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# When will my mouse die? Life span prediction based on immune function, redox and behavioural parameters in female mice at the adult age

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ABSTRACT

The identification of predictive markers of life span would help to unravel the underlying mechanisms influencing ageing and longevity. For this aim, 30 variables including immune functions, inflammatory-oxidative stress state and behavioural characteristics were investigated in ICR-CD1 female mice at the adult age (N = 38). Mice were monitored individually until they died and individual life spans were registered. Multiple linear regression was carried out to construct an Immunity model (adjusted $R^2$ = 75.8%) comprising Macrophage chemotaxis and phagocytosis and Lymphoproliferation capacity, a Redox model (adjusted $R^2$ = 84.4%) involving Reduced Glutathione and Malondialdehyde concentrations and Glutathione Peroxidase activity and a Behavioural model (adjusted $R^2$ = 79.8%) comprising Internal Locomotion and Time spent in open arms indices. In addition, a Combined model (adjusted $R^2$ = 92.4%) and an Immunity-Redox model (adjusted $R^2$ = 88.7%) were also constructed by combining the above-mentioned selected variables. The models were also cross-validated using two different sets of female mice (N = 30; N = 40). Correlation between predicted and observed life span was 0.849 ($P < 0.000$) for the Immunity model, 0.691 ($P < 0.000$) for the Redox, 0.662 ($P < 0.000$) for the Behavioural and 0.840 ($P < 0.000$) for the Immunity-Redox model. Thus, these results provide a new perspective on the use of immune function, redox and behavioural markers as prognostic tools in ageing research.

## 1. Introduction

It is known that the pace of ageing and the expected life span varies among individuals of the same chronological age (Collier and Coleman, 1991). Individual trajectories of ageing depend on the genetic background, the environment and on interactions at the epigenetic level. As a consequence, the biological age is in part dissociated from the chronological age of an organism (Finkel et al., 1995). Construction of models for life span prediction is a fundamental challenge in ageing research given that it would provide a useful tool to study the effect that a given intervention has on the ageing rate of a subject, in less than his/her lifetime. Since life expectancy data are difficult to collect in humans due to their long life span, mice, which have a mean longevity of around two years, are more suitable for developing such models. In addition, the identification of markers that predict life span would help to disentangle the factors influencing ageing and longevity (Swindell et al., 2008). However, the question that remains is what type of parameters should be used for life span prediction. In mice, previous studies have identified early and mid-life markers that correlate with life span (such as low body weight, T-cell subsets, serum hormone levels, cataract scores…) and models for life span prediction have been constructed (Miller, 2001; Miller et al., 2002; Harper et al., 2003; Anisimov et al., 2004; Harper et al., 2004; Swindell et al., 2008). However, most of them have been focused on phenotypic traits that are more the result than the cause of the ageing process.

Many theories have been proposed to explain ageing. Out of all of them, the oxidative-inflammatory theory of ageing (De la Fuente and Miquel, 2009) links the age-related increase in oxidative stress

*Abbreviations:* AGEs, Advanced Glycated End-products; BCA, Bicinchoninic Acid; Con, A Concanavalin A; EPM, Elevated Plus Maze; GPx, Glutathione Peroxidase; GR, Glutathione Reductase; GSH, Reduced Glutathione; GSSG, Glutathione disulfide; HB, Hole board; IL, Interleukin; MDA, Malondialdehyde; MLR, Multiple Linear Regression; NADPH, Reduced Nicotinamide Adenine Dinucleotide Phosphate; NBT, Nitro Blue Tetrazolium; NF-kB, Nuclear Factor Kappa B; OPT, o-phthalaldehyde; ROS, Reactive Oxygen Species; TBA, Thiobarbituric Acid; TNF-α, Tumour necrosis factor-α

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(Harman, 1956; Barja, 2002) with the chronic low-grade inflammation, so-called "inflamm-ageing" (Franceschi et al., 2000), through the interplay of the immune system. Thus, the age-related increase in oxidative stress experienced by immune cells, impairs their adequate functioning and results in an increased release of pro-inflammatory mediators. Given that oxidation and inflammation are interlinked processes, there is a positive feedback loop between both processes resulting in the establishment of the age-related chronic oxidative and inflammatory stress situation. Accordingly, a relationship has been found between the immune function, the oxidative and inflammatory stress of immune cells and life span (De la Fuente and Miquel, 2009; Martínez de Toda et al., 2016, 2017; Martínez de Toda et al., 2019). However, it is still not known which of these parameters or which combination of them could be used in the construction of a mathematical model in order to predict life span.

In the present study, we decided to focus on the adult, mid-life age for forecasting life span, which in our mice strain is 40 weeks and is equivalent to 40 years in humans. Previous studies have demonstrated that the inter-individual differences in ageing trajectories can already be quantified at the adult age both in humans (Rockwood et al., 2011; Belsky et al., 2015) and in mice (Whitehead et al., 2014; Rockwood et al., 2017; Antoch et al., 2017). In addition, the sooner an accelerated ageing rate is detected, the earlier lifestyle strategies can be implemented to slow it down.

In relation to this, several conditions have been described to cause an accelerated ageing, among them an inadequate response to stress or an anxiety-like behaviour (Perna et al., 2015; De La Fuente, 2018). Accordingly, it has been demonstrated that mice with chronic hyperreactivity to stress show a premature immunosenescence, a premature frail status and they have a shorter life span (Viveros et al., 2007; Martínez de Toda et al., 2016, 2018). Several behavioural tests are used to assess anxiety levels in mice, the most used being the elevated plus maze (EPM) (Walf and Frye, 2007) and the hole-board (HB) (Brown and Nemes, 2008) tests. However, it is not known which of the behavioural indices derived from these tests, if any, can be used to forecast the life span of a given mouse. Thus, we also decided to investigate the potential predictive power of the behavioural indices of adult mice exposed to the hole-board (HB) test and the elevated plus maze (EPM) test towards life span.

