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LinAge2: providing actionable insights and benchmarking with epigenetic clocks

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Biological aging is marked by a decline in resilience at the cellular and systemic levels, driving an exponential increase in mortality risk. Here, we evaluate several clinical and epigenetic clocks for their ability to predict mortality, demonstrating that clocks trained on survival and functional aging outperform those trained on chronological age. We present an enhanced clinical clock that predicts mortality more accurately and provides actionable insights for guiding personalized interventions. These findings highlight the potential of mortality-predicting clocks to inform clinical decision-making and promote strategies for healthy longevity.

Biological aging is characterized by the progressive decline in intrinsic biological resilience that is associated with an exponential increase in mortality, expressed in the demographic “Gompertz mortality law”¹. Not all humans age at the same rate since genetics, lifestyle, and stochastic factors significantly affect future mortality and morbidity trajectories. Consequently, individual true biological age (BA) is not identical to calendar or chronological age (CA). The true BA of an individual can be uniquely defined as the age at which subjects of a reference cohort have the same risk of age-dependent disease and all-cause mortality as the subject in question. Tools to accurately track changes in true BA are essential for the development and validation of novel life- and healthspan-optimizing diet, lifestyle, supplement, and drug interventions.

Biological aging “clocks” are computational tools that estimate individual true BA based on demographic, clinical, and/or molecular data. CA itself is widely used for both clinical prognostication and decision-making, and can be viewed as a first-order approximation of true BA. The ideal BA clock should predict individual Gompertz mortality risk with higher accuracy than CA alone. Some aging clocks, including most clinical clocks, explicitly include CA as a covariate, using biological features to estimate a correction factor aimed at providing a better estimate of true BA. CA, in this case, is used as a proxy for effects and mechanisms, such as entropic damage, not captured by the clock itself. Of course, the ideal clock would include all relevant processes, wherein the model would assign zero or negligible weight to CA.

Aging clocks are generalizations of current clinical risk markers that predict disease-specific morbidity and, in some cases, mortality. Aging clocks should similarly enable early detection of hidden or subclinical diseases, surpassing the capabilities of diagnostics by identifying disease processes years or decades before overt disease is present. Secondly, to inform risk-to-benefit estimates (clinical equipoise), aging clocks should capture all-

