria and pyuria) were excluded until treated.

506

#### 507 **Intervention**

508 The investigational veterinary product (IVP), LY-D6/2 was a proprietary combination of a  
509 senolytic and an NAD+ precursor. Dogs were administered placebo, low or full dose LY-D6/2  
510 starting the day following Day 0 assessment. The doses were determined in pharmacokinetic  
511 and safety studies performed by Animal Bioscience. Owners were instructed to administer the  
512 NAD+ precursor (or placebo) capsule(s) once daily and the senolytic (or placebo) capsule(s) on  
513 two consecutive days each month. Owners were given customized calendars to record  
514 administration of the capsules throughout the study. Both these calendars and capsule counts  
515 (checked at each visit) were utilized to determine compliance.

516

#### 517 **Recruitment and Eligibility**

518 Dogs were recruited from the local community through postings on the NC State CVM clinical trials  
519 website and social media from January 2022 through June 2023. Dogs were initially screened via an  
520 online survey platform (Qualtrics) and those meeting age ( $\geq 10$  years), weight ( $\geq 8$  kgs) and  
521 cognitive status (CADES category mild or moderate) requirements proceeded to a telephone  
522 interview to confirm cognitive changes were age-related and that the owner understood the  
523 clinical trial design and commitments. Eligible participants proceeded to an in-person screening  
524 at the NC State College of Veterinary Medicine. Owners reviewed and signed informed consent  
525 forms at this time. The screening visit included physical examination, lab work (CBC and serum  
526 biochemistry panel) and behavioral assessment to confirm eligibility prior to enrollment. A PAM  
527 (WGT3X-BT monitor, Actigraph, Pensacola, FL) was placed on the dog's collar and the owners  
528 were provided with a log to record times where the collar/PAM was removed and/or changes

529 from routine activity. The appointment for the first day of the trial was scheduled two weeks after  
530 the screening appointment in order to allow for adequate PAM data collection prior to beginning  
531 treatment (Supplementary Figure S8). If a UTI was detected at screening, dogs were treated  
532 with an appropriate antibiotic by their primary veterinarian and had to have a clear urinalysis  
533 prior to entering the clinical trial.

534

### 535 **Data collection**

536 Study data were collected and managed using REDCap electronic data capture tools hosted at  
537 NC State University [82,83].

538

539 Questionnaires were distributed to owners via REDCAP( <https://www.project-redcap.org/>) seven  
540 days prior to each appointment. Basic signalment and medical history were obtained. Owners  
541 were also instructed to indicate any changes in pet health, medication, environment and  
542 attitude. RedCap questionnaires included CCDR and frailty phenotype. The CCDR  
543 Questionnaire consists of 13 behavioral items related to orientation, memory, apathy, olfaction  
544 and locomotion. (Supplementary Figure S6). Scores from each item are summed (16-80),  
545 where a score of 50 or greater indicates CCDS[80]. The frailty phenotype screening tool  
546 assesses age-related deficits in mobility, exhaustion, social activity, muscle condition, and  
547 nutritional status and predicts six-month mortality[78]. Criteria have been established to classify  
548 an individual as impaired in each of these five domains and the individual was classified as frail  
549 if criteria are met for three or more of these domains. (Supplementary Figure S6) These data  
550 were expressed both as frail, yes or no and as scores from 0-5 (number of impaired domains).  
551 In addition to chronological age, fractional lifespan was also calculated for each dog using an  
552 equation that incorporates dog height and weight [84]. In order to evaluate the owner's  
553 perception of efficacy, each was asked to categorize their dog's mobility and their level of  
554 happiness as improved, the same or worse at months three and six.

555

556 Dogs underwent a physical, neurological and orthopedic examination (Supplementary Figure  
557 S6). Joint and spinal pain was quantified using an established scale, where scores are  
558 summated to give a score from 0-76 [85,86]. Off leash gait speed over a five-meter distance  
559 was measured in triplicate and the mean value was calculated [30]. Cognitive testing included  
560 the sustained gaze task and two tests of executive function (cylinder and detour tasks). In the  
561 sustained gaze task, dogs are asked to focus on a treat held by the handler's face and the time  
562 they maintain the gaze is recorded, performed in triplicate for calculation of the mean value in  
563 seconds [21]. The cylinder and detour tasks both generate outcomes as a percentage of  
564 correct choices in a set of trials (n=8). All tasks are described in detail elsewhere [79]. All  
565 findings from examinations, mobility tests and cognitive tests were recorded in the REDCap  
566 database.

567

568 These assessments were performed at time 0, and at one, three and six months. Blood work  
569 and urinalysis was repeated at one and six months, and a urine dipstick test was performed at  
570 three months. All lab work performed during the study are provided in Supplementary Table S9.  
571 The PAM device was placed for two weeks at the screening appointment and again at months  
572 one, three and six. It was removed after two weeks and returned to the research group along

573 with the owner log that recorded collar removal or unusual activity. Data were downloaded for  
574 analysis after each time period using ActiLife software (version 6.13.3; Actigraph, Pensacola,  
575 FL) All raw activity data are provided in Supplementary Table S10.

576  
577 Changes in the dog's environment (e.g. address change, addition of another pet or other  
578 household changes) were categorized as present or absent at one, three and six months as  
579 were changes in medications the dogs were receiving. Adverse events (changes in blood work  
580 and/or new clinical signs) were categorized as grade 1 through 5 according to the VCOG-  
581 CTCAEv2 guidelines [87]. Those classified as grade 2 or above were reviewed by the safety  
582 monitor and the treatment mask broken if considered appropriate.

583

#### 584 **Statistical analysis**

585 All statistical analyses were performed with patient and group identity masked. Analyses were  
586 performed using JMP Pro 16 (SAS Institute, Cary, NC). Summary statistics were generated for  
587 dog demographics in each group at time of study entry. Continuous data were reported as mean  
588 and standard deviation (SD) or median and range depending on data distribution; categorical  
589 and ordinal data were reported as population proportion. Data were examined for normality  
590 based on inspection on Q-Q plots, histogram and Shapiro-Wilks test. Baseline demographic  
591 differences between groups were assessed using one-way ANOVA or Wilcoxon Rank Sum test  
592 (based on data normality) for continuous data. For ordinal/categorical data (Sex, BCS, Frailty  
593 Status [Y/N], Frailty Score) contingency tables were constructed and a chi-square test was  
594 performed with Fisher Exact test performed if needed. Changes in household and changes in  
595 medication were assessed in a similar manner, utilizing a chi-square test to assess significance  
596 ( $p<0.05$ ).

597

598 Changes in our owner-based assessments (CCDR and frailty phenotype) from time zero to the  
599 primary endpoint (month three) were categorized as success (unchanged or decreased score)  
600 or failure (increased score). These categorical changes in CCDR and frailty were compared  
601 between groups by constructing contingency tables and performing a chi-square test. I.

602

603 Changes in CCDR score, cognitive testing performance and off-leash gait speed over the study  
604 were compared between groups with repeated measures ANOVA. Repeated measures ANOVA  
605 requires approximately normal distribution, so the residuals of these data within the model were  
606 assessed for distribution and outliers based on inspection of Q-Q plots and histogram. To  
607 account for covariates that might influence outcomes in senior dogs, univariate analyses of age,  
608 fractional lifespan, weight, sex (M or F), BCS, baseline frailty status (frail in 3 domains, yes or  
609 no), change in household and change in medication was performed for each of the outcome  
610 measures. Those covariates that reached significance of  $p<0.1$  were incorporated into the  
611 repeated measures ANOVA model for that outcome in addition to treatment effect. Overall  
612 differences between groups over time were assessed via wilk's lambda using a threshold of  
613  $p<0.05$ .