Therefore, several immune function (macrophage chemotaxis and phagocytosis, natural killer activity, lymphocyte chemotaxis and proliferation), inflammatory (basal release of IL-6, IL-1β, TNF-α and IL-10) and redox parameters (catalase, glutathione peroxidase, glutathione reductase activities, reduced and oxidized glutathione, superoxide anion and malondialdehyde concentrations) as well as behavioural responses to the HB and EPM tests, were investigated in one group of female mice at the adult age (40 weeks-old). Mice were monitored individually along the ageing process and individual life spans were recorded. Therefore, based on the obtained data, it was possible to construct one model based on immune functionality and inflammatory mediators, other based on redox parameters, another based on behavioural indices and a fourth one by combining redox and immune parameters using multiple linear regression (MLR). Afterwards, mathematical models were cross-validated in a different group of female mice than the one used for model construction. This approach, allowed us firstly to discover the parameters most implicated in longevity; secondly, to assess which cohort of parameters (if immune function, redox or behavioural) is most relevant for life span prediction and thirdly, which model has a higher reproducibility by verifying their predictive power in different groups of female mice.

## 2. Material and methods

### 2.1. Experimental animals and extraction of peritoneal leukocytes

Three different groups of female outbred ICR-CD-1 ex-reproductive mice (*Mus musculus*) were used for this study. All of them were acquired from Janvier Labs (Germany) when they were 32 ± 4 weeks old. The first group of mice (N = 40) was used for data collection and development of model construction. Another group of mice (N = 30) was used for validation of the Immunity, Redox and Immunity-Redox models. Another different group of mice (N = 40) was used for validation of the Behavioural model. Behavioural tests described below and collection of peritoneal cell suspensions were performed at the adult age of 40 ± 4 weeks; following a previously described method (Martínez de Toda et al., 2016). The measurement of markers was performed using unfractionated peritoneal leukocytes to better reproduce the *in vivo* situation. Animals were monitored individually along the ageing process and each individual achieved life span was recorded.

### 2.2. Analysis of immune function and inflammatory parameters

#### 2.2.1. Chemotaxis

Cell suspensions were adjusted to $0.5 \times 10^6$ cells (macrophages or lymphocytes)/ml in Hank's medium and placed into a Boyden chamber. The number of cells that migrated towards formyl-Met-Leu-Phe were counted and expressed as Chemotaxis Index, as previously described (Martínez de Toda et al., 2016).

#### 2.2.2. Phagocytosis

Cell suspensions were adjusted to $0.5 \times 10^6$ macrophages/ml in Hank´s medium and placed into migration inhibition factor (MIF) plates for 30 min. After washing, latex beads were added into the plates and the number of latex beads phagocyted by 100 macrophages were counted and expressed as Phagocytic Index, as previously described (Martínez de Toda et al., 2016).

#### 2.2.3. Natural killer cytotoxicity

Cell suspensions were adjusted to $10^6$ total cells/ml in RPMI 1640 medium and placed into 96-well plates. Murine YAC-1 lymphoma cells were added into wells and Natural killer activity was assessed by quantifying released lactate dehydrogenase into the medium (Cytotox 96 T M Promega, Germany). The results were expressed as the percentage of tumour cells killed (% lysis), as previously described (Martínez de Toda et al., 2016).

#### 2.2.4. Lymphoproliferative capacity

Cell suspensions were adjusted to $0.5 \times 10^6$ lymphocytes/ml in RPMI 1640 medium supplemented with foetal bovine serum (FBS) and placed into 96-well plates. The mitogen concanavaline A (Con A) or complete medium were added into wells and incubated for 48 h. Then $^3$H-thymidine was also added and incubated for 24 h. $^3$H-thymidine uptake was quantified in a beta counter both in basal and stimulated conditions and results are expressed as lymphoproliferation capacity (%) being 100% the counts per minute (cpm) in basal conditions, as previously described (Martínez de Toda et al., 2016).

#### 2.2.5. Cytokine measurement

After incubation of peritoneal immune cells during 48 h in the absence of any mitogen (basal conditions), supernatants were collected. Basal release of IL-1β, IL-6, TNF-α and IL-10 was measured simultaneously in those supernatants by multiplex luminometry (Beadlyte mouse multiplex cytokine detection system, MHYSTOMAG-70 K, Upstate, Millipore).

### 2.3. Determination of oxidative stress parameters

#### 2.3.1. Catalase activity

Cell suspensions were adjusted to $10^6$ total cells/ml in Hank´s medium, centrifuged and cell pellets resuspended in oxygen-free phosphate buffer 50 mM. Then, they were sonicated and supernatants

were used for the enzymatic reaction together with 14 mM $H_2O_2$ as substrate. Decomposition of $H_2O_2$ was measured at 240 nm as previously described (Martínez de Toda et al., 2019). The results were expressed as units (U) of catalase activity/mg protein.

#### 2.3.2. Glutathione peroxidase activity

Cell suspensions were adjusted to $10^6$ total cells/ml in Hank´s medium, centrifuged and cell pellets resuspended in oxygen-free phosphate buffer 50 mM. Then, they were sonicated and supernatants were used for the enzymatic reaction together with cumene hydroperoxide as a substrate (cumene − OOH) as previously described (Martínez de Toda et al., 2019). Oxidation of NADPH was measured at 340 nm. The results were expressed as units (U) of glutathione peroxidase activity/mg protein.

#### 2.3.3. Glutathione reductase activity

Cell suspensions were adjusted to $10^6$ total cells/ml in Hank´s medium, centrifuged and cell pellets resuspended in oxygen-free phosphate buffer 50 mM EDTA 6.3 mM. Then, they were sonicated and supernatants were used for the enzymatic reaction together with GSSG 80 mM as substrate. Oxidation of NADPH was measured at 340 nm, as previously described (Martínez de Toda et al., 2019). The results were expressed as units (U) of glutathione reductase activity/mg protein.

#### 2.3.4. Glutathione concentration

Cell suspensions were adjusted to $10^6$ total cells/ml in Hank´s medium, centrifuged and cell pellets resuspended in phosphate buffer 50 mM EDTA 0.1 M pH 8. Then, they were sonicated and supernatants were used for the quantification of both reduced (GSH) and oxidized (GSSG) glutathione by the reaction capacity that GSSG and GSH have with o-phthalaldehyde (OPT) at pH 12 and pH 8, respectively, resulting in the formation of a fluorescent compound. Fluorescence was measured at 350 nm excitation and 420 nm emission, as previously described (Martínez de Toda et al., 2019). Results were expressed as nmol of GSSG and GSH per milligram of protein. Moreover, the GSSG/GSH ratio was calculated for each sample.

