

# 1 **A toolbox for the longitudinal assessment of healthspan in ageing mice**

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41 **Keywords:** aging, healthspan, phenotyping, geroprotectors, cardiovascular function,  
42 cognitive function, neuromuscular function, metabolic health, glucose tolerance test, insulin  
43 tolerance test, body composition analysis

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45 EDITORIAL SUMMARY A series of techniques (echocardiography, novel object recognition, grip  
46 strength, rotarod, glucose and insulin tolerance tests, body composition, and energy expenditure)  
47 are used to assess the health of mice.

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49 TWEET Assessing the health of aging mice

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51 COVER TEASER Aging mice health assessment

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55 RELATED LINKS

56 Key reference(s) using this protocol

57 Martin-Montalvo, A. *et al.* Metformin improves healthspan and lifespan in mice. *Nature*  
58 *communications* **4**, 2192, doi:10.1038/ncomms3192 (2013)

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63

64 **Abstract**

65 The number of people aged over 65 is expected to double in the next 30 years. For many,  
66 living longer will mean spending more years with the burdens of chronic diseases such as  
67 Alzheimer's, cardiovascular disease, and diabetes. Although researchers have made rapid  
68 progress in developing geroprotective interventions that target mechanisms of ageing and  
69 delay or prevent the onset of multiple concurrent age-related diseases, a lack of  
70 standardized techniques to assess healthspan in preclinical murine studies has resulted in  
71 reduced reproducibility and slowed progress. To overcome this, major centres in Europe and  
72 the USA skilled in healthspan analysis came together to agree upon a toolbox of techniques  
73 which can be used to consistently assess the healthspan of mice. Here, we describe the  
74 agreed toolbox which contains protocols for echocardiography, novel object recognition, grip  
75 strength, rotarod, glucose and insulin tolerance tests, body composition, and energy  
76 expenditure. They can be performed longitudinally in the same mouse over a period of 4-6  
77 weeks to test how candidate geroprotectors affect cardiac, cognitive, neuromuscular and  
78 metabolic health.

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As the average age of the world population increases, for many living longer will not mean living healthier. The number of healthy years is predicted to increase at a slower pace or not at all compared to lifespan. The result is that people are expected to spend an increasing number of years suffering from the burdens of multiple chronic age-associated diseases such as heart disease, Alzheimer's disease, and diabetes. Over 60% of those aged 65 and over have more than one chronic condition (multimorbidity), often leading to frailty (an accumulation of multiple deficits and loss of resilience to adverse events) and a resulting loss of independence<sup>1</sup>. This continuously growing trend will mean a substantial increase in health and social care costs<sup>1</sup>. For example the European Commission has estimated that every year of health lost will cost to the European Union 0.3 percentage point of GDP, equivalent to 54 billion euros<sup>1</sup>.

For this reason, studies on how to maintain health with age and how to compress the period of multimorbidity have become an emerging priority<sup>2</sup>. Recent research suggests that ageing, the major risk factor for the development of many deleterious chronic conditions, may be modifiable by both lifestyle (e.g., diet, exercise) and pharmacological interventions (geroprotectors), which in turn may be able to simultaneously delay the onset of multiple age-related diseases and reduce the risk of developing multimorbidity and frailty<sup>1</sup>. This has stimulated great interest in developing interventions to promote healthspan<sup>3-6</sup>.

Mice are the model organisms most often used for preclinical analysis of interventions due to their mammalian physiology, which resembles that of humans in many aspects, the relatively inexpensive cost to house and treat the animals, and their relatively short lifespan. However, measuring healthspan (the portion of life that is relatively healthy and free from major deficits that impair quality of life) is challenging because of the multiple parameters which need to be assessed. Despite this difficulty, it is widely agreed that measuring healthspan comprehensively is imperative<sup>1,7</sup>. Maximizing the robustness, reproducibility, and utility of these measurements of healthspan requires a consensus on which outcomes should be measured, the ages at which mice should be examined, and the frequency of examination.

At present the majority of laboratories do not comprehensively assess healthspan, and testing is not all-inclusive in relation to organ systems but rather focused on one or two specific organs of choice. Further, there are differences in the experimental design and the way the techniques are performed. In combination, these result in both a knowledge gap about the effectiveness of candidate geroprotective agents, and a lack of reproducibility. Therefore, a consensus on the best practices to assess healthspan and development of a standard operating procedure (SOP) is required.

### **Development of the toolbox**

In an attempt to overcome these limitations, experts in healthspan assessment of mice gathered to compare practices in their laboratories. The toolbox was developed via a series of meetings and conference phone calls, culminating in in-person meeting at a MouseAGE/INFRARONTIER-sponsored workshop in Athens, Greece in October of 2017. Here, we present the consensus toolbox for the measurement of healthspan in ageing mice using a battery of tests, which assess the major organ systems in the same animals longitudinally. The toolbox consists of protocols assessing cardiac function (echocardiography), muscle strength and neuromuscular function (grip strength, hanging bar, and rotarod), metabolic health (glucose and insulin tolerance test, body composition, and energy balance), and cognitive function (novel object recognition). We also recommend mice are assessed for frailty. The protocols in this toolbox can be repeated longitudinally during the lifespan of a mouse, or during the course of treatment of aged mice to which geroprotective agents have been administered. At the time of euthanasia, collection of

148 tissues for histological analysis is also recommended for studies with a planned euthanasia  
149 age.

150 The assays were chosen based on the evaluation of a number of criteria: clinical  
151 relevance, good reproducibility, a low level of invasiveness or exposure to radiation and  
152 stress for the animal (e.g. no need for anaesthesia when possible), and a low level of  
153 technical difficulty.

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155 For the evaluation of cardiac function, echocardiography was selected over blood  
156 pressure measurement despite the need for anaesthesia and for specialized personnel to  
157 perform the analysis because the latter procedure has very high variability, likely due to  
158 stress, and there is significant difficulty in reproducing results.

159

160 In the case of neuromuscular function, we chose a panel of easy to perform tests  
161 with very low impact on animal welfare. Among these assays, grip strength, cage top and  
162 hanging wire were chosen over the testing of forced treadmill running capacity (FTR). Wire  
163 hang is also known as wire suspension latency test, wire hang, hang test or body  
164 suspension in the literature. This combination of tests was chosen because they are less  
165 demanding for the aged animals than the treadmill running test. Aged animals may be lost  
166 during the latter and therefore it is preferable to perform it on a dedicated cohort of animals.  
167 We have also chosen rotarod because, in addition to serving as another measure of  
168 endurance, it also assesses coordination and balance. Alternative assays such as beam  
169 balance and beam walking assay are less effective at detecting mild impairments than  
170 rotarod<sup>8</sup>. When performing grip strength it is worth remembering that it has the significant  
171 limitation of being very sensitive to the ability of the operator. In addition, several of these  
172 assays are heavily influenced by the weight of the animal. For this reason, the evaluation of  
173 the neuromuscular system relies on more than one test, and where possible, it is advisable  
174 to verify conclusions by post-mortem histological analysis of muscle. We also suggest to  
175 perform follow-up studies if there is any evidence of a change in muscle function, using FTR  
176 and evoked leg muscle strength and fatigue<sup>3,5,9,10</sup>.

177

178 For metabolic function, glucose and insulin tolerance tests were selected to measure  
179 glucose homeostasis over the hyperinsulinemic-euglycemic clamp due to the requirement for  
180 surgery and euthanasia following the clamp procedure. This precludes longitudinal  
181 assessment and is very stressful to the animals. We selected intraperitoneal (IP) dosing of  
182 glucose due to the ease of training individuals and the consideration that oral glucose  
183 delivery is considered more stressful. Body composition analysis by MRI was selected over  
184 DXA due to the requirement for anaesthesia, the speed of measurement, and accuracy, as  
185 MRI provides a better estimate of both fat mass and lean tissue mass<sup>11</sup>. Metabolic  
186 chambers to assess energy balance via indirect calorimetry, activity tracking, and food intake  
187 measurement are the gold standard method to track energy balance and can be used  
188 longitudinally with a minimum of stress to the animals. They were selected over alternative  
189 apparatus that can only track a subset of these measurements, and thus provide only a  
190 partial snapshot of energy balance.

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192 A separate session was dedicated to the choice of behavioural tests. Consensus  
193 versions of protocols for each technique were modified from those already published by the

194 individual authors<sup>3-6,12-21</sup>. A key requirement for cognitive assays suitable for longitudinal  
195 analyses is that order effects need to be avoided as much as possible. A number of  
196 commonly used cognitive tasks in rodents, such as the Morris water maze or contextual fear  
197 conditioning, have limited value for longitudinal assessments because performance on these  
198 assays will be strongly influenced by previous experiences in these tasks. We chose novel  
199 object recognition as it is well suited for multiple longitudinal assessments of the same sets  
200 of mice using different object pairs. Other behavioural assays, such as the recently  
201 developed touchscreen-based automated battery system<sup>22</sup>, would also be suitable for  
202 longitudinal assessments of cognitive function. However, novel object recognition has  
203 particularly low demands regarding the availability of specialized equipment and can hence  
204 be readily implemented in a broad range of laboratories. Moreover, novel object recognition  
205 places minimal stress on the animals tested as it does not involve exposure to aversive  
206 stimuli, food restriction, single housing or extensive periods of pre-training. More detailed  
207 explanations of novel object recognition can be found in Benice and Raber (2008)<sup>23</sup>.

208  
209 We also recommend uses assess mice according to the frailty index, based on the  
210 Rockwood frailty score including 31 different parameters, which we do not describe here due  
211 to the extremely detailed description available from the developers<sup>24-26</sup>. This is our preferred  
212 method to score frailty compared to measuring frailty using a Fried Frailty Criteria-like score  
213 because the latter is based on the assessment of only 4 criteria (grip-strength, walking  
214 speed, physical activity, and endurance) and was considered more a measure of  
215 musculoskeletal fitness rather than an accumulation of deficits in several organ systems.  
216 The Rockwood-like frailty index better captures the potential benefits of geroprotectors  
217 across multiple systems.

218 At the end of the experiments we recommend harvesting as many tissues as  
219 possible, as well as blood for clinical biochemistry and to perform analysis of bone, e.g. by  
220 X-Ray microCT. It was decided that analysis of bone would be done only after terminal  
221 anaesthesia to avoid potential detrimental effects due to radiation exposure. Longitudinal  
222 analysis of bone requires exposure of mice to repetitive doses of radiation and anaesthesia.  
223 Although the dose received at each imaging session is small and only in the region of  
224 interest, the cumulative effects are unknown.

225 An alternative approach to the testing of healthspan was proposed by Sukoff-Rizzo *et*  
226 *al.*<sup>27</sup> However, this approach was primarily focused on the in-depth study of motor, sensory,  
227 and cognitive function, and does not evaluate important parameters of healthspan such as  
228 cardiac and metabolic function<sup>27</sup>. Considering the longitudinal nature of these studies and  
229 considering that some of the tests reported by the protocol of Sukoff-Rizzo are taxing for  
230 ageing animals, we advise investigators to perform some of the tests described in this  
231 protocol in a second, parallel cohort of animals when alterations in neuromuscular function  
232 or behaviour are found using our pipeline.

233 Using the procedures included in this toolbox, our laboratories have successfully  
234 characterized the effectiveness of several compounds, including metformin<sup>18</sup>, nicotinamide<sup>20</sup>,  
235 rapamycin<sup>4,6</sup>, and senolytics<sup>28,29</sup>, as well as other interventions including diet<sup>30-32</sup> and  
236 exercise<sup>33</sup> as geroprotective agents on mice, and determined the effect of specific genetic  
237 modifications<sup>12,19,34</sup> on the health and longevity of mice. Consensus across laboratories was  
238 reached by performing a comparison of the protocols in use in each laboratory, together with  
239 evidence of best practice and consideration of animal welfare, particularly when animals  
240 reach old age.

## 242 Application of the toolbox

243 Healthspan assessment can be used when studying gene function using genetically  
244 modified animals, or for testing new compounds (e.g. geroprotectors), diet or exercise  
245 regimens. For example, we have used this toolbox to identify new genes involved in the  
246 development of age-related diseases, which can be used as novel drug targets<sup>12</sup>. In addition,  
247 we have studied the efficacy, side effects, and mechanism of action of geroprotectors using  
248 several tests in this toolbox. These drugs have emerged for their ability to delay the onset of  
249 multiple concurrent age-related diseases and boost resilience by modulating mechanisms of  
250 ageing such as senescence, autophagy, and inflammation<sup>1</sup>. More than 200 compounds have  
251 been described as geroprotectors (<http://geroprotectors.org/>). Our studies, collectively, have  
252 shown that drugs such as rapamycin, resveratrol, metformin, senolytics (e.g. fisetin,  
253 dasatininb, and quercetin<sup>35,36</sup>) which remove senescent cells, or dietary interventions can  
254 slow the development of cataracts, osteoarthritis, osteoporosis, the loss of muscle mass and  
255 can improve cardiac function<sup>3-6,13-21,35-42</sup>. Similarly, we have shown that decreased  
256 consumption of protein or of specific dietary amino acids improves metabolic health<sup>31,43</sup>. We  
257 recommend the use of this toolbox for a first assessment of healthspan whenever testing  
258 geroprotectors or assessing the function of specific genes in healthy ageing. These studies  
259 can serve as a guide for a subsequent, in-depth assessment of specific organ systems and  
260 healthspan effects.

261 The toolbox has been designed with C57BL/6J mice in mind because of the amount  
262 of data already available for this commonly used strain. However, with some modification it  
263 can be used for other strains such as UM-HET3 mice (the F2 progeny of (BALB/cJ x  
264 C57BL/6J) mothers and (C3H/HeJ x DBA/2J) fathers; parental strains from The Jackson  
265 Laboratory). Some procedures have also been tested on mice of different genetic  
266 backgrounds, including PWK/PhJ (The Jackson Laboratory) and C57BL/6NHsd,  
267 DBA/2NHsd, 129P2/OlaHsd, and C3H/HeNHsd (Envigo), and many of the toolbox  
268 procedures have been tested on genetically modified mice, including dwarf and progeroid  
269 animals<sup>15,34,44-46</sup>.

## 271 Comparison with other approaches

272 To date, the vast majority of healthspan studies have taken an *ad hoc* approach to  
273 the collection of longitudinal data that has been limited by the interests of the investigators in  
274 one or two specific tissues. This has limited the amount of data collected in a single study,  
275 slowed scientific advance overall and limited the translation of geroscience discoveries. For  
276 example, it was only recently shown that short-term calorie restriction and rapamycin can  
277 rejuvenate the aging mouse heart, reversing pre-existing age-dependent cardiac  
278 hypertrophy and diastolic dysfunction<sup>47,48</sup>. If these types of studies were routinely performed  
279 during longevity studies, the beneficial effects of rapamycin on cardiac diastolic function  
280 would have been identified several years earlier. For example, while there have been  
281 several studies demonstrating that late-life treatment with rapamycin extends lifespan<sup>6,49-51</sup>,  
282 none of these studies examined cardiac function. Likewise, there are studies testing  
283 geroprotectors and focusing on cognition and metabolic health, showing beneficial effects on  
284 cognition in mouse models of Alzheimer's disease or aged mice<sup>52,53</sup>, and improving the  
285 metabolic health of aged mice<sup>20,29,54</sup>. For the greater part, however, those studies that  
286 examined cognition did not assess metabolic health, and vice-versa. It would also be  
287 interesting to know if the beneficial effects of these short-term interventions persist or are  
288 temporary.

