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Two phases model of ageing in mice: towards a better identification of age-related and late-life metabolic decline.

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Abstract

Since being described in *Drosophila melanogaster* in 2011, the Smurf phenotype, has been seen to be evolutionarily conserved in nematode and zebrafish, and has helped to identify the discontinuous nature of ageing and predict impending death from natural causes as well as from environmental stresses. This phenotype allowed us to model ageing as being made of two successive phases: a phase A where individuals are healthy and have no risk of mortality but an age-dependent increasing risk of entering phase B, followed by a phase B where individuals show the so-called hallmarks of ageing and a high risk of death. We will test here whether these two consecutive phases of ageing separated by the Smurf transition are a conserved feature of ageing in the classical mammalian laboratory model *Mus musculus*. Thanks to a longitudinal longevity study using both males and females from two different mouse genetic backgrounds and by integrating physiological, metabolic and molecular measurements with the life history of approximately 150 mice, we are attempting to identify a phenotypic signature typical of the last phase of life, observable at any chronological age. Validating the two-phase ageing model in a mammalian organism would allow the high risk of imminent death to be better characterized in this model and would extend its implications to a broader range of species for aging research.
Introduction

In 2015, building upon the first description of a pre-death phenotype dubbed Smurf, we described a new mathematical model for analyzing longevity curves. This model of ageing, first described in Drosophila, is characterized by two successive and necessary phases, a phase A where individuals are healthy and have no risk of mortality but an age-dependent increasing risk of entering phase B, and a phase B where individuals show so-called hallmarks of ageing as well as a high risk of death. The discovery of this model in Drosophila was made possible by a singular characteristic of phase B, its increased intestinal permeability. To assess intestinal permeability in Drosophila, a non-absorbed and non-toxic blue food dye (FD&C blue dye #1) is added to the medium of adult individuals. In phase A, this dye does not pass the intestinal barrier and remains in the digestive tract. In contrast, for individuals in phase B, the dye crosses the intestinal epithelium by mechanisms that are still unknown and reaches the hemolymph. The blue color is visible to the naked eye and blue individuals were hence dubbed "Smurfs." Individual centered studies in females and males using flies demonstrated that about 50% to 100% of individuals go through these two consecutive phases. More importantly, individuals in phase B show typical hallmarks of ageing such as decreased energy storage (triglycerides, glycogen), locomotor activity and fertility, a deregulation of insulin signaling, and an increase in systemic inflammation. This powerful predictor of death strongly supports that ageing can be described not as a progressive and continuous age-related alteration of the physiology in all individuals from a population but as an abrupt alteration in an age-dependent growing proportion of individuals with high mortality risk.

The Smurf two-phase ageing model reinforces several important ideas with respect to ageing: (1) not all individuals of the same chronological age have the same physiological age, (2) although some parameters may evolve gradually, the transition from phase A to phase B is abrupt, and (3) it is possible to identify physiologically old individuals, i.e. at higher risk of impending death than the rest of the population. Interestingly, this model of ageing is evolutionarily conserved as it was also
described in nematode⁶ (Caenorhabditis elegans) and fish⁶ (Danio rerio). Additionally to its ability to predict a high-risk of impending natural death in Drosophila, the Smurf phenotype was shown to allow for death prediction in non-naturally occurring causes of death such as traumatic brain injury Drosophila model⁷. The reports regarding age-dependent increase of intestinal permeability in aged rodents⁸–¹⁰, aged primates¹¹ as well as in critically ill humans, septic or not, in Intensive Care Unit¹²,¹³ led us to investigate whether this “Smurf” two-phase ageing model is also conserved in mammals. Moreover, in humans, certain phenotypic markers such as physical capability¹⁴, sense of smell¹⁵ and facial appearance¹⁶ have been described as predictors of death. Recently, studies have identified metabolic¹⁷, protein¹⁸ and inflammatory biomarkers¹⁹ that can identify, within a population of the same chronological age, individuals with a high risk of mortality from any causes. These arguments suggest that ageing could be separated into two phases in humans and that some typical parameters of phase B in Drosophila could be found in humans.

In the present study, we therefore aim to determine whether the “Smurf” two-phase ageing model described in insects, nematodes and zebrafish is also conserved in the classical mammalian laboratory model, Mus musculus. We are searching for a phenotypic signature that would be associated with an increased intestinal permeability in the last phase of life, and observable at any chronological age. For this, we use two mouse lines, with different lifespans: the AKRJ line with a T⁵₀ (the age at which 50% of a population has died) of 9 months and the C57Bl6/J line with a T⁵₀ of 30 months²⁰. We regularly assess intestinal permeability, metabolic parameters, inflammatory status and microbiota homeostasis throughout the life of each individual until their natural death. These measurements allow us to answer the following four research questions: (1) Is the end of life in mice characterized by an increase in intestinal permeability? (2) Is there a specific physiological signature of the end of life in mice? (3) Do defined biomarkers allow us to discriminate between two subpopulations in mice characterized by different mortality risk at any chronological age? (4) How well can we predict impending death in mice?
Validating the “Smurf” two-phase ageing model in mice would represent multiple benefits for the ageing research field. First, because it would provide arguments to pursue this research in humans. Secondly, it would make possible the assessment of an individual’s physiological age using a method validated in multiple model organisms. Third, the multiple physiological parameters that will be followed longitudinally until the natural death of individuals, will allow the construction of a new multiparametric predictive model\textsuperscript{21}, based on metabolic criteria likely to be relevant across model organisms. Finally, it would also allow a better understanding of the underlying mechanisms of ageing by offering the possibility to study the onset of the aforementioned hallmarks of ageing in healthy, phase A individuals.
Materials and Methods