#### 2.3.5. Intracellular superoxide anion concentration

Cell suspensions were adjusted to $10^6$ macrophages/ml in Hank´s medium and mixed with NBT (1 mg/ml). After 60 min incubation the reaction was stopped with HCl 0.5 M, samples were centrifuged and supernatants discarded. Intracellularly reduced NBT was extracted with dioxan and absorbance was measured at 525 nm, as previously described (Martínez de Toda et al., 2019). Results were expressed as nmol superoxide anion/$10^6$ macrophages.

#### 2.3.6. Malondialdehyde concentration

Determination of malondialdehyde (MDA) concentration was evaluated using the commercial kit “Lipid peroxidation (MDA) Assay Kit” (Biovision, CA, USA). Cell suspensions were adjusted to $10^6$ total cells/ml in Hank´s medium, centrifuged and cell pellets resuspended in MDA lysis buffer containing BHT. Then, they were sonicated and supernatants were incubated with thiobarbituric acid (TBA) for 60 min in a water bath at 95 °C. Then, samples were centrifuged, supernatants collected and dispensed into 96-well plates for spectrophotometric measurement at 532 nm, as previously described (Martínez de Toda et al., 2019). Results were expressed as nmol MDA/mg protein.

### 2.4. Exploratory and anxiety-like behavioural tests

#### 2.4.1. Hole board test

The hole board consisted of a box (60 × 60 x 45 cm) with matte-painted metallic walls, divided into 36 squares (10 x 10 cm), bearing four equally spaced holes (3.8 cm of diameter) and brightly illuminated (75 W). All but the 20 peripheral squares were considered internal. White plastic objects were placed in each hole to attain mice attraction and drive their “goal-directed behaviour”. Mice were placed in a corner of the box and allowed to explore for 5 min. Exploration was recorded and then analysed by reviewing video recordings. Calculated behavioural indices were: total locomotion (total number of squares that the animal crosses), internal locomotion (total number of internal squares that the animal crosses), external locomotion (total number of external squares that the animal crosses), percentage of internal locomotion (total number of internal squares that the animal crosses divided by total locomotion), percentage of external locomotion (total number of external squares that the animal crosses divided by total locomotion). All these parameters reflect the horizontal activity of the animal. For vertical activity parameters, total number of rearing and time (in seconds) of each rearing were analysed. Furthermore, the total number of head-dipping and the time (in seconds) of each head-dipping were evaluated as “goal-directed behaviour”. Finally, grooming and freezing behaviours (number and duration, in seconds) were also calculated.

#### 2.4.2. Elevated plus maze

The elevated plus-maze, which was acquired from Panlab, Spain, consisted of two closed arms (CA, 30 x 5 cm, black walls) and two open arms (OA; 30 x 5 cm) forming a square cross with a 5 x 5 cm square central piece. The apparatus was elevated 40 cm above the floor. Mice were individually placed on the central platform facing an enclosed arm and were allowed to freely explore the maze for 5 min. Exploration was recorded and then analysed by reviewing video recordings. Calculated parameters were: time spent in open arms, time spent in closed arms, time spent in central platform, number of entries (four paws criteria) in open arms, closed arms and central platform as well as total number of grooming and freezing behaviours (number and duration).

### 2.5. Model construction and statistical analysis

All the investigated parameters were grouped into three sets, the first one including immune function and inflammatory mediators, the

**Table 1**
Set of variables used for model construction.

| Immune function and inflammatory parameters | Antioxidant and Oxidant parameters | Behavioural parameters | |
|---|---|---|---|
| Natural Killer activity | Catalase activity | **Elevated Plus Maze (EPM) test** | Time in open arms |
| Macrophage Chemotaxis | Glutathione Peroxidase activity | | Number of entries in open arms |
| Macrophage Phagocytosis | Glutathione Reductase activity | | Time in closed arms |
| Lymphocyte Chemotaxis | Reduced Glutathione (GSH) | | Number of entries in closed arms |
| Lymphoproliferation Capacity | Xanthine Oxidase activity | | Time in central platform |
| Basal release of IL-1β | Anion Superoxide concentration | | Number of entries in central platform |
| Basal release of IL-6 | Malondialdehyde concentration | **Hole Board (HB) test** | Total, internal and external locomotion |
| Basal release of TNF-α | Oxidised Glutathione (GSSG) | | Percentage of internal and external locomotion/total |
| Basal release of IL-10 | GSSG/GSH ratio | | Number and time of “rearings” |
| | | | Number and time of “groomings” |
| | | | Number and time of “head-dippings” |
| | | | Number and time of “freezings” |

second one involving redox parameters and the third one comprising behavioural parameters (Table 1). The power to predict remaining life span of immune, redox and behavioural parameters recorded at 40 weeks of age was investigated by multiple linear regression (MLR).

Recorded life span was used as a dependent variable and all other variables were used as predictor variables. The step-wise forward method procedure for generation of the model was chosen. It first selects the predictor variable that explains the dependent variable the most, then the next one and so on. The threshold criteria for variable selection into the model construction was $P < 0.05$. Generation of models was performed using SPSS 21.0 and Statgraphics Centurion XVIII. Five models were generated, one for immune function and inflammatory parameters called the "Immunity model", another one for redox parameters called the "Redox model" and a third one based on behavioural indices called the "Behavioural model". Moreover, the "Combined model" including immune, redox and behavioural variables and the "Immunity-Redox model" including both immune and redox variables were produced. For each adjusted model, the normality of residuals, the constant variability of residuals (homoscedasticity) and the independence of residuals were checked, in order to verify the Gauss-Markov hypothesis, through the corresponding graphic and analytical analysis. In addition, for validation and cross-validation of the models, Pearson´s correlation coefficients were calculated between the observed and predicted life span in different sets of female mice.