289 The major problem of older age is that "it comes as a package"<sup>55</sup> and the major  
290 health challenges are due to the accumulation of deficits and the presence of concurrent  
291 diseases. Therefore, a systematic and holistic approach which goes beyond the study of the  
292 single disease is required. In addition the use of a standardised toolbox allows the

293 comparison of measure across laboratories, improving reproducibility. For example, the  
294 effects of interventions including intermittent fasting and rapamycin have alternatively been  
295 reported to either have minimal or significant effects on aging and healthspan<sup>4,32,56,57</sup>; it is  
296 difficult to determine whether the differences are due to the differences in regimen or to the  
297 specific endpoints considered. While a few studies have provided a comprehensive  
298 assessment of healthspan measures across different physiological systems<sup>4</sup>, these studies  
299 employed cross-sectional designs, and were not suited for an examination of the longitudinal  
300 development of health measures during ageing in individual mice. A principle advantage of  
301 the toolbox here is its simplicity, ready training of investigators, the minimal stress placed on  
302 the animals, and its applicability even to very old mice<sup>29</sup>. While there has been concern  
303 about phenotyping of mice possibly altering their lifespan, we have found that mice subject  
304 to longitudinal metabolic phenotyping, for example, have a similar lifespan to wild-type  
305 C57BL/6J mice not subject to such studies<sup>6,30,58</sup>. Overall, use of the toolbox will speed the  
306 prioritization of possible geroprotectors for further preclinical and clinical testing.

307

## 308 **Experimental design**

309 When testing geroprotectors, a minimum of two groups of mice is required: a  
310 group treated with the geroprotector and a vehicle-treated control group; Treating young  
311 mice is also often desirable to control for effects which are independent of age or may be  
312 detrimental such those seen with rapamycin<sup>4,59</sup>. When testing genetically modified  
313 animals, there may also be a need to include additional control groups based on how the  
314 mice were generated and the precise experimental question to be answered. Every  
315 intervention should be tested in male and female mice as there are many cases of sexual  
316 dimorphism<sup>60-63</sup>. In our experience, groups of at least 20 animals per sex are required to  
317 detect physiologically relevant changes in assays included in this toolbox, such as rotarod  
318 performance, glucose tolerance, and grip strength<sup>4,6,16,17,31,43,64,65</sup>. These have been  
319 obtained using in house and published data on mean and standard deviation for each  
320 test, using biological relevant effect sizes with a power of 80% and alpha of 0.05. We  
321 have considered the same size of effect following exercise, an intervention known to have  
322 positive effects in humans. We recommend that animals be randomly assigned to groups  
323 at the cage level, using computing generated assignment of mice to the treated and  
324 control group and stratified by weight; we typically observe that stratification by weight is  
325 sufficient to match most baseline measures of health in young wild-type animals.  
326 Stratification based also (or instead) on frailty may be considered when group assignment  
327 is performed in aged mice to reduce variability and group size.

328 We also recommend blinding of the experiments, i.e. that the allocation of the  
329 treatment groups is performed by a different researcher and should not be revealed to the  
330 people conducting the experiment until the data have been analysed. This is particularly  
331 important for tests such as frailty or grip strengths, which are operator dependent. It may not  
332 always be possible as some phenotypes (i.e. mice treated with calorie restriction) are  
333 visually obvious.

334 For both cross-sectional and longitudinal deaths, it is important to include sufficient  
335 numbers of mice to allow for age-related mortality, particularly after 18 months of age. In  
336 the hands of some of the investigators, female mice on a C57BL/6J genetic background  
337 or from the NIA Aging Mouse Colony experience 30-36% mortality between 20 and 27  
338 months of age (50-66% mortality between 20 and 30 months of age), while male mice  
339 experience 8-18% mortality between 20 and 27 months of age (and 30-36% mortality  
340 between 20 and 30 months of age) (<sup>6</sup> and data not shown). The overall mortality rate also  
341 varies based not only on strain and genetic background, but also diet and animal facility;  
342 for example, the NIA's Intervention Program has found a persistent difference in lifespan



343 between mice test sites despite standardized mice, diet, and husbandry<sup>66</sup>. If local data for  
344 the survival of a strain is not available, the mortality between any two ages can be  
345 estimated for many strains using lifespan data published by the NIA and by The Jackson  
346 Laboratory<sup>58,67</sup>.

347 Fig. 1 is a schematic representation of the experimental design for testing in  
348 C57BL/6J mice, the frequency of assessment and the type of tests. The sequence with  
349 which the tests are performed is also important and the time in between tests to avoid  
350 stress to the animals as much as possible. We recommend to perform the multiple assays  
351 over a period of 4-6 weeks using the following order, novel object recognition, frailty  
352 index, grip strength, cage top and hanging bar, followed by rotarod, body composition,  
353 energy expenditure, GTT, ITT and echocardiography.

354 **Limitations of the approach** Testing healthspan involves complex experiments, which are  
355 costly and requires an in-depth knowledge of multiple organ systems combined with  
356 knowledge of the ageing process and welfare issues in mice. The protocols described in this  
357 toolbox summarize the knowledge collected from years of experience in the area of  
358 healthspan assessment and murine ageing biology. Their availability will reduce animal  
359 usage and costs by reducing the need to optimize each technique when establishing  
360 healthspan assessment platforms in other laboratories. It will also ensure that the data are  
361 comparable and reproducible across laboratories, speeding up progress.

362  
363 **Regulatory requirements.** These vary in each country and between institutions, and  
364 therefore it is always advisable to check with the relevant veterinary authorities, and gain  
365 regulatory approval as may be required, before performing any procedure. At each  
366 institution, all authors performing any of the described procedures received regulatory  
367 approval and oversight as appropriate. This included approval/oversight by the UK Home  
368 Office (IB, PP), the Institutional Animal Care and Use Committee of the William S. Middleton  
369 Memorial Veterans Hospital (DL), Landesamt für Natur, Umwelt und Verbraucherschutz  
370 Nordrhein-Westfalen, Recklinghausen, Germany (DE), Mayo Clinic Institutional Animal Care  
371 and Use Committee (TT, JDM), the Committee of Ethics and Animal Experimentation of the  
372 Cajal Institute, Ethics Committee of the CSIC, and the Animal Protection Area of the Ministry  
373 of Environment of the Community of Madrid (JLT), and the Animal Care and Use Committee  
374 of the National Institute on Aging, NIH, US (RdC, CDG, SJM, IN).

375  
376 **Requirements for staffing, expertise, and equipment.** While the majority of the  
377 techniques in the toolbox do not require an abundance of highly specialized staff, training is  
378 required, particularly for the grip strength test and echocardiography. It is advisable that PhD  
379 students and post-docs visit a lab which is running these tests routinely. This is particularly  
380 important for grip strength as this test is highly operator dependent. For echocardiography a  
381 specialized facility and extensive specialized training of staff is required for the acquisition,  
382 analysis and interpretation of the images. Some techniques such as the glucose tolerance  
383 test require a significant investment of hands-on staff time. Likewise, performing several  
384 informative protocols in the toolbox, including determination of body composition, cardiac  
385 function, and energy balance, requires an upfront investment in single-purpose equipment,  
386 and an ongoing commitment to maintain that equipment.

387  
388  
389 **Handling of aged mice.** Working with aged animals presents additional difficulties when  
390 compared to working exclusively with young animals. Animal care, laboratory, and veterinary  
391 staff should be familiar with the care and handling of aged mice, and be able to distinguish  
392 between an old mouse, a sick mouse and an old, sick mouse. Older mice tend to be less  
393 active, may lose weight, and appear less healthy, and humane endpoints developed for

394 younger mice may not be appropriate for older mice. With the time, expense and ethical  
395 implications of ageing cohorts of mice it is important that aged mice are not culled  
396 unnecessarily, thus affecting cohort size, or allowed to exceed humane endpoints or ethical  
397 standards. Staff should become familiar with how the strain(s) or line(s) age, their care, and  
398 the treatment of spontaneous phenotypes. The age of breeding stock can have effects on  
399 the offspring<sup>13,68-72</sup>; therefore knowledge of the age of breeding mice and even limited  
400 phenotyping such as body composition or weight, would be useful metadata. Ideally, the age  
401 of breeding stock would be standardised to minimise variation in offspring. Phenotypic data  
402 in aged mice tends to exhibit a greater variance than that in young mice<sup>65</sup>, which may reflect  
403 the differences in ageing between individuals, even within inbred strains. With the increased  
404 variance of data from aged mice the standardisation of protocols down to the finest detail is  
405 critical as is the recording of metadata as small variations can be amplified thus confounding  
406 the output of experiments. This includes time of day of phenotyping and the operator.

407  
408 **Anaesthesia.** The cardiac function protocol requires anaesthesia. Repeated anaesthesia –  
409 as would be required for longitudinal examination of cardiac function in aged mice - has  
410 been suggested to be linked to cognitive impairment in humans. However, repeated  
411 anaesthesia with isoflurane does not result in prolonged cognitive deficits at least in mice  
412 that are approximately 18 months old<sup>73</sup>. In this toolbox, the animal is euthanized after the last  
413 echocardiography at 24 months of age. Aged animals may also be more likely to have  
414 negative reactions to anaesthesia<sup>74-76</sup>.

415  
416 **Timing may vary.** Timings and frequency are for ageing C57BL/6J mice, but may vary for  
417 other strains or genetically modified models. For example, it may prove appropriate to begin  
418 phenotyping at an early age for very short-lived mice or for genetically modified mice  
419 expected to begin developing phenotypes at an early age<sup>34,44</sup>. Conversely, when mice are  
420 expected to develop phenotypes at an advanced age, or an intervention is begun only late in  
421 life, it is likely a more efficient approach to not begin phenotyping mice until later in their  
422 lifespan or immediately prior to the start of an intervention<sup>6,47,49</sup>.

423  
424 **Suitability for progeroid mice.** Many of the metabolic phenotyping protocols in the toolbox  
425 are not suitable, or are suitable only with modification, for the use of progeroid mice, which  
426 are often small, extremely frail, and prone to hypothermia if singly housed. When using  
427 metabolic chambers, in which animals are single-housed without bedding, some  
428 investigators suggest that animals which may be more sensitive to cold stress be analysed  
429 for a shorter time period than suggested below, typically approximately 24-26 hours, or the  
430 temperature in the room or specifically in the metabolic chamber (if housed in a temperature  
431 controlled cabinet) can be raised to prevent hypothermia.

432  
433 **Limitations of intraperitoneal administration of glucose.** While we have provided  
434 instructions below for performing a glucose tolerance test using glucose administered via  
435 intraperitoneal injection (IPGTT), glucose can also be administered via oral gavage (OGTT).  
436 An OGTT is likely more physiological since glucose is administered orally, and incretin  
437 hormones are engaged. Incretin hormones (GIP and GLP1) are secreted from the intestine  
438 in the blood stream and influence the insulin secretory response and pancreatic beta cells;  
439 they also have effects on glucagon response and fat deposition<sup>77-79</sup>. If investigators are  
440 specifically asking questions that involve incretins, it is likely they will need to perform  
441 OGTTs.

442  
443 However, drawbacks to OGTT include that it needs to be performed by a very well trained  
444 operator to reduce stress to the animals, which may influence the outcome of the study.  
445 Further, in some countries, oral gavage is considered a highly invasive procedure for the

446 animal and needs to be very well justified for its use. In contrast, IPGTT is a technique that  
447 can be rapidly taught and involves minimal stress for the animals. Finally, IPGTT and OGTT  
448 provide comparable results in young and aged C57BL/6J mice<sup>80</sup>. The majority of our labs  
449 administer IPGTTs unless a specific question requires the use of OGTT.

450  
451 **Limitations of the novel object recognition task.** It involves only a single training  
452 session<sup>81,82</sup> and hence it is not possible to resolve potential effects on learning rate which  
453 would require repeated training. It should also be noted that the level of object exploration by  
454 the animals can be low or inconsistent which may require the exclusion of animals in order to  
455 avoid skewing of results. Also, it is necessary to carry out careful pilot studies to ensure that  
456 training period, delay intervals and test duration are optimized for suitable behavioural  
457 performance within a given experimental context. Moreover, one needs to carefully evaluate  
458 all object pairs used in the task to ensure that there is no intrinsic difference in object  
459 exploration by the animals. Conditions should always be counterbalanced to minimize any  
460 effect of differential intrinsic object preference.

461 **The influence of the environment.** The standardization of protocols is critical to minimize  
462 variation due to environmental differences or gene-environment interactions. Recent studies  
463 have highlighted how apparently minor differences, including timing, temperature, bedding,  
464 as well as variation in the microbiome can affect the outcome and reproducibility of mouse  
465 experiments<sup>83</sup>. The experience of the National Institute on Aging's Interventions Testing  
466 Program has demonstrated that even when mouse source, diet, and husbandry conditions  
467 are intentionally coordinated to be identical, there remain site-specific variations in murine  
468 lifespan<sup>84</sup>. While these studies demonstrate the difficulty of achieving perfect reproducibility  
469 in experiments conducted at disparate sites, they also demonstrate that by matching known  
470 variables as closely as possible, reproducibility can be enhanced by minimizing differences.  
471 Thus, we encourage investigators to control for those variables that are most likely to impact  
472 the experiment, such as the operator, the testing room, the time of the day, and the  
473 equipment utilized. Some factors such as housing conditions (number of animals per cage,  
474 type of enrichment, and noise) or diet may not always be under the control of individual  
475 researchers because it is determined at an institutional level. These factors should be  
476 carefully recorded as metadata along with experimental data.

## 477 478 **MATERIALS**

### 479 **REAGENTS**

#### 480 **Laboratory mice**

481 The procedures described here were primarily developed with mice on a C57BL/6  
482 genetic background (specific variants include but are not limited to C57BL/6J from  
483 The Jackson Laboratory, C57BL/6J from Janvier Labs, C57BL/6N from Taconic  
484 Biosciences, and C57BL/6J.Nia from the National Institute on Aging Aged Rodent  
485 Colony).

486  
487 The procedures can be readily carried in healthy animals of a wide range of ages;  
488 cautions regarding risks of specific procedures to old animals (C57BL/6J mice over  
489 approximately 24 months of age) are noted in specific procedures.

490  
491 **CAUTION:** All experiments should be performed in accordance with relevant  
492 guidelines and regulations and approval of the relevant institutional committees.

#### 493 **General reagents**

- 494 • Cleaning supplies

495 CRITICAL All of the equipment utilized in the procedures below must be cleaned  
496 after use, using procedures suitable to the equipment and in accordance with both  
497 the manufacturer's instructions and institutional policies.

- 498 • Paper towels

#### 499 **Cardiac function**

- 500 • Isoflurane for use in isoflurane vaporizer

501

502 CAUTION: Isoflurane is a respiratory depressant and chronic exposure can  
503 negatively impact health, resulting in effects including hypotension, tachycardia,  
504 respiratory depression, and elevated blood glucose level. Isoflurane should be used  
505 in combination with equipment designed to minimize exposure (e.g. vaporizer with an  
506 activated carbon scavenging system), and institutional standard operating  
507 procedures to ensure safe use should be followed.