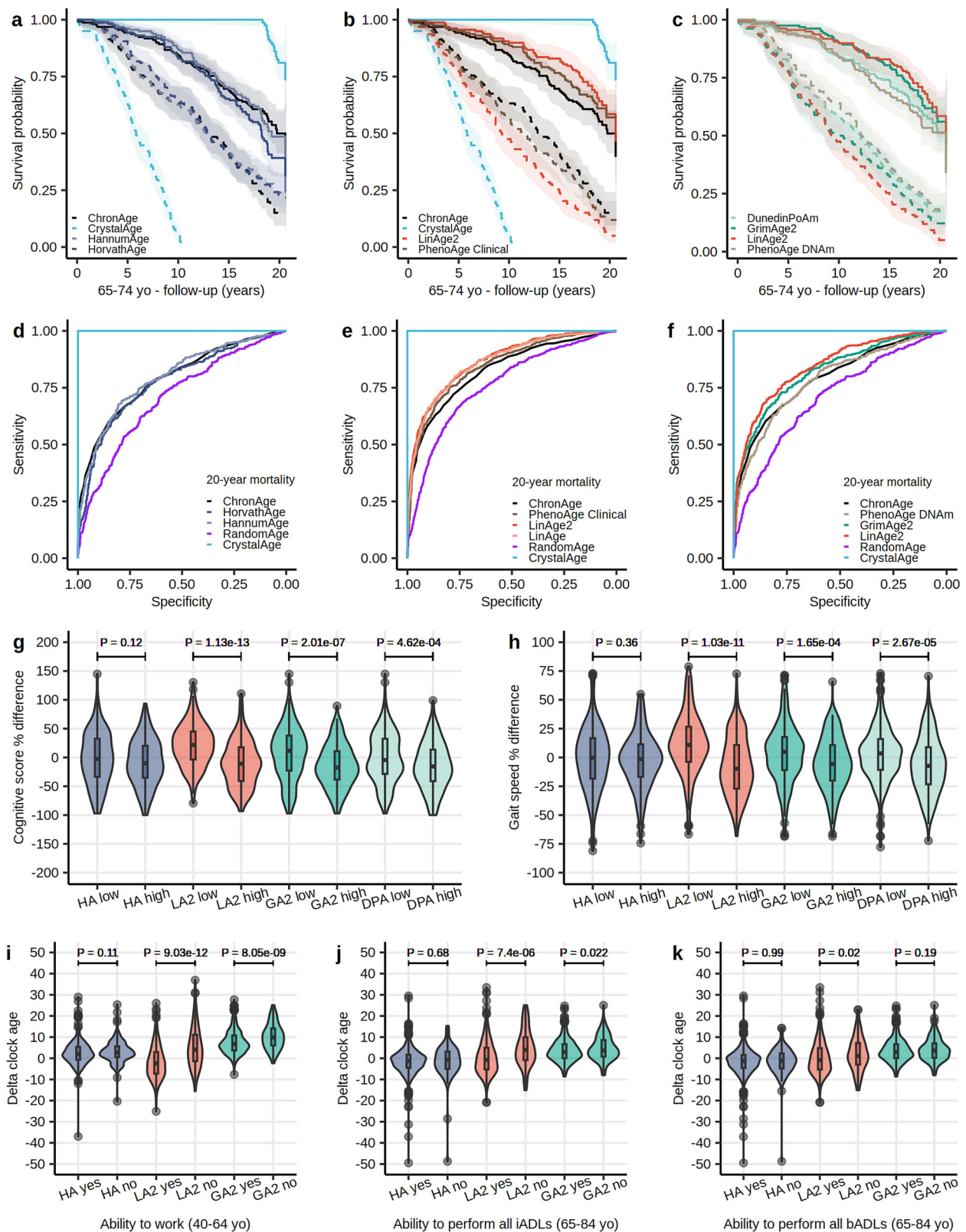
cause mortality holistically, providing value beyond organ or disease-specific risks. Thirdly, aging clocks must be sensitive to individual variations in biological resilience. Finally, aging clocks should provide tools for mechanistic interpretation and provide actionable insights, facilitating targeted interventions. To date, none of the existing clocks meet all these criteria.

To evaluate the performance of aging clocks, we can compare them to a hypothetical “ideal” clock, which we term “CrystalAge”. This optimal clock would predict disease-specific and all-cause mortality at the individual level with near-perfect accuracy, essentially forecasting an individual’s date of death (Fig. 1a and d). While practically impossible, in retrospective studies, we can determine the theoretically optimal performance of CrystalAge and use it as a benchmark to evaluate the performance of existing aging clocks.

Taking inspiration from Levine’s PhenoAge clinical clock², we recently developed and validated clinical aging clocks (PCAge, LinAge) based on linear dimensionality reduction by matrix factorization (singular value decomposition) and demonstrated them to be highly predictive in terms of future disease-specific and all-cause mortality³. These clocks have since been applied in a range of clinical settings and, taking advantage of user feedback, we have implemented several improvements, creating an updated version of these clocks (LinAge2). Like LinAge, we trained LinAge2 in the National Health and Nutrition Examination Survey (NHANES) IV 1999–2000 wave before testing it in the 2001–2002 wave. LinAge2 further reduces the number of parameters (eliminating the need for serum fibrinogen, gamma glutamyl transferase, total cholesterol, high-density lipoprotein, and triglycerides) and emphasizes interpretability.

As expected, LinAge2 was highly correlated with CA (Supplementary Fig. 1a). Clinical clocks can be very sensitive to disruptions of homeostasis caused by disease, leading to excessively high estimates of BA in ill subjects, as was for instance shown by Neytchev et al.⁴ However, in our study, even

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participants with serious health conditions, such as heart failure, recent cancer diagnosis, or those undergoing long-term dialysis, while showing significantly accelerated aging as measured by LinAge2, had BA deltas of at most 35 years with estimated BAs that never exceeded 105 years (Supplementary Fig. 1). For a detailed description of LinAge2's features and construction, refer to Methods.

