Biological Age in Healthy Elderly Predicts Dementia in the Absence of a Brain Marker

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Abstract

Application of biological age as a measure of an individual´s health status offers new perspectives into extension of both lifespan and healthspan. While algorithms predicting mortality and most aging-related morbidities have been reported, the major shortcoming has been an inability to predict dementia. We present a community-based cohort study of 1930 participants with a mean age of 72 years and a follow-up period of over 7 years, using two variants of a phenotypic blood-based algorithm that either excludes (BioAge1) or includes (BioAge2) neurofilament light chain (NfL) as a neurodegenerative marker. BioAge1 and BioAge2 predict dementia equally well, as well as lifespan and healthspan. Each one-year increase in BioAge1/2 was associated with 11% elevated risk (HR=1.11; 95%CI 1.08-1.14) of mortality and 7% elevated risk (HR=1.07; 95%CI 1.05-1.09) of first morbidities. We additionally tested the association of microRNAs with age and identified 263 microRNAs significantly associated with biological and chronological age alike. Top differentially expressed microRNAs based on biological age had a higher significance level than those based on chronological age, suggesting that biological age captures aspects of aging signals at the epigenetic level. We conclude that biological age is a predictor of major age-related diseases, including dementia, among healthy elderly.

Introduction

Prevention of aging-related diseases (ARDs) is paramount in the current era of population aging. Delaying the aging process can potentially extend healthspan and reduce ARDs burden. It has long been observed that the pace of aging varies from person to person. This highlights a key concept that due to underlying biological mechanisms, biological age at an individual level can be separated from chronological age.[1−3] Biological age, defined by clinical and molecular biomarkers, indeed predicts overall mortality and ARDs, sometimes even better than chronological age.[4] Similarly, modifiable risk factors predict mortality reasonably well over time, even though independent genetic factors predict mortality only modestly.[5] Investigating biological age can help identify individuals at higher risk of disease and death, before clinical manifestation of disease. Biomarkers of biological age have great potential in evaluating healthy-aging intervention programs, selecting suitable candidates for clinical trials, as well as indicating levels of personal health, and predicting risk of aging-related diseases. These applications may provide insight on how to extend both lifespan and healthspan and cope with the burden of ARDs worldwide.

The concept of biological age has been constructed and validated in large human cohort studies for a panel of physiological biomarkers,[6−8] differentially methylated sites in DNA (DNAm age),[9,10] circulatory metabolites (metabo-age), the levels of messenger-RNAs [11] and microRNAs (miRNAs) in both whole blood[12] and plasma samples (Wu J.W. et al., submitted). Physiological biomarkers tend to be stronger predictors of mortality and aging-related morbidity outcomes than molecular biological age measures,[4] indicating the complexity of human aging processes. Most of these measures rely on the association between composite biomarkers and chronological age and might be imperfect because of the practice of minimizing the deviation from chronological age through regression and not estimating this entity of interest empirically.[4] The main knowledge gap is to what extent the deviation of biological age measure from chronological age predicts risks of mortality and major ARDs onsets, especially dementia, in advanced age populations. We sought to ask the following question: is it possible to build a physiological marker-based biological age algorithm in the absence of a brain marker that predicts elevated risk of dementia for a given chronological age?

In a previous study[13], we have validated the approach of Klemera and Doubal[14] and showed that over a median follow-up of 11 years, biological age at baseline was superior to chronological age and traditional biomarkers, in predicting mortality, morbidity and onset of specific diseases such as stroke and cancer. Furthermore, compared to chronological age alone or combined with the individual biomarkers, adding a brain biomarker for neurological degeneration (plasma NfL, total-tau, amyloid beta-40 and – 42) further improved the association of biological age with dementia, including Alzheimer disease (AD). On the other hand, the Klemera and Doubal-based biological age did not predict mortality or morbidity when adjusting for chronological age, and had stronger associations with chronological age than with risk of dementia. It became evident

that a completely new model for biological age, beyond chronological age, was needed to improve both the association with prediction of mortality and dementia, including AD.

In this study, we aimed to improve biological age algorithms through a population-risk-based framework. Recent work by Levine et al.,[4] has introduced a novel measure of 'phenotypic age', developed and cross-validated on NHANES III (n = 9,926). A Gompertz proportional hazards regression[14] was applied to account for the hazard of mortality when selecting clinical biomarkers in the training dataset. This approach has the advantage of capturing the incremental risk of death and morbidities due to accelerated biological aging, whereas traditional biological age algorithms largely aimed to model chronological age. However, Levine's "phenotypic age" algorithm was unable to establish a significant link between accelerated aging and elevated risk for AD. We hypothesized that this caveat was not due to the population-risk-framework itself, but because the "phenotypic age" was trained in the NHANES data, which has a relatively younger age distribution, than is common for neurological outcomes. Furthermore, we investigated whether by including markers of neurodegeneration, we could improve the prediction of neurological outcomes in advanced-aged cohorts.