614

615 In order to assess whether any treatment effect was maintained from month three to month six,  
616 changes in individual CCDR, Frailty, cognitive testing and off leash gait speed were calculated

617 (month 6 - month 3) and compared across groups. Changes were assessed via a Wilcoxon test,  
618 with p<0.05 indicating significance. Owner's perception of mobility and happiness level  
619 (categorized as improved, static or worse) were evaluated at months three and six via a chi-  
620 square test  
621  
622 Activity data for weekdays and weekends were analyzed separately given the impact of owner  
623 schedule on dogs' activity [16,74]. Data underwent quality control to identify the first five  
624 weekdays and two weekend days with complete data. Data from days that owners reported  
625 removal of the collar or unusual activity and days that included more than three consecutive  
626 hours with no (zero) activity detected were excluded. The mean activity per minute was  
627 calculated from five weekdays and from two weekend days and the change in mean  
628 activity/minute was calculated for each individual dog between study start and the primary end  
629 point at month three. The change between three and six months was also calculated. Baseline  
630 and change in activity were analyzed using functional linear modeling (FLM) in order to facilitate  
631 modeling of activity data over time, thus avoiding the data loss that occurs when periods of time  
632 are averaged [16,81]. This was performed using RStudio 2021 (PBC, Boston, Ma) package  
633 "Actigraphy" version 1.4.0, which uses a Fourier expansion model to transform raw data into  
634 smoothed activity curves. Differences between groups were evaluated using a non-parametric  
635 permutation F-test with 1000 permutations performed. This generates a curve of the F-statistic  
636 over time. Point-wise (a curve with the F permutation proportion at each time point) and global  
637 (single number referring to the proportion of maximized F values from each permutation) critical  
638 F values are calculated to set thresholds for statistical significance [88].  
639 The FLM analysis only allows the incorporation of a single covariate (treatment group) and  
640 therefore cannot account for other potential confounding influences that we predict exist in the  
641 senior pet population. For this reason, we also analyzed total day time (5:00am - 10:59pm) and  
642 night time (11:00pm-4:59am) activity in a repeated measures analysis (similar to the rest of the  
643 outcome measures). Covariates (age, fractional lifespan, weight, sex, BCS, frailty status,  
644 change in household and change in medication) were incorporated if they reached significance  
645 (p<0.1) in a univariate analysis. As with the FLM analysis, weekday and weekend data were  
646 assessed separately and wilks lambda values were assessed for significance (p<0.05). Change  
647 in cumulative activity during days and nights on weekdays and weekends across groups was  
648 assessed via a Wilcoxon test with p<0.05 representing significance.  
649

## 650 **References**

- 651  
652 1. Selman, C., Nussey, D.H. & Monaghan, P. Ageing: it's a dog's life. *Current Biology*. **23**,  
653 PR451-R453 (2013).  
654 2. Lewis, T. W., Wiles, B. M., Llewellyn-Zaidi, A. M., Evans, K. M., & O'Neill, D. G. Longevity  
655 and mortality in Kennel Club registered dog breeds in the UK in 2014. *Canine genetics and*  
656 *epidemiology*. **5**, 1-17 (2018).  
657 3. Sarver, A.L., Makielski, K.M., DePauw, T.A., Schulte, A.J. & Modiano, J.F. Increased risk of  
658 cancer in dogs and humans: A consequence of recent extension of lifespan beyond  
659 evolutionarily determined limitations? *Aging and Cancer*. **3**, 3-19 (2022).

- 660 4. Creevy, K. E., Austad, S. N., Hoffman, J. M., O'Neill, D. G., & Promislow, D. E. The  
661 Companion Dog as a Model for the Longevity Dividend. *Cold Spring Harbor perspectives in*  
662 *medicine*. **6**, a026633 (2016).
- 663 5. Jin, K., Hoffman, J. M., Creevy, K. E., O'Neill, D. G., & Promislow, D. E. Multiple morbidities in  
664 companion dogs: a novel model for investigating age-related disease. *Pathobiology of aging &*  
665 *age related diseases*. **6**, 33276 (2016)
- 666 6. Mazzatorta, A., Carluccio, A., Robbe, D., Giulio, C. D., & Cellerino, A. The companion dog as  
667 a unique translational model for aging. *Seminars in cell & developmental biology*. **70**, 141–153  
668 (2017).
- 669 7. McKenzie, B. A., Chen, F. L., Gruen, M. E., & Olby, N. J. Canine Geriatric Syndrome: A  
670 Framework for Advancing Research in Veterinary Geroscience. *Frontiers in veterinary science*.  
671 **9**, 853743 (2022).
- 672 8. Ruple, A., MacLean, E., Snyder-Mackler, N., Creevy, K. E., & Promislow, D. Dog Models of  
673 Aging. *Annual review of animal biosciences*. **10**, 419–439 (2022).
- 674 9. Salt, C., Saito, E. K., O'Flynn, C., & Allaway, D. Stratification of Companion Animal Life  
675 Stages from Electronic Medical Record Diagnosis Data. *The journals of gerontology: Series A*.  
676 **78**, 579–586. (2023).
- 677 10. Hoffman, J. M., Creevy, K. E., Franks, A., O'Neill, D. G., & Promislow, D. E. L. The  
678 companion dog as a model for human aging and mortality. *Aging cell*. **17**, e12737 (2018).
- 679 11. Dias-Pereira P. Morbidity and mortality in elderly dogs - a model for human aging. *BMC*  
680 *veterinary research*. **18**, 457 (2022).
- 681 12. Inouye, S. K., Studenski, S., Tinetti, M. E., & Kuchel, G. A. Geriatric syndromes: clinical,  
682 research, and policy implications of a core geriatric concept. *Journal of the American Geriatrics*  
683 *Society*. **55**, 780–791 (2007).
- 684 13. Ferrucci, L., Cooper, R., Shardell, M., Simonsick, E. M., Schrack, J. A., & Kuh, D. Age-  
685 Related Change in Mobility: Perspectives From Life Course Epidemiology and Geroscience.  
686 *The journals of gerontology: Series A*. **71**, 1184–1194 (2016).
- 687 14. Demnitz, N. et al. Cognition and mobility show a global association in middle- and late-  
688 adulthood: Analyses from the Canadian Longitudinal Study on Aging. *Gait & posture*, **64**, 238–  
689 243 (2018).
- 690 15. Bray, E. E. et al. Associations between physical activity and cognitive dysfunction in older  
691 companion dogs: results from the Dog Aging Project. *GeroScience*, **45**, 645–661 (2023).
- 692 16. Mondino, A. et al. Activity patterns are associated with fractional lifespan, memory, and gait  
693 speed in aged dogs. *Scientific reports*. **13**, 2588 (2023).
- 694 17. Casagrande, M., Forte, G., Favieri, F., & Corbo, I. Sleep Quality and Aging: A Systematic  
695 Review on Healthy Older People, Mild Cognitive Impairment and Alzheimer's Disease.  
696 *International journal of environmental research and public health*. **19**, 8457 (2022).
- 697 18. Tapp, P. D. et al. Frontal lobe volume, function, and beta-amyloid pathology in a canine  
698 model of aging. *The Journal of neuroscience*. **24**, 8205–8213 (2004).
- 699 19. Kirova, A. M., Bays, R. B., & Lagalwar, S. Working memory and executive function decline  
700 across normal aging, mild cognitive impairment, and Alzheimer's disease. *BioMed research*  
701 *international*. **2015**, 748212 (2015).
- 702 20. Commodari, E., & Guarnera, M. Attention and aging. *Aging clinical and experimental*  
703 *research*. **20**, 578–584 (2008).