Many aging clocks have been developed, with epigenetic or DNA methylation (DNAm) clocks most widely recognized and well-established. Several epigenetic clocks have been commercially licensed for applications, including estimating CA (HorvathAge⁷, HannumAge⁶), optimizing life insurance policies (PhenoAge DNAm⁷, GrimAge⁸), and monitoring the rate of aging (DunedinPoAm⁹)¹⁰. Recently, a dataset of pre-calculated epigenetic

Fig. 1 | LinAge2 predicts 20-year all-cause mortality and tracks with healthspan markers. **a–c** Kaplan–Meier survival curves showing 20-year survival in the 65–74 CA bin ($n = 631$). For each clock, subjects were stratified by selecting the lowest (best, solid line) and highest (worst, dotted line) 25% quartiles for BA. Clocks within the same quartile were compared using log-rank tests with Benjamini–Hochberg correction. Areas shaded indicate 95% error bands for lines of the same color. **b** Compared to ChronAge, use of LinAge2 BA results in a significant survival difference for the lowest 25% BA quartile ($P = 6.16E-04$), but not for the highest 25% quartile ($P = 0.07$). PhenoAge Clinical did not significantly outperform ChronAge in predicting survival in this age bin. **c** LinAge2 significantly outperformed DunedinPoAm ($P = 1.09E-02$) and PhenoAge DNAm ($P = 1.37E-03$) in the lowest 25% BA quartile, but not GrimAge2 ($P = 0.22$). In the highest 25% quartile, while LinAge2 significantly outperformed PhenoAge DNAm ($P = 0.03$), the differences between LinAge2 and DunedinPoAm ($P = 0.11$) and GrimAge2 ($P = 0.58$) did not reach statistical significance. **e** ROC analysis revealed that LinAge2 (area under the curve (AUC) = 0.8684) was significantly more informative than PhenoAge Clinical (AUC = 0.8479, $P = 6.35E-05$) and ChronAge (AUC = 0.8288, $P = 3.16E-10$) in predicting future mortality ($n = 2036$). LinAge2 performed similarly to LinAge (AUC = 0.8647). **f** LinAge2 (AUC = 0.8440) also outperformed PhenoAge DNAm (AUC = 0.7859, $P = 4.44E-07$) and GrimAge2 (AUC = 0.8233, $P = 0.016$) in

predicting 20-year mortality ($n = 1,065$). Although GrimAge2 outperformed ChronAge (AUC = 0.7933, $P = 2.74E-03$) in predicting 20-year mortality, PhenoAge DNAm did not ($P = 0.47$). **a, d** HorvathAge, HannumAge, and ChronAge did not significantly differ in predicting mortality risk (AUCs = 0.7776, 0.7978, and 0.7933, respectively, $n = 1065$). ROC curves were compared using DeLong's test.

a, b, d–f CrystalAge, a theoretical perfect clock shown for reference, accurately identifies individuals at risk of dying (AUC = 1), whereas RandomAge adds random Gaussian noise of ± 10 years to CA. **g, h** Violin plots for each clock categorized into low (biologically younger/best 25% quartile) and high (biologically older/worst 25% quartile) groups plotted against cognitive score (digit symbol substitution test) and gait speed. **i–k** Violin plots for each clock categorized by ability to perform (“yes” group) versus inability to perform (“no” group) employment work, all instrumental or all basic activities of daily living (iADLs and bADLs). Delta clock age refers to the age difference between an individual's BA and CA. Groups were compared using two-sided *t*-tests. Median value, lower (25th) and upper (75th) percentiles are indicated. Lines extend to ± 1.5 times interquartile range, with points outside this range drawn individually. The violin shape indicates the probability density function. yo years old, HA HorvathAge, LA2 LinAge2, GA2 GrimAge2, DPA DunedinPoAm.

clock age estimates has been published for the NHANES 1999–2002 waves, permitting direct comparison of the predictive power of CA, the original LinAge, LinAge2, and PhenoAge clinical clocks, and the HorvathAge, HannumAge, PhenoAge DNAm, GrimAge2¹¹, and DunedinPoAm epigenetic clocks.