Our approach to improve the biological age models comprised of two steps. First, we validated Levine's "phenotypic age" algorithm in the Rotterdam Study (n = 1,930). Second, we developed and cross-validated new biological age algorithms in the Rotterdam Study using the same Gompertz proportional hazards regression framework plus neurodegenerative markers (NfL, total-tau, amyloid beta – 40 and – 42). MicroRNAs (miRNAs) age signature was found to be not only predictive of the actual age, but useful as a biomarker of all-cause mortality in both whole blood[12] and plasma samples (Wu J.W. et al., submitted). We assessed the deviation of biological age from chronological age through the plasma miRNA expression signature.

Results

Development of BioAge1 and BioAge2 algorithms

This study included a subset of participants from the Rotterdam Study, a prospective community-based cohort study (see Figure S1). In total 1,930 participants from RS-I and RS-II were followed-up between 2002–2012, with a mean follow-up time of 7.2 (SD = 1.8) years, with ascertainment of 411 all-cause mortalities. The mean age for this study sample was 71.6 years (SD = 7.6), representing an advanced age population. We fitted Gompertz proportional hazard models on a training subset (n = 1158), and developed two new biological age algorithms: one including the same set of ten predictors as in the "phenotypic age" algorithm (BioAge1, summarized in Table 1) and the other including brain marker NfL in addition to the set of ten predictors (BioAge2, summarized in Table 2). We also explored biological age algorithms including either total-tau, amyloid beta – 40, or -42, but none of these brain markers were significant.

Table 1
Gompertz coefficients developed in the Rotterdam study for BioAge1:
without brain markers

Variable	Marker of	Units	Weight
Albumin	Liver	g/L	-0.043
Creatinine	Kidney	umol/L	0.013
Glucose, serum	Metabolic	mmol/L	0.153
C-reactive protein (log)	Inflammation	mg/dL	0.015
Lymphocyte percent	Immune	%	-0.017
Mean (red) cell volume	Immune	fL	0.035
Red cell distribution width	Immune	%	0.083
Alkaline phosphatase	Liver	U/L	0.008
White Blood Cell count	Immune	1000 cells/uL	0.072
Age		Years	0.089

Table 2
Gompertz coefficients developed in the Rotterdam study for BioAge2: with
brain marker nFL.

Variable	Marker of	Units	Weight
Albumin	Liver	g/L	-0.044
Creatinine	Kidney	umol/L	0.009
Glucose, serum	Metabolic	mmol/L	0.150
C-reactive protein (log)	Inflammation	mg/dL	0.006
Lymphocyte percent	Immune	%	-0.017
Mean (red) cell volume	Immune	fL	0.034
Red cell distribution width	Immune	%	0.059
Alkaline phosphatase	Liver	U/L	0.007
White Blood Cell count	Immune	1000 cells/uL	0.074
Neurofilament level (log)	Brain	pg/ml	0.529
Age		Years	0.071

BioAge1 and BioAge2 predict risk of all-cause mortality

In the validation subset (n = 720) of the Rotterdam study data, we cross-validated the two new biological age measures: BioAge1 and BioAge2. BioAge1 was highly correlated with chronological age (r = 0.85) and PhenoAge (r = 0.96). Table 3 shows the association between BioAge1 and risk of all-cause mortality, based on Cox proportional hazard models in this validation subset. Each one-year increase in BioAge1 was associated with 11% (HR = 1.11; 95%CI 1.08−1.14; p < 0.0001) elevated risk of mortality, after adjusting for chronological age, *APOE* status and gender. When restricting the outcome within the first 5-year and 3-year time intervals, each one-year increase in BioAge1 was associated with 15% and 16% elevated risks of mortality respectively, after adjusting for chronological age, *APOE* status and gender. Similarly, BioAge2 was highly correlated with chronological age (r = 0.85) and PhenoAge (r = 0.95). Table 4 shows the association between BioAge2 and

risk of all-cause mortality, based on Cox proportional hazard models in this validation subset. Each one-year increase in BioAge2 was associated with 11% (HR = 1.11; 95%CI 1.08–1.14; p < 0.0001) elevated risk of mortality, after adjusting for chronological age, *APOE* status and gender. When restricting the outcome within the first 5-year and 3-year time intervals, each one-year increase in BioAge2 was associated with 14% and 16% elevated risks of mortality respectively, after adjusting for chronological age, *APOE* status and gender.

Table 3
Association of BioAge1, 3-Year, 5-Year and total all-cause Mortality: The Rotterdam Study, 2002–2012.