## 3. Results

In the first group of mice (N = 38), used for data collection, life span varied from 45 to 136 weeks and mean life span was 74 ± 4 weeks. In the other group of mice (N = 30) used for cross-validation of the Immunity, Redox and Immunity-Redox models, life span varied from 50 to 128 weeks. Mean life span was 93 ± 3 weeks. In the other group of mice (N = 40), used for validation of the Behavioural model, life span varied from 40 to 130 weeks and mean life span was 81 ± 4 weeks.

Model construction of the Immunity model is shown in Table 2. The first variable selected (macrophage chemotaxis) accounts for almost 50% of the variance in life span. When introducing the second one (macrophage phagocytosis) the explained variance increased up to almost 70%, and when the third one was added (lymphoproliferation capacity) the explained variance increased to 76%.

The steps for the Redox model construction are shown in Table 3. The first selected variable (GSH concentration) explains 65% of the life span achieved. When introducing the next one (MDA concentration) the explained variance increased to almost 79%, and when the third one was added (glutathione peroxidase activity) the explained variance increased to 84%.

**Table 2**
Immunity Model construction through step-wise forward method. Predicted remaining life span = $\hat{\beta}_0$ + $\hat{\beta}_1$ x Macrophage chemotaxis + $\hat{\beta}_2$ x Macrophage phagocytosis + $\hat{\beta}_3$ x Lymphoproliferation.

| | Model 1 | Model 2 | Model 3 |
|---|---|---|---|
| Constant ($\hat{\beta}_0$) | 22.023 (8.963) | 1.171 (8.143) | −0.071 (7.295) |
| Macrophage Chemotaxis ($\hat{\beta}_1$) | 0.094*** (0.015) | 0.069*** (0.013) | 0.055*** (0.012) |
| Macrophage Phagocytosis ($\hat{\beta}_2$) | | 0.049*** (0.010) | 0.040*** (0.009) |
| Lymphoproliferation ($\hat{\beta}_3$) | | | 0.068** (0.022) |
| $R^2$ | 51.3% | 71.3% | 77.7% |
| Adjusted $R^2$ | 49.9% | 69.7% | 75.8% |

Each value shows the estimated coefficient and the standard error for each coefficient is shown in brackets.
** $P < 0.01$.
*** $P < 0.001$.

**Table 3**
Redox Model construction through step-wise forward method. Predicted remaining life span = $\hat{\beta}_0$ + $\hat{\beta}_1$ x GSH + $\hat{\beta}_2$ x MDA + $\hat{\beta}_3$ x Glutathione Peroxidase.

| | Model 1 | Model 2 | Model 3 |
|---|---|---|---|
| Constant ($\hat{\beta}_0$) | 46.659 (4.655) | 85.894 (9.530) | 74.459 (8.846) |
| GSH ($\hat{\beta}_1$) | 5.134*** (0.681) | 3.506*** (0.645) | 2.532*** (0.624) |
| MDA ($\hat{\beta}_2$) | | −10.224*** (2.297) | −8.934*** (2.004) |
| Glutathione Peroxidase ($\hat{\beta}_3$) | | | 0.021** (0.006) |
| $R^2$ | 66.2% | 80.2% | 86.0% |
| Adjusted $R^2$ | 65.0% | 78.8% | 84.4% |

Each value shows the estimated coefficient and the standard error for each coefficient is shown in brackets.
** $P < 0.01$.
*** $P < 0.001$.

**Table 4**
Behavioural Model construction through step-wise forward method. Predicted remaining life span = $\hat{\beta}_0$ + $\hat{\beta}_1$ x Internal Locomotion + $\hat{\beta}_2$ x Time spent in open arms.

| | Model 1 | Model 2 |
|---|---|---|
| Constant ($\hat{\beta}_0$) | 13.763 (8.245) | 11.833 (6.001) |
| Internal Locomotion ($\hat{\beta}_1$) | 0.554*** (0.071) | 0.378***(0.060) |
| Time spent in open arms ($\hat{\beta}_2$) | | 0.202*** (0.035) |
| $R^2$ | 62.8% | 80.9% |
| Adjusted $R^2$ | 61.7% | 79.8% |

Each value shows the estimated coefficient and the standard error for each coefficient is shown in brackets.
*** $P < 0.001$.

The construction of the Behavioural model is shown in Table 4. The first chosen variable (internal locomotion in the hole board test) accounts for 62% of the variance. When introducing the second one (time spent in open arms in the elevated plus maze test) the explained variance increased to almost 80%. A third variable was not introduced given that none of the other variables fulfilled the $P < 0.05$ criteria.

Furthermore, an additional model, denominated the "Combined model", was built including all the selected variables for the Immunity, Redox and Behavioural models. Variables chosen were: internal locomotion, macrophage chemotaxis, GSH concentration, macrophage phagocytosis, time spent in open arms and MDA concentration. All variables included fulfilled the $P < 0.05$ criteria and the explained variance of this model was almost 93% (Table 5).

However, given that the reproducibility of this Combined Model could not be validated because we did not have any group of mice in which all different sets of variables were investigated, an additional model was built, including all the selected variables in the Immunity and the Redox models. All variables included fulfilled the $P < 0.05$ criteria and the explained variance of this model was almost 89% (Table 6).

In order to ascertain the individual contribution of each variable in the different adjusted models, the standardized beta values were calculated for each variable within each model and shown in Fig. 1. Standardization of the coefficient is usually used to answer the question about which of the independent variables have a greater effect on the dependent variable in a multiple regression analysis, when the variables are measured in different units of measurement, as it is the case of the variables used in this study.