To compare efficacy in predicting mortality, we performed survival and receiver operating characteristic (ROC) analyses on 20- and 10-year mortality in the NHANES 2001–2002 test cohort. Compared to CA, LinAge2 demonstrated significant survival differences across all age bins, whereas PhenoAge Clinical did not in the 65–74 age bin (Fig. 1b, Supplementary Fig. 2b and e). LinAge2 performed similarly to LinAge and demonstrated better predictive power for future mortality compared to PhenoAge Clinical and CA itself (Fig. 1e and Supplementary Fig. 3b). Surprisingly, LinAge2 also outperformed PhenoAge DNAm and DunedinPoAm in predicting age-specific survival differences (Fig. 1c, Supplementary Fig. 2c and f) and future mortality (Fig. 1f and Supplementary Fig. 3c). In contrast, PhenoAge DNAm, HorvathAge, and HannumAge did not significantly differ from CA in predicting future mortality (Fig. 1a, d and f, Supplementary Fig. 2a and d, and Supplementary Fig. 3a and c). Even though LinAge2 and GrimAge2 performed similarly in predicting future mortality (Fig. 1f and Supplementary Fig. 3c) and survival across all age bins (Fig. 1c, Supplementary Fig. 2c and f), GrimAge2 age deltas were correlated only moderately with some, but not all, of the PCs used by the LinAge2 model (Supplementary Fig. 4 and 5).

While mortality prediction is an important function of aging clocks, it is essential to evaluate if clock ages are similarly predictive of functional status and healthspan. We tested this for the same clinical and epigenetic clocks by comparing markers of functional and health status. Our analysis revealed that lower LinAge2 BAs were associated with superior healthspan markers, including higher cognitive scores, faster gait speed, and vice versa. Subjects who reported that they were able to engage in employment work and those able to perform all instrumental and basic activities of daily living (iADLs and bADLs), on average, had significantly lower LinAge2 BA than those reporting deficits in these domains (Fig. 1g–k). Similar trends were observed for GrimAge2 and DunedinPoAm, with statistically significant differences between the groups with low and high BAs across most healthspan markers, except for the ability to perform all bADLs (Fig. 1g–k and Supplementary Fig. 6). In contrast, no statistically significant differences were found between low and high HorvathAge BAs across healthspan markers (Fig. 1g–k). Our findings on healthspan markers and mortality, for HorvathAge, HannumAge, PhenoAge DNAm, and GrimAge2, corroborate similar findings for 10-year survival in 490 subjects of the Irish Longitudinal Study on Aging¹².

A significant drawback of many existing aging clocks is that they lack interpretability and actionable insights. A key benefit of clinical clocks is that they are built from parameters directly related to the underlying disease mechanisms, enabling easier interpretation of clock residuals and allowing development of tools that can provide more actionable pathophysiological insights. Individual age-associated principal components (PCs) may identify clusters of features that change in a coordinated manner. Analyzing individual PCs can provide insights into the underlying aging trajectories. Using heatmaps, we visualized the association of individual PCs relative to clinical outcomes, including sex-specific causes of death and chronic diseases (Fig. 2).

To facilitate interpretation of individual PCs, we provide an R script (see Code availability) that enables the calculation of LinAge2 BA and visualization of the relevant PC values based on user-supplied data (Fig. 3). Supplementary Table 5 offers more detailed information on each PC, including associations with causes of death, chronic diseases, sociological factors, and potential aging mechanisms, along with some potential intervention strategies, based on current best medical practice, that may impact individual PCs to lower LinAge2 BA. This integrated approach aims to empower clinicians and patients to explore factors affecting LinAge2 and to target interventions.

Accurately predicting patient outcomes and allocating healthcare resources is a significant challenge in clinical practice¹³. Currently, clinicians rely heavily on CA to make these decisions. However, here we show that mortality-predicting clocks, such as LinAge2 and GrimAge2, outperform CA in predicting mortality risk across timeframes, ranging from 2 to 20 years (Fig. 1, Supplementary Fig. 7). Moreover, clinical clocks can also predict specific causes of death within a 5-year window (Fig. 2c and d). This illustrates that clock-based BAs are more accurate and informative estimates of true BA than CA itself. By providing a more precise metric of biological status than CA alone, BA can enable clinicians to better support patients and their caregivers in navigating healthcare choices, including end-of-life care.