	3-year Mortality				5-year Mortality				Total Mortality			
	Univariate		Final Model		Univariate		Final Model		Univariate		Final Model	
Variables	HR	P	HR	P	HR	P	HR	P	HR	P	HR	P
Age	1.13	< .0001	0.95	0.156	1.14	< .0001	0.99	0.555	1.13	< 0.0001	1.02	0.368
Female	0.49	0.020	0.89	0.746	0.59	0.017	1.21	0.455	0.64	0.004	1.10	0.594
APO E	0.83	0.577	0.75	0.398	0.85	0.522	0.81	0.392	1.03	0.846	0.99	0.968
BioAge1	1.13	< 0.0001	**1.16**	< 0.0001	1.14	< 0.0001	**1.15**	< 0.0001	1.12	< 0.0001	**1.11**	< 0.0001

Table 4
Association of BioAge2, 3-Year, 5-Year and total all-cause Mortality: The Rotterdam Study, 2002–2012.

	3-year Mortality				5-year Mortality				Total Mortality			
	Univariate		Final Model		Univariate		Final Model		Univariate		Final Model	
Variables	HR	P	HR	P	HR	P	HR	P	HR	P	HR	P
Age	1.13	< .0001	0.957	0.193	1.14	< .0001	0.99	0.816	1.13	< 0.0001	1.02	0.198
Female	0.49	0.020	0.854	0.656	0.59	0.017	1.13	0.630	0.64	0.004	1.04	0.838
APO E	0.83	0.577	0.728	0.357	0.85	0.522	0.78	0.324	1.03	0.846	0.97	0.840
BioAge2	1.13	< 0.0001	**1.16**	< 0.0001	1.14	< 0.0001	**1.14**	< .0001	1.12	< 0.0001	**1.11**	< 0.0001

BioAge1 and BioAge2 predict risks of aging-related morbidities including dementia

We explored whether BioAge1 and BioAge2 predict additional risks of aging-related morbidities/ARDs, for people with fixed chronological age. Figure 1 shows the association between BioAge1 and risk of aging outcomes (BioAge2 yielded similar results; see **Figure S2**). Each additional year increase in BioAge1 was associated with 7% elevated risk of first morbidities, after adjusting for chronological age, *APOE* status and gender. Note that each one-year increase in BioAge1 and BioAge2 was associated with 3% (HR = 1.03; 95%CI 1.00-1.06; p = 0.08) and 5% (HR = 1.05; 95%CI 1.02–1.08; p = 0.001) elevated risk of dementia, respectively, after adjusting for chronological age, *APOE* status and gender. PhenoAge was associated with 2% elevated risk of dementia (HR = 1.02; 95%CI 0.99–1.04; p = 0.24) for the same adjustment with lower significant level.

Plasma-based microRNA aging signature: biological age vs. chronological age

DNA methylation-based biological age (DNAm age) has been widely recognized as an epigenetic clock. In addition to DNA methylation and histone modifications, miRNAs have been classified as important epigenetic markers. Using the more robust quantification of RNA-seq we determined expression levels of 2083 plasma miRNAs. Of these, 591 miRNAs remained as well-expressed after normalization. We identified 291 miRNAs that were differentially expressed in relation to chronological age, at the Bonferroni-corrected $p < 8.5 \times 10^{-5}$ (0.05/591 well-expressed miRNAs), **Table S1** summarizes the top 20 miRNAs among them. Similarly, by using biological age, specifically BioAge1 and PhenoAge, we also identified 296 and 291 miRNAs respectively that were differentially expressed. **Tables S2** and **S3** summarize the top 20 miRNAs in relation to BioAge1 and PhenoAge. These three sets of significant miRNAs were highly overlapping (Fig. 2), with 263 miRNAs presented in all three sets, indicating the similarity of the miRNA aging signatures in relation to biological age vs. chronological age. In comparison to models including just chronological age, the top differentially expressed miRNAs had much higher significance levels (Fig. 3) in biological age models. This suggests that biological age models (or algorithms) are capable of condensing aging signals and predicting mortality for people of a fixed chronological age.

Discussion

We explored the application of Gompertz proportional hazard regression[15], as a platform integrating multiple biological system data accounting for every person's observed mortality risk. We developed and cross-validated two new biological age algorithms in the advanced age Rotterdam Study (n = 1930). We have shown that increase of biological age, for a given chronological age, gender and *APOE* status combination, predicts elevated risks of all-cause mortality as well as major ARDs such as coronary heart disease, diabetes, cancer, stroke, COPD and dementia. To investigate the deviation of biological age from chronological age, we compared the miRNA expression signatures based on biological age and chronological age.