Protocol

Animals

Fifty 10-week-old AKRJ female mice (Charles Rivers, US) were used for the pilot study. Fifty 20-24-month-old C57BL/6J male mice (Janvier Labs, France) and fifty 20-24-month-old C57BL/6J female mice will be used for the pre-registered part of the study. Mouse age depends on the animal supplier's capabilities, as older animals are not always accessible due to low demand. Mice are grouped house (five animals / cage) or isolated in PET plastic cages (EasyCage® mouse, Allentown Inc, France) on corn cob bedding (Lab cob 12, Serlab, France) at 21 ± 1°C and 55 ± 5 % air humidity with a 12 hours' light / 12 hours’ dark cycle and are acclimatized 2-3 weeks before experiments are performed. Animals have access to water and standard diet ad libitum (3.339 Kcal/g with 19.3% from proteins,8.4% from lipids and 72.4% from carbohydrates; SAFE#A04). Animals are monitored daily. As defined in the project's ethics approval, when an animal shows (1) clinical signs of pain and/or (2) a visible tumor that ulcerates, prevents it from moving normally or if it exceeds 1.2 cm in diameter, the animal is killed by neck dislocation with prior isoflurane anesthesia. AKRJ females were kept group-housed during the whole duration of the experiment, except for the indirect calorimetry measurements where mice had to be individualized transiently. After having worked on this first group of AKRJ females, we wanted to limit the slight weight loss induced by the individualization at each measurement. Therefore, 40-50% of C57BL/6J mice will be isolated upon receipt and will remain so until their natural death. The percentage of initially isolated mice depends upon the amount of calorimetric cage material that will be available at the initiation of the experiment. These isolated mice will be the ones that go into the indirect calorimetry cages every 3-4 weeks. The rest of the group will be isolated as the isolated group of mice dies in order to maintain a sufficient number of mice for the calorimetry recordings. In this case, the mice will be isolated at least 1 week before in order to limit the effects of the isolation on the physiological parameters measured. For all C57BL/6J
mice males and females, body weight and composition, glycemia, plasma and feces sampling, intestinal permeability assessment, rectal temperature will be collected every 2-3 weeks from their arrival to their natural death. The individual or grouped status of each mouse for each measure will be reported in the data table.

**0.8 kDa FITC-Dextran synthesis**
Glucosamine hydrochloride (5.4 mg, 0.025 mmol) and fluorescein isothiocyanate (FITC, 10 mg, 0.025 mmol) were dissolved in DMF (1mL). DIPEA (9 μL, 0.05 mmol) was added and the mixture was stirred at 40°C for 24 hours. The mixture was added to DCM dropwise and the precipitate was collected by filtration and crystallized on MeOH/Cyclohexane 1/1 to give the product as a yellow/red solid (8.8 g, 62%). 1H NMR (400 MHz, D2O) δ 8.16 (d, J = 1.9 Hz, 1H), 7.93 (dd, J = 8.1, 1.9 Hz, 1H), 7.27 (d, J = 8.1 Hz, 1H), 6.79 (dd, J = 8.1 Hz, 2.3Hz, 1H), 6.77 – 6.69 (m, 3H), 6.65 – 6.60 (m, 2H), 5.71 (d, J = 2.2 Hz, 1H), 4.87 (d, J = 6.0 Hz, 1H), 4.53 (d, J = 7.8 Hz, 1H), 4.52 (d, J = 6.0 Hz, 1H), 4.05 (m, 2H), 3.9-3.8 (dd, J =3.3-10.8 Hz, 2H), 3.78-3.65 (m,4H).

**FITC-Dextran solution preparation**
The smallest size of FITC-Dextran on the market is 4kDa. For the experiments performed on the AKRJ group we used the solution A prepared from a mix of FITC-Dextran of different sizes: 4kDa, 20 kDa and 70 kDa. Since we did not observe a massive increase in intestinal permeability, we redesigned our protocol and collaborated with a chemist to make a FITC with a size close to the one of the Blue#1 used in the Drosophila protocol. This new FITC of 568 Da will be used to make the solution B in the experiments performed on the C57BL/6J mouse line. Solution A : fluorescein isothiocyanate–dextran powder (FITC-Dextran, TdB labs., Sweden) 4kDa, 20kDa and 70kDa (60mg/ml) are diluted together in sterile water. Solution B : FITC-Dextran 0.8 Da (See “0.8 Da FITC-Dextran production” section) is kept at a concentration of 280mg/ml in DMSO (Dimethyl sulfoxide BioUltra, for molecular biology, ≥99.5%, Sigma-Aldrich®, US) at -80°C and diluted (7.25mg/ml) in
sterile water for the gavage. Both solutions are prepared the same day of the gavage in a 15 ml Falcon™ tube (Corning, US), just before the test and are protected from light using foil.