Overall, our analysis reveals that aging clocks trained to predict mortality or functional aging outcomes provide more predictive value in terms of clinical decision-making. Surprisingly, clinical aging clocks still outperform several prominent mortality-predicting and functional epigenetic clocks, including PhenoAge DNAm and DunedinPoAm, in predicting future mortality. A key advantage of LinAge2 lies in its interpretability. Because principal component analysis is a linear matrix factorization technique, the resulting model is easier to interpret than nonlinear alternatives¹⁴. Latent variables based on linear dimensionality reduction (PCs), especially those based on clinical parameters, are comparatively easy to understand and interpret, making them potentially more actionable. This may enable clinical aging clocks like LinAge2 to detect hidden or subclinical

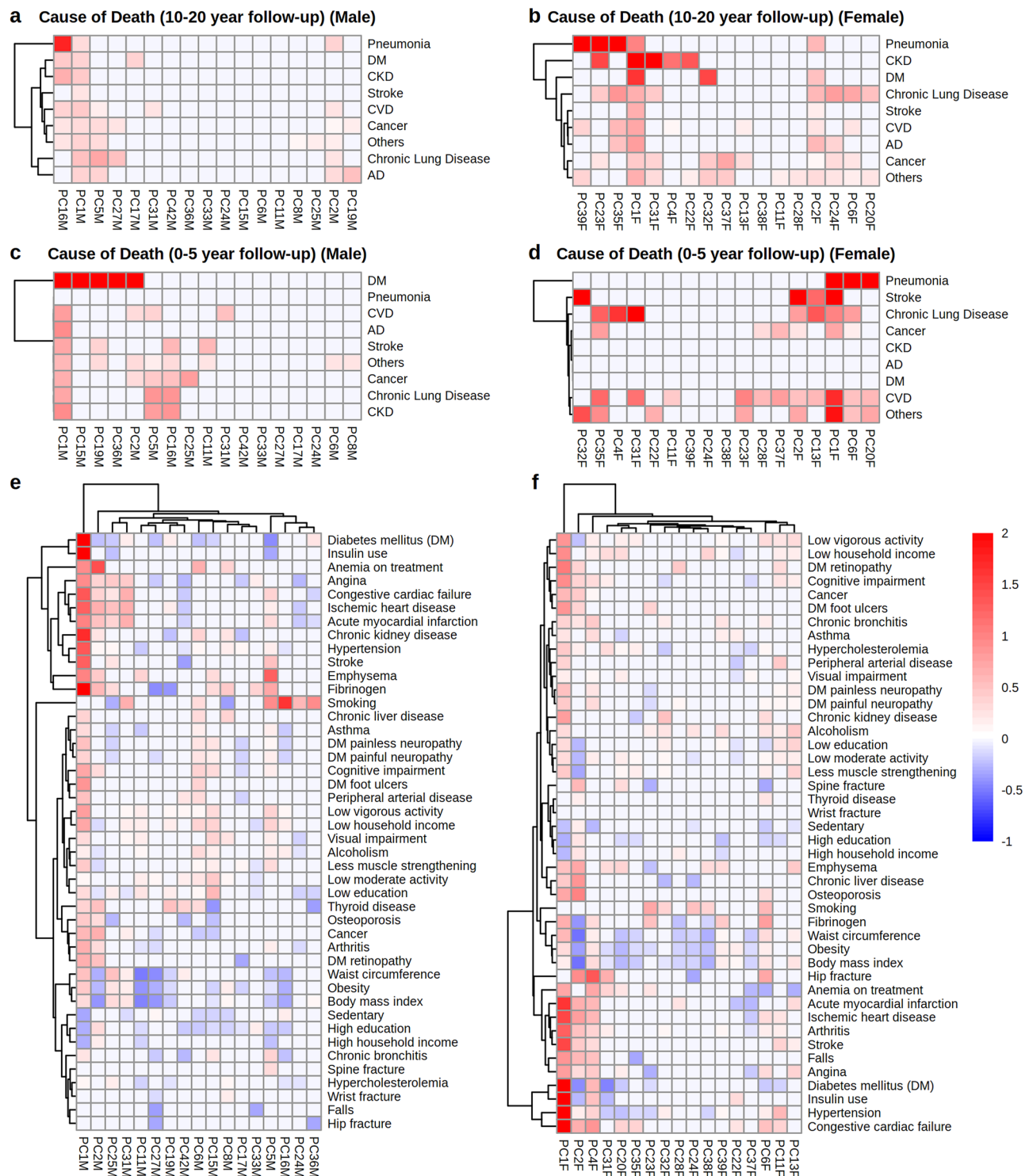


Fig. 2 | Heatmaps illustrating the associations between clinical outcomes and PCs analyzed using multivariate logistic regression. Associations of PCs with specific causes of death at (a, b), 10–20 year and (c, d), 0–5 year follow-up for male and females, respectively. Strength of association (log risk ratio) is represented using a red color scale. PCs that are strongly positively associated with a specific cause of death are bright red. For cause of death, negative associations were truncated by

setting their values to zero (i.e., no harm). e, f Association of PCs with specific chronic diseases and measures of sociological factors. Strength of association is represented using a blue-red scale ranging from –1 (blue, negative association) to 2 (red, positive association). PCs that are strongly positively associated (positive log risk ratios) with a specific disease are bright red, whereas PCs that are strongly negatively associated (negative log risk ratios) with a specific disease are bright blue.