- 704 21. Hoel, J. A. et al. Sustained Gaze Is a Reliable In-home Test of Attention for Aging Pet Dogs.  
705 *Frontiers in veterinary science*. **8**, 819135 (2021).
- 706 22. Piolatto, M., Bianchi, F., Rota, M., Marengoni, A., Akbaritabar, A., & Squazzoni, F. The  
707 effect of social relationships on cognitive decline in older adults: an updated systematic review  
708 and meta-analysis of longitudinal cohort studies. *BMC public health*. **22**, 278 (2022).
- 709 23. Chapagain, D., Wallis, L. J., Range, F., Affenzeller, N., Serra, J., & Virányi, Z. Behavioural  
710 and cognitive changes in aged pet dogs: No effects of an enriched diet and lifelong training.  
711 *PLoS one*. **15**, e0238517 (2020).
- 712 24. Xia, X., Jiang, Q., McDermott, J., & Han, J. J. Aging and Alzheimer's disease: Comparison  
713 and associations from molecular to system level. *Aging cell*. **17**, e12802 (2018).
- 714 25. Cummings, B. J., Head, E., Ruehl, W., Milgram, N. W., & Cotman, C. W. The canine as an  
715 animal model of human aging and dementia. *Neurobiology of aging*. **17**, 259–268 (1996).
- 716 26. Cotman, C. W., & Head, E. The canine (dog) model of human aging and disease: dietary,  
717 environmental and immunotherapy approaches. *Journal of Alzheimer's disease*. **15**, 685–707  
718 (2008).
- 719 27. Chapagain, D., Range, F., Huber, L., & Virányi, Z. Cognitive Aging in Dogs. *Gerontology*.  
720 **64**, 165–171 (2018).
- 721 28. Ehrenzweig, J., & Hunter, R. P. Canine cognitive decline and Alzheimer disease: clinical  
722 insights to solve a shared one-health problem. *Journal of the American Veterinary Medical  
723 Association*. **261**, 1–8 (2023).
- 724 29. Studenski, S. et al. Gait speed and survival in older adults. *JAMA*. **305**, 50–58 (2011).
- 725 30. Mondino, A. et al. Winning the race with aging: age-related changes in gait speed and its  
726 association with cognitive performance in dogs. *Frontiers in veterinary science*. **10**, 1150590  
727 (2023).
- 728 31. Dao, T. et al. Sarcopenia and Muscle Aging: A Brief Overview. *Endocrinology and  
729 metabolism*. **35**, 716–732 (2020).
- 730 32. Yarmohammadi, S., Mozafar Saadati, H., Ghaffari, M., & Ramezankhani, A. A systematic  
731 review of barriers and motivators to physical activity in elderly adults in Iran and worldwide.  
732 *Epidemiology and health*. **41**, e2019049 (2019).
- 733 33. Loeser, R. F., Collins, J. A., & Diekman, B. O. Ageing and the pathogenesis of osteoarthritis.  
734 *Nature reviews. Rheumatology*. **12**, 412–420 (2016).
- 735 34. Ge, L., & Mordiffi, S. Z. Factors Associated With Higher Caregiver Burden Among Family  
736 Caregivers of Elderly Cancer Patients: A Systematic Review. *Cancer nursing*. **40**, 471–478  
737 (2017).
- 738 35. McKenzie, B. A., Chen, F., & LaCroix-Fralish, M. L. The phenotype of aging in the dog: how  
739 aging impacts the health and well-being of dogs and their caregivers. *Journal of the American  
740 Veterinary Medical Association*. **260**, 963–970 (2022).
- 741 36. Spitznagel, M. B. et al. Relationships among owner consideration of euthanasia, caregiver  
742 burden, and treatment satisfaction in canine osteoarthritis. *Veterinary journal*. **286**, 105868  
743 (2022).
- 744 37. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. Hallmarks of aging:  
745 An expanding universe. *Cell*. **186**, 243–278 (2023).
- 746 38. Li, Z. et al. Aging and age-related diseases: from mechanisms to therapeutic strategies.  
747 *Biogerontology*. **22**, 165–187 (2021).

- 748 39. Di Micco, R., Krizhanovsky, V., Baker, D., & d'Adda di Fagagna, F. Cellular senescence in  
749 ageing: from mechanisms to therapeutic opportunities. *Nature reviews. Molecular cell biology*.  
750 **22**, 75–95 (2021).
- 751 40. Zhang, L., Pitcher, L. E., Prahalad, V., Niedernhofer, L. J., & Robbins, P. D. Targeting  
752 cellular senescence with senotherapeutics: senolytics and senomorphics. *The FEBS journal*.  
753 **290**, 1362–1383 (2023).
- 754 41. Covarrubias, A.J. et al. Senescent cells promote tissue NAD<sup>+</sup> decline during ageing via the  
755 activation of CD38<sup>+</sup> macrophages. *Nature metabolism*. **2**, 1265–1283 (2020).
- 756 42. Kirkland, J. L., & Tchkonia, T. Senolytic drugs: from discovery to translation. *Journal of  
757 internal medicine*. **288**, 518–536 (2020).
- 758 43. Xu, M. et al. Senolytics improve physical function and increase lifespan in old age. *Nature  
759 medicine*, **24**, 1246–1256 (2018).
- 760 44. Yousefzadeh, M. J. et al. Fisetin is a senotherapeutic that extends health and lifespan.  
761 *EBioMedicin*. **36**, 18–28 (2018).
- 762 45. Chaib, S., Tchkonia, T., & Kirkland, J. L. Cellular senescence and senolytics: the path to the  
763 clinic. *Nature medicine*. **28**, 1556–1568 (2022).
- 764 46. Katsyuba, E., Romani, M., Hofer, D., & Auwerx, J. NAD<sup>+</sup> homeostasis in health and  
765 disease. *Nature metabolism*. **2**, 9–31 (2020).
- 766 47. Chu, X., & Raju, R. P. Regulation of NAD<sup>+</sup> metabolism in aging and disease. *Metabolism:  
767 clinical and experimental*. **126**, 154923 (2022).
- 768 48. Lautrup, S., Sinclair, D. A., Mattson, M. P., & Fang, E. F. NAD<sup>+</sup> in Brain Aging and  
769 Neurodegenerative Disorders. *Cell metabolism*. **30**, 630–655 (2019).
- 770 49. Xie, N. et al. NAD<sup>+</sup> metabolism: pathophysiologic mechanisms and therapeutic potential.  
771 *Signal transduction and targeted therapy*. **5**, 227 (2020).
- 772 50. Nadeeshani, H., Li, J., Ying, T., Zhang, B., & Lu, J. Nicotinamide mononucleotide (NMN) as  
773 an anti-aging health product - Promises and safety concerns. *Journal of advanced research*. **37**,  
774 267–278 (2021).
- 775 51. Rajman, L., Chwalek, K., & Sinclair, D. A. Therapeutic Potential of NAD-Boosting Molecules:  
776 The In Vivo Evidence. *Cell metabolism*. **27**, 529–547 (2018).
- 777 52. Yoshino, J., Baur, J. A., & Imai, S. I. NAD<sup>+</sup> Intermediates: The Biology and Therapeutic  
778 Potential of NMN and NR. *Cell metabolism*. **27**, 513–528 (2018).
- 779 53. Song, Q., Zhou, X., Xu, K., Liu, S., Zhu, X., & Yang, J. The Safety and Antiaging Effects of  
780 Nicotinamide Mononucleotide in Human Clinical Trials: an Update. *Advances in nutrition*. **14**,  
781 1416–1435. (2023).
- 782 54. Cardoso, D., Barthélémy, I., Blot, S., & Muchir, A. Replenishing NAD<sup>+</sup> content reduces  
783 aspects of striated muscle disease in a dog model of Duchenne muscular dystrophy. *Skeletal  
784 muscle*. **13**, 20 (2023).
- 785 55. Sharma, A., Chabloz, S., Lapidès, R. A., Roïder, E., & Ewald, C. Y. Potential Synergistic  
786 Supplementation of NAD<sup>+</sup> Promoting Compounds as a Strategy for Increasing Healthspan.  
787 *Nutrients*. **15**, 445 (2023).
- 788 56. Tapp, P. D. et al. Size and reversal learning in the beagle dog as a measure of executive  
789 function and inhibitory control in aging. *Learning & memory*. **10**, 64–73 (2003).