508

- 509 • Medical Oxygen (e.g. BOC) or Air source for isoflurane vaporiser
- 510 • Conductive electrocardiography (ECG) Gel
- 511 • Aqueous medical ultrasound gel (e.g. AquaSonic)
- 512 • Micropore Tape (e.g. 3M)
- 513 • Electric small animal hair clippers
- 514 • Hair removal lotion (Veet)

#### 515 **Energy Balance**

- 516 • Respiratory gasses. Gasses for the calibration of the O<sub>2</sub> and CO<sub>2</sub> sensors will be  
517 specified by the equipment vendor, but must be ordered from gas vendors.

#### 518 **Glucose tolerance test**

519 30% glucose: Prepared by dissolving 30% (30g/100mL) glucose (G7021,  
520 MilliporeSigma) and 0.9% NaCl (S5886, MilliporeSigma) in high purity water, and  
521 then filter-sterilized using a 0.2µM filter (430626, Corning) and ~10mL aliquoted into  
522 15mL tubes (12565268, Fisher Scientific). Aliquots may be stored at 4°C or frozen at  
523 -20°C. This solution should be prepared at least one day prior to the assay.

524 CRITICAL: 30% glucose must be prepared for the treatment of any mice that develop  
525 hypoglycaemia during the insulin tolerance test also.

526

- 527 • Syringe: 1mL sterile disposable syringe (309659, Becton Dickinson) – one syringe is  
528 required per mouse

- 529 • Needle: 27G1/2 sterile needle (305109, Becton Dickinson) – one sterile needle is  
530 required per mouse.

531 CRITICAL: The procedure described here is an intraperitoneal (I.P.) glucose  
532 tolerance test. As discussed in the limitations above, some investigators, particularly  
533 those investigating incretins, will prefer to perform an oral glucose tolerance; in such  
534 a case, an oral gavage needle is required instead.

- 535 • Glucose test strips: Bayer Contour Blood Glucose Test Strips (Various) – six glucose  
536 test strips per mouse are required; extra glucose test strips in case of mishaps,  
537 misreads, or defective test strips is a good idea

- 538 • Razor blades: 55411050 (Andwin Scientific via VWR) – one razor blade per mouse  
539 cage

540 • Spreadsheet: One or more datasheets per experimenter, with space for entering the  
541 weight, volume of glucose to be administered, blood glucose levels at each of six  
542 time points, and room for other notes.

543 • EDTA Blood Collection Tubes: 20.1288.100 (Sarstedt Inc via Fisher) CRITICAL  
544 Required if also collecting blood for insulin or other hormone measurements.

545 • Insulin ELISA Kit: 90080 (Crystal Chem) CRITICAL Required if also collecting blood  
546 for insulin or other hormone measurements.

547

#### 548 ***Insulin tolerance test***

549 • Insulin: Prepared by diluting 22.5uL of sterile 100U/mL Humilin-R (Lily) in 10mL of  
550 filter-sterilized (using a 0.2µM filter (430626, Corning)) 0.9% NaCl.

551 CRITICAL: Do not use long-acting insulins.

552 CRITICAL: Diluted insulin does not retain activity indefinitely, and must be freshly  
553 prepared (within ~2 hrs of use). Do not filter insulin after dilution.

554

#### 555 ***Grid hanging test***

556 • Spreadsheet with a list of mice, with space for entering the weight and the time to fall  
557 for up to three attempts.

558

### 559 **EQUIPMENT**

#### 560 **General equipment**

561 • Marker – A black or other dark color marker capable of temporarily marking the tail of  
562 mice, e.g. 52877-310 (VWR).

563 • Scale – an electronic balance with sensitivity to at least 0.1 g. Some of us use an  
564 Ohaus SP-402 Scout Pro Balance (Various), but many brands are available.

565 • Timer – A lab or kitchen style timer that can count upwards from zero while  
566 displaying both minutes and seconds, e.g. 76204-504 (VWR).

567 • Weighing container – a glass or plastic container of 300-500mL volume for placing  
568 mice in while being weighed.

#### 569 **Cardiac function**

570 • Inhaled anaesthesia system with small nose cone to allow easy access to thoracic  
571 area with transducer.

572 • Vevo 770 / 1100 / 2100 / 3100 System with the recommended transducer for the  
573 specific system used, e.g. the MX550D (32-55 MHz linear array) for mice with the  
574 Vevo 3100 system. Lamp (e.g. 250 Watt Infra-Red warming bulb) to maintain body  
575 temperature.

576 • Gel warmer (available from Visualsonics) or 37°C waterbath to ensure gel is warmed  
577 to body temperature.

#### 578 **Cognitive function**

579 • A dedicated room big enough to allocate a 42 x 32 x 31 centimetre arena and for the  
580 experimenter to move freely around the cage with access to a computer. The

581 behavioural room should be in proximity to the place where the animals are housed  
582 to limit stress associated with transportation of the mice. A video camera attached to  
583 the ceiling, thus providing overhead footage on the very centre of the arena and  
584 connected to the computer. Room lights should be indirect or at least located in a  
585 way that no reflection appears in the area to be tracked and should preferably be  
586 adjustable in brightness, as illumination should be around 150 lux at the floor of the  
587 arena.

588 CRITICAL: One of the most damaging distractions in the behavioural laboratory is  
589 unexpected noise, which should be avoided. Similarly, the room environment should  
590 be free from interference by external or internal lights or odours. A running air purifier  
591 (e.g. Honeywell True HEPA Air Purifier 50250) in the testing room can be used to  
592 generate a constant low level of white background noise to help to mask unexpected  
593 external noise sources.

- 594 • An open wooden or methacrylate box of 42 x 32 x 31 centimetres used as an open-  
595 field arena. Although novel object recognition performance has not been directly  
596 compared across boxes with different dimensional characteristics, this is unlikely to  
597 impact the result of the test. Indeed other mouse behavioural measures in an open  
598 field setting have been found to be insensitive to different box dimensions<sup>85</sup>. The box  
599 should not be covered with sawdust and should be located on the floor or on a table.
- 600 • Small plastic objects, e.g. toys or cans. We use objects sized 5 x 5 x 5 cm but a  
601 variety of object dimensions can be used. The objects should not have mobile parts  
602 and must be easy to attach and remove from the floor of the arena (usually by means  
603 of an adhesive tape). They should be located around the centre of the arena, in an  
604 opposite and symmetrical way (see below).
- 605 • CRITICAL: It should be established empirically for all pairs of objects to be used for  
606 the assessment of novel object recognition that they induce indistinguishable levels  
607 of exploratory behaviour when encountered for the first time by animals of the  
608 specific mouse strain used for experimentation. Objects should be sufficiently distinct  
609 for successful discrimination, yet similar enough (e.g. with regards to size, shape,  
610 presence of protrusions/intrusions, texture, brightness) to avoid inherent preference  
611 biases for one of the objects within the object pair. For further consideration of object  
612 feature influences on the performance in novel object recognition tasks, see<sup>86,87</sup>.
- 613 • A computer connected to an overhead video camera and able to run commercially  
614 available animal behaviour tracking software. The software needs to be able to track  
615 the animal in the arena, by a multiple body points tracking system, and based on the  
616 tracking data, compute time spent, as well as distance travelled in different user-  
617 defined sections of the arena, considering the nose point and center point of the  
618 animal separately<sup>23</sup>. We use Ethovision XT (Noldus) or Smart video tracking software  
619 (Panlab). Video footage should be provided for potential additional analyses. Careful  
620 arrangement of the environment of the behaviour room should be observed  
621 (especially illumination). This should follow the instructions by the software  
622 manufacturer, to assure adequate tracking of the animals' nose point. We  
623 recommend the use of automated video tracking. While manual scoring may be  
624 necessary in some circumstances, the process is labour-intensive and the exact  
625 determination of whether the nose point is in the target area is more subjective<sup>23</sup>. For  
626 automated video tracking, it is critical to maintain continuity of nose tracking. This can  
627 be easily achieved with careful illumination combined to adequate contrast detection  
628 parameters allowed by the software.

629 **Body Composition**

- 630 • A variety of systems are available such as the EchoMRI-700 system with an A-100  
631 insert antenna, which enables the measurement of animals up to 100g of body  
632 weight, and the Bruker's minispec Whole Body Composition Analyzer LF90.

633 CRITICAL: While any system based on nuclear magnetic resonance (NMR) can be  
634 utilized, dual-energy X-ray absorptiometry (DXA) based body composition analysis is  
635 not preferred, as anaesthesia is required, DXA uses a small dose of ionizing  
636 radiation, the length of procedure is longer, and NMR is more accurate than DXA for  
637 the determination of both fat mass and lean mass<sup>11</sup>. However, if a laboratory only has  
638 access to DXA, the investigator will need to balance these factors vs. the need to  
639 acquire body composition data.

- 640 • An animal holder, sized appropriately to the weight of the animal. A tube sized for  
641 mice of approximately 40g (e.g., EchoMRI part 600-E25130R-40) is appropriate for  
642 the majority of longitudinal healthspan studies in wild-type mice.

643 **Energy Balance**

- 644 • A metabolic chamber system equipped to measure food consumption, spontaneous  
645 activity, and respiratory gasses (O<sub>2</sub> consumption, CO<sub>2</sub> production). The  
646 Oxymax/CLAMS (Comprehensive Lab Animal Monitoring System) manufactured by  
647 Columbus Instruments is the most commonly used, but alternative and functionally  
648 equivalent systems are also available from other vendors, e.g. TSE Systems or  
649 Sable Systems. Other options are available, including measurement of water intake,  
650 running wheels (to assess voluntary activity), urine collection, and temperature  
651 telemetry. Some systems can also assess respiratory gasses during forced running  
652 with the use of a treadmill.

653 CRITICAL: The system should be installed in a location where disturbances from  
654 animal facility staff and laboratory personnel is minimal, ideally in a room not used for  
655 the housing of other animals and not routinely entered by others while the chambers  
656 are in use. The system can be installed in an optional environmental chamber, which  
657 we highly recommend as it aids in the isolation of the animals from external stimuli. In  
658 particular, it permits finer control of temperature and lighting than is typically possible  
659 in an animal facility, and places it under computer control.

- 660  
661  
662 • Food processor – if the metabolic chamber system has a feeder that dispenses  
663 ground food rather than pellets, a commercial food processor to pulverize pelleted  
664 feed into a powder is extremely convenient. Many different options are available such  
665 as the Hamilton Beach 12-Cup Stack and Snap Food Processor (#70725A, Amazon).  
666 Small quantities of food may also be pulverized by hand using a mortar and pestle.

667 **Glucose and insulin tolerance tests**

- 668 • Glucometer – a human glucose meter capable of utilizing the test strips purchased in  
669 reagents such as a Bayer Contour Blood Glucose Meter (Various), but many brands  
670 are available

671 CRITICAL: Different models of glucometers consistently produce slightly different  
672 blood glucose readings. As a consequence, all strips and glucometers used during  
673 an assay should be of the same brand and model.

674

675 **Grid hanging test**

- 676 • The apparatus used can basically be as simple as a metal cage top from the home  
677 cage of the mouse or any other kind of mesh/grid (squares no bigger than 1cm x  
678 1cm) that allows the mouse to grip and hang upside down.

679 **Grip strength**

- 680 • A Grip Strength Meter – similar and functionally equivalent grip strength meters are  
681 available from several vendors, including Columbus Instruments (e.g., 1027CSM,  
682 Single Computerized Sensor with Standard Pull Bars for Mice) and Harvard  
683 Apparatus (e.g., Bioseb Grip Strength Meter).

684 **Hanging bar**

- 685 • Hanging bar apparatus - A 50 cm wide, 2 mm thick metallic wire tightly secured  
686 between 2 vertical stands approximately 37-50 cm above a layer of bedding.

687 **Rotarod**

- 688 • A variable speed, accelerating rotarod –e.g. Columbus Instruments (e.g., 0890M,  
689 Rotamex-5 4 Lane Rota-Rod for Mice with RS-232 and Software) but similar  
690 equipment is also available from a variety of vendors.

691 **PROCEDURE**

692 **Methods for assessment of cardiac function**

- 693 1. Place ultrasound gel in the gel warmer.  
694 2. Turn on the ultrasound machine. Enter animal ID, date and time, and other  
695 relevant information, including body weight, age and experimental intervention (if  
696 any).

697 CRITICAL: Use high frequency ultrasound transducer appropriate to the size and  
698 weight of the animal; e.g. a 40 MHz transducer for imaging mice less than ~20 grams  
699 or a 30 MHz transducer for mice weighing more than ~20 grams.

700 CRITICAL: In order to maintain normothermia during the imaging session, turn on  
701 the warming mechanism on heated platforms, as well as an ancillary warming lamp if  
702 the room temperature is cool (e.g., < 72°F/22°C). It is helpful to have a warming  
703 lamp and heat pad also focused on the cages used for recovery (with mice  
704 recovering on a paper towel in an otherwise empty cage) to prevent hypothermia and  
705 accelerate recovery. Turn these devices on at this time as well.

706 CRITICAL: Ensure that all hardware/tubing for scavenging of isoflurane gas is  
707 connected/intact, functional, and turned on (e.g., suction for active scavenging if  
708 present).

- 709 3. Gently pick up the mouse by the tail and firmly grasp the animal by its nape.  
710 Quickly and accurately insert the animal's nose and mouth into the nosecone and  
711 begin administration of 2-3% isoflurane. Once anesthetized (10-30 seconds), lay  
712 the animal on the platform in supine position making sure the forefeet and  
713 hindfeet lay on the ECG sensors of the platform.

714 CRITICAL: Methods for induction of anaesthesia are a major source of variability when  
715 imaging rodents. If you are using an "induction box" prior to placement of the mouse on  
716 the platform/nosecone, be sure to standardize the concentration of isoflurane used for  
717 induction and use the animal's individual response to anaesthesia (i.e., immediately



718 upon immobilization and clear ability to be transferred safely to platform) rather than a  
719 fixed period of time for induction.

720 4. Gently secure the animal with adhesive tape on all four limbs and the tail, and  
721 lightly also apply adhesive tape to stabilize the head in the nose cone apparatus.

722 5. Check heart rate (HR). Ensure that the baseline HR in is between 500 to 700  
723 bpm.

724 CRITICAL: Ensure that the HR does not fall below 450 bpm under any  
725 circumstances. Should HR fall below 450 bpm, progressively reduce anaesthesia by  
726 0.1% increments every 15 seconds until HR recovers. If HR does not recover and/or  
727 continues to decrease, immediately remove the animal from the nose cone and  
728 transfer to a warming pad for recovery. Adjust anaesthesia flow by small increments  
729 accordingly (~0.1 % increments every 15 seconds) until a stable state of anaesthesia  
730 is reached. While the final level of anaesthesia can vary based on the type and  
731 design of the isoflurane delivery/evacuation system used, experienced imaging  
732 personnel can successfully acquire data using level of isoflurane as low as 0.8%  
733 (with commonly used ranges of 1.0-1.5%). Critically, this should not be a  
734 standardized level but tailored to the physiological response of each animal to  
735 anaesthesia. Also, this should be an "imaging plane" of anaesthesia (responsive to  
736 toe pinch, no cardiorespiratory depression), not a "surgical plane" of anaesthesia  
737 (non-responsive to toe pinch, often cardiorespiratory depression).