**FITC-Dextran gavage test**

All mice are food-deprived for 3 hours at the beginning of the light cycle. Home bedding and water bottles are kept in the cage. When the 3 hours of food deprivation are up, body weight and glycaemia are measured and 75 µL of blood are collected in awake animals at the tail tip after incision. Once the pre-test measurements and samples have been taken, the test starts with the gavage of the mice with the FITC-Dextran solution A or B (100µl/10g of body mass) in awakened animals at the tail tip. Glycaemia is expressed in ng/ml and measured using a glucometer (Glucofix® Sensor and Glucofix® Premium, A.Menarini diagnostics, France). Blood is collected using haematocrit capillaries coated with heparin (Hirschmann® Laborgeräteann, Germany). Blood samples are kept on ice before centrifugation in 0.5 ml Eppendorf® Safe-Lock tubes (Eppendorf®, Germany). After centrifugation (10 000x g for 7 min at 4°C), plasma samples are collected and immediately frozen at ~80°C and kept for further analyses. Mice are left in their home cages. If they are grouped or isolated at the beginning of the test, they will remain so during the test. At the end of the test, after the last sampling, the food is put back in the cage of the mice. 1, 3 and 5 hours after the gavage, 20 µl blood samples are collected

**Fluorescent Plasma Measurements** Plasma samples are diluted 5:95 (v/v) in DPBS (Dulbecco’s Phosphate Buffered Saline, Gibco™, ThermoFisher, US). Fluorescence is measured spectrophotometrically (FlexStation® 3, Molecular Devices, US) in 96-well dark plates (Non-Treated Surface, Non-Sterile, Thermo Scientific™, US) using the following parameters: excitation = 493 / emission = 525 / cut off = 515 / room temperature. Fluorescence is expressed in arbitrary units.
**Indirect calorimetry measurements**

Oxygen consumption ($V_{O2}$, mL/15min), carbon dioxide production ($V_{CO2}$, mL/15min), food intake (g/15min), water intake (mL/15min) and locomotor activity (counts/h) are measured using indirect calorimetry apparatus (PhenoMaster, TSE Systems GmbH, Germany; and Promethion™, Sable Systems International, US). Mice are individually housed with ad libitum access to food and drink. Measurements included a minimum of 3 up to 5 days of data recording. The first day and the first night of the recording is not taken into account and is considered as a period of habituation of the animal to the indirect calorimetry apparatus.

Using values from the indirect calorimetry apparatus, a number of different calculations can be performed to derive multiple indices such as respiratory exchange ratio (RER), energy expenditure (EE) and fatty acid oxidation, but not exclusively\(^{23}\). The measurements of food and water intake, $V_{O2}$, $V_{CO2}$, infrared beams crossing the X, Y and Z axes taken every 15 minutes will be summed up to evaluate their hourly-based values and their daily-based values. $V_{O2}/V_{CO2}$ ratio, corresponding to the RER, will allow to assess the quality of energy substrate used by animals. Total EE will be calculated according to the Weir equation\(^{24}\), energy expenditure when the animal is resting (RE, resting metabolism) was calculated according to Péterfi et al\(^{25}\) and manufactured recommendations. Fatty acid oxidation will be calculated according to the following equation\(^{26}\) : \[ FA_{oxidation} (kcal/h) = EE \times (1-RER/0.3) \] . The X, Y and Z axes of infrared beams will allow plotting their ambulatory and fine motor movements, as well as rearing behavior.

**Body temperature measurements**

The mouse is hand-restrained and a temperature probe (Physitemp thermal TH5, Phymep, France) covered with Glycerin (DUREX® Gel lubrifiant naturel, France) is gently inserted into the rectum to a fixed depth: 2 cm. The body temperature was always taken at the same time of day, between 10 and 11 am.
**Body composition analysis**

Body mass composition, lean mass, fat mass, free water and total water content are analyzed by magnetic resonance imaging with the EchoMRI 100 system or Minispec LF90 (Whole Body Composition Analyzers, EchoMRI, Bruker, US) according to the manufacturer’s instructions in awake mice.

**Metabolic biomarkers, cytokines and chemokines quantification in plasma**

A U-Plex multiplex assay (K152ACL-2, Metabolic Group1 (ms) assay, Meso Scale Diagnostics, US) is used to measure the levels of metabolic biomarkers, cytokines and chemokines (insulin, leptin, FGF-21, active GLP-1, IL1-beta, IL-6, IL-10, MCP-1, RANTES, TNF-alpha) in mice plasma according to the manufacturer's protocol.

**Microbiota analysis**

Fecal samples are directly collected at the exit of the anus of the hand-restrained mouse in 0.5 ml Eppendorf® Safe-Lock tubes (Eppendorf®, Germany) that are instantly placed in liquid nitrogen and kept at −80°C for further analyses. Fecal samples were always collected at the same time of day, between 10 and 11 am. Total microbial DNA is extracted from ~40 mg of fecal sample using a kit for isolation of genomic DNA from stool samples according to the manufacturer's protocol (15573626, Macherey-Nagel™ NucleoSpin™ DNA Stool, Fisher Scientific, France). DNA concentration and integrity are assessed by spectrophotometry using the Qubit dsDNA HS (High Sensitivity) assay kit (Q32851, Qubit™ dsDNA HS, Invitrogen™, US). The V3-V4 hypervariable region of the bacterial 16S rDNA was amplified by PCR using forward primer: 5' - CTTTCCCTACACGACGCTCTTTCCGATCTACGGRAGGCAGCAG-3', reverse primer: 5' - GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT-3'. Amplifications were carried out using the following ramping profile: 1 cycle at 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 1 min before a final step at 72°C for 10 min. After quality
checking by electrophoretic 2% agarose gel migration, obtained amplicons were sequenced using Illumina MiSeq technology (GenoToul platform, Toulouse, France). Reads obtained after 16S rRNA gene MiSeq sequencing will be filtered and taxonomic affiliation (SILVA database version 132) will be performed using the Galaxy-supported pipeline named FROGS (Find, Rapidly, OTUs (operational taxonomic units) with Galaxy Solution)\textsuperscript{27}. Samples will be rarefied to even sampling depths, and then diversity measures (Shannon index from raw data and Bray-Curtis distance from normalized data) as well as composition analysis will be performed using Phylseq package, ggplot2, and DESeq2 in R\textsuperscript{28–30}. Raw, un-rarefied OTU counts will be used to identify taxa abundances and characterize gut microbiota composition.
Sampling plan