diseases and inform primordial prevention strategies. By identifying individuals at high risk of developing specific diseases, healthcare providers can implement targeted interventions early and proactively. By casting specific risk in terms of BA acceleration, aging clocks can significantly increase compliance and adherence with specific health recommendations¹⁵.

All current aging clocks, regardless of feature space (e.g., clinical, methylation, proteomics, etc.) and target (mortality, functional outcomes, disease, or CA) share significant limitations. Most importantly, many current clocks employ linear techniques (e.g., principal component analysis/singular value decomposition, regression-based predictions), which limit

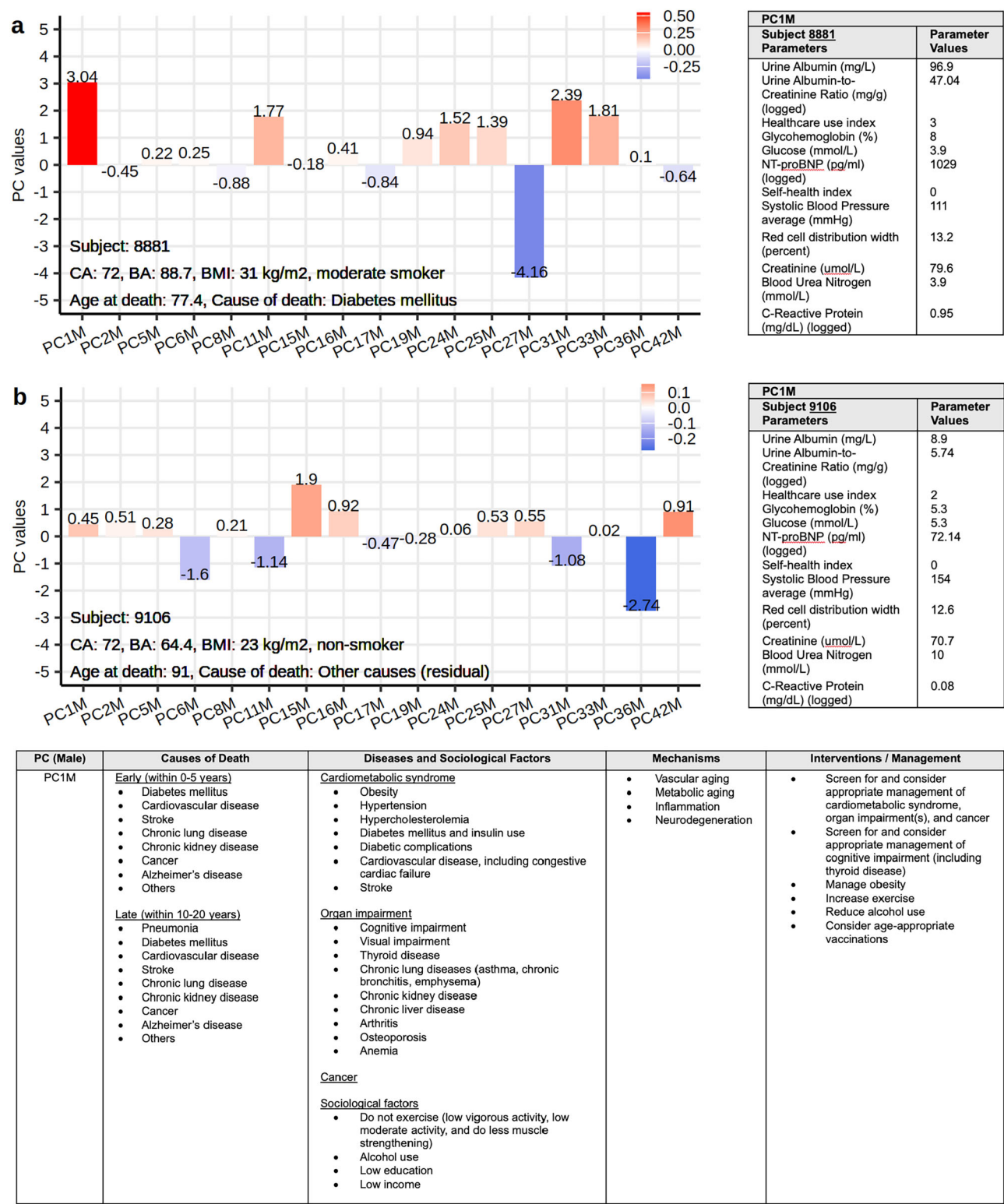


Fig. 3 | Interpretation of LinAge2 PCs for personalized interventions. We present two case studies from the NHANES dataset, both featuring chronologically 72-year-old males. **a, b** Individual PC values (covariates) of the Cox model are shown as bars and attached values. Each bar is colored by the PC value multiplied by the weight of the Cox model (see Supplementary Table 4). Blue bars indicate PCs that contribute negative age deltas (younger), while red bars are positive age deltas (older). Tables adjacent to bar graphs display selected clinical parameters, with loadings ≥ 0.1 in PC1M (see Supplementary Table 3), for each subject. The bottom table, extracted from Supplementary Table 5, facilitates interpretation of high PC1M values, including associations with causes of death, chronic diseases, sociological factors, potential aging mechanisms, and candidate interventions. **a** Subject 8881 is an obese

smoker, who at the time of survey in 2000, is biologically more than two mortality rate doubling times (16 years) older than his CA. This age delta is largely driven by PC1M (associated with cardiometabolic syndrome) and PC31M (associated with smoking). This subject died from diabetes mellitus 5.4 years after the samples were taken. Personalized targeted interventions, including both non-pharmacological and pharmacological interventions, for example, treatment with a glucagon-like peptide 1 agonist, for weight loss, as well as smoking cessation, would be expected to lower LinAge2 contributions driven by PC1M and PC31M. **b** Subject 9106 is a non-smoker with an ideal body mass index (BMI), unremarkable PC1M, and LinAge2 BA 7.6 years lower than his CA. This subject died 19 years later at an age of 91 years. NT-proBNP N-terminal pro-brain natriuretic peptide.