738 TROUBLESHOOTING:.

739 6. Place ECG gel on contact pads, with a narrow strip of tape to attach the back of  
740 the fore paws to the contact pads and the pads of hind paws to the contact pads.

741 7. Insert temperature probe in rectum and tape in place. Document baseline body  
742 temperature and ensure that the warming lamps and platform are sufficient to  
743 maintain body temperature throughout initial imaging sessions (particularly after  
744 shaving).

745 8. Shave off hair on chest using an electric clipper designed for use with fine hair.  
746 Wipe clean chest with a damp paper towel. Clip fur from the top of the sternum  
747 down to the xiphoid process then down either side of the rib cage. Shaving lotion  
748 may be used if helpful.

749 9. Cover clipped area with hair removal lotion, leave for 30 seconds to a minute for  
750 mice. Remove lotion with warm wet swabs.

751 CAUTION: Make sure all hair removal lotion is removed as chemical burns will form if  
752 any is left.

753 10. Apply a generous amount of warmed ultrasound gel on the transducer or directly  
754 on the animal's chest.

755 11. Position the transducer parasternally about 90 degrees perpendicular with the  
756 long axis of the heart with image index marker of the transducer pointing  
757 posteriorly. While in B-mode, slide the transducer cephalad until the aortic valve  
758 (AV) comes into view.

759

760 TROUBLESHOOTING:

761 12. Press the color Doppler control key in the control panel. Apply color Doppler to  
762 region of the aortic valve and carefully sweep back and forth across the aortic  
763 root to observe for aortic valve regurgitation (mosaic-patterned, high velocity jet in

- 764 the ventricle during diastole) or stenosis (mosaic-patterned, high velocity jet  
765 during systole). Importantly, neither of these phenotypes generally emerge with  
766 chronological aging in mice.
- 767 13. Place the transducer in the apical position in the B-mode. Position the transducer  
768 so that it is angled towards the head of the mouse. Observe the right ventricle  
769 (RV), left ventricle (LV), right atrium (RA) and left atrium (LA) on the image  
770 display.
- 771 From the apical 4-chamber view, bring the mitral valve in focus by reducing the  
772 image width.
- 773 14. Using the apical 4-chamber view, apply color Doppler to image flow from the left  
774 atrium through the mitral valve during diastole. Observe for mitral valve  
775 regurgitation.
- 776 15. From the apical long axis view, tilt or point the transducer tip with a rocking  
777 motion so that the right ventricle is at the center of the image display. Reduce the  
778 image width so that only the right ventricle is visible in the image display.
- 779 16. Apply colour Doppler in the region of the tricuspid valve. Observe for tricuspid  
780 valve regurgitation.
- 781 17. Move the transducer in the parasternal position at the level of aortic valve.  
782 Observe the pulmonic valve in the parasternal short axis views.
- 783 18. Rotate the transducer clockwise to a modified parasternal long axis position.  
784 Then tilt the transducer slightly upward to obtain a short axis view of the pulmonic  
785 valve.
- 786 19. Apply colour Doppler in the region of the pulmonic valve to assess for valvular  
787 regurgitation (mosaic-patterned, high velocity jet during diastole) and stenosis  
788 (mosaic-patterned, high velocity jet during systole). Importantly, neither of these  
789 phenotypes generally emerge with chronological aging in mice
- 790 20. Obtain short axis view of the LV in B-mode with the transducer in parasternal  
791 short axis position at the level of papillary muscles.
- 792 21. From a parasternal short axis view of the left ventricle, press the M-mode button  
793 located in the control panel. Using the track ball, position the M-mode cursor at  
794 the level of the papillary muscles, and obtain M-mode images.
- 795 22. Measure left ventricular cavity dimension at end-diastole where the distance  
796 between the anterior wall and posterior wall is largest, and in end-systole where  
797 inward motion of the both anterior and posterior walls is maximum
- 798 23. Place the transducer in B-mode imaging modality and move the transducer to the  
799 apical window (make sure the transducer is angled towards the head of the  
800 mouse). Often times, placing the platform so that the animal is in a slight "head  
801 down tilt" can facilitate image acquisition from this window.
- 802 24. Place sample volume at the tips of the mitral valve leaflets. Derive the isovolumic  
803 relaxation time, isovolumic contraction time, left ventricular ejection time and peak  
804 mitral inflow velocity from the spectral display of pulsed-wave Doppler velocities  
805 across the mitral valve
- 806 25. Perform tissue Doppler imaging (TDI) of the mitral annulus in the apical long axis  
807 view. Press the TDI control key and place sample volume at the medial aspect of  
808 the mitral annulus. Make sure that the sample volume does not encroach on the

809 mitral leaflets. Keep Doppler sample volume size between 0.21 mm to 0.27 mm.  
810 Measure early diastolic velocity ( $e'$ ) of the mitral annulus.

811 CRITICAL: Review acquired images. Ascertain that all required images were  
812 obtained.

813 26. Remove the animal from anaesthesia and place the animal on an absorbent  
814 paper towel (not bedding, which can be aspirated/block airways during recovery).  
815 Observe the animal until sternal recumbency is attained. If the anaesthesia is  
816 administered appropriately, recovery should occur within 30 to 60 seconds.

817

### 818 **Assessment of cardiac function.**

819 **CRITICAL:** Generally speaking, high resolution echocardiography systems designed for use  
820 in small animals have multiple tools that facilitate calculation of cardiac function parameters.  
821 Consequently, this section will focus predominantly on critical aspects of *post hoc* analyses  
822 that are central to the acquisition of reproducible cardiac function data.

823

824 27. *Ejection fraction, systolic function, and ventricular wall thickness from M-mode*  
825 **(Figure 2A).** We typically assess ejection fraction from M-mode images (step  
826 21), as the data tend to be more reproducible and less affected by subtle  
827 alterations in probe orientation and/or ventricular movement/orientation  
828 throughout the cardiac cycle. For assessment of cardiac dimensions and  
829 automated calculation of ejection fraction, measure distance between the anterior  
830 and posterior ventricular walls at its largest point during end-diastole, and at its  
831 smallest point at end systole. Subsequently measure the anterior and posterior  
832 wall thicknesses at end systole and end diastole. While it is essential to use the  
833 papillary muscles as an anatomical landmark to ensure an appropriate imaging  
834 plane (which should be assessed on each M-mode measurement), it is critical to  
835 exclude them from any measurements of ventricular dimension or wall thickness.  
836 When possible, it is ideal to take 5 or more measurements during consecutive  
837 cardiac cycles to ensure accuracy of the data.

838 28.

839

840 a.

841 *Assessment of diastolic function using pulsed-wave Doppler measurements (Figure*  
842 **2B, C).** We generally do not assess diastolic function using E/A ratios due to the  
843 fusion of these two peaks at physiological heart rates in mice. Our preferred method  
844 is to use the E/ $e'$  method, which is derived from the blood and tissue Doppler images  
845 acquired in steps 24-25. It has also recently been shown that isovolumic relaxation  
846 time (IVRT) can serve as an additional tool to detect changes in diastolic function in  
847 the diseased hearts of young mouse models of diastolic dysfunction<sup>88</sup>. Given the high  
848 degree of context dependence of most models (and the general lack of validation in  
849 models of chronological aging to date), we strongly recommend the acquisition of at  
850 least two measurements of diastolic function (e.g., E/ $e'$  and IVRT) in studies of aging  
851 mice. Future work assessing the utility and validity of other variables in detecting  
852 subclinical and overt diastolic dysfunction (e.g., peak longitudinal strain rates, etc.)  
853 will be essential to advancing the field of biogerontology. For assessment of peak  
854 mitral inflow velocity (E), open the apical long-axis view of the mitral valve acquired in  
855 step 24. Place the sample volume for the pulsed-wave Doppler at the tips of the

856 mitral valve leaflets and acquire the spectral display over several cardiac cycles.  
857 Measure the peak inflow velocity, and if desired, the isovolumic contraction,  
858 isovolumic relaxation, and ejection times for the left ventricle. For assessment of the  
859 septal mitral annulus tissue velocity ( $e'$ ), place the Doppler sample volume  
860 (approximately 0.21-0.27 mm) at the septal region of the mitral annulus. Be sure that  
861 the sample volume does not encroach on the mitral leaflets. Measure and record the  
862 tissue velocity.

### 863 **Cognitive function**

864 CRITICAL This novel object recognition test is based on the one developed by  
865 Ennaceur and Delacour<sup>81</sup> and adapted from the protocol by Leger *et al.*<sup>82</sup>. This assay is of  
866 special translational interest given existing implementations for rodents and humans<sup>90</sup>. The  
867 test described here uses the recognition and association of salient cues, specifically the  
868 comparison between similar but not equal objects, in order to recognize which of the objects  
869 is already known and which one is novel. By adjusting the number of cues, the salience of  
870 cues, and the time allowed to learn and to memorize the information provided, the test can  
871 efficiently be adapted to mice of different ages and genetic background. In the same way, by  
872 adequately changing the type, form, and brightness of the objects in the object recognition  
873 test, the test can be adapted to be performed more than one time.

874

875 1. Before the assay. Plan the experiment by considering the sex of animals and the  
876 time of day behavioural assays will be undertaken. Identical procedures can be used  
877 on both male and female mice<sup>91</sup>. Notably, while it has previously been thought that  
878 female performance is sensitive to the phase of the estrous cycle, recent analyses  
879 have found that female variability when studied without regard for the estrous phase  
880 is actually not any greater than male variability; thus, female mice may be studied  
881 without monitoring of the estrous cycle<sup>92-94</sup>. Most laboratories perform behavioural  
882 tests during the daytime, which is the resting time for mice. We recommend that  
883 behavioural experiments are executed during the daytime, but avoiding the period of  
884 time leading up to the end of the light period when the animals start to become more  
885 active. The preferred time to perform the behavioural analysis is between 9 am  
886 (approximately two-three hours after the end of the dark cycle) and not after  
887 approximately 4-5 pm (the two hours leading up the beginning of the dark cycle).

888

889

890 2. Ensure animals have been handled as this decreases stress and anxiety induced by  
891 contact with the investigator. The experimenter can handle the animals while  
892 moving them from an old to a new cage every day over the course of two-three  
893 days. Gently take the animal by its tail (being careful not to suspend by the tail for  
894 too long) and place the animal with one hand on the experimenter's arm covered  
895 with a waterproof sleeve, or alternatively on a paper towel in your gloved hand, for  
896 15 s. Once all of the animals in one cage have been handled once, repeat in the  
897 same order 5 more times. It is also recommended that the animals are labelled by  
898 marking the tail.

899 3. For aged mice, determine visual acuity as the protocol below relies heavily on the  
900 vision of animals, and aged animals may suffer from blindness or reduced vision.  
901 Consequently, it is mandatory to perform a short visual discrimination test prior to all  
902 stages of the longitudinal analysis. Protocols for visual discrimination test for ageing

903 mice can be found elsewhere; we recommend the efficient visual discrimination test  
904 for ageing mice described by Brooks and colleagues<sup>95</sup>, which is based on the  
905 animal's normal ability to discriminate between bright and matte objects. Animals  
906 showing significantly reduced vision compared with the group mean should not be  
907 utilized for the remaining stages of the longitudinal analyses.

908 4. Prepare the arena and objects by cleaning with an ethanol solution (70%). If a  
909 methacrylate arena is used, no alcohol should be used, but a soft detergent solution.  
910 This step must also be carried out between all trials to avoid animals being  
911 influenced by the scents of previously tested mice.

912  
913 5. Prepare software. Most software requires the experimenter to draw or locate  
914 predefined areas into the arena, which will consist of small areas around both novel  
915 and known objects. We consider the mice exploring objects when their nose point,  
916 but not their center point<sup>23</sup>, is within a predefined area with a distance equal to or  
917 less than 2 cm from the object<sup>81</sup>. The maximum time for each trial will need to be set  
918 (see below). For specific steps, follow the manufacturer instructions.  
919

920

921

922 CRITICAL Ensure that one specific object is not used only as the novel object or  
923 as the familiar object. Change the use of each object within a pair, such that each  
924 object is equally used as novel object or familiar object within each experimental  
925 group. Similarly, one specific side of the box should not contain always the novel  
926 or the familiar objects. However, the assigned locations of the objects during  
927 training should be maintained during the test sessions.

928

929

930

931 6. Habituation and training. To Habituate the animals, place the animals in the room  
932 for at least 30 minutes prior to commencing habituation to the arena. Then allow  
933 animals to explore the open-field arena freely individually in the absence of objects  
934 for five minutes<sup>23,96</sup>. Note that center occupancy measures in the open field arena  
935 during habituation can be used to gauge stress- and anxiety-related behaviours in  
936 the mice<sup>97</sup>. Remove the animals and place back in their home cage.

937 7. The day after habituation, place two identical objects (objects A and A) in the arena.  
938 Individually introduce the animals into the open-field arena facing one of the corners  
939 and allow the animal to explore the objects freely (for five-fifteen minutes depending  
940 on the desired difficulty). Then remove each animal and put back in its home cage.

941

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#### TROUBLESHOOTING:

947

948 CRITICAL: The time spent exploring the objects will greatly impact the efficiency  
949 with which the animal is able to recognize new objects subsequently. This influences

950 the discrimination index (DI, see below for definition), and allows the experimenter to  
951 adjust the difficulty of the test according to the age, mouse strain, and model of  
952 ageing. In the same way, the time between training and test phases can be adjusted  
953 to modify the difficulty of the task. It is highly recommended to establish in a pilot  
954 study how normal ageing and/or geroprotectors may affect novel object recognition in  
955 the experimenter's specific mouse model. Special attention should be paid to the time  
956 between training and test phase, and the time allowed to explore the objects. The  
957 shorter the time allowed to explore, and the longer the delay between training and  
958 test phases, the more difficult the test. This timing can then be used as a reference,  
959 adjusting the difficulty of the test to the specific mouse model and the intervention.

- 960
- 961 8. *Test phase.*– Introduce each animal into the open-field arena containing a known  
962 object (object A) and a novel object (object B) and allow the animal to explore them  
963 freely for 10 minutes. Return all animals to their home cage after the test. CRITICAL  
964 To assess short-term object recognition memory (STM), perform the test one hour  
965 after the training phase. To assess long-term object recognition memory (LTM),  
966 perform the test 24 hours after the training phase or repeat the test with a different  
967 novel object (object C) 24 hours after the STM test.  
968  
969  
970

#### 971 TROUBLESHOOTING:

972  
973  
974 CRITICAL: At different times after the first test phase, new objects can efficiently be  
975 included as new unfamiliar objects, thereby permitting a longitudinal analysis of the  
976 cognition abilities of the animals. However, the DI will not be exactly comparable to  
977 those obtained at the first time, as prior experience with the testing environment (the  
978 arena) and the procedural aspects of the task is expected to influence behavioural  
979 outcomes.  
980

#### 981 **Metabolic Health *Body Composition assay***

- 982  
983
- 984 1. Plug the EchoMRI machine in at least 24 hours prior to the assay in order to charge  
985 the magnet.
  - 986 2. Calibrate the machine following the manufacturer's protocol.  
987 CRITICAL: The manufacturer advises (and we have noticed) that the presence of  
988 large masses of metal near the machine will interfere with (and may prevent)  
989 calibration, and may decrease the accuracy of measurements. The machine must be  
990 positioned away from large metal objects, including heavy metal tables, chairs, and  
991 gas cylinders.
  - 992 3. Using a scale, weigh each animal.
  - 993 4. Place awake mice into an appropriately sized holder, sliding the adjustable barrier to  
994 minimize the area of movement for each animal.