Power calculation
For this a priori power analysis, we consider the case of detection of a mean difference between the "Smurf" and the "non-Smurf" groups at T₅₀, when 50% of the population is dead, assuming 30% of "Smurf" individuals are alive based on drosophila studies and our mathematical model. A total sample size N = 50 would correspond to 17 "non-Smurf" and 8 "Smurf" individuals at T50. An effect size of 1.6 can be detected with $\alpha = 0.05$ and $1-\beta = 0.95$, (G*Power 3.1.9.731, Sensitivity analysis) at 80% POWER. This is considered an extra-large effect according to Cohen's criteria, but is consistent with results obtained in drosophila3. Thus, our proposed sample size of N=50 is adequate for this study, and has already been approved by the Animal Care Committee (see Ethical Approval Plan for details). G*Power 3.1.9.7 uses the equations described in Cohen, J. Statistical Power Analysis for the Behavioral Sciences (Academic Press, 2013)32.

Criteria for data exclusion
Regarding body weight and composition, glycemia, body temperature, plasmatic fluorescent level, as well as metabolic biomarkers, cytokines and chemokines quantification in plasma and microbiota composition no data will be excluded. When possible, at the time of the measurement, if a value fails to be within its range value, another measurement will immediately be performed to validate the first one. In the case where the second measurement does not correspond to the first, a third measurement is performed and the average of the two closest values is taken into account. In very rare cases, if the blood glucose level is below 20mg/dL, the glucometer cannot measure this out-of-range value and shows "LOW" on the display. In this case only, the blood glucose value will be assumed to be 20 mg/dL. Concerning the measurements of O₂, CO₂, locomotor activity, food and water intake, simultaneous analysis of metabolic and behavioral data allows to detect inconsistencies and errors preventing the apparition of false datas. In this case the data will not be replaced but removed from the study. In the event that an animal dies of accidental death induced
by an external factor, it is removed from the entire study. If an animal near death presents a degraded clinical condition that requires ethical killing, its whole data can be retained for certain analyses. Finally, fitting the segmented linear mixed effect model (SLME) requires at least 4 data points per mouse (otherwise the model is misspecified and no solution can be found). Consequently, for each outcome variable we remove all mice that have 3 or fewer data points for that variable. In any case, this will be specified in the figure legend.

**Cessation of data collection**

Data collection would stop when all individuals in each group have died naturally or been euthanized for ethical reasons defined in APAFIS#18333-2018112915281820v6.
**Proposed analysis pipeline**

**Description of the variables**

We will analyze a series of variables longitudinally as a function of both chronological age of individuals and remaining days before their natural death. These variables are: the evolution of body weight and composition, blood glucose concentration, body temperature, intestinal permeability assessed by plasmatic fluorescence quantification, O\textsubscript{2} and CO\textsubscript{2} volumes, RER, EE, RE, FA oxidation, weight of food and water intake, X/Y/Z dimensional activities of infrared beams analysis as well as metabolic biomarkers, cytokines and chemokines plasmatic concentrations, gut microbiota composition.

**Distinguish the two phases A and B of aging in the mouse**

To identify a two-phase evolution of the biomarkers, we use the R package “segmented”, which fits a segmented linear regression (SLM) to the data. The model can be fitted with zero, one, or multiple breakpoints. If the model with one breakpoint provides a good fit ($R^2_{\text{segmented}} > R^2_{\text{linear}}$ and p-value (absence of 1 breakpoint (BP)) > 0.05)) and if the slopes before and after are estimated to be significantly different, this confirms a two-phase evolution.

Since the measurements are longitudinal data, one needs to account for the mixed effects of each mouse when fitting the SLM. Hence we use the following procedure: (1) Fit a segmented linear mixed effect model (SLME) and segmented linear model (SLM) and compare the variance of breakpoint estimates; (2) If the mixed effect is significant, use the estimated mixed effects to compute a modified explanatory variable where the mixed effect of each mouse has been zeroed-out (intercept, slope, and slope difference), else, go directly to the next step; (3) Fit an SLM and statistically test the non-nullity of the slope difference at the breakpoint (i.e. the presence of 1 breakpoint versus 0 breakpoint) and that there is only one breakpoint (i.e. the presence of 1 breakpoint versus 2 breakpoint). These analyses will allow us to confirm or reject the increased intestinal permeability before death and the existence of two phases during ageing with a specific physiologic signature of the end of life in the
mouse model. Change in data behavior before and after the breakpoint point can also be tested using a repeated measures analysis with all measurements by splitting data into two groups: "phase A" group before the breakpoint and "phase B" group after the breakpoint, and treating mice as a random variable to take into account native differences among mice, and including an autocorrelation structure. We will add the variable “single/group housed” as a covariate in both the SLM and SLME models. This will allow us to test for the presence of a breakpoint and to estimate its position while taking into account the potential effect of single/grouped housing.