- 790 57. Guarino, A., Forte, G., Giovannoli, J., & Casagrande, M. Executive functions in the elderly  
791 with mild cognitive impairment: a systematic review on motor and cognitive inhibition, conflict  
792 control and cognitive flexibility. *Aging & mental health*. **24**, 1028–1045 (2020).
- 793 58. Dolopikou, C. F. et al. Acute nicotinamide riboside supplementation improves redox  
794 homeostasis and exercise performance in old individuals: a double-blind cross-over study.  
795 *European journal of nutrition*. **59**, 505–515 (2020).
- 796 59. Niu, K. M. et al. The Impacts of Short-Term NMN Supplementation on Serum Metabolism,  
797 Fecal Microbiota, and Telomere Length in Pre-Aging Phase. *Frontiers in nutrition*. **8**, 756243  
798 (2021).
- 799 60. Huang H. A Multicentre, Randomised, Double Blind, Parallel Design, Placebo Controlled  
800 Study to Evaluate the Efficacy and Safety of Uthever (NMN Supplement), an Orally  
801 Administered Supplementation in Middle Aged and Older Adults. *Frontiers in aging*. **3**, 851698  
802 (2022).
- 803 61. Chini, C. C. S., Cordeiro, H. S., Tran, N. L. K., & Chini, E. N. NAD metabolism: Role in  
804 senescence regulation and aging. *Aging cell*. **23**, e13920 (2024).
- 805 62. Madari, A. et al. Assessment of severity and progression of canine cognitive dysfunction  
806 syndrome using the CAnine DEmentia Scale (CADES). *Applied Animal Behaviour Science*. **171**,  
807 138–145 (2015).
- 808 63. Schwartz, D., & Lellouch, J. Explanatory and pragmatic attitudes in therapeutical trials.  
809 *Journal of chronic diseases*. **20**, 637–648 (1967).
- 810 64. Ford, I., & Norrie, J. Pragmatic Trials. *The New England journal of medicine*. **375**, 454–463  
811 (2016).
- 812 65. Neilson, J. C., Hart, B. L., Cliff, K. D., & Ruehl, W. W. Prevalence of behavioral changes  
813 associated with age-related cognitive impairment in dogs. *Journal of the American Veterinary  
814 Medical Association*. **218**, 1787–1791 (2001).
- 815 66. Azkona, G., García-Belenguer, S., Chacón, G., Rosado, B., León, M., & Palacio, J.  
816 Prevalence and risk factors of behavioural changes associated with age-related cognitive  
817 impairment in geriatric dogs. *The Journal of small animal practice*. **50**, 87–91 (2009).
- 818 67. Yarborough, S., Fitzpatrick, A., Schwartz, S. M., & Dog Aging Project Consortium.  
819 Evaluation of cognitive function in the Dog Aging Project: associations with baseline canine  
820 characteristics. *Scientific reports*. **12**, 13316 (2022).
- 821 68. Conzemius, M. G., & Evans, R. B. Caregiver placebo effect for dogs with lameness from  
822 osteoarthritis. *Journal of the American Veterinary Medical Association*. **241**, 1314–1319 (2012).
- 823 69. Milgram, N. W. et al. Learning ability in aged beagle dogs is preserved by behavioral  
824 enrichment and dietary fortification: a two-year longitudinal study. *Neurobiology of aging*. **26**,  
825 77–90 (2005).
- 826 70. Pop, V. et al. Synergistic effects of long-term antioxidant diet and behavioral enrichment on  
827 beta-amyloid load and non-amyloidogenic processing in aged canines. *The Journal of  
828 neuroscience*. **30**, 9831–9839 (2010).
- 829 71. Fahnestock, M. et al. BDNF increases with behavioral enrichment and an antioxidant diet in  
830 the aged dog. *Neurobiology of aging*. **33**, 546–554 (2012).

- 831 72. Degeling, C., Burton, L., & McCormack, G. R. An investigation of the association between  
832 socio-demographic factors, dog-exercise requirements, and the amount of walking dogs  
833 receive. *Canadian journal of veterinary research*. **76**, 235–240 (2012).
- 834 73. Lee, H. S., Song, J. G., & Lee, J. Y. Influences of Dog Attachment and Dog Walking on  
835 Reducing Loneliness during the COVID-19 Pandemic in Korea. *Animals*. **12**, 483 (2022).
- 836 74. Dow, C., Michel, K. E., Love, M., & Brown, D. C. Evaluation of optimal sampling interval for  
837 activity monitoring in companion dogs. *American journal of veterinary research*. **70**, 444–448  
838 (2009).
- 839 75. Hua, J. et al. Assessment of frailty in aged dogs. *American journal of veterinary research*.  
840 **77**, 1357–1365 (2016).
- 841 76. Banzato, T. et al. A Frailty Index based on clinical data to quantify mortality risk in dogs.  
842 *Scientific reports*. **9**, 16749. (2019).
- 843 77. Melvin, R. L., Ruple, A., Pearson, E. B., Olby, N. J., Fitzpatrick, A. L., & Creevy, K. E. A  
844 review of frailty instruments in human medicine and proposal of a frailty instrument for dogs.  
845 *Frontiers in veterinary science*. **10**, 1139308 (2023).
- 846 78. Russell, K. Establishing a Frailty Phenotype for Aging Dogs. Oral Presentation. ACVIM  
847 Forum. (2022).
- 848 79. Fefer, G. et al. Use of Cognitive Testing, Questionnaires, and Plasma Biomarkers to  
849 Quantify Cognitive Impairment in an Aging Pet Dog Population. *Journal of Alzheimer's disease*.  
850 **87**, 1367–1378 (2022).
- 851 80. Salvin, H. E., McGreevy, P. D., Sachdev, P. S., & Valenzuela, M. J. The canine cognitive  
852 dysfunction rating scale (CCDR): a data-driven and ecologically relevant assessment tool.  
853 *Veterinary journal*. **188**, 331–336 (2011).
- 854 81. Woods, H. J., Li, M. F., Patel, U. A., Lascelles, B. D. X., Samson, D. R., & Gruen, M. E. A  
855 functional linear modeling approach to sleep-wake cycles in dogs. *Scientific reports*. **10**, 22233  
856 (2020).
- 857 82. Harris, P.A., Taylor, R., Thielke, R., Payne, J., Gonzalez, N., Conde, J.G. Research  
858 electronic data capture (REDCap) – A metadata-driven methodology and workflow process for  
859 providing translational research informatics support, *Journal of biomedical informatics*. **42**, 377–  
860 81 (2009).
- 861 83. Harris, P. A. et al. The REDCap consortium: Building an international community of software  
862 platform partners. *Journal of biomedical informatics*. **95**, 103208 (2019).
- 863 84. Greer, K.A., Canterbury, S.C. & Murphy, K.E. Statistical analysis regarding the effects of  
864 height and weight on life span of the domestic dog. *Research in Veterinary Science*. **82**, 208–14  
865 (2007).
- 866 85. Knazovicky, D., Helgeson, E. S., Case, B., Gruen, M. E., Maixner, W., & Lascelles, B. D. X.  
867 Widespread somatosensory sensitivity in naturally occurring canine model of osteoarthritis.  
868 *Pain*, **157**, 1325–1332. (2016).
- 869 86. Mondino, A. et al. Static posturography as a novel measure of the effects of aging on  
870 postural control in dogs. *PloS one*. **17**, e0268390. (2022).
- 871 87. LeBlanc, A. K. et al. Veterinary Cooperative Oncology Group-Common Terminology Criteria  
872 for Adverse Events (VCOG-CTCAE v2) following investigational therapy in dogs and cats.  
873 *Veterinary and comparative oncology*. **19**, 311–352 (2021)

874 88. Wang, J. et al. Measuring the impact of apnea and obesity on circadian activity patterns  
875 using functional linear modeling of actigraphy data. *Journal of circadian rhythms*. **9**, 11 (2011).

876

## 877 **Acknowledgements**

878 This clinical trial was funded by Animal Bioscience, Boston MA. Animal Bioscience played no  
879 role in data acquisition, analysis or presentation.

880 We would like to acknowledge all the dogs and their owners and Amanda Valentino and  
881 Katerina Slaughter for assistance with dog handling.

882

## 883 **Author Contributions**

884 KES, NJO, MEG and KR were responsible for the design, analysis, and primary writing of the  
885 manuscript for this study. KES, AM, KR, BC, CW, ZA, CY all participated in the data acquisition;  
886 all authors participated in editing and review of the manuscript.