Of the various methodologies deployed for biological age development, most were based on the association between composite biomarkers and chronological age, which did not account for the deviation from chronological age, the exact entity that biological age was expected to capture. In 2018, Levine et al proposed a revolutionary approach of incorporating observed mortality risk using large cohort data. In their model, the biological age reflected the putative age in the general population that corresponds to the risk of mortality.[4] Using this innovative modeling, the authors developed and validated a phenotypic age construct in NHANES cohorts to predict all-cause mortality and all major aging-related morbidities, with the exception of cerebrovascular disease and dementia. Here we confirmed our hypothesis that the caveat was not due to the population-risk-approach itself, but because the "phenotypic age" was trained in the NHANES data with a relative younger age structure than common for neurological outcomes. Biological age algorithms trained in advanced age cohorts indeed predict the elevated risk for neurological disorders, associated with accelerated aging.

We investigated whether we could improve the prediction of the biological age model, particularly for dementia and stroke, by including markers of neurodegeneration. We have incorporated NfL, the only significant neurodegenerative marker in our study, into our novel BioAge2 algorithm. Previous studies[16–18] as well our own analysis of a longitudinal study[19] strongly indicate that NfL, a relatively unspecific marker of neuroaxonal damage, was the best predictor of neurological outcomes. Our findings show that incorporating NfL into the biological age algorithm further improved the prediction of the elevated risk for dementia, although the added value was not impressively large, relative to the high cost of measuring NfL. Its small incremental gain was possibly because the ten blood markers had already largely explained the variance that NfL otherwise would have explained. Indeed, in our validation set our newly developed BioAge1 and BioAge2 were highly correlated with NfL. We therefore focused on BioAge1 and not BioAge2 for further evaluations.

The finding that the new biological age algorithms were predictive of dementia including AD has important implications. Accelerated biological age indeed predicts an elevated risk of mortality and all major age-related morbidities including dementia outcomes for people of a fixed chronological age, supporting the key concept of biological age and its important role in the field of healthy aging. A major strength of our study is the usage of a large community-based advanced- age cohort study data with a novel RNA-sequencing method to assay the levels of a wide array of miRNAs in stored plasma

samples. The mortality and morbidity outcomes were well-ascertained. Nevertheless, there were several limitations with our study. First, we have not used an independent cohort outside of the Rotterdam Study to replicate our results even though we have conducted cross-validation and established internal validity. Secondly, one additional year of accelerated aging at age 70 might not be equivalently detrimental to that of age 80. However, we did not model this non-linearity as we did not want to make the model overly difficult to understand. This differentiation was not our main focus, and what we presented should be considered as an average effect across age groups. Third, previous studies have shown the importance of omic-based biological age such as DNAm-age and metabo-age. In this study we did not have DNA methylation, gene expression or metabolomics data available from the same time point to calculate biological age based on such omics data. However, miRNA aging signatures were identified in relation to biological age vs. chronological age. The top differentially expressed miRNAs in relation to biological age had much higher significance level, suggesting that biological age algorithms capture some aspect of aging signals at the epigenetic level other than DNA methylation.

We have shown that in a large community-based advanced-age population, biological age predicts lifespan, healthspan and risks for all major age-related morbidities including neurologic outcomes. Similar plasma miRNA aging signatures were identified in relation to biological age vs. chronological age. The top differentially expressed miRNAs in relation to biological age had much higher significance level, suggesting epigenetic links with biological age algorithms beyond DNA methylation.

Methods

Study Settings

This study included participants from the Rotterdam Study, a prospective community-based cohort study.[20] In 1990, residents aged 55 years and older residing in Ommoord, a district of Rotterdam, the Netherlands, were invited to participate in the study. Of 10,215 invited inhabitants, 7,983 agreed to participate in the baseline examinations. In 2000, 3,011 participants (of 4,472 invitees) who had reached 55 years of age or moved into the study district since the start of the study were added to the cohort. In 2006, a further extension of the cohort was started in which 3,932 participants, of 6,057 invited, aged at least 45 years living in Ommoord were included. Follow-up examinations take place every 3–4 years. The Rotterdam Study was approved by the Institutional Review Board at Erasmus Medical Center, the Netherlands, and performed in accordance with the Declaration of Helsinki. For this study, 2,000 individuals were randomly selected from the fourth round of Rotterdam Study-I (RS-I-4) and second round of Rotterdam Study-II (RS-II-2) between 2002–2005. The criteria for inclusion included the availability of informed consent and valid serum samples that were both available at the visits in the Rotterdam Study. Data analyzed in this study concern 1,930 participants with physiological and neurological function information available (**Figure S1**). All research was performed in accordance with relevant guidelines/regulations.

Calculation of biological age

We used the following two-fold approach for developing our biological age models: 1) We first validated Levine's "phenotypic age" algorithm in the Rotterdam Study (n = 1,930); 2) We then developed and cross-validated new