For better visualization and to graphically illustrate the trend in our data points (not to infer any model parameter) figures may also include a nonparametric local regression estimate of each variable, using locally estimated scatterplot smoothing (LOWESS33).

**Differentiate individuals with the same chronological age but with different life expectancy**

If one or more breakpoint points are identified, an average breakpoint point between the different physiological variables will be calculated. Physiological markers showing a breakpoint point will be chosen to perform a principal component analysis (PCA). This PCA will allow us to identify the parameters that are best explaining the physiological status of the mice - i.e. whether they are in phase A or in phase B. PCA results will be used to discriminate whether individuals are in phase A or B of ageing at any given chronological age using the score of the dimension(s) that best explain similarities between individuals. After fitting SLM on the score of the dimension of interest the calculation of threshold PCA value is done as follows: \( \text{PCA}_{\text{threshold}} = (\text{Mean of Dimension Score in phase A}) +/-(2 \times (\text{Standard deviation of (Mean of Dimension Score in phase A)}))). \) Depending on data behavior between phase A and B of ageing, individuals scored below or under this PCA\(_{\text{threshold}}\) value will be named “non-Smurf” if they are in phase A and “Smurf” if they are in phase B of aging at any given chronological age. In the event of an insufficient number of physiological parameters with a breakpoint, we will use the physiological parameter values to define the phase in which an individual is. These analyses will help determine whether longitudinal analyses
of biomarkers allow us to identify individuals with the same chronological age but with different mortality risks. In order to ensure that the two-phase ageing mouse model matches the one observed in Drosophila, the proportion of smurf individuals will be analyzed as a function of chronological age using linear or non-linear regression. Moreover, the amount of time spent for each individual in both phase A and phase B will be calculated and represented.

**Predict impending death independently from chronological age**

Finally, we will build a predictive model for high risk of impending death and assess whether it performs better than chronological age alone. To do so, we will develop a joint model of biomarker trajectories and survival\(^\text{34}\). Included biomarkers will be the physiological variables previously identified as displaying a breakpoint point in their values before death. By calculating the mean of the pre- and post-breakpoint point variable values, we will be able to estimate the threshold value of the chosen biomarker as the estimator in our model.
Statistics

We use the R package “segmented” to fit an SLM and an SLME. The graphical representation of the model residuals displays an (approximately) normal distribution. The implementation of the SLM comes with a statistical test for the presence of one breakpoint versus zero breakpoint (pseudo score statistic test\textsuperscript{35}); selection criteria for the correct number of breakpoints (Akaike Information Criterion (AIC)\textsuperscript{36}); a statistical test for the nullity of the difference of slope at the breakpoint (Davie’s test\textsuperscript{35,37}). However, SLME provide no such tests. So, in order to test for the presence of a breakpoint while taking mixed effects into account, we use the estimated mixed effects to compute a modified explanatory variable where the mixed effect of each mouse has been zeroed-out. This modified dataset presents the same pattern as the data, except that the effect of each mouse of the intercept, slope, and slope difference has been negated. Additionally, we can compute the adjusted $R^2$ coefficient for the segmented fit and compare it to that of the LM to assess the benefit of using the latter model. The width of the 95% confidence interval around the pointwise estimate of the breakpoint also helps to assess the confidence in the model fit.

It is worth noting that from a statistical standpoint, we use the modified data as if it were the real data, without taking into account the uncertainty stemming from the estimation of the mixed effect. This boils down to slightly underestimating the uncertainty of the estimation provided by the SLM. We reckon that this has minimal practical implications as the p-values obtained for the non-nullity of the slope difference are usually very small.

We use “lnme” R package to perform Repeated Measures ANOVA and “FactoMineR” R package for PCA analysis. To visualize data behavior LOWESS curves were created using GraphPad Prism 9. with the "coarse" parameter which takes into account 5 points in the smoothing window (To create the LOWESS smoothed curves Prism uses the equations explained in detail in section 4.11 of JC Chambers, et. al, Graphical Methods for Data Analysis, Wadsworth & Brooks, 1983). Comparison between “non-Smurf” and “Smurf” groups will be carried out using Mann-Whitney $U$ test (unpaired) in GraphPad Prism (cross-sectional analysis). If the test requires longitudinal comparisons with
repeated measurements from an animal’s “Smurf” and “non-Smurf” periods, then non-independence must be accounted for. The Smurf Increase Rate (SIR) will be determined by regression of the “Smurf” proportion as a function of chronological age as implemented in GraphPad Prism. Estimated size effect and Bayes factor will be calculated *a posteriori* with G*Power and JASP. All tests are going to be performed using R 3.6.3, GraphPad Prism 6, Microsoft Office Excel 2016, G*Power 3.1.9.7 and JASP 0.15.0.038. Numbers of animals are given in the legends.

The measurement tools we use ensure that we cover the whole range of values that each longitudinal variable can adopt. This guarantees the absence of floor or ceiling effects in their distributions. The only sensitive parameter is the measurement of blood glucose by the glucometer, which does not allow measurements below 20ng/ml. However, this is not a problem because a blood sugar level below this value is very rare and indicates imminent death, which will not bias our interpretations.

**Timeline**

Completion of the study estimated in April 2024.
Data Availability Plan and Conflict of Interest

All data and codes will be made available on GitHub. This applies to all data and other information about mice (IDs, data on single- versus group-housed status, physiological and metabolic data…). The authors declare no conflict of interest.