887

## 888 **Data Availability Statement**

889 All data analyzed in this study has been provided in the supplementary data as Supplementary  
890 Data files Supplementary Tables: S5, S9 and S10.

891

## 892 **Competing Interests Statement**

893 The Authors declare no competing interests

894

## 895 **Figure Legends**

896

897 Figure 1: Participant Flowchart. Diagram featuring patient screening, treatment allocation,  
898 attrition, and exclusion throughout the trial. GDV- Gastric Dilation-Volvulus. UTI- Urinary Tract  
899 Infection.

900

901 Figure 2: Repeated Measures Analysis of CCDR Score (Adjusted for Baseline Frailty Status)  
902 Mean CCDR scores by group adjusted for individual baseline frailty status over time from  
903 repeated measures model (MANOVA analysis in JMP). Wilks' lamda value was evaluated, with  
904 p<0.05 indicating a significant difference between groups. All original outcome measure data is  
905 provided in Supplementary Table S5.

906

907 Figure 3: Baseline Activity. Baseline activity by treatment group prior to starting treatment on  
908 weekdays and weekends. The graphs (a,c) on the left illustrate average weekday/weekend  
909 activity levels over a 24-hour period. The graphs on the right (b,d) indicate the level of  
910 significance between groups. When the observed statistic (red line) is above the dashed and  
911 dotted lines (blue line), it indicates a global or pointwise significant difference between groups  
912 respectively. No significant differences were observed during weekdays. A global threshold for  
913 significance is reached around 12pm on weekends. A pointwise significant difference is  
914 observed around 12am on weekends. All original activity data is provided in Supplementary  
915 Table S10.

916

917 Figure 4: Change in activity from month 0 to month 3. Change in activity from baseline to  
918 primary endpoint on weekdays and weekends by treatment group. Activity at baseline was  
919 subtracted from activity at month three to obtain the change in activity utilized in the FLM  
920 analysis. The graphs on the left (a,c) illustrate change in weekday/weekend activity levels over a  
921 24-hour period. The graphs on the right (b,d) indicate the level of significance between groups.  
922 When the observed statistic (red line) is above the dashed and dotted blue lines, it indicates a  
923 global or pointwise significant difference between groups respectively. There was no significant  
924 difference between group activity over the weekdays. On the weekends, pointwise significance  
925 was reached at approximately 6am and 10pm. All original activity data is provided in  
926 Supplementary Table S10.

927

928

929 Figure 5: Change in activity from month 3 to month 6. Change activity from primary endpoint  
930 (month three) to secondary endpoint (month six) on weekdays and weekends by treatment  
931 group. Activity at month three was subtracted from activity at month six to obtain the change in  
932 activity utilized in the FLM analysis. The graphs on the left illustrate average change in  
933 weekday/weekend activity levels over a 24-hour period. The graphs on the right indicate the  
934 level of significance between groups. When the observed statistic (red line) is above the dashed  
935 and dotted lines (blue line), it indicates a global or pointwise significant difference between  
936 groups respectively. Pointwise significance is observed to reach threshold level on weekdays  
937 around 2am and 2pm. Weekend activity reaches the global significance level at 6am as well as  
938 a pointwise significance level around 9pm. All original activity data is provided in Supplementary  
939 Table S10.

940

941 Figure 6: Repeated measures analyses of secondary outcome measures (Cylinder Task,  
942 Detour, Sustained Gaze, Off Leash Gait Speed). Mean values (adjusted as necessary) are  
943 reported at each timepoint by group. Group scores over time were compared in a repeated  
944 measures model (MANOVA analysis in JMP). The Wilks' lamda value was evaluated with  
945 p<0.05 indicating a significant difference between groups. All original outcome measure data is  
946 provided in Supplementary Table S5.

947

948 Figure 7: Total number of adverse events (VCOG grade 2 or higher) during the trial by group.  
949 Events were reported by the owner to be new during the trial (not present during/before  
950 baseline) and separated by system. Events were summed based on the system affected. All  
951 original adverse event data is provided in Supplementary Table S7.

952

953 Supplementary Figure S3: Repeated measures analysis of summated activity monitor activity by  
954 group. Repeated measures analysis of summated activity across month 0, 1 and 3. Day (5am-  
955 10:59pm) and night (11pm-4:59am) were assessed separately, as were weekdays and  
956 weekends. Mean summated activity levels by group were obtained from repeated measures  
957 models (MANOVA analysis in JMP) with adjustment for covariates when necessary. The Wilks'  
958 lamda value was evaluated, with p<0.05 indicating a significant difference between groups. All  
959 original activity data is provided in Supplementary Table S10.

960

961      Supplementary Figure S6: Study Exam Forms and Questionnaires. Forms are displayed in the  
962      following order: physical examination form, neurological examination form, orthopedic  
963      examination form, Canine Cognitive Dysfunction Rating (CCDR) Questionnaire, Frailty  
964      Assessment, Changes since last visit.

965

966      Supplementary Figure S8: Study Timeline. The timeline of all visits in the study are provided  
967      along with all assessments performed at each visit. Activity monitors were placed at specific  
968      visits in order to obtain two week increments of activity data for each timepoint. Intervention  
969      was started the day following an individual's baseline (month zero) visit.

970

971

972

973

974

975

976

977

	Placebo (n=23)	Low Dose (n=23)	Full Dose (n=21)	P value	Fisher's Exact Test
Age (years)	Mean: 12.94 St Dev: 1.45	Mean: 12.25 St Dev: 1.42	Mean: 13.63 St Dev: 1.29	0.01	
Fractional Lifespan	Median: 1.10 Range: 0.76-1.24	Median: 1.03 Range: 0.72-1.22	Median: 1.12 Range: 0.87-1.23	0.19	
Sex	Female: 10 (43.5%) Male: 13 (56.5%)	Female: 9 (39.1%) Male: 14 (60.9%)	Female: 12 (57.1%) Male: 9 (42.9%)	0.393	
Weight (kg)	Median: 24.5 Range: 7.9-39.5	Median: 27.7 Range 6.5-45	Median: 22.9 Range: 8.3-33.2	0.25	
Body Condition Score	3/9: 1 (4.3%) 4/9: 3 (13.0%) 5/9: 13 (56.5%) 6/9: 3 (13.0%) 7/9: 3 (13.0%)	4/9: 2 (8.7%) 5/9: 13 (56.5%) 6/9: 8 (34.8%)	4/9: 5 (23.8%) 5/9: 12 (57.1%) 6/9: 2 (9.5%) 7/9: 1 (4.8%) 8/9: 1 (4.8%)	0.15	0.19
Frailty Status	Non-frail: 15 (65.2%) Frail: 8 (34.8%)	Non-frail: 12(52.2%) Frail: 11 (47.8%)	Non-frail: 11 (52.4%) Frail: 10 (47.6%)	0.59	
Frailty Score	0/5: 1 (4.3%) 1/5: 6 (26.1%) 2/5: 9 (39.1%) 3/5: 4 (17.4%) 4/5: 3 (13.0%) 5/5: 1 (4.3%)	1/5: 5 (21.7%) 2/5: 6 (26.1%) 3/5: 8 (34.8%) 4/5: 1 (4.3%) 5/5: 2 (8.7%)	1/5: 3 (14.3%) 2/5: 8 (38.1%) 3/5: 9 (42.9%) 4/5: 1 (4.8%)	0.42	0.56
Pain Score	Median: 7 Range: 0-20	Median: 5 Range: 1-15	Median: 6 Range: 2-21	0.61	
CCDR Score	Median: 38 Range: 34-46	Median: 40 Range: 34-57	Median: 39 Range: 34-58	0.28	
Inhibitory Control (Cylinder Task)	Median: 87.5 Range: 0-100	Median: 87.5 Range: 0-100	Median: 75 Range: 0-100	0.57	
Detour	Median: 37.5 Range: 0-87.5	Median: 25 Range: 0-100	Median: 12.5 Range:0-100	0.52	
Sustained Gaze (sec)	Median: 22.78 Range: 5.92-60	Median: 17.74 Range: 4.28-55.56	Median: 15.09 Range: 2.12-60	0.22	
Off Leash Gait Speed (m/s)	Median: 1.51 Range: 0.54-3.56	Median: 1.39 Range: 0.59-3.03	Median: 1.44 Range: 0.66-3.81	0.55	