995 CRITICAL: The mouse needs to be snug, but should not be held tightly. The  
996 investigator should not attempt this procedure with progeroid mice or aged mice that  
997 are visibly frail unless the scientific goals of the project absolutely require it.

- 998 5. Measure whole body fat mass, lean mass, and (if desired) water mass using the  
999 instrument in accord with the standard operating procedure for the machine. We  
1000 typically take duplicate one-minute measurements on each animal, omitting the water  
1001 stage. CRITICAL: While scanning, the investigator should ensure that the sum total  
1002 fat mass, lean mass and water mass measured is within ~5% or less of the body  
1003 weight of the animal determined using a scale. If not, the investigator should re-  
1004 calibrate the machine, ensuring metal objects are not nearby, and repeat the scan; if  
1005 this does not solve the problem, the machine may need to be serviced.
- 1006 6. Average the measurements from the duplicate readings, and express lean, fat and  
1007 water mass data either as an absolute quantity (mass in grams) or as a percentage  
1008 of total body weight.

1009  
1010  
1011 ***Metabolic Health Energy Balance Assay***

- 1012 1. Arrange a room containing only the metabolic chambers in which the mouse can be  
1013 undisturbed for two to three days. If arranging a room containing only the chambers  
1014 is not possible, running experiments when no cage changes are schedule to occur  
1015 may help minimize disturbances.
- 1016  
1017  
1018  
1019 2. Turn on the system– ideally for an hour – in order to equilibrate to the desired  
1020 temperature and to remove humidity within the tubing and chambers.
- 1021 3. As water vapor needs to be removed from the air before it is flowed over the O<sub>2</sub> and  
1022 CO<sub>2</sub> sensors, check the water vapor removing system, which differs between  
1023 systems. Some systems may need a desiccant (e.g. Drierite) replenished or a  
1024 condensation trap emptied.
- 1025 4. Calibrate the system (following the manufacturer's instructions), using cylinders of  
1026 gas with known concentrations of O<sub>2</sub> and CO<sub>2</sub>.
- 1027 5. Fill feeders and water bottles.
- 1028 •
- 1029
- 1030 •
- 1031
- 1032 6. Weigh each animal. If desired, also determine body composition prior to placing  
1033 mice into the chamber to enable normalization to lean mass instead of body weight.
- 1034 7. Place one mouse into each chamber, sealing it according to manufacturer's  
1035 instructions, and entering the mouse ID and weight into the control program's  
1036 software.
- 1037 CRITICAL: If multiple chamber runs are to be combined, do not always load mice of  
1038 the same group into the same chamber(s) each time. Randomize mice such that  
1039 genotypes and sexes are evenly distributed. This eliminates any biases resulting  
1040 from individual chamber differences (e.g. in noise or lighting).
- 1041 8. Run the chambers, checking the availability of food and water once per day. As mice  
1042 are being temporarily single housed in the new environment of the metabolic testing  
1043 chamber, a degree of habituation is required. Typically, discard data collected during  
1044 the first 24 hours of chamber time to account for this acclimatization.

1046 CRITICAL STEP: When checking on the animals the first day, the data from the  
1047 habituation period should be visually inspected to ensure proper functioning of the  
1048 machine. In particular, the O<sub>2</sub> in, CO<sub>2</sub> in, O<sub>2</sub> out, and CO<sub>2</sub> out values and the RER  
1049 should be checked to ensure that gas concentration values are fluctuation as  
1050 expected and the RER values are physiologically reasonable.

### 1051 1052 **Glucose tolerance test**

- 1053 1. Fast the mice by placing them in new, clean cage with *ad libitum* access to water, but  
1054 without food. The length of this fast – and the time of day the experiment is  
1055 performed – depends upon the question being asked. If primarily interested in  
1056 pancreatic beta cell function, a short daytime fast of 6 hours is sufficient. However, if  
1057 interested in hepatic glucose metabolism and insulin sensitivity, a longer overnight 16  
1058 hour fast should be performed. As the longer fast provides more information, and the  
1059 short-term fast is often more variable and uninformative as C57BL/6J mice do not  
1060 have an age-dependent decrease in beta cell function<sup>99</sup>, most aging researchers are  
1061 best served by performing an overnight fast for 16 hours. The 16h fast should start in  
1062 the evening (approximately 4-5 PM local time), with the glucose tolerance test  
1063 performed the following morning, approximately 16 hours later (approximately 8-9  
1064 AM local time). If a short fast is performed, the fast should start as soon as the light  
1065 cycle starts (approximately 6 AM local time), with the glucose tolerance test  
1066 performed 6 hours later. Special challenges are posed by studies involving once-a-  
1067 day feeding such as calorie restriction (CR). While there is no perfect answer, if an  
1068 overnight fast is to be performed, the CR animals should be fed prior to lights out  
1069 (approximately 4pm). As CR animals typically consume their food within 2 hours,  
1070 both CR and *ad libitum* fed animals will therefore be fasted for approximately 16  
1071 hours the following morning.

1072 CAUTION: Fasting aged mice overnight (as for a glucose tolerance test) can be  
1073 stressful, and should be avoided in aged mice (for C57BL/6J mice, we define this as  
1074 male mice over 30 months of age and female mice over 28 months of age). We  
1075 recommend ceasing routine glucose tolerance tests after approximately 24 months of  
1076 age.

1077 CRITICAL: The bedding should not be caloric; we recommend aspen bedding. The  
1078 use of commercial sterile water gels (e.g. hydrogel) instead of water bottles is  
1079 discouraged due to the small number of calories contained within the gel.

- 1080  
1081  
1082  
1083 2. Move the mice to the procedure room for acclimatization at least one hour prior to the  
1084 start of the glucose tolerance test. During this time period, you can continue to work  
1085 preparing the room and the mice (steps 3-5 below), but unnecessary noise should be  
1086 avoided. Weigh each mouse, marking the tail of each animal with a sharpie or similar  
1087 marker such that each animal can be readily distinguished from its cage mates  
1088 without picking it up.
- 1089  
1090 3. Calculate the volume of glucose in  $\mu\text{L}$  to be administered to each mouse. Using 30%  
1091 glucose, a dose of 1g/kg of glucose can be rapidly calculated by dividing the body



1092 weight in grams by 30, and then multiplying by 100 (Example: (30 g mouse/30) x 100  
1093 = 100 $\mu$ L). Enter this value on the spreadsheet.

1094 4. Prepare one syringe for each mouse. For either an I.P. or oral glucose tolerance test,  
1095 attach the appropriate needle, load a 1mL syringe with more than the required  
1096 volume of glucose, and then remove all bubbles and extra volume from the syringe.

1097 5. To begin the glucose tolerance test, pick up the first mouse, and make a transverse  
1098 nick across the tail using the edge of a fresh razor blade.

1099 6. Immediately collect a fresh drop of blood with a new glucometer test strip.

1100 7. Gripping the mouse firmly, administer the glucose and start the timer, which will  
1101 count upwards from 0. Return the first mouse to its cage.

1102 8. Proceed to each mouse in sequence, nicking the tail, collecting a fresh drop of blood  
1103 with a glucometer test strip, and administering glucose as in steps 6-7. For each of  
1104 these subsequent mice, note the time of injection of glucose for each mouse on the  
1105 spreadsheet.

1106 TROUBLESHOOTING:

1107 9. Determine glucose levels in each mouse 15, 30, 45, 60, and 120 minutes after  
1108 glucose administration. I.e. when the timer reads "15:00", it is time to take a second  
1109 reading from the first mouse that was injected.

1110 CRITICAL: It is important to collect a fresh drop of blood that is not contaminated with  
1111 blood from the previous time point; this can be readily achieved by wiping the surface  
1112 of the tail and then gently stroking the tail until a fresh drop is released.

1113 10. At the end of the assay, ensure that bleeding has stopped in the mice. In rare cases,  
1114 use of a silver nitrate pencil or styptic powder may be necessary to stop the flow of  
1115 blood.

1116

### 1117 ***Insulin tolerance test***

1118 1. Fast the mice by placing them in new, clean cage with *ad libitum* access to water, but  
1119 without food.

1120 Many aging researchers routinely fast their mice overnight prior to conducting an  
1121 insulin tolerance test. However, we believe that this is often unnecessary, and that a  
1122 short fast – long enough to ensure that all of the mice have not fed recently – is  
1123 usually sufficient to assess insulin tolerance, whilst being less stressful. Also, mice  
1124 fasted for 4-6 hours have a relatively high fasting blood glucose relative to mice  
1125 fasted overnight. This widens the dynamic range of the assay, increasing its power,  
1126 and also is safer, as it lessens the risk of hypoglycaemia.

1127 CAUTION: C57BL/6J male mice over 30 months of age and C57BL/6J female mice  
1128 over 28 months of age should not be fasted overnight. We recommend ceasing  
1129 routine insulin tolerance tests after 24 months of age.

1130 CRITICAL: The bedding should not be caloric; we recommend aspen bedding. The  
1131 use of commercial sterile water gels (e.g. hydrogel) instead of water bottles is  
1132 discouraged due to the small number of calories contained within the gel.

1133 2. Move the mice to the procedure room for acclimatization at least one hour prior to the  
1134 start of the insulin tolerance assay. During this time period, you may continue to work  
1135 preparing the room and the mice (steps 3-5 below), but unnecessary noise should be  
1136 avoided. Weigh each mouse, marking the tail of each animal with a sharpie or similar

1137 marker such that each animal can be readily distinguished from its cage mates  
1138 without picking it up.

- 1139
- 1140 3. Prepared sterile diluted insulin for injection into the mice as described in “Reagents”.

1141 CRITICAL: The insulin dose must be adjusted empirically for different strains and  
1142 conditions; for example, while we utilize 0.75U/kg for *ad libitum* fed C57BL/6J mice,  
1143 0.5U/kg is sufficient for CR mice fasted overnight.

1144 CRITICAL: While this is an insulin tolerance test, at least 10 mL of sterile 30%  
1145 glucose should also be prepared and brought to the procedure room for the  
1146 emergency treatment of hypoglycaemic mice.

- 1147 4. Calculate the volume of insulin in  $\mu\text{L}$  to be administered to each mouse.

1148 We typically dilute 22.5  $\mu\text{L}$  of stock insulin in 10mL of 0.9% sterile saline; using this  
1149 concentration, the volume of insulin to achieve a final dose of 0.75U/kg can be  
1150 rapidly calculated by dividing the body weight in grams by 30, and then multiplying by  
1151 100 (Example:  $(30 \text{ g mouse}/30) \times 100 = 100\mu\text{L}$ ). Enter this value on the spreadsheet.

- 1152 5. Prepare one syringe for each mouse, loading a single 1mL syringe with more than  
1153 the required volume of diluted insulin, and then removing all bubbles and extra  
1154 volume from the syringe.

- 1155 6. To begin the insulin tolerance test, pick up the first mouse, and make a transverse  
1156 nick across the tail using the edge of a fresh razor blade.

- 1157 7. Immediately collect a fresh drop of blood with a new glucometer test strip.

- 1158 8. Gripping the mouse firmly, administer the insulin and start the timer, which will count  
1159 upwards from 0. Return the first mouse to its cage.

1160 CRITICAL STEP: Insulin that was not correctly injected into the I.P. space will  
1161 aggregate and not enter the circulation; the experimenter should note any possibly  
1162 misinjected animals during this step.

- 1163 9. Proceed to each mouse in sequence, nicking the tail, collecting a fresh drop of blood  
1164 with a glucometer test strip, and administering insulin as in steps 7-8. For each of  
1165 these subsequent mice, note the time of injection of insulin for each mouse on the  
1166 spreadsheet.

1167 TROUBLESHOOTING:

1168 Glucose levels should be determined in each mouse 15, 30, 45, 60, and 120 minutes  
1169 after glucose administration. Thus, when the timer reads “15:00”, it is time to take a  
1170 second reading from the first mouse that was injected. CRITICAL: It is important to  
1171 collect a fresh drop of blood that is not contaminated with blood from the previous  
1172 time point; this can be readily achieved by wiping the surface of the tail and then  
1173 gently stroking the tail until a fresh drop is released.

1174

1175 CRITICAL: Continual assessment of the animal’s health is required throughout the  
1176 course of the assay in order to avoid hypoglycaemia. Mice with a blood glucose level  
1177 that rapidly drops below 20 mg/dL, that become cool to the touch, or that become  
1178 unresponsive should be removed from the assay, injected with approximately 1.5  
1179 g/kg sterile glucose, and placed in a new cage by themselves with food on the cage  
1180 bottom, and monitored until recovered.

1181 CRITICAL STEP: If blood glucose levels do not fall in an animal following insulin  
1182 administration and misinjection is suspected as the cause, I.P. insulin can be  
1183 administered a second time immediately following the end of the ITT, and the blood  
1184 glucose tested 15 minutes later. If the animal now responds robustly, indicating the  
1185 first dose of insulin was likely misinjected, the ITT data for that animal should be  
1186 discarded.

- 1187 10. At the end of the assay, ensure that bleeding has stopped in the mice. In rare cases,  
1188 use of a silver nitrate pencil or styptic powder may be necessary to stop the flow of  
1189 blood. Any mice that appear unresponsive or weak can be given 1g/kg glucose.  
1190 Return food to the cage.

1191 CRITICAL: Place some food pellets on the bottom of the cage so animals can reach  
1192 easily.

### 1193 **Grid hanging test to determine Muscle strength and neuromuscular function**

1194 CRITICAL The cage top test, also called grid hanging test or four limbs hanging test (as  
1195 opposed to the wire hanging test where only the forelimbs are used) is a quantitative,  
1196 inexpensive, non-invasive test used to measure the muscular strength and endurance of  
1197 mice in opposing the gravitational force with their four limbs. In fact, mice will endure to their  
1198 maximum strength to instinctively avoid falling. The advantage over the two limbs test is that  
1199 mice cannot prolong hanging time by balancing or using any other behaviour that may bias  
1200 the outcomes, so results tend to be more consistent and reliable<sup>100</sup>. The test can be used to  
1201 measure decline in strength with age, if repeated measurement throughout life is conducted.  
1202 The ability to hang depends on sex, strain, and age<sup>18,20</sup>.