Ethical Approval Plan

All protocols are carried out in accordance with French standard ethical guidelines for laboratory animals and with approval of the Animal Care Committee (Comité d’éthique en expérimentation animale Buffon C2EA-40; Comité d’éthique pour l’expérimentation animale Charles Darwin CEEACD/N°5; APAFIS#18333-2018112915281820v6; APAFIS#30102-2021022517524316v4; “Non-technical summaries of notified files” n°10766 (www.enseignementsup-recherche.gouv.fr))
Pilot Data

Proof of concept

Intestinal permeability and different metabolic parameters were measured regularly on a group of AKRJ mice throughout their lifespan as indicated by the grey arrows intersecting the survival curve of the cohort (Figure 1a). As described in the timeline section, we started our study with a group of AKRJ female mice in order to obtain preliminary results in a relatively short time frame. Indeed, AKRJ mice have a T50 of ~9 months which is a considerable advantage over C57BL/6J mice which have a T50 of ~30 months\(^{20}\). AKRJ mice are viremic from birth which leads to the inevitable evolution towards leukemia or lymphoma\(^{39}\), which is why their T50 is much lower than that of C57B/6J mice. Nevertheless, existing data obtained in flies show that this should not be a confounding factor since pathological conditions\(^{7}\) and environmental conditions increasing mortality\(^{3}\) lead to an accelerated appearance of Smurfs. The design of the study made us start with females in order to avoid fights between individuals as mice were regularly isolated for measurements in the metabolic cages and subsequently regrouped for housing. Preliminary results on AKRJ mice need to be validated in a mouse strain with different mortality drivers, which is why we also include a group of male C57Bl6j and a group of female C57Bl6j in our study. We will then discuss the limitations of the mouse model as well as of each strain.

Is the end of life in mice characterized by an increase in intestinal permeability?
The visualization of nonparametric local regression (LOWESS) of intestinal permeability data as a function of individuals’ chronological age appears stable throughout the study (Figure 1b). prior to death (Figure 1c) In order to detect a possible change in data behavior before death, scatter plot from Figure 1c was analyzed using the R package “segmented” to fit an Segmented Linear Model (SLM). SLM analysis reveals a breakpoint point 15.4 days before death (Figure 1d). “Adjusted \(R^2\)segmented” value higher than “Adjusted \(R^2\)linear” show that the fit is improved with SLM,
compared to the linear model. The "p−value absence of 1 breakpoint (BP)" is 0.0821 meaning that we can exclude the absence of a breakpoint and that the slope difference before and after the breakpoint is non-null. These results suggest that mouse intestinal permeability increases before death, as previously described in Drosophila\textsuperscript{3}. However, the SLM requires each mouse to have at least 4 data points, otherwise the model would be statistically misspecified and no solution can be found. Consequently, all mice that have 3 or fewer data points for that variable were removed. Then, if we consider all individuals, only 64% of the individuals exceed the "normal range" for intestinal permeability. The data on males and females C57Bl6j will allow us to draw more robust conclusions.

**Is there a specific metabolic signature of the end of life in mice?**

Analysis of physiological markers as a function of remaining time before death highlights two phases. Indeed, the visualization of LOWESS of data of blood glucose (GLY, Figure 2a), body weight (BW, Figure 2b), percentage of fat mass (%FAT, Figure 2c), energy balance (EB, Figure 2d) food intake (FI, Figure 2e), locomotor activity (ACT, Figure 2f), energy expenditure (EE, Figure 2g) and rectal temperature (RT, Figure 2h) shows a plateau followed by a decrease in their values just before an individuals' death. The SLM analysis (Figure 3) detects a change in data behavior for all physiological parameters between 48.6 and 19 days prior to death (Figure 3a: GLY=20.5; Figure 3b: BW=25; Figure 3c: %FAT=48.6; Figure 3d: EB=44.6; Figure 3e: FI=35.1; Figure 3f: ACT=35.2; Figure 3g: EE=26.6; Figure 3h: RT=19). In fact, for all variables, the “Adjusted $R^2$-segmented” value is higher than “Adjusted $R^2$ linear” indicating that the fit is improved with SLM, compared to the linear model (Figure 3). $R^2$ $R^2$ And, for all variables, The “p−value absence of 1 BP”>0.05 means that we can exclude the absence of a breakpoint and that the slope difference before and after the breakpoint is non-null (Figure 3). These findings indicate that there is a metabolic signature of the end of life in mice characterized by the concomitant association of a decrease in glycemia, body weight and locomotor activity (Figure 2 and 3 a,b,f). The decrease in body weight is mostly explained by a decrease in fat content likely due to a negative energy balance (Figure 2 and 3 c,d) as a result of
reduced food intake (Figure 2 and 3 e). Those dramatic changes might concourse to diminished
glycemia and capacity for the animal to maintain his body temperature (Figure 2 and 3 a,h).

Preliminary analysis of glycemia data from male HET3 mice from Palliyaguru et al. (PMID34508697)
presented in Figure 5 clearly shows two phases in the aging process of mice, characterized by a
decreased glycemia before death. The fact of collecting physiological data at two different points of
the world while obtaining the same results as well as the demonstration that a group of 50 mice can
be sufficient to highlight some of the aspects of aging are issues that will be discussed in the final
version of the paper.