After model construction, Pearson´s correlation coefficients were

**Table 5**
Combined Model including the previously selected variables from the Immunity, Redox and Behavioural models. Predicted remaining life span $= \hat{\beta}_0 + \hat{\beta}_1$ x Internal Locomotion $+ \hat{\beta}_2$ x Macrophage Chemotaxis $+ \hat{\beta}_3$ x GSH concentration $+ \hat{\beta}_4$ x Macrophage Phagocytosis $+ \hat{\beta}_5$ x Time in open arms $+ \hat{\beta}_6$ x MDA concentration.

| | Model 1 |
|---|---|
| Constant ($\hat{\beta}_0$) | 13.454 (9.661) |
| Internal Locomotion ($\hat{\beta}_1$) | 0.228*** (0.044) |
| Macrophage Chemotaxis ($\hat{\beta}_2$) | 0.029** (0.029) |
| GSH concentration ($\hat{\beta}_3$) | 1.304* (0.477) |
| Macrophage Phagocytosis ($\hat{\beta}_4$) | 0.020** (0.006) |
| Time in open arms ($\hat{\beta}_5$) | 0.070* (0.029) |
| MDA concentration ($\hat{\beta}_6$) | −3.008* (1.411) |
| $R^2$ | 93.6% |
| Adjusted $R^2$ | 92.4% |

Each value shows the estimated coefficient and the standard error for each coefficient is shown in brackets.
* P < 0.05.
** P < 0.01.
*** P < 0.001.

**Table 6**
Immunity-Redox Model including the previously selected variables from the Immune and the Redox models. Predicted remaining life span $= \hat{\beta}_0 + \hat{\beta}_1$ x GSH $+ \hat{\beta}_2$ x MDA $+ \hat{\beta}_3$ x Macrophage Phagocytosis $+ \hat{\beta}_4$ x Glutathione Peroxidase $+ \hat{\beta}_5$ x Lymphoproliferation $+ \hat{\beta}_6$ x Macrophage Chemotaxis.

| | Model 1 |
|---|---|
| Constant ($\hat{\beta}_0$) | 37.196 (10.434) |
| GSH ($\hat{\beta}_1$) | 2.006*** (0.518) |
| MDA ($\hat{\beta}_2$) | −5.150** (1.621) |
| Macrophage Phagocytosis ($\hat{\beta}_3$) | 0.020* (0.008) |
| Glutathione Peroxidase ($\hat{\beta}_4$) | 0.013* (0.006) |
| Lymphoproliferation ($\hat{\beta}_5$) | 0.035* (0.016) |
| Macrophage Chemotaxis ($\hat{\beta}_6$) | 0.021* (0.011) |
| $R^2$ | 90.5% |
| Adjusted $R^2$ | 88.7% |

Each value shows the estimated coefficient and the standard error for each coefficient is shown in brackets.
* P < 0.05.
** P < 0.01.
*** P < 0.001.

calculated between observed and predicted life span using the data from the mice that were used for model development (Fig. 2). Pearson´s correlation coefficient for the Immunity Model was 0.883, for the Redox Model was 0.923, for the Behavioural Model was 0.899, for the Combined Model 0.951 and for the Immunity-Redox 0.963 (P = 0.000000 in all cases).

Next step was to validate their predictive power in a different set of ICR/CD-1 female mice. The Immunity, Redox and Immunity-Redox models were validated in one set of 30 mice whereas the Behavioural model was validated in another set of 40 mice (Fig. 3). For the Immunity model, Pearson´s correlation coefficient obtained between predicted and observed life span was 0.849 (P = 0.000000), (n = 30). For the Redox model, Pearson´s correlation coefficient obtained between predicted and observed life span was 0.691 (P = 0.000024), (n = 30). For the Behavioural model Pearson´s correlation coefficient obtained between predicted and observed life span was 0.662 (P = 0.000006), (n = 40) and for the Immunity-Redox model, Pearson´s correlation coefficient between predicted and observed life span was 0.840 (P = 0.000000) (n = 30).

## 4. Discussion

Models that predict individual life span can provide a valuable tool for ageing research and the development of such models represents a platform for understanding the mechanisms that influence longevity. Full-length survivorship experiments require many years to be accomplished and consequently they are a rate-limiting step in the study of mammalian ageing. Therefore, well-developed and validated predictive models can generate preliminary data years in advance, and the output of such models, as an integration of multiple variables that may predict life span individually, can provide a surrogate target for ageing research that is faster to evaluate than life span (Swindell et al., 2008). Thus, instead of waiting 30 months in order to investigate the effect that a given intervention has on the life span of mice, by investigating a few, easy and inexpensive to quantify variables, such as the ones taken into account in this study, longevity can be predicted in less than one month. Moreover, these models can be useful to quantify baseline heterogeneity in studies testing geroprotective interventions. Therefore, the capacity to control for baseline heterogeneity in ageing at the beginning of an intervention would boost power and could shrink minimum sample size in those studies.

In previous studies, different variables have been shown to correlate with longevity such as tight wire clinging ability, open field activity, collagen denaturation rate, hair regrowth, wound healing and blood haemoglobin concentration in certain mouse genotypes (Harrison and Archer, 1988). The most important step when developing predictive models is the variable selection criteria. Chosen variables should be known to be related to the ageing process but also should be implicated in it. Many biomarkers that are moderately correlated with age may be totally irrelevant to ageing. For example, degree of baldness increases with age especially in men, but men who grow bald early do not necessarily show signs of accelerated ageing nor is baldness a risk factor for early death (Hochschild, 1989). In the present study, we decided to focus on immune functionality, inflammatory and redox parameters of immune cells due to several reasons. First, the immune system has been proposed to be a marker and modulator of the rate of ageing (De la Fuente and Miquel, 2009; Martínez de Toda et al., 2016). Therefore, the appearance of age-related changes in those parameters at the adult age was hypothesized as influencing final achieved life span. In addition, the age-related changes regarding functional and redox state of immune cells have been shown to follow the same pattern in both humans and mice (Martínez de Toda et al., 2016, 2019), and therefore, the results would have the potential to be extrapolated to humans. In addition, anxiety-like behaviours were also evaluated at the adult age given that they have been associated with an acceleration of the ageing rate (Perna et al., 2015).

The second most important step when developing a life span predictive model is the election of an appropriate approach to do so. In the present study, the multiple linear regression method was chosen. The advantage of this approach is the strong statistical foundation of least-square regression procedures, which allows for robust statistical inferences when parametric assumptions are satisfied (Swindell et al., 2008). The main disadvantage, however, is that a complex and non-linear relationship may not be adequately captured by linear regression models (Neter et al., 1996). However, for most of the variables investigated in the present study, there was a linear relationship between them and life span allowing us to choose this procedure.