- 1204 1. Before starting the test, it is important to reduce to a minimum the stress of the  
1205 animal, so it is suggested to avoid changing cage bedding in the previous 24h.
- 1206 2. Acclimate the mice for one hour to the testing room, and record the bodyweight of the  
1207 mouse.
- 1208 3. Train the mice before performing the test for the first time. To do this....., CRITICAL  
1209 STEP In longitudinal studies, usually training the first time is enough.
- 1210 4. Place the mouse on the grid, allow him to grip securely, then quickly turn the grid  
1211 upside down. The grid can be placed onto a holding apparatus or held by hand at a  
1212 fixed distance from the floor (80 cm) with a soft pad underneath to avoid injuries after  
1213 the fall.

1214 CRITICAL: If the mice let go quickly because they lose their fear of falling, the  
1215 operator may hold the apparatus a bit higher.

- 1216 5. Start the timer when you invert the grid to record the latency time to fall (in seconds).
- 1217 6. If the mouse falls before 10 seconds, allow it to repeat the test. If it still falls before 10  
1218 seconds on the third trial, record the best latency out of the three.
- 1219 7. When finished, place the mouse back in the home cage, and test the next mouse.  
1220 Depending on how the apparatus is set up, it may be possible to test multiple mice at  
1221 once, especially for young ones that tend to hold on longer. It is not advisable to test  
1222 multiple old mice, as they tend to fall quickly and it is difficult to properly track all of  
1223 them. Also, ensure to wipe the grid and the underneath pad with 70% ethanol or  
1224 chlorine dioxide based sterilant (Clidox) before starting the next cage.

- 1225 8. Repeat steps 4-7 until each mouse has done the test three times. Allow the mice to  
1226 rest for 30 minutes in between. **CRITICAL:** In longitudinal studies, when the mice are  
1227 still relatively young it is advisable to consider the maximum hanging time (young  
1228 females can hang for more than 3 hours at 3-6 months) to have a more  
1229 discriminating power, whilst when comparing the effects of different treatments in  
1230 mice older than 12 months, usually setting a threshold at 7 minutes is sufficient.
- 1231 9. Average the three trials and perform a two-way ANOVA and use weight as a  
1232 covariant.

1233  
1234  
1235 ***Grip strength to determine Muscle strength and neuromuscular function***

- 1236 1. As general good practice acclimatize mice to the novel environment for 30-45  
1237 minutes prior to testing if the testing is carried out in a different room than their  
1238 normal housing. This test should be carried out on an open bench, as disruption from  
1239 the air flow in hoods can affect the results.

1240 **CRITICAL:** For longitudinal studies, always perform the test at the same time of day.  
1241 Mice with missing or injured digits should not be tested.

- 1242 2. Remove a mouse from the cage, gripping the base of the tail between the thumb and  
1243 the forefinger with the thumb below the forefinger.

- 1244 3. Gently lower the mouse over the top of the grid so that only its front paws can grip  
1245 the grid. Stop when you can feel some pulling tension from the mouse

- 1246 4. Keeping the torso horizontal, pull the mouse back steadily (not jerking) down the  
1247 complete length of the grid until the grip is released. Do not allow the rear paws to  
1248 touch the grid. If the rear paws do touch, then re-do the measurement. If the mouse  
1249 does not grip the grid properly then repeat the measurement. Similarly, if the mouse  
1250 turns backwards during the pull, or leaves the bar without resistance.

1251 **CRITICAL STEP:** High variability in results can result from inconsistent performance  
1252 of the procedure by the operator. The operator may hold the mouse at an incorrect  
1253 angle, and the reading may be influenced by the forces generated by the operator  
1254 when pulling.

- 1255  
1256 5. Record the value from the meter. Repeat 3 times at 1-minute intervals and select the  
1257 highest value. Do not repeat the measurement more than 5 times.

- 1258 6. Front paws alone are generally sufficient, but if time permits, grip strength  
1259 assessment can be repeated with all four paws on the grid. However, with old mice  
1260 the risk of fatigue is greater during multiple testing; allowing mice to rest at least 15  
1261 minutes before testing all four paws is recommended.

- 1262 7. Normalize grip strength values by lean mass, or if lean mass is not available, use  
1263 body weight. Major differences in body weight can influence the results substantially,  
1264 potentially leading to erroneous conclusions. An alternative approach to analyze  
1265 these data is to use ANCOVA analysis using body weight or lean mass as a  
1266 covariate, which is widely accepted as a more statistically rigorous method to  
1267 address confounding factors such as body weight than simply normalizing to the  
1268 weight of the animals <sup>101</sup>. As shown in **Fig. 6A**, the senolytic cocktail dasatinib plus  
1269 quercetin improves the grip strength of mice in this assay.

1270

1271 ***Hanging bar to determine Muscle strength and neuromuscular function***

1272 1. Acclimate animals by placing them on the wire of the hanging bar apparatus for  
1273 several minutes watching that they don't use their back legs or tail to climb on the  
1274 wire. Sometimes, the mice are difficult to position such that they use only the front  
1275 limbs. This is a matter of patiently training the mice by gently repositioning it on the  
1276 wire in the correct way until they learn. They may need to be repositioned several  
1277 times because they tend to use only their hind limbs.

1278 CRITICAL: In longitudinal studies young mice that have not been previously tested  
1279 need to be trained and acclimated by placing on the wire each day for 5 days. Old  
1280 mice (24 months and older) usually are not so active, and do not jump on the wire or  
1281 run on it, and thus can be trained only once prior to testing. The outcome of results  
1282 will depend on how well they are trained.

1283 TROUBLESHOOTING:

1284 CRITICAL STEP: if the mice let go quickly even when they are young, bring the wire  
1285 to a slightly higher position to induce a fear of falling.

- 1286 2. On the day of the test, bring all mice to the procedure room 30 min prior to the test to  
1287 allow animals to acclimatize to local environmental cues.
- 1288 3. Carefully take mouse at the base of its tail and bring it in proximity to the wire.
- 1289 4. Let the mouse grasp the wire with the two forepaws only, and slowly lower the hind  
1290 limbs in such a way that the mouse hangs on the wire only supported by its  
1291 forepaws.
- 1292 5. Start the timer as soon as the mouse is released.
- 1293 6. When a mouse shows improper behaviour (like balancing on or deliberately jumping  
1294 off the wire, grabs the wire with four paws, or reaches the end of the wire) reposition  
1295 the mouse on the wire without stopping the timer.
- 1296 7. If mouse falls before 10 seconds have elapsed, remember what time they fell and  
1297 quickly repeat the test. If on the 3rd try the mouse still falls before 10 seconds, record  
1298 the best latency out of 3 attempts.
- 1299 8. When a mouse falls off the wire, stop the timer and record the hanging time. When  
1300 mice are able to hang for 5mins take them off the wire and return them to the cage.
- 1301 9. Record hanging time.
- 1302 10. Once one trial is completed for all the mice, complete a second trial by repeating  
1303 steps 3-9. There should be approximately 30 minutes break between trials for each  
1304 mouse. Repeat steps 3-9 again so each mouse has done a total of 3 trials.
- 1305 11. Take the best time out of the 3 trials for analysis and normalize results to lean mass  
1306 or body weight, or perform ANCOVA using weight or lean mass as covariant. As  
1307 shown in **Fig. 6B**, the senolytic cocktail dasatinib plus quercetin improves the hang  
1308 time of mice in this assay. In young mice and mice up to 18 month old, test every 6  
1309 months. After that, we recommend testing mice monthly.

1310

1311 ***Rotarod to determine Muscle strength and neuromuscular function***

- 1312 1. CRITICAL Training mice prior to conducting the rotarod assay is essential. An  
1313 example training schedule is in steps 1 and 2. Place mice on the rotarod at a

1314 constant speed of 4rpm for a minimum of 60s; and up to 300s. If they fall place them  
1315 back on. Use a timer to count the 60-300s.

1316 2. On the next two dates (Days 2 and 3), train the mice again by placing them on the  
1317 rotarod at constant speed at 4 rpm for 60-300s.

1318

1319

1320

1321 CAUTION: As it is the case for behavioural experiments, it is important to not make  
1322 the mice anxious, and thus careful handling is recommended.

1323 CRITICAL STEP If mice have been previously tested (as in a longitudinal study),  
1324 perform a refresher training run of the mice as above on just the day before testing.  
1325 Do not train mice on the day of the test in order to avoid fatigue. If the mice fall then  
1326 place them back on. Nothing needs to be recorded during this refresher.

1327

1328 3. The day after the last day of training bring the mice into the testing room, allowing  
1329 them to acclimatise to the room for 30 minutes. During this time, weigh all of the  
1330 mice, marking tails if desired for easier animal identification.

1331 4. Whilst the mice are acclimatising, clean the Rotarod apparatus in accordance with  
1332 facility protocols and manufacturer's instructions.

1333 5. Put some padding at the bottom to ensure mice are not harmed during the fall. Some  
1334 of us fill a Ziplock bag with clean aspen bedding; this has the advantage of being  
1335 sanitizable.

1336 6. Start the rotarod at 4 rpm, and quickly place each mouse in a separate lane of the  
1337 rotarod; mice should be placed facing away from the experimenter. The maximum  
1338 number of mice that can be analysed at a single time is determined by the number of  
1339 lanes on the equipment, but is typically four-five.

1340 7. Once all the mice have settled and are facing away from the investigator, accelerate  
1341 the rotarod. The acceleration speed and interval can be determined by the  
1342 investigator; but accelerating from 4 to 40 rpm in 300 sec is typical.

1343 8. The timers for all five lanes will start counting up. If the mouse falls within 10 second  
1344 from the start put it back on. If it fails three times to remain on the Rotarod as it slowly  
1345 rotates, remove them from the trial and record the time as 0s.

1346 CRITICAL: if a mouse has not fallen by the end of the 300s, the experiment should  
1347 be ended and the time recorded as 300 seconds. This maximum time may need to  
1348 be adjusted in different strains to fully capture the dynamic range of the animals'  
1349 abilities.

1350 CRITICAL: When the mouse falls, it will usually (but not always) stop the timer. While  
1351 recording with the computer is a useful timesaver, a paper record should also be kept  
1352 such that data can be rapidly captured if the time fails to stop.

1353 9. As soon as a mouse falls, carefully remove the mouse from the apparatus and return  
1354 the mice to the home cage. In the case of passive rotation (the mice hang the rotarod  
1355 and complete a rotation), reposition the mice once; if this behaviour is repeated,  
1356 consider this as the time to fall (but make a note).

1357 10. Repeat the test (steps 6-9) so each mouse does it 3 times. CRITICAL: Trials of the  
1358 same animal should be separated by a minimum of 15 minutes. If comparisons  
1359 between cohorts analysed on separate days are desired, the resting time should be  
1360 similar between each cohort.

1361 11. Plot rotarod performance as time to fall, or as maximal speed. A major  
1362 consideration is that lighter animals, if healthy, typically can stay on the  
1363 rotarod longer than obese animals simply due to body weight. Rotarod data  
1364 can be normalized to body weight, although the most rigorous analysis is to  
1365 perform a 2-way ANCOVA with body weight or lean mass as a covariant. As  
1366 shown in **Fig. 6C**, the senolytic cocktail dasatinib plus quercetin improves the  
1367 rotarod performance of mice in this assay.

## 1371 **TIMING**

1372 Timing varies each assay in the toolbox, and for most assays in the toolbox timing will  
1373 increase as the number of animals increase. We have given indicative times for each test for  
1374 n=10 mice and two operators in table 1. Table 1 also provides a schedule of when to carry  
1375 out tests over a 6 week period. Conducting any of the above assays requires advance  
1376 planning!

## 1378 **TROUBLESHOOTING**

1379 We have selected these tests for their relative simplicity. Therefore it is unlikely that they do  
1380 not work. However, variability may be high and differences may not be detected. To address  
1381 this we have highlighted all the critical steps in each protocol. If any problem is encountered  
1382 we recommend to carefully re-examine those steps.

1383 Table 2 also gives guidance regarding some of the most commonly encountered problems.

## 1385 **ANTICIPATED RESULTS**

### 1386 **Cardiac function**

1387 Generally speaking, mice are remarkably resistant to chronic left ventricular pressure  
1388 overload and have relatively preserved systolic function (but a reasonable hypertrophic  
1389 response) during all but the most severe insults (e.g., transverse aortic constriction). As a  
1390 general guideline, we exclude animals that have evidence of significant aortic or mitral valve  
1391 regurgitation (which is almost always associated with a phenotype of cardiac hypertrophy  
1392 and dilatation) unless that is a specific aim of the study. Importantly, measures of cardiac  
1393 mass (either echocardiographic estimates or direct wet weights after euthanasia) should be  
1394 evaluated as both absolute weights and when normalized by bodyweight.

1395 In our experience, severe systolic cardiac dysfunction is an uncommon phenotype with  
1396 unstressed chronological aging (i.e., ejection fraction is generally well-preserved and greater  
1397 than 75% in most strains). One may observe subtle reductions in ejection fraction, however,  
1398 which are generally associated with increases in end-diastolic operating volumes and larger  
1399 increases in end-systolic volumes and slight increases in ventricular thickness/mass.

1400 Diastolic dysfunction can be observed with aging in mice, with cardiac hypertrophy and  
1401 decreased myocardial performance observed during aging<sup>47</sup>, and significant changes in the  
1402 E/e' ratio can become evident with long-term pathophysiological stressors. As is the case  
1403 with numerous patient populations, increases in the E/e' ratio are indicative of diastolic  
1404 dysfunction. While our experience to date would suggest that E/e' values ranging from 15-  
1405 25 are typical in young, unstressed animals, there can be significant variability across  
1406 strains. With prolonged stressors combined with chronological aging, we have observed  
1407 values of E/e' that exceed 75 units, and can be associated with increases in ventricular wall  
1408 thickness and overall mass.

1409 Collectively, these approaches are readily applicable to assessment of cardiac  
1410 function with aging in small animals/rodents. The procedure described in this toolbox  
1411 provides a simply way to assess cardiovascular function longitudinally in aging mice. While  
1412 inbred mouse strains are quite resistant to the development of atherosclerosis<sup>102</sup>, mice can  
1413 and do develop both systolic and diastolic cardiac dysfunction with age<sup>103</sup>. We expect that  
1414 many geroprotectors will prevent or reverse pre-existing age-dependent cardiac hypertrophy  
1415 and diastolic dysfunction<sup>35,47,48</sup>. This can be seen by an improvement in left ventricular  
1416 ejection fraction, improved systolic function (LVIDd) or decreased LVIDs.

1417 Further comprehensive assessments of cardiac and heart valve function (as described  
1418 previously by Verzosa and colleagues<sup>89</sup>) generally require significant training and  
1419 experience, and should not be attempted without extensive training to ensure appropriate  
1420 technique and rigorous/reproducible results.

1421

## 1422 **Cognitive function**

1423 The tracking software can output a range of parameters, such as velocity, time spent  
1424 exploring both objects (novel and known) and time not exploring objects. Tracking software  
1425 including nose-point detection is more reliable than tracking tools based only on the  
1426 detection of the center point of the animal<sup>23</sup>. We consider a behaviour as active object  
1427 exploration if the nose point of the mouse is within the pre-defined target zone with a  
1428 distance to the object equal to or less than 2 cm<sup>81</sup>. Climbing on the objects itself is not  
1429 considered object exploration unless it is combined with sniffing the object<sup>82</sup>. When  
1430 automatically scoring object exploration, climbing behaviours can be excluded by removing  
1431 data in which the center point of the mouse is located within the target zone<sup>23</sup>. Despite the  
1432 use of automated systems, it is recommended that an experienced researcher review the  
1433 output of the software to detect potential misbehaviour of the animals during the test, which  
1434 cannot be distinguished by the tracking software.