their ability to distinguish between aging signatures and those of age-dependent diseases, and to learn U-shape response patterns. Current clocks, therefore, inherently conflate intrinsic biological aging with disease-specific signatures (hidden sickness, primordial disease signatures, and disease risk factors). Nonlinear approaches, including generative artificial intelligence and artificial neural networks, are being investigated and could offer improved models, but their increased complexity pose a significant challenge for interpretation^{16,17}. Next-generation clocks will need to differentiate between disease signatures and intrinsic aging and quantify intrinsic biological resilience. Further theoretical work will be required to deconvolute these disease-centric signatures from determinants of intrinsic resilience and entropic aging^{18–20}. Data availability remains a major bottleneck, with some of the most promising methods requiring long mortality follow-up, large sample numbers and, ideally, longitudinal data. Advancing next-generation clocks is crucial to equip healthcare providers with the essential tools needed to make informed decisions regarding targeted interventions that support healthy longevity in populations where healthcare needs are increasingly dominated by aging.

Methods

Motivation for enhancing LinAge2

The original PCAge and LinAge³ both utilized some parameters that are not routinely collected. LinAge has been utilized by several clinics worldwide, and we have received informal feedback regarding its use. Common suggestions for enhancing the clock include: (i) improving handling of outliers and threshold effects, (ii) further refining the clinical parameters, especially removing serum fibrinogen due to the need for a specialized sodium citrate tube, (iii) providing additional tools to improve the interpretability of PCs, and (iv) providing specific strategies to optimize each PC to lower BA. PCs can also be sensitive to batch effects. We developed LinAge2 in response to these concerns.

We followed the same workflow, as previously described³, to construct LinAge2 but with several modifications. To enhance LinAge2, we refined the clinical parameters by reducing the total number to 60 (Supplementary Table 2). We also addressed outliers and thresholding by capping outliers at six standard deviations and log-transforming additional parameters (Supplementary Table 2). Batch effects were mitigated through z-score normalization by median and median absolute deviation to a younger, generally healthy cohort (age 40–50 years), separately for males and females (Supplementary Table 2), generating sex-specific PCs. The loadings for male and female PCs are provided in Supplementary Table 3, and sex-specific weights of the Cox proportional hazards models are listed in Supplementary Table 4. The null model fits a Cox proportional hazards model using only CA as a covariate. The resulting mortality rate doubling time of approximately 7.8 years is in good agreement with the literature value of approximately 8 years²¹.