**Do identified biomarkers allow us to discriminate between two subpopulations characterized
by different mortality risk?**

The principal component analysis of physiological markers yields Dimension 1 (Dim1) and
Dimension 2 (Dim 2), which explain 61.98% and 12.48% of the data variance respectively (Figure
4a). The graphical representation of individual values on these two dimensions shows that data are
clustering along with the first dimension Dim1 in two groups which are characterized by a lifespan
higher or lower than 30 days (Figure 4a). Thus, the physiological markers described above enable
us to separate individuals of the same chronological age into two groups according to whether their
life expectancy is higher or lower than 30 days. This breakpoint point of 30 days was calculated as
the mean value of the breakpoint points described above for each physiological marker.

By fitting SLM on the score of DIM1 expressed as a function of remaining days before death, we
calculate the PCA threshold value as follows: \( \text{PCA\_threshold} = (\text{Mean of Dimension 1 Score before}
\text{breakpoint}) = 0.75) +/-(2 \times (\text{Standard deviation of (Mean of Dimension 1 Score before breakpo}nt =
1.13)) = -1.51. \) We label individuals with a PCA\_threshold value >-1.51 as “non-Smurf” and
individuals scored <-1.51 as “Smurf”, regardless of chronological age. Thus, we can observe that as
described in Drosophila\(^3\), the life expectancy of “Smurf” individuals is much lower than that of "non-
Smurf" individuals of the same chronological age (Figure 4c) and that the proportion of "Smurf"
individuals in the population increases exponentially with chronological age (Figure 4d). Interestingly, the duration of the Smurf phase (approx. 30 days) represents the same proportion of the mice life expectancy at birth as what was previously described in other organisms⁶.

We represented mice followed up from adulthood to their natural death as individual life histories centered on the moment of the Smurf transition (Figure 4e). This representation helps visualizing that (i) individuals become Smurf at random times, (ii) there is no correlation between the age at Smurf transition and the life expectancy of a Smurf and (iii) the remaining lifespan of individual in Phase B of ageing (after “Smurf” transition) is significantly decreased compared to that of Phase A of ageing (before “Smurf” transition).

**Feasibility of proposed methods**

The results from our pilot study highlight the feasibility of our method but have led us to rethink certain aspects, particularly regarding FITC-Dextran gavage protocol. The smallest size of FITC-Dextran on the market is 4kDa. In our protocol we used a mix of FITC-Dextran of different sizes: 4kDa, 20 kDa and 70 kDa. However, in the protocol applied to Drosophila³, the molecule Blue#1 allowing to evaluate intestinal permeability is 792.58 Da. We therefore assumed that the problem in the measurement of intestinal permeability observed at the end of life in mice came from the fact that the size of the FITC-Dextran was too large. We therefore collaborated with a chemist to manufacture a custom FITC-Dextran as close as possible to the size of Blue#1. This new FITC of 568 Da will be used in the experiments performed on the C57BL/6J mouse line.

Moreover, on this first group of AKRJ, we had established to choose the animals which passed in metabolic cages for each recording by using their intestinal permeability as criterion of selection in order to make sure to be able to record "Smurfs" individuals. Animals were isolated at each passage in metabolic cages and then grouped for housing. However, we observed a transient effect on body weight at each isolation. We therefore decided to modify our protocol for the C57BL/6J groups. Indeed, from the beginning, we isolate a group of mice that will be dedicated to the passage in the
metabolic cages so we can follow a mouse from the beginning of the experiment until its death without affecting its body weight. In addition, this prevents the risk of fighting within the group of males. As mice die, previously grouped mice are individualized to maximize recording in the metabolic cages.

Finally, this first group of AKRJ mice allowed us to demonstrate that in the last 30 days of life, animals develop disorders that lead to severe distress. For ethical reasons, we had to euthanize about 30% of our animals. Based on these observations we have undertaken several actions: (1) we decided not to repeat the experiment on a male AKRJ group as originally planned but to continue using only C57BL/6J mice only, (2) we reclassified our ethical protocol as severe and (3) we decided to keep the data from euthanized animals to characterize the evolution of physiological parameters before death because we considered that the death of the animal would have occurred at most 24 hours later.

To conclude this manuscript is submitted using the “Pre Registered Studies” format. Here we tend to provide evidence about the relevance of this study. We do not believe that our results are sufficient to state that our model is generalizable to humans. For the moment we are unable to state anything. We are still in the process of collecting and analyzing all the results. The relevance of our results and their limitations in light of the existing literature will certainly be strongly addressed in the final discussion of the article.
Figures legends

Figure 1: Assessing pre-death intestinal permeability in a mouse cohort. Survival curve of 50 AKRJ female mice cohort. Dashed vertical grey arrows intersecting the survival curve of the cohort represent the time points where physiological parameters were monitored (a). Fluorescence unit in plasma 3h after FITC-dextran gavage indicating intestinal permeability as a function of chronological age of individuals (b) and as a function of days remaining before natural death of individuals (c,d). Full black line depicts the nonparametric local regression (LOWESS) of longitudinal intestinal permeability values (b,c). The dataset was modified to alleviate the impact of inter-individual variability by negating the intercept, slope, and slope differences. This treatment does not affect the pre-death pattern of the data. The vertical dashed blue line and the red dot show the breakpoint point identified by segmented linear model (SLM) analysis of longitudinal intestinal permeability values. Red lines represent the results of SLM analysis before and after the breakpoint point (d). Results of subsequent calculations and statistic tests (p-values) are written on the graph: adjusted $R^2$ for the linear and segmented fit; statistical test for the absence of 1 breakpoint (BP) (Davies’ test); statistical test for the absence of a 2nd BP (Pseudo-score test). n=44 AKRJ female mice.