1435

1436 Results should be presented as a Discrimination Index (DI). The DI is the difference  
1437 between the time exploring the novel and the familiar object, divided by the total amount of  
1438 exploration of both objects. Exploration is defined as directing the nose to the object at a  
1439 maximum distance of one centimetre. Some animals may explore very few times, or not  
1440 explore one of the objects during any of the training or the test phases. Those animals are  
1441 excluded from the analysis. A positive score means more time spent exploring the novel  
1442 object and a negative score indicates more time spent exploring the familiar object. A score  
1443 of 0 indicates no preference for any of the objects. However, a minimum DI threshold of  
1444 +0.20 was agreed to indicate meaningful novel object discrimination. It is expected that the  
1445 vast majority of animals will explore the novel object for longer than the known object. An  
1446 example of data is presented in **Fig.3**.

1447 Geroprotectors such as rapamycin have shown to improve cognitive function with age.  
1448 Particularly in the context of mouse models of Alzheimer's disease, there is growing



1449 evidence that geroprotectors may be able to treat or prevent the disease and its associated  
1450 cognitive deficits<sup>52,53,104-107</sup>. We expect that young mice will spend more time exploring new  
1451 objects as opposed to already known objects. This effect is lost as the mouse ages, and old  
1452 mice, which will spend equal time with old and new objects. An improvement in this function  
1453 would be shown by the recovery of this ability to explore new objects for longer.

1454

### 1455 ***Body Composition assay of metabolic health***

1456 The procedure described in this toolbox is a simple way to measure body  
1457 composition using magnetic resonance imaging (MRI). Many longevity-promoting  
1458 interventions are associated with reduced adiposity, including diets in which total calories,  
1459 protein, or methionine are restricted<sup>12,17,18,108-111</sup>. Some pharmaceutical interventions which  
1460 extend lifespan, including rapamycin<sup>112</sup> and acarbose<sup>113</sup>, are likewise associated with  
1461 reduced adiposity. On the other hand, senescence cell clearance restores adipose tissue  
1462 function and prevents fat loss in aged mice<sup>28</sup>. A geroprotector would likely help to preserve  
1463 and maintain both muscle mass and adipose tissue function, and limit increases in ectopic  
1464 lipid accumulation during aging.

### 1465 ***Energy Balance to assay metabolic health***

1466 In this toolbox, we describe a simple procedure for the measurement of energy balance –  
1467 simultaneous evaluation of food consumption, activity, and energy expenditure as assessed  
1468 by indirect calorimetry using an open flow respirometry system. Very precise CO<sub>2</sub> and O<sub>2</sub>  
1469 sensors measure the difference in CO<sub>2</sub> and O<sub>2</sub> concentrations in air volumes flowing through  
1470 control or animal cages. The amount of oxygen consumed, and the amount of CO<sub>2</sub>  
1471 produced, over a given period of time can thus be determined.

1472 Specific measurements of interest obtained from the energy balance assay usually include  
1473 the VCO<sub>2</sub> and VO<sub>2</sub>, the Respiratory Exchange Ratio (RER), activity, food consumption, and  
1474 energy expenditure (heat). While differences between groups can be measured at multiple  
1475 time points, typically averages during the course of the light and dark cycles are calculated  
1476 and compared between groups. As shown in **Fig. 4**, RER, food consumption, and energy  
1477 expenditure are higher at night, as expected since mice are nocturnal, and these values are  
1478 affected by dietary composition. There are specific issues for each type of data:

- 1479 • Activity: The Oxymax software for the Columbus Instrument CLAMS system  
1480 distinguishes between all activity and ambulatory activity; reporting the sum of  
1481 ambulatory along the X and Y axis may provide the most accurate assessment of  
1482 activity.
- 1483 • Energy expenditure: may be normalized to either body weight or lean mass. While  
1484 many investigators believe normalizing to lean mass is best, this may be  
1485 inappropriate as it assumes that fat mass has a minimal contribution to metabolism<sup>98</sup>.  
1486 Analysing both group and body composition effects using analysis of covariance is  
1487 widely considered to be the gold-standard<sup>98</sup>.
- 1488 • Food consumption: It is very important to inspect the food consumption data and  
1489 remove any data that reports food consumption of less than zero, as this indicates  
1490 either a scale problem or the mouse “playing” with food.

1491

1492 We expect that the value of the RER will vary between approximately 0.7, which  
1493 reflects the exclusive utilization of fats, and 1.0, which reflects the exclusive utilization of  
1494 carbohydrates<sup>3,6,18,114</sup>. In actual fact, the measured RER can temporarily exceed 1.0 in  
1495 animals as well as humans that are exercising very vigorously or are calorie restricted.

1496 While the effect of geroprotectors on RER and other readout may vary, healthy  
1497 young animals have increased “metabolic flexibility” – the ability to adapt and respond to  
1498 changes in metabolic demand<sup>115</sup> – including the switch between fasting and fed states in  
1499 response to the day-night cycle. Unhealthy conditions, including aging and metabolic  
1500 disease, impairs metabolic flexibility and the ability of RER to rapidly switch as mice cycle  
1501 between the dark and light portions of the day. We expect that young and healthier animals  
1502 – including aged mice treated with effective geroprotectors – will exhibit better metabolic  
1503 flexibility, and thus a greater difference in RER measured during the dark and light portions  
1504 of the day.

### 1505 ***Glucose tolerance test as an assay of metabolic health***

1506

1507 The protocol in this toolbox is an effective way to determine glucose tolerance, which when  
1508 performed after an overnight fast provides insight into hepatic gluconeogenesis and insulin  
1509 sensitivity, as well as pancreatic beta cell function. It is expected that glucose will be cleared  
1510 more rapidly, and hepatic glucose production suppressed more rapidly, if a geroprotector is  
1511 effective. Calorie restriction, as well as many other geroprotective interventions including  
1512 protein restriction, metformin, and acarbose improve glucose tolerance in both young and  
1513 aged mice, reducing fasting blood glucose levels and decreasing area under the  
1514 curve<sup>3,12,14,17,18,60,62,116</sup>. If important for the analysis, groups of mice can be compared at  
1515 individual time points using two-way repeated measures ANOVA. However, it is usually  
1516 more biologically meaningful ([https://www.graphpad.com/support/faq/when-does-it-make-  
1517 sense-to-use-repeated-measures-two-way-anova/](https://www.graphpad.com/support/faq/when-does-it-make-sense-to-use-repeated-measures-two-way-anova/)), as well as more powerful, to calculate  
1518 area under the curve (AUC) and use AUC as the basis for the statistical analysis. As shown  
1519 in **Fig. 5A**, the geroprotector rapamycin impairs glucose tolerance – increasing the AUC –  
1520 when injected daily, but when dosed intermittently has no significant effect on glucose  
1521 tolerance.

### 1522 ***Insulin tolerance test as an assay of metabolic health***

1523 The protocol in this toolbox is an effective way to determine insulin sensitivity, which is often  
1524 – although not always – increased in interventions that promote metabolic health. As with  
1525 glucose tolerance, calorie restriction and a number of other geroprotective diets and  
1526 interventions in mammals are strongly associated with improved insulin sensitivity  
1527 <sup>3,12,17,61,111,113</sup>. Glucose data for each animal should be normalized to its fasting (basal) blood  
1528 glucose level (fasting blood glucose = 100% of basal glucose), and plotted as % of basal  
1529 blood glucose. As with glucose tolerance tests, groups of mice can be compared at  
1530 individual time points using two-way repeated measures ANOVA. However, it is usually  
1531 better to calculate the area under the curve (AUC) of the normalized blood glucose values,  
1532 and use AUC as the basis for the statistical analysis. As shown in **Fig. 5B**, intermittent  
1533 administration of the geroprotector rapamycin does not impair insulin sensitivity; this is in  
1534 contrast to daily administration, which does impair insulin sensitivity and increases the AUC  
1535 <sup>15</sup>.

1536

### 1537 **Muscle strength and neuromuscular function**

1538 Assessment of muscle and neuromuscular function can be challenging as  
1539 measurement of these phenotypes may be influenced heavily by the operator, or by how  
1540 cooperative the individual animal is. In addition severe muscle defects occur at 28-30  
1541 months in C57BL6/J mice<sup>117,118</sup>. For this reason improvements are small and difficult to  
1542 detect. It is advisable that muscle function is tested using multiple measures of outcome to  
1543 overcome these issues.

1544

1545 **Grid hanging test and Grip strength.** Grip strength is “a universal measurement  
1546 used to assess physical competency in older adults” humans<sup>119</sup>, and the decline in grip  
1547 strength with age is predictive for both the risk of inability to perform activities of daily living,  
1548 an important component of healthspan, and mortality in humans<sup>120</sup>. For this reason it is used  
1549 in mice<sup>4,12</sup>. Grip strength as measured using a grip strength meter may decline with age in  
1550 mice, but this has not been observed by every group<sup>119,121</sup>. Interventions which benefit the  
1551 muscle system should result in an increase in grip strength, and this has been observed by  
1552 some groups<sup>29,122</sup>. However, this is not always the case.

1553 We believe the variability associated with this assay, and the small size of the effect  
1554 means that most studies are underpowered. We have observed that minor variations can  
1555 confound the results, and that different operators even within the same lab can see different  
1556 results. We have tried to describe the procedure in detail. However, for grip strength it is  
1557 advisable that new operators are trained by someone experienced.

1558 The grid hanging test, also known as the cage top test, four limbs hanging test, grid  
1559 wire hang, or inverted cling test, is a widely used and easy to perform assay; grip strength as  
1560 measured by this assay also declines with age in mice<sup>18,123,124</sup>, and we expect it to increase  
1561 as a result of an geroprotective intervention. This can be a more robust assay than grip  
1562 strength.

1563

1564 **Hanging bar.** This test seeks to evaluate motor function measuring forelimb muscle  
1565 strength of the mouse. It is expressed as latency to fall from the wire. Neuromuscular  
1566 abnormalities in mice can be detected as well with this test<sup>18,125</sup>. We expect that  
1567 geroprotective interventions will often increase wire hang time in aged mice<sup>18,19</sup>.

1568

1569 **Rotarod** The rotarod assay is one of the most commonly used tests in the study of  
1570 aging mouse healthspan. It is measured as the latency (time) to fall. This time declines with  
1571 age, and neuromuscular abnormalities in mice can be detected as well with this test<sup>44,125,126</sup>.  
1572 Rotarod running time is positively impacted by interventions including calorie restriction<sup>34</sup>,  
1573 metformin<sup>3</sup>, nicotinamide<sup>20</sup>, and rapamycin<sup>127</sup>, and it is expected that the latency time to fall  
1574 is increased with the use of geroprotectors<sup>19,122</sup>.

1575

## 1576 **TABLE 2: TROUBLESHOOTING**

### 1577 **Cardiac function**

Step	Problem	Possible reason	Possible Solution
5	No heart rate/ECG signal	Poor contact with platform, lack of signal conduction	Check foot placement and use electroconductive gel if needed, check connections of platform to system
11	Initial poor image quality	Ineffective skin preparation, insufficient ultrasonic gel for contact	Check skin for effective and thorough hair removal, add additional warmed

			gel,
11	Excess LV dilation/poor systolic function with slow, gradual recovery	Excess anesthesia upon induction resulting in anesthesia-induced cardiodepression	Practice rapidly placing the animal on the platform, inducing under the lowest level of isoflurane possible, and rapidly lowering to a minimal plane of anesthesia. Avoid use of anesthesia box for induction when possible (particularly with obese mice)
All steps	Significant bradycardia (HR<450) and poor LV function	Excess administration of anesthesia, excess pressure on chest	Lower anesthesia concentration; ensure negligible pressure is applied to the chest while imaging
All steps	Profound bradycardia (HR<250), LV dilation, and arrhythmias	Adverse response to anesthesia	Remove animal from nose cone and allow to recover fully. Attempt imaging again after full recovery or on another day.

1578

1579 **Cognitive function**

Step	Problem	Possible reason	Possible Solution
5	Some animals might spend most of the time in a corner, immobile and without exploring	Stress	Special care in the behaviour room environment. If it happens in individual animals only, these should be removed from the experiment. If this is seen in many animals, it may be necessary to identify and eliminate stressor present in the behavioural testing or animal housing environment. Stress related to the experimental procedure can also be attenuated by

			increasing handling and/or habituation time.
5	Some objects promote animals to play with the object including climbing on top of the object and remaining there for a while	This is most often due to mobile pieces in the object or a big surface on top of the object	Pre-select a different pair of objects
6	Lack of increased exploration time of novel vs. familiar object	Stress  Insufficient training  Lack of interest in objects	Identify and eliminate possible stressors in the behavioural testing or animal housing environment, increase handling and/or habituation time  Increase training time  Pre-select a different pair of objects

1580

1581 **Metabolic Health**

1582 ***Body Composition***

Step	Problem	Possible reason	Possible Solution
2	Machine unable to calibrate, takes an unusually long time to calibrate, or takes an unusually long time to read a single animal.	Too much metal mass within a 6-foot radius of the MRI.	Increase separation between metal objects and the MRI.
5	Sum of the fat mass, lean mass, and water weight differ by more than 5% from the weight as assessed using a scale.	Too much metal mass within a 6-foot radius of the MRI; alternatively, calibration standard may have leaked.	Increase separation between metal objects and the MRI, replace calibration standard if necessary, and recalibrate the machine.

1583

1584 ***Energy Balance***

Step	Problem	Possible reason	Possible Solution
5	RER results are non-physiological	If the reference cage is only read at the	Set the system to read the reference

	(continuously > 1.0 or < 0.7) and O <sub>2</sub> /CO <sub>2</sub> in readings are constant.	beginning of each experimental run, but room air O <sub>2</sub> /CO <sub>2</sub> concentrations change, the delta O <sub>2</sub> and delta CO <sub>2</sub> will be calculated incorrectly.	cage (and update O <sub>2</sub> in and CO <sub>2</sub> values) every time it cycles through the experimental chambers. once before each reading
5	RER results are non-physiological (continuously > 1.0 or < 0.7)	Valves on one or more of the calibration air tanks may have been shut during the calibration procedure; alternatively, the calibration air may have settled or the tank may be empty.	Recalibrate system, checking that there is adequate gas flow from the calibration tanks.
5	The gas values read from all of the chambers (O <sub>2</sub> out/CO <sub>2</sub> out) becomes "stuck" on the same value	A bubble may have formed in the ammonia traps.	Replace the ammonia traps.
Data analysis	Food consumption values are negative or absurdly high.	Mice may have greater than intended access to the feeder, and either deposit waste products or remove food without eating it; or the scale may be defective.	Discard data from cages with negative food consumption values, and inspect the cage bottom to determine if food was removed from the feeder and not consumed. The cage floor may be able to be reset to limit the access of small/lean mice. Verify that the scale is functioning properly and replace/repair as needed.