In the Immunity model, the two most important variables selected were macrophage chemotaxis and phagocytosis. The fact that both macrophage functions predict almost 70% of final achieved life span agrees with the hypothesis of phagocytes being the main cell type responsible for the chronic oxidative and inflammatory stress associated with immunosenescence, and therefore, responsible for the ageing rate of a subject, as it has been previously suggested (Vida et al., 2017a, b).

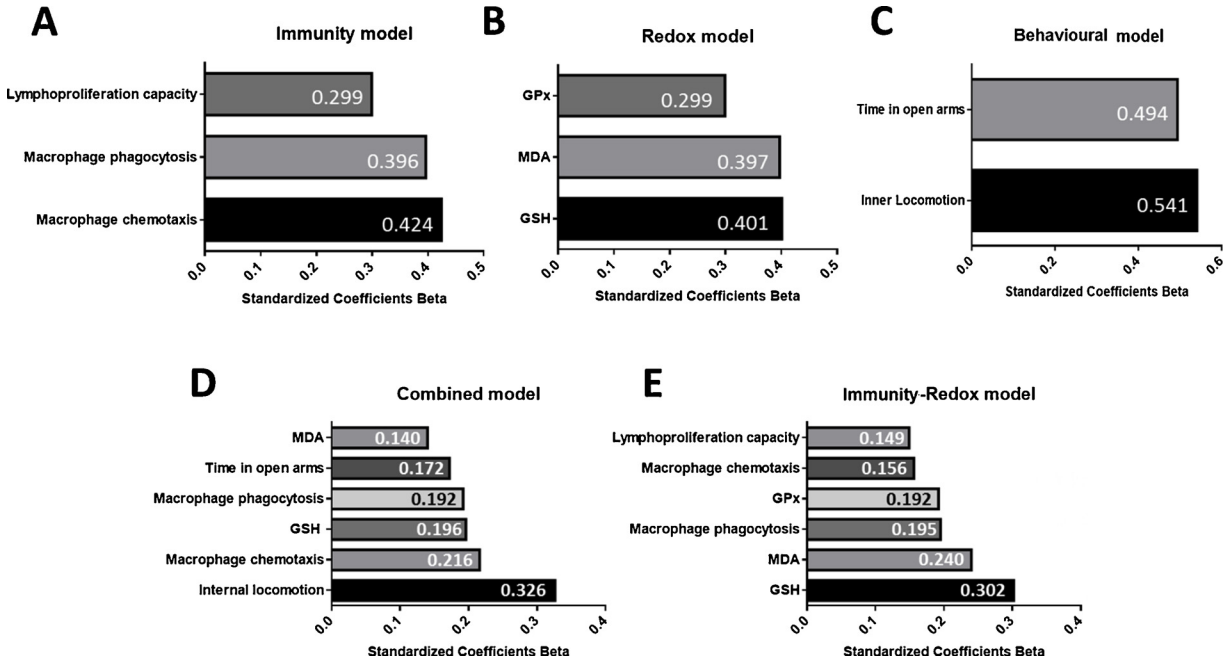


**Fig. 1.** Relative contribution of each variable towards life span prediction in A) Immunity Model; B) Redox Model; C) Behavioural Model; D) Combined Model and E) Immunity-Redox Model. The contribution of each variable is expressed as its standardized beta coefficient.