978 Table 1: Population demographics and outcome measures of study participants at enrollment by  
979 group.

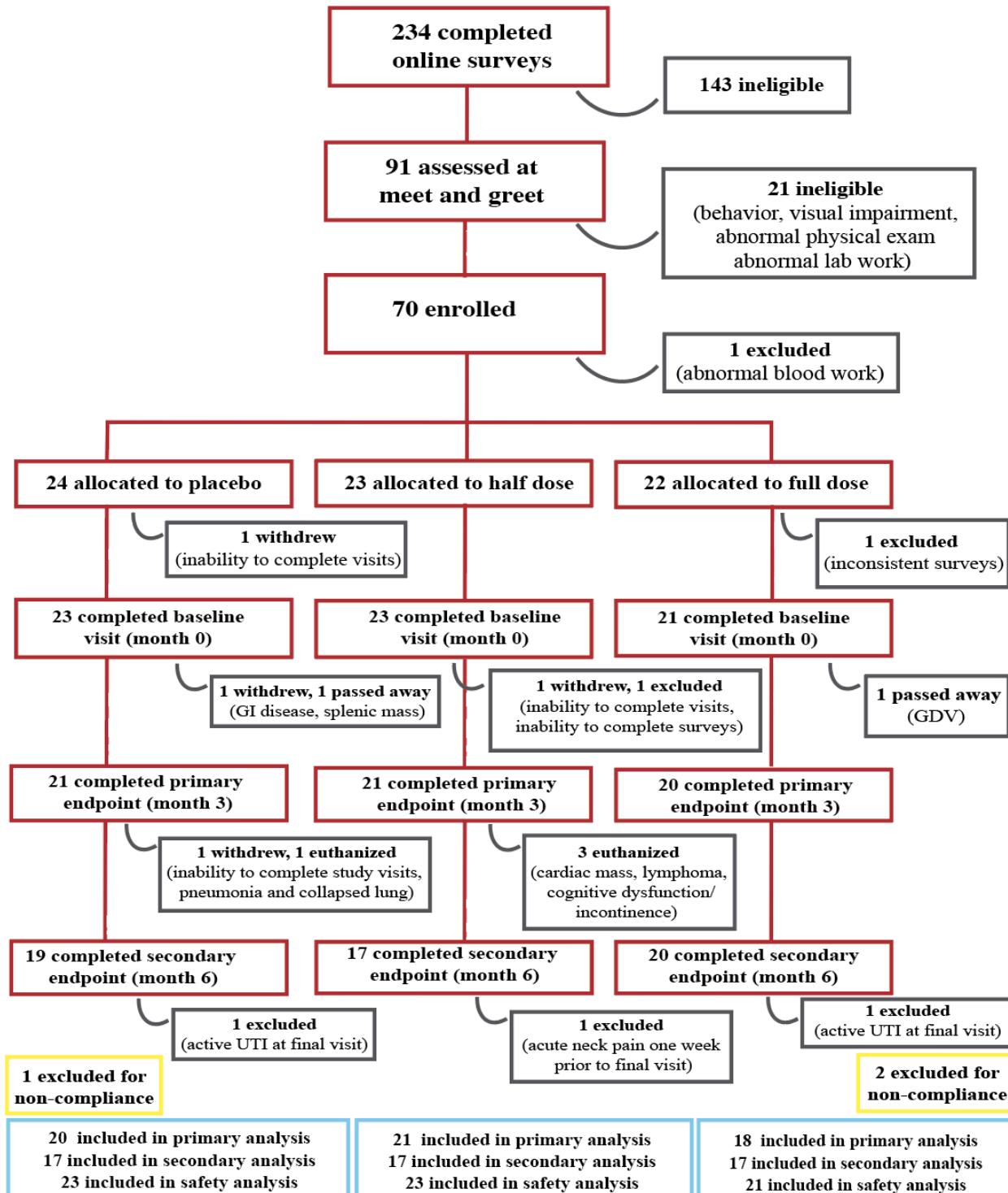


Figure 1: Participant Flowchart. Diagram featuring patient screening, treatment allocation, attrition, and exclusion throughout the trial. GDV- Gastric Dilation-Volvulus. UTI- Urinary Tract Infection.

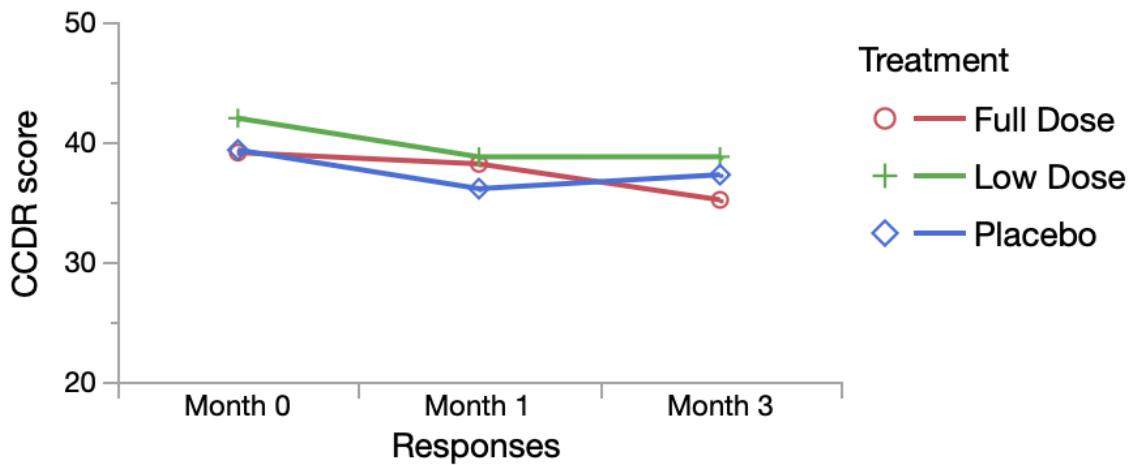


Figure 2: Repeated Measures Analysis of CCDR Score (Adjusted for Baseline Frailty Status). Mean CCDR scores by group adjusted for individual baseline frailty status over time from repeated measures model (MANOVA analysis in JMP). Wilks' lambda value was evaluated, with  $p<0.05$  indicating a significant difference between groups. All original outcome measure data is provided in Supplementary Table S5.