A parametrized version of LinAge2 is provided as previously described³ (Supplementary Table 2). The baseline characteristics of the study participants are listed in Supplementary Table 1.

PhenoAge Clinical and epigenetic clocks

PhenoAge Clinical was implemented using the equation from the original publication. The dataset of pre-calculated epigenetic clock ages published for the NHANES 1999–2002 waves was obtained from <https://www.cdc.gov/nchs/nhanes/dnam/> and analyzed.

Construction of healthspan markers

The digit symbol substitution test score (NHANES variable ‘CFDRIGHT’) was used as a cognitive measure. Gait speeds were obtained by taking the total distance walked (20 feet or 6.096 meters) divided by the time taken (NHANES variable ‘MSXWTIME’). Differences in cognitive scores and gait speeds were calculated as the percent difference from a control group (middle 50% of all subjects), for younger (best 25% quartile) and older (worst 25% quartile) groups. The ability to work was established using the NHANES variable ‘PFQ048’. The ability to perform all instrumental

activities of daily living (iADLs) was a combination of the NHANES variables ‘PFQ060A’, ‘PFQ060F’, ‘PFQ060G’, ‘PFQ060Q’, ‘PFQ060R’ and ‘PFQ060S’, while the ability to perform all basic activities of daily living (bADLs) was a combination of the NHANES variables ‘PFQ060B’, ‘PFQ060C’, ‘PFQ060H’, ‘PFQ060I’, ‘PFQ060J’, ‘PFQ060K’ and ‘PFQ060L’. Participants had to have either no difficulty or some difficulty in all the variables to be deemed able to perform all iADLs or all bADLs.

Heatmap analysis

Using the ‘nnet’²² (version 7.3-19) R package, heatmaps were generated to evaluate the predictive values for PCs included in LinAge2. For each parameter, we attempted to predict status (diseased/compromised or not) using multivariate logistic regression with the clock PCs as covariates. PCs that received a statistically insignificant ($P \geq 0.05$) weight in the logistic regression model were assigned zero weights (white). The remaining PCs ($P < 0.05$) were assigned color values according to their weight in the model (see Fig. 2f legend for color mapping).

Statistics and reproducibility

For the NHANES IV 1999–2002 waves, we excluded participants top-coded at age 85 years, as we could not ascertain the exact CAs of these adults, and participants who died from accidental deaths, as these were deemed to be not age-related.

Survival analyses were performed using log-rank tests with Benjamini-Hochberg correction. Receiver operating characteristic curves were compared using DeLong’s test. For healthspan markers, two-sided t-tests were used to compare between the low and high clock groups. For the analysis of diseased subjects, Wilcoxon signed-ranked tests were used to compare between groups. Correlation analyses were performed using linear regression, and the strength of correlation was determined using Pearson correlation coefficient. All statistical analyses were performed using R version 4.2.0 (<https://www.R-project.org/>).

Data availability

All datasets used are publicly available online at <https://www.cdc.gov/nchs/nhanes/Default.aspx>. There were no restrictions on data availability. This study was reported according to STROBE guidelines for cohort studies.

Code availability

Code to generate individual LinAge2 BA and visualize the corresponding PCs is available as a zipped R archive. The archive should be uncompressed and the R script (linAge2.R) executed in the same folder using R version 4.2.0. User input is provided by editing the input file (userData.csv), replacing the two NHANES example subjects (8881 and 9106, see Fig. 3) with the appropriate user data in NHANES units (see Supplementary Table 2). Sequence numbers (SEQN) can be chosen freely but should be numeric. The linAge2.R script provides log output to the R console and returns LinAge2 BA and individual PC values for each subject provided in the userData.csv file. Results are appended to the original input data and saved as userData_out.csv.

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Author contributions

S.F., B.K.K., and J.G. conceived, conceptualized, and designed the study. S.F., K.A.D., A.A.N., and J.G. analyzed and interpreted the data. S.F., B.K.K., and J.G. wrote the first draft of the paper. S.F. and J.G. wrote the revised manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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