Figure 2: Longitudinal representation of physiological variables as a function of remaining days before natural death of individuals. Variables are: glycemia (a), body weight (b), fat mass expressed as a percentage of body weight (c), average daily energy balance (d, food intake - energy expenditure), food intake (e), locomotor activity (f), energy expenditure (g) and rectal temperature (h) all expressed as a function of time before recorded death. Full black line depicts the nonparametric local regression (LOWESS) of longitudinal variables values. n=44 AKRJ female mice.
**Figure 3**: Segmented linear model analysis of physiological variables as a function of remaining days before natural death of individuals. Variables are: glycemia (a), body weight (b), fat mass expressed as a percentage of body weight (c), average daily energy balance (d, food intake - energy expenditure), food intake (e), locomotor activity (f), energy expenditure (g) and rectal temperature (h). All parameters expressed as a function of time before recorded death and corrected for inter-individual variability. Vertical dashed blue lines and red dots show the breakpoint point identified by segmented linear model (SLM) analysis of longitudinal values (a,b,c,d,e,f,g,h). Red lines represent the result of SLM analysis before and after the breakpoint point (a,b,c,d,e,f,g,h). Results of subsequent calculations and statistic tests (p-values) are written on the graph: adjusted $R^2$ for the linear and segmented fit; statistical test for the absence of 1 breakpoint (BP) (Davies' test) ; statistical test for the absence of a 2nd BP (Pseudo-score test). n=44 AKRJ female mice.

**Figure 4**: Identifying individuals with the same chronological age but different mortality risks in mice. Graphical representation of individual values on dimension 1 (Dim 1, x-axis) and dimension 2 (Dim 2, y-axis) of the principal component analysis (PCA) of longitudinal variables that have a breakpoint point: intestinal permeability (Permeability), glycemia, body weight, fat and lean masses expressed as a percentage of body weight, food intake (Food int.), locomotor activity (Activity), energy expenditure (Energy exp.) and rectal temperature (Rectal temp.) (a). Individuals with a remaining lifespan under or equal to 30 days are depicted in red and those with a remaining lifespan over 30 days are depicted in green (a). Correlation graph from PCA analysis of the variables that have a breakpoint point (b). PCA results were used to discriminate whether individuals are in phase A or B of ageing at any given chronological age using the score of Dim1 for each individual at any chronological age. Remaining lifespan of “non-Smurfs” (black boxes, points) and “Smurfs” (blue boxes, points) individuals as a function of chronological age of individuals (c). The boxes extend from the 25th to 75th percentiles; the line in the box is the median; the whiskers span the smallest and largest values; each point represents an individual. * p<0.05 by the Mann-
Whitney U test on mean remaining lifespan values in “non-Smurf” and “Smurf” individuals (c). Proportion of “Smurf” individuals in the mice cohort (d). Grey line represents exponential growth regression analysis of “Smurf proportion” and grey dotted lines enclose the area that contains the true curve with 95% certainty (d). Representation of mice followed up from adulthood to their natural death as individual life histories centered on the moment of the Smurf transition (e). Grey squares display mice life before “Smurf” transition and blue squares display mice life after “Smurf” transition (e). 7 individuals were never scored as Smurf and 2 out of 37 showed a reversion or a mischaracterization of their Smurf state at least once but within 90 days of their natural death (grey squares with blue squares). n=44 AKRJ female mice

**Supplementary figure 1 :** Longitudinal representation of physiological variables as a function of individuals’ chronological age. Variables are : glycemia (a), body weight (b), fat mass expressed as a percentage of body weight (c), average daily energy balance (d, food intake - energy expenditure), food intake (e), locomotor activity (f), energy expenditure (g) and rectal temperature (h) all expressed as a function of chronological age of individuals. Full black line depicts the nonparametric local regression (LOWESS) of longitudinal variables values. n=44 AKRJ female mice.

**Supplementary figure 2 :** Segmented linear model analysis of plasmatic glucose concentration data from Palliyaguru et al. (PMID34508697) as a function of remaining days before natural death of individuals. Variable is glycemia. The dataset is obtained by independent measurements on individual mice. Dashed blue line and red point shows the breakpoint point identified by segmented linear model (SLM) analysis of longitudinal plasmatic glucose concentration values. Red lines represent SLM analysis before and after the breakpoint point. Results of subsequent calculations and statistic tests (p value) are written on the graph : adjusted $R^2$ for the linear and segmented fit ; statistical test for the absence of 1 breakpoint (BP) (Davies' test)
; statistical test for the absence of a 2nd BP (Pseudo-score test). n=135 HET3 male mice. Data from:
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Bibliography


Figure 1
Figure 3

a) Glycemia
b) Body weight
c) Fat mass
d) Energy balance
e) Food intake
f) Locomotor activity
g) Energy expenditure
h) Rectal temperature
**Figure 4**

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**a)** PCA graph of individuals
- Remaining lifespan ≤ 30 days: red squares
- Remaining lifespan > 30 days: green circles

**b)** PCA graph of variables
- Dim 1 (61.98%)
- Dim 2 (12.48%)

**c)** Remaining lifespan (days)
- Non-smurf: black bars
- Smurf: blue bars

**d)** Smurf individuals (%)
- Exponential reg.
- 95% confidence band

**e)** Time to Smurf transition (days) vs. Time as Smurf (days)
Figure sup 1
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Figure sup 2