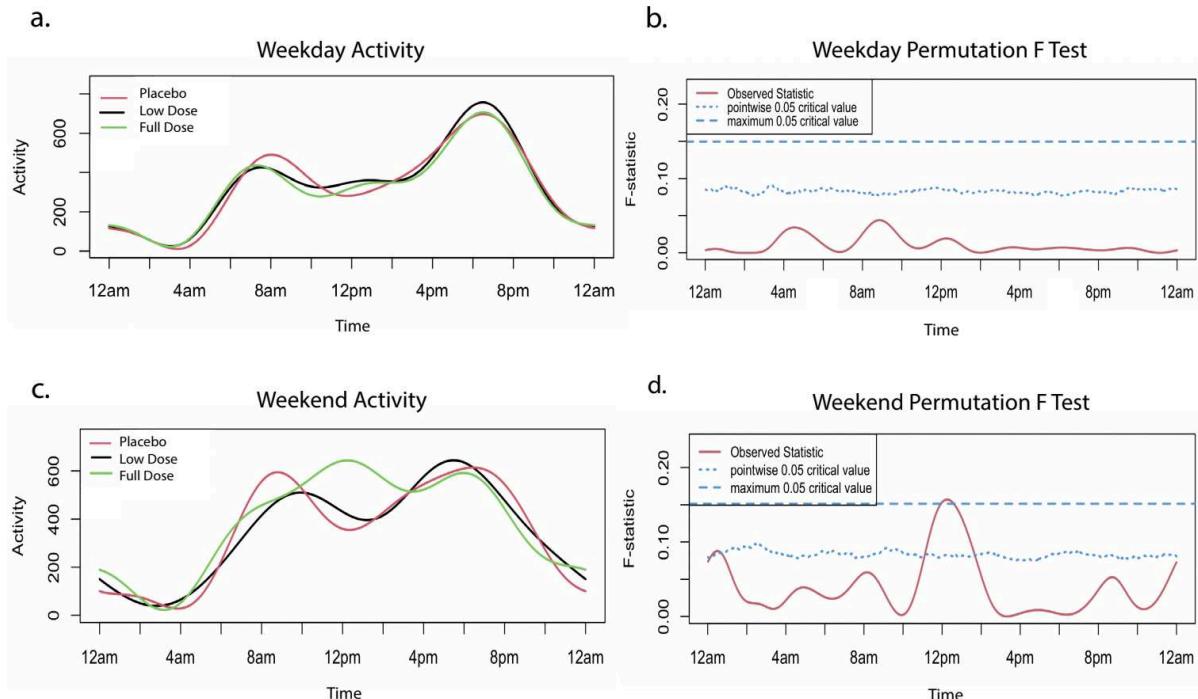


Figure 3: Baseline Activity. Baseline activity by treatment group prior to starting treatment on weekdays and weekends. The graphs (a,c) on the left illustrate average weekday/weekend activity levels over a 24-hour period. The graphs on the right (b,d) indicate the level of significance between groups. When the observed statistic (red line) is above the dashed and dotted lines (blue line), it indicates a global or pointwise significant difference between groups respectively. No significant differences were observed during weekdays. A global threshold for significance is reached around 12pm on weekends. A pointwise significant difference is observed around 12am on weekends. All original activity data is provided in Supplementary Table S10.

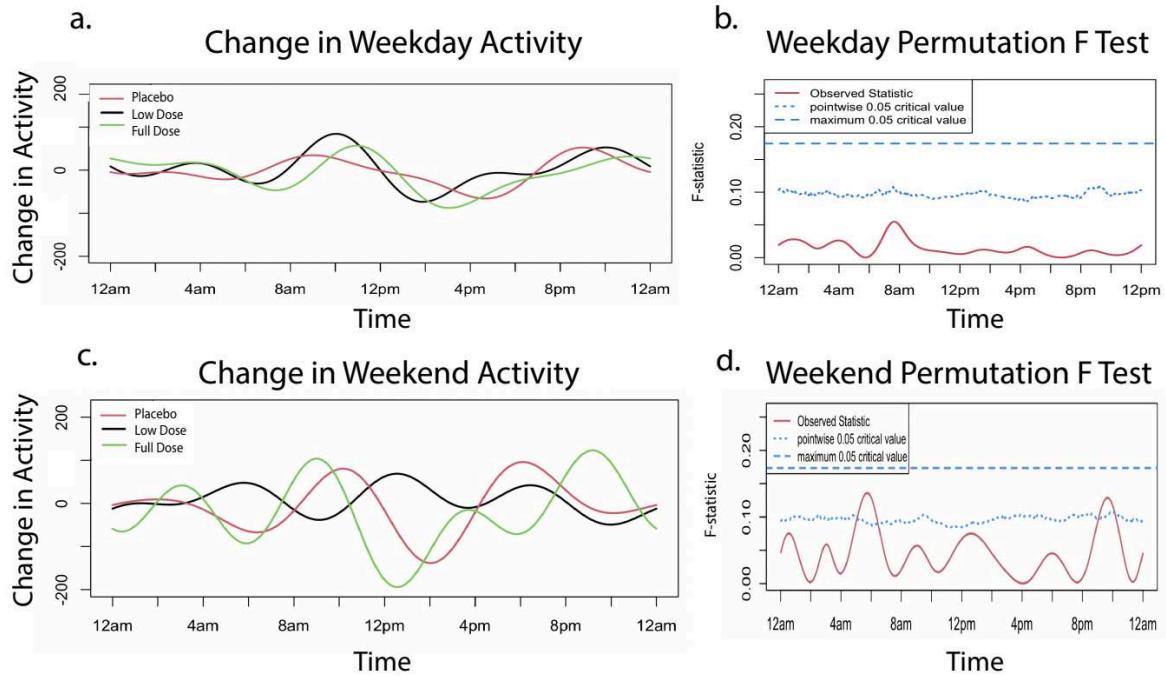


Figure 4: Change in activity from month 0 to month 3. Change in activity from baseline to primary endpoint on weekdays and weekends by treatment group. Activity at baseline was subtracted from activity at month three to obtain the change in activity utilized in the FLM analysis. The graphs on the left (a,c) illustrate change in weekday/weekend activity levels over a 24-hour period. The graphs on the right (b,d) indicate the level of significance between groups. When the observed statistic (red line) is above the dashed and dotted blue lines, it indicates a global or pointwise significant difference between groups respectively. There was no significant difference between group activity over the weekdays. On the weekends, pointwise significance was reached at approximately 6am and 10pm. All original activity data is provided in Supplementary Table S10.

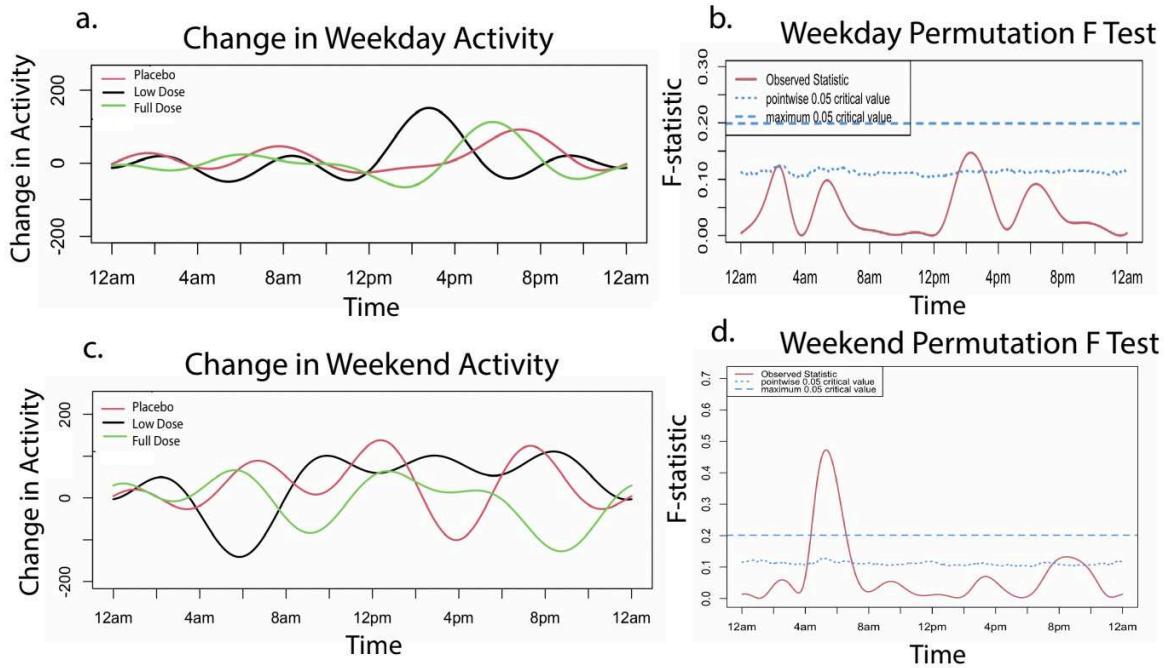


Figure 5: Change in activity from month 3 to month 6. Change activity from primary endpoint (month three) to secondary endpoint (month six) on weekdays and weekends by treatment group. Activity at month three was subtracted from activity at month six to obtain the change in activity utilized in the FLM analysis. The graphs on the left illustrate average change in weekday/weekend activity levels over a 24-hour period. The graphs on the right indicate the level of significance between groups. When the observed statistic (red line) is above the dashed and dotted lines (blue line), it indicates a global or pointwise significant difference between groups respectively. Pointwise significance is observed to reach threshold level on weekdays around 2am and 2pm. Weekend activity reaches the global significance level at 6am as well as a pointwise significance level around 9pm. All original activity data is provided in Supplementary Table S10.