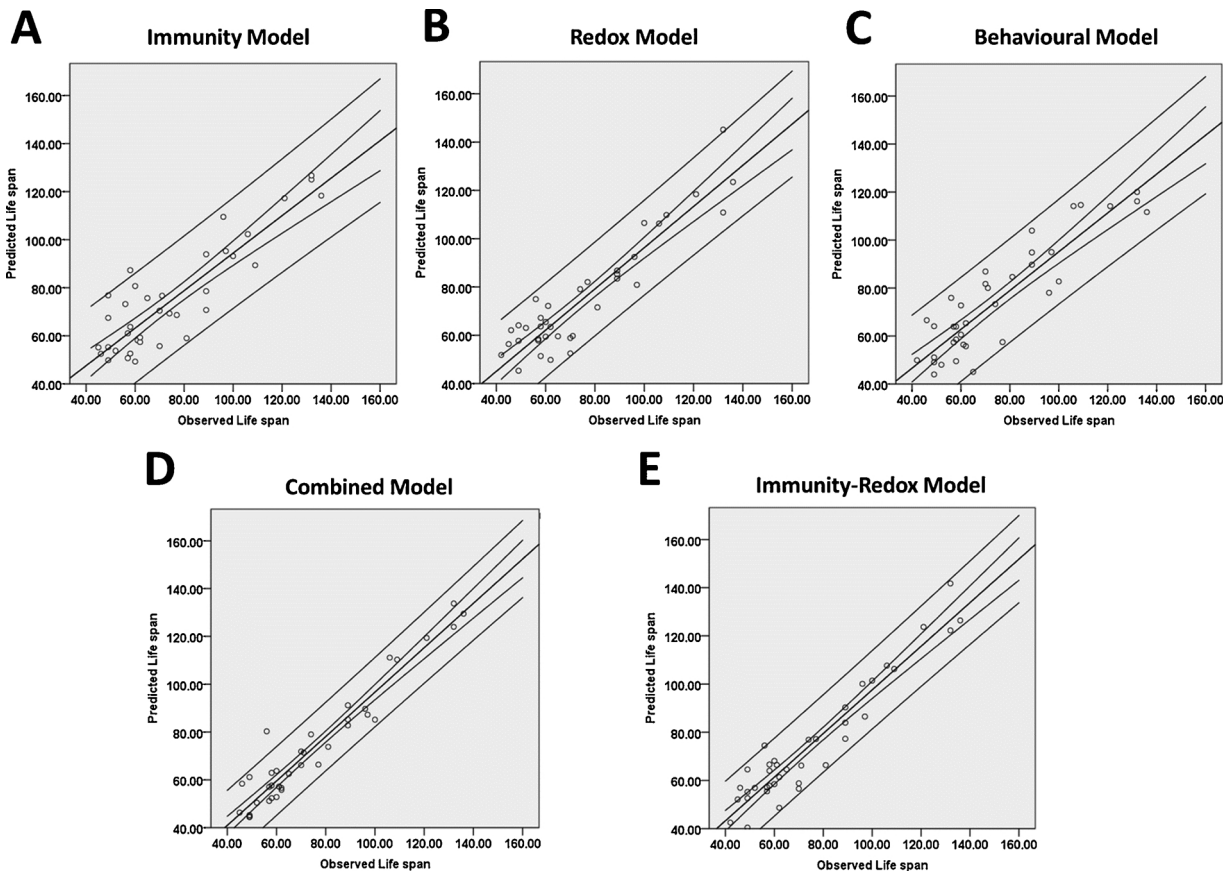


**Fig. 2.** Validation of A) Immunity model, B) Redox model, C) Behavioural model, D) Combined model and E) Immunity-Redox model in the same set of mice used for model construction (N = 38). The narrowest lines indicate the mean confidence interval and the wider lines indicate the individual confidence interval, with a 95% confidence.

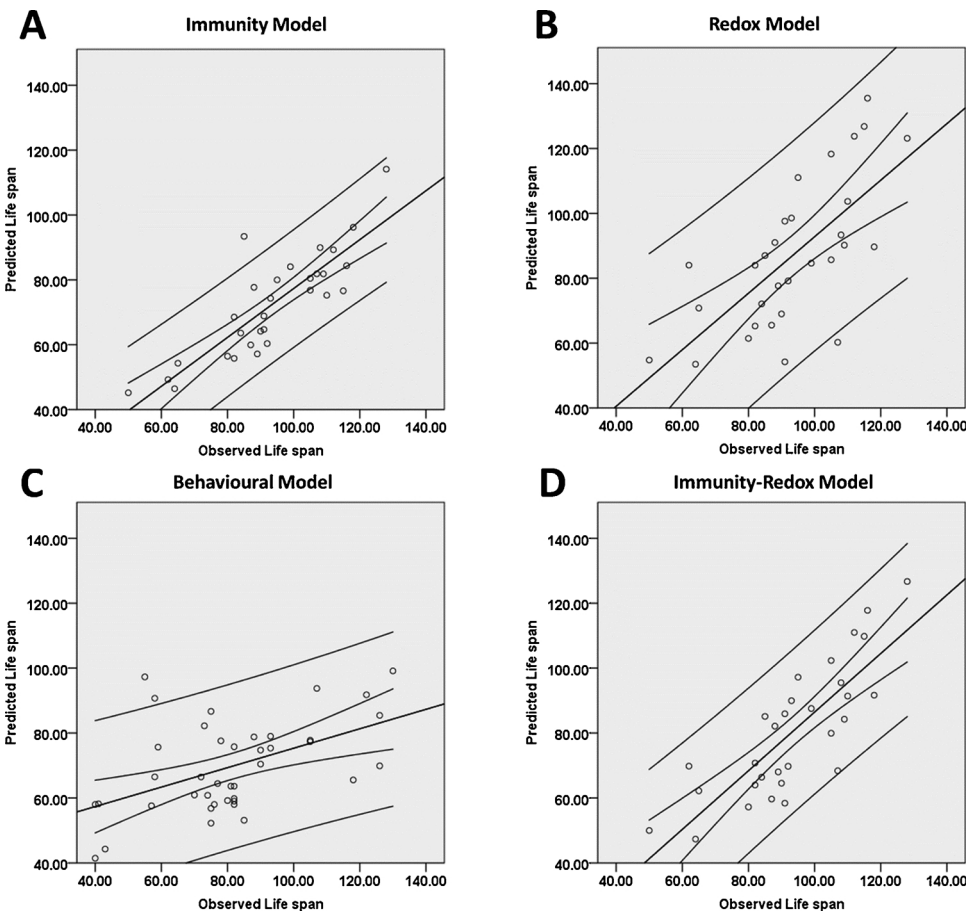


**Fig. 3.** Cross-validation of A) Immunity model, B) Redox model and D) Immunity-Redox model in another set of 30 female mice, and C) Behavioural model in a different set of 40 female mice. The narrowest lines indicate the mean confidence interval and the wider lines indicate the individual confidence interval with a 95% confidence.

Selection of the third variable, the proliferative capacity of lymphocytes, also agrees with previous studies in which this marker has been used to ascertain the Immune Risk Phenotype in humans, which relates to mortality (DelaRosa et al., 2006).

With respect to the Redox model, GSH and MDA concentrations were found to be predictive of almost 80% of the final achieved life span. It is known that immune cells are among the cell types in which an optimal redox balance is most critical for proper functioning, given that they need to constantly produce oxidant and pro-inflammatory compounds in order to exert their defensive function against inner and foreign insults (De la Fuente and Miquel, 2009). Thus, it is understandable to think that the higher the GSH concentration and the higher Glutathione Peroxidase activity in immune cells together with the lower one of MDA, the better the redox balance of these cells, and therefore their function. In fact, GSH has been shown to be essential for proper immune cell functioning given that even a moderate depletion of GSH has been shown to impair several leukocyte functions (Dröge and Breitkreutz, 2000). MDA, which is an end-product of reactive oxygen species (ROS)-induced peroxidation and therefore is used as a marker of oxidative stress, has also been shown to play an active role by inducing the cross-links in proteins and forming irreversible advanced glycated end-products (AGEs) (Esterbauer et al., 1990). These AGEs increase the expression of the membrane anchored receptor for AGEs and activate nuclear factor kappa B (NF-κB) inducing the production of a variety of pro-inflammatory cytokines, including IL-6 and TNF-α (Cai et al., 2018). In addition, glutathione peroxidase activity is known to disarm hydrogen peroxide, limiting its harmful effects and therefore playing a critical role against oxidative stress establishment. However, it has also been demonstrated that this enzyme can inhibit degradation of the inhibitory subunit α of nuclear factor-kappa B (NF-kB) (Li et al., 2000). Thus its activity would maintain NF-kB repressed, also playing an essential role against the establishment of a chronic inflammatory stress.