1585

1586 **Glucose tolerance test**

Step	Problem	Possible reason	Possible Solution
8	Blood glucose reading seems extraordinarily high or low based on reading at prior time point.	Approximately 2% of reads are unusual, due to causes that include defective or contaminated glucose test strips or	Wipe the tail, and with a fresh drop of blood immediately repeat the reading with a fresh test strip. If value differs by

		samples, or samples that are too small or partially clotted.	more than 10% from the first reading, use the data from this second reading; otherwise, keep the first reading.
8	Blood glucose monitor says all or most glucose test strips are defective.	Monitor failure.	Replace blood glucose monitor. As many monitors are inexpensive, spares can often be kept on hand.

1587

1588 **Insulin tolerance test**

Step	Problem	Possible reason	Possible Solution
9	Blood glucose reading seems extraordinarily high or low based on reading at prior time point.	Approximately 2% of reads are unusual, due to causes that include defective or contaminated glucose test strips or samples, or samples that are too small or partially clotted.	Wipe the tail, and with a fresh drop of blood repeat the reading with a fresh test strip. If value differs by more than 10% from the first reading, use the data from this second reading; otherwise, keep the first reading.
9	Blood glucose monitor says all or most glucose test strips are defective.	Monitor failure.	Replace blood glucose monitor. As many monitors are inexpensive, spares can often be kept on hand.
8 and 10	Blood glucose level does not drop or rises following insulin administration.	Insulin that is not correctly injected into the I.P. space will aggregate and not enter the circulation.	If misinjection is suspected, I.P. insulin can be administered a second time immediately following the end of the ITT, and the blood glucose tested 15 minutes later. If the animal now responds robustly, the ITT data for that animal should be discarded as a result of misinjection.

1589

1590 **Muscle strength and neuromuscular function**

1591 ***Grid hanging test***

Step	Problem	Possible reason	Possible Solution
4	Mice let go very quickly	Grid not high enough/loss of fear of falling	Place the grid slightly higher

1592

1593 ***Grip strength***

Step	Problem	Possible reason	Possible Solution
6	Very variable readings	Operator holding the mouse at an angle	Ensure the mouse is kept horizontally when pulling and the force applied is proportionate
6	Very variable readings	Claws are too long	Claw length may be affected by activity or environment (e.g. bedding). Change bedding or clip claws

1594

1595

1596 ***Hanging bar***

Step	Problem	Possible reason	Possible Solution
1	Mouse is difficult to position	Insufficient training	Gently reposition the mouse until they have learnt
1	Mouse let go quickly even if they are young	They have lost fear of falling	Position the bar higher

1597

1598 ***Rotarod***

Step	Problem	Possible reason	Possible Solution
7	Mouse change position on the rotarod,	Insufficient training	Gently reposition the mouse.
7	Mice fall from the rotarod too quickly.	Old, frail mice, background noise.	Gently position mouse on the track again. Reduce exposure to possible distractions. Remove extremely frail mice from the assay.



7	Mouse turns around in the middle of the experiment and start walking in the opposite direction	Insufficient training	Gently reposition the mouse until they have learnt
7	Mouse "hugs" the rod and starting looping	Insufficient training	Gently reposition the mouse until they have learnt

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## **CONCLUSIONS**

1608 A lack of standardized techniques to assess healthspan in mice has resulted in  
 1609 reduced reproducibility of results and slowed progress in developing geroprotective agents  
 1610 to extend healthy life. The toolbox described here describes a comprehensive set of  
 1611 techniques that can be used to consistently and reproducibly assess the healthspan of mice.  
 1612 We expect that the use of this toolbox will permit investigators to rapidly and robustly identify  
 1613 candidate geroprotectors that may potentially be of clinical use in the treatment of many age-  
 1614 related diseases in humans.

1615

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1935

1936 **Data availability**

1937 All data shown in this paper are available from the authors upon reasonable request.

1938

1939 **Acknowledgments**

1940 This article is based upon work from COST Action (BM1402: MouseAGE), supported by  
1941 COST (European Cooperation in Science and Technology). Part of this work has been  
1942 funded by the European Union Research and Innovation Program Horizon 2020 (Grant  
1943 Agreement Number 730879). The Lamming laboratory is supported in part by the NIH  
1944 (AG051974, AG056771, and AG062328) and by the U.S. Department of Veterans Affairs  
1945 (I01-BX004031), and this work was supported using facilities and resources from the William  
1946 S. Middleton Memorial Veterans Hospital. RdC, CDG and IN are supported by the Intramural  
1947 Research Program of the NIA/NIH. This work does not necessarily represent the official  
1948 views of the NIH, the Department of Veterans Affairs or the United States Government.

1949

1950 **Author contributions**

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1955

1956 **Competing interests**

1957 The authors declare no competing financial interests.

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## Figure legends

**Figure 1.** A graphic representation of the experimental design for healthspan assessment in response to a geroprotector of choice in C57BL6/J using the recommended toolbox. At each time point the multiple tests are performed over a period of 4-6 weeks, leaving those which may impact more on the animal at the end. In this case we recommend the following order, novel object recognition, frailty index, grip strength, cage top and hanging bar followed by rotarod, body composition, energy expenditure, glucose tolerance test (GTT), Insulin tolerance test (ITT) and echocardiography. Mo, Month

**Figure 2. Representative images from high resolution echocardiography systems. A)** Representative image of M-mode image used for assessment of cardiac systolic function (AW = anterior wall, PW = posterior wall, LVEDD = left ventricular diastolic dimensions, LVESD = left ventricular systolic dimensions; arrows denote the thickness/dimension of interest for each variable). **B)** Representative image of pulsed-wave Doppler assessment of peak mitral valve inflow (E) using color Doppler guidance (IVRT = isovolumic relaxation time, IVCT = isovolumic contraction time, LVET = left ventricular ejection time). **C)** Representative image of tissue Doppler assessment of the septal mitral annulus velocity ( $e'$ ) using the spectral velocity display. White arrows denote the point of measurement. Images are reproduced from Verzosa and colleagues<sup>89</sup> with permission. Animal procedures were performed following approval by the Mayo Clinic Institutional Animal Care and Use Committee.

**Figure 3. NOR memory enhancement in exercised fathers and their offspring. (A–B)** NOR memory enhancement in exercised (RUN) vs. sedentary (SED) animals. Results represent as Discrimination index (DI). A DI score of  $>0.20$  was set ad hoc to determine proper novel-object discrimination; statistically significant within-group differences were also considered between the training phase and the test phases. (A) Before exercising, mice were unable to discriminate the novel object in a difficult protocol. (B) After 6 wk of physical exercise, exercised animals showed memory enhancement. For comparisons between independent groups,  $*P < 0.05$ ; for intragroup differences (LTM vs training),  $+P < 0.05$ ; tendencies  $0.05 > \#P < 0.09$ . Extreme values were removed from the analysis. SED A,  $n = 4$ ; RUN A,  $n = 5$ . Figure adapted with permission from McGreevy et al, 2019, PNAS<sup>33</sup>. The animal procedures were performed following approval by the Committee of Ethics and Animal Experimentation of the Cajal Institute, Ethics Committee of the CSIC, and the Animal Protection Area of the Ministry of Environment of the Community of Madrid. LTM, Long term memory, STM short term memory

**Figure 4. Effects of dietary protein level on energy balance.** Metabolic chambers were used to assess A) Food Consumption, B) Spontaneous activity (as assessed by wheel running), C-E) Respiration and F) Energy Expenditure over a 24-hour period after approximately 8 weeks on the indicated diets ( $n=8-9$ /group, two-tailed t-test,  $* = p < 0.05$ ). Error bars represent standard error. Figure is adapted from<sup>64</sup> with permission. Animal procedures were performed following approval by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital.

**Figure 5. Glucose and insulin tolerance tests.** A) Glucose tolerance test conducted on male C57BL/6J mice treated with either vehicle or with 2 mg/kg rapamycin (1x/day or 1x/5 days) for 2 weeks ( $n = 9$  per treatment; for GTT, Tukey–Kramer test following two-way repeated measures ANOVA,  $a = P < 0.05$  vehicle vs rapamycin 1x/day,  $b = P < 0.05$  vehicle vs. rapamycin 1x/5 days; for AUC, means with the same letter are not significantly different from each other, Tukey–Kramer test following one-way ANOVA,  $P < 0.05$ ). Figure is adapted from<sup>16</sup> with permission. B) Insulin tolerance test on female C57BL/6J.Nia mice treated with either vehicle or rapamycin (2 mg/kg) once every 5 days for 8 weeks ( $n = 7-10$  vehicle,  $n =$

2013 10 rapamycin, two-tailed t-test). Test was performed 5 days after the last administration of  
2014 either vehicle or rapamycin, at the conclusion of an overnight fast. Figure is adapted from  
2015 <sup>6</sup>with permission. Error bars represent standard error. Animal procedures were performed  
2016 following approval by the Institutional Animal Care and Use Committee of the William S.  
2017 Middleton Memorial Veterans Hospital.

2018

2019 **Figure 6. Assessment of muscle strength and neuromuscular function.** Physical  
2020 function measurements in 20-month-old male mice treated with dasatinib and Quercetin  
2021 (D+Q) once every 2 weeks (bi-weekly) for 4 months. (a) Grip strength, (b) hanging  
2022 endurance, and (c) maximal walking speed on rotarod (relative to baseline) 4 months after  
2023 drug initiation ( $n = 20$  for D+Q;  $n = 13$  for V). Results are shown as box and whiskers plots,  
2024 where a box extends from the 25th to 75th percentile with the median shown as a line in the  
2025 middle, and whiskers indicate smallest and largest values. \* $P < 0.05$ ; n.s., not significant;  
2026 Two-tailed Student's t-tests. Figure is adapted from <sup>29</sup>with permission. Animal procedures  
2027 were performed following approval by the Mayo Clinic Institutional Animal Care and Use  
2028 Committee.

2029

2030

2031 **Table 1**

2032 Estimated timing for each test for n=10 animals and n=2 operators.

2033

2034

Week #	Test	Comments
1	<p><b>NOVEL OBJECT RECOGNITION</b></p> <ul style="list-style-type: none"> <li>• <b>Day 1.</b> <u>Habituation</u>: 30 min/mouse</li> <li>• <b>Day 2.</b> <u>Training phase</u>: 5-15 min/mouse <u>Test phase</u>: For STM, after 1 hour of training phase. 10 min/mouse.</li> <li>• <b>Day 3.</b> <u>Test phase</u>: For LTM, after 24h of training phase. 10min/mouse.</li> </ul>	<p>For <u>Day 1</u>, estimated time is 5-6 hours. For <u>Day 2</u>, estimated time is between 3:30h and 5:15h with STM same day or 2:00h - 3h without STM. For <u>Day 3</u>, estimated time is 2h.</p>
2	<p><b>MUSCLE STRENGTH AND NEUROMUSCULAR FUNCTION</b></p> <ul style="list-style-type: none"> <li>• <b>Day 1.</b> <u>Grid Hanging</u>. 3 trials per animal allowing 30 min in between trials. *</li> <li>• <b>Day 2.</b> <u>Grip Strength</u>. 3 trials per animal allowing 30 min in between trials. Time estimate is 3 min per animal.</li> <li>• <b>Day 2.</b> <u>Frailty index</u>. 15min/animal</li> <li>• <b>Day 3.</b> REST Day</li> <li>• <b>Day 4.</b> <u>Hanging Bar</u>. 3 trials per animal allowing 30 min in between trials. ***</li> <li>• <b>Day 5.</b> <u>Rotarod</u>. 3 trials per animal allowing 30 min in between trials. Time estimate is 3 min per animal.</li> </ul>	<p>*depending the age/sex of the animals, estimated total time might vary between 1h and 4h. ** This will take 2-3h *** depending the age/sex of the animals, estimated total time might vary between 1h and 3h. SIDE NOTE: in some tests, total time estimated will depend of the number of different set-ups available. Some apparatus allow the operator to run multiple animals at a time like multi-channel rotarod or grid hanging test. However the operator must be continually scanning and monitoring all animals during this time.</p>
3	<p><b>BODY COMPOSITION and ENERGY BALANCE</b></p> <ul style="list-style-type: none"> <li>• <b>Day 1.</b> <u>Body Composition Test</u>. Experiment takes 2 min/mouse. Estimated time is 1h.</li> <li>• <b>Day 2.</b> <u>Energy balance test</u>. Prepare the animals, calibrating and setting up the experiment and run the experiment. Estimated time 2h.</li> <li>• <b>Day 3.</b> <u>Energy balance test</u>. The software is up and running recording in vivo metabolic data. *</li> <li>• <b>Day 4.</b> <u>Energy balance test</u>. The software is up and running recording in vivo metabolic data. *</li> <li>• <b>Day 5.</b> <u>Energy balance test</u>. Stop the experiment, exporting the data, cleaning the equipment and putting animals back in home</li> </ul>	<p>*schedule based on a 72h experiment set-up with two light cycles and two dark cycles of 12h/each. Side note: During these recording days, animals have to be checked by the operator to make sure they are in good condition and have access to food and water. Also software needs to be checked in order to make sure data is being collected accurately. Two people can expedite the set up of these tests. One person does the body composition measurement, the</p>

	cages and room. Estimated time 2h.	other sets up the <i>in vivo</i> metabolic machine for calibration and measurement.
4	<p><b>GLUCOSE TOLERANCE TEST</b></p> <ul style="list-style-type: none"> <li>• <b>Day 0.</b> <u>Fast animals</u> 6 or 16 hours before the start of the test</li> <li>• <b>Day 1.</b> <u>Test day.</u> The last of the time points is at 2 hours after the beginning of the test. To allow for setup and clean up, an additional 1.5-2h is needed. Therefore, total time required is 3.5-4h.</li> </ul>	Due to the invasive and stressful character of the test, we let the animals rest the whole week after the test. A skilled person can inject between 20-30 mice within 15min. After the conclusion of the test, animals are returned to their home cage. Mice should be checked after 4-6h to ensure they have returned to normal.
5	<p><b>INSULIN TOLERANCE TEST</b></p> <ul style="list-style-type: none"> <li>• <b>Day 1.</b> <u>Fast animals.</u> Animals need to be fasted 3-6h before the experiment.*</li> </ul> <p><u>Test.</u> The last of the time points is at 2 hours after the beginning of the test. To allow for setup and clean up, an additional 1.5-2h is needed. Therefore total time required is 3.5-4h.</p>	*Fasting periods might vary between different studies. We propose for longitudinal studies a fasting between 3h and 6h. Due to the invasive and stressful nature of this test, we let the animals rest the whole week after the test. A skilled person can inject between 20-30 mice within 15min. After the conclusion of the test, animals are returned to their home cage. Mice should be checked after 4-6h to ensure they have returned to normal.
6	<p><b>CARDIAC FUNCTION</b></p> <p><b>Day 1.</b> Echocardiogram. Mice are anesthetized using isoflurane-oxygen Echocardiograms can take up to 30-45min per animal depending on the age, strain and bodyweight. Obese mice can be harder to image given their excess amounts of fat.</p> <p><b>Day 2.</b> <u>Data processing.</u> 1h</p>	Caution must be taken for old mice who are more vulnerable to death from isoflurane. A skilled operator can process up to 10 animals per day. Mice should be given at least 5 days (longer for aged mice) to recover from the isoflurane.