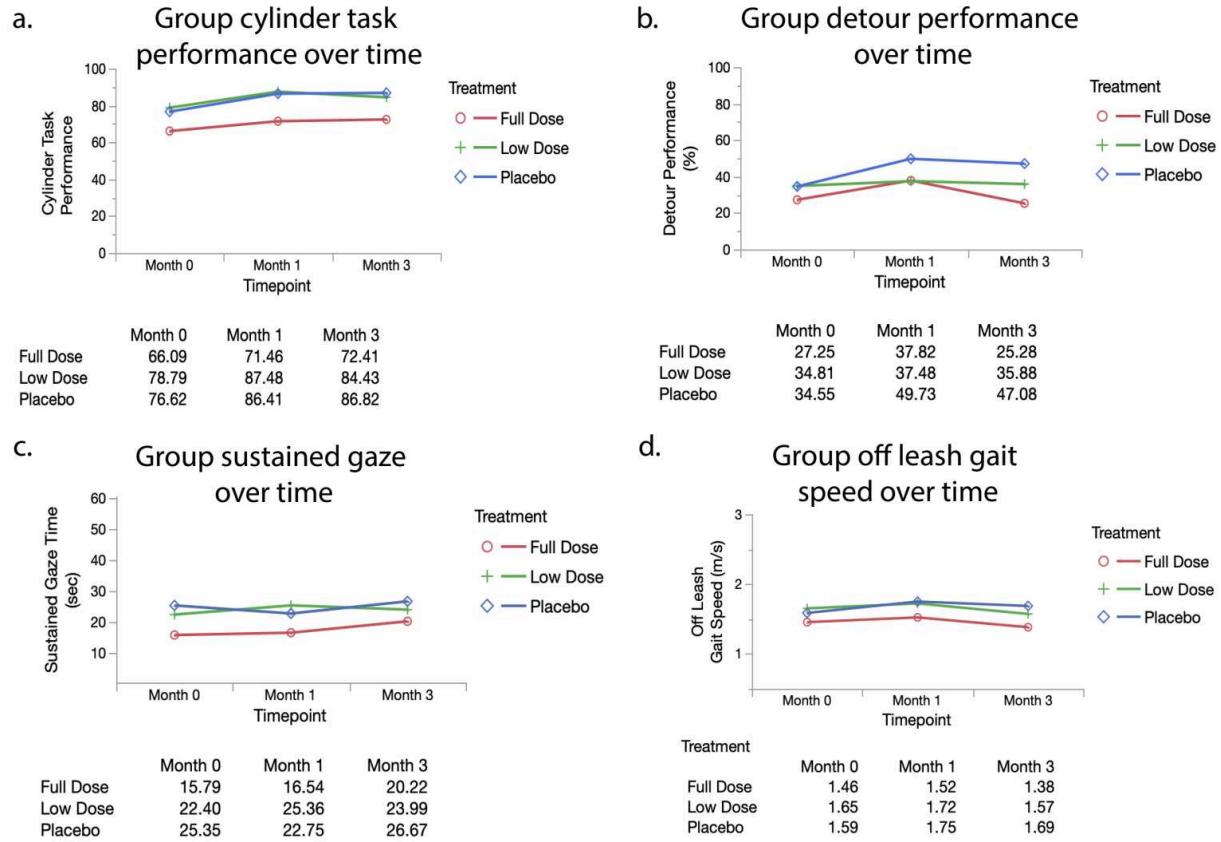


Figure 6: Repeated measures analyses of secondary outcome measures (Cylinder Task, Detour, Sustained Gaze, Off Leash Gait Speed). Mean values (adjusted as necessary) are reported at each timepoint by group. Group scores over time were compared in a repeated measures model (MANOVA analysis in JMP). The Wilks' lambda value was evaluated with  $p<0.05$  indicating a significant difference between groups. All original outcome measure data is provided in Supplementary Table S5.

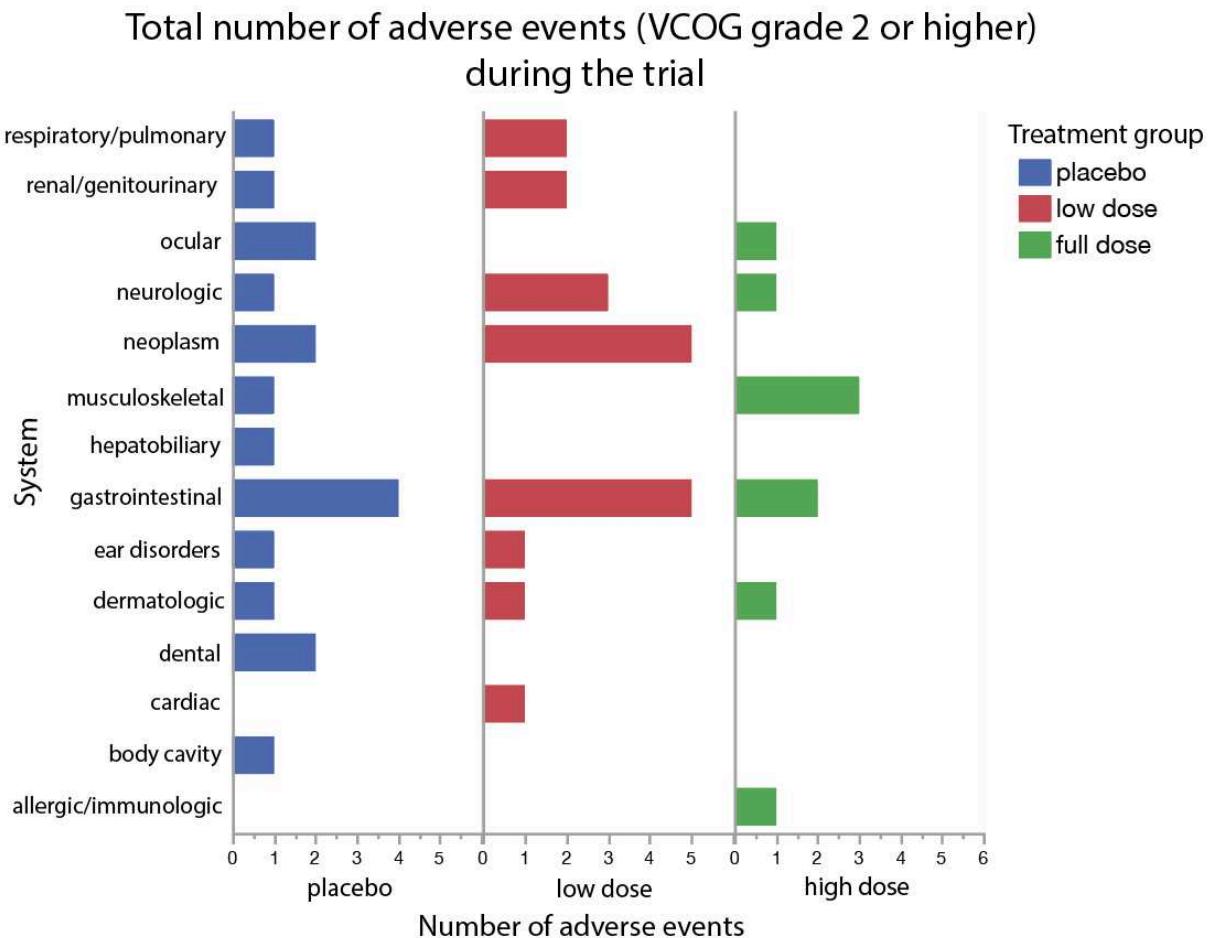


Figure 7: Total number of adverse events (VCOG grade 2 or higher) during the trial by group. Events were reported by the owner to be new during the trial (not present during/before baseline) and separated by system. Events were summed based on the system affected. All original adverse event data is provided in Supplementary Table S7.