In the Behavioural model, both the variables that correlated the most with life span, explaining 80% of the variance, were inner locomotion and time spent in open arms. Because mice have a natural aversion to the brightly lit centre of an open field, exploration of the inner space in the hole board test as well as exploration of an open arm in the elevated plus maze test are indicators of an anti-anxiety like behaviour (Crawley, 2000; Kassed and Herkenham, 2004). This draws the conclusion that the less anxiety-like behaviour a mouse shows at the adult age, the longer it lives. These results agree with a previous study in which behavioural indices in old males were used for life span prediction, and the variables that positively correlated the most with remaining life span were distance in open field and number of entries in open arms (Fahlström et al., 2012).

The next step was to develop another model, which would take into account more than one dimension for life span prediction, and as such it was constructed by using the variables selected from the Immunity, Redox and Behavioural models. Thus, the Combined model selected 6 variables: Macrophage phagocytosis and chemotaxis, GSH and MDA concentrations, as well as time spent in open arms and internal locomotion. This model had an adjusted r-squared value of 92.4%.

In addition, we decided to focus on the selected variables from the Immunity and the Redox models to construct the Immunity-Redox model. These variables were prioritized over the Behavioural ones in order to develop a model with the potential to be extrapolated to humans and with an easier application. According to this, it has been previously demonstrated that the age-related changes in immune and redox parameters follow a similar pattern in humans and mice (Martínez de Toda et al., 2016, 2019). Moreover, and despite the fact that anxiety traits have also been linked to an accelerated ageing in

humans (De La Fuente, 2018), the behavioural tests performed in the present study in mice cannot be directly translated to humans. Thus, the Immunity-Redox model including Macrophage Phagocytosis and Chemotaxis, Lymphoproliferation capacity, GSH, MDA and GPx activity was found to explain almost 90% of the total variance.

It is important to highlight that the adjusted r-squared values obtained from the models are high comparing with those previously published. Thus, in a study carried out on 29 months-old C57/BL6 male mice, the best model including haematocrit, haemoglobin levels and collagen denaturation rate, explained about 40% of life span variance (Ingram et al., 1982). In another study, a different set of variables including T-cell subsets, hormone levels, body weight and cataract scores were investigated in female and male genetically heterogeneous mice at different months of age in order to predict life span quartile by using machine learning (Swindell et al., 2008). They found after cross-validation an accuracy of around 30%–35% depending on the algorithm carried out. In a more recent study carried out on 22 months-old C57/BL6 male mice in which exploratory capacity and motor skill indices were used to construct models for life span prediction. It was found that the best single indices could explain 60–65% of the observed variances in life span. Combining two indices generated linear models that could account for 70–80%, and models with three indices up to 92–93% of the variances in remaining life span (Fahlström et al., 2012). However, none of the models was validated using a different group of mice.

Even though the potential value of constructed models that accurately predict life span is substantial and efforts directed towards formulating such models will advance our understanding of the factors regulating longevity, the most important step is to evaluate how accurately mouse life span is predicted when models are applied to a new set of animals, which have not been used for model construction. Accordingly, the reproducibility of the models constructed (Immunity, Redox, Behavioural and Immunity-Redox models) was verified using a different set of mice. This provides an assessment of model reproducibility, which is of great importance for determining the usefulness of models in practical contexts (Duda et al., 2001). Unfortunately, the cross-validation of the Combined model could not be performed given that we do not have a set of mice in which the three different sets of variables were measured. Nevertheless, the other four models were shown to be useful for life span prediction in a different set of mice and the more robust ones were the Immune and the Immunity-Redox models. It is important to highlight that the mice used for the present study are outbred, which means that they are much more heterogeneous within themselves than other strains more commonly used such as C57/BL6. However, as a limitation of the study, these models were constructed and validated using only female mice. Female mice were prioritised over males because of the ease of caging them together. In fact, male mice show aggressive and dominant behaviour when caged together when they are adults and even when they are young. To avoid this, the animals can be isolated, however, it has been shown that social isolation causes alterations in the neuroimmunoendocrine communication (Cruces et al., 2014). Nevertheless, validation of the models in males could be achieved by housing them when they are 3 or 4 weeks of age, especially when they are littermates. Thus, further research is needed to ascertain if these models could also be valid for males or if other parameters could be more robust in males than those depicted here for female life span prediction. Likewise, the models need to be investigated in other strains of mice. In this sense, previous studies from our research group have demonstrated that parameters such as macrophage phagocytosis at the adult age are associated with life span in both ICR-CD1 and Balb/C mice (Guayerbas and De La Fuente, 2012). Therefore, we believe that immune function parameters have the potential to be predictive of life span in other strains. However, with respect to behavioural indices, it has been demonstrated that anxiety-like behaviours can vary between different strains (An et al., 2011). Therefore, and although anxiety-like behaviours have been shown to be predictive of mortality in strains such as C57/BL6 (Fahlström et al., 2012), the constants that affect the variables included in those models, should probably be adjusted in order to be applied to other strains.

Nevertheless, in order to shed light on the causative or consequence role that these variables have on the ageing rate of mice, the investigated parameters should vary in response to a certain intervention, which is known to extend life span. In this context, previous studies from our research group have shown that several interventions (antioxidant supplementation, moderate exercise and social relationships) carried out at the adult age, improve the function, redox state of peritoneal leukocytes and some behavioural indices, and thus increase life span (De la Fuente et al., 2011; De la Fuente, 2014). However, the fact that antioxidant supplementation, which increases GSH concentration in peritoneal leukocytes, also extends life span, does not demonstrate that it is due to a causal process. It could be that antioxidant supplementation triggers other changes, apart from those in GSH concentrations, that account for life span differences. In order to test this, interventions such as genetic manipulation of GSH levels should be performed and its effects on life span variation investigated.

Regardless, the results shown in the present study highlight the importance of an appropriate function and redox state of immune cells as well as a non-anxiety like behaviour for a high longevity, and provide a novel benchmark for future work aimed at prediction of life span.

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## Declaration of Competing Interest

The authors have no conflict of interest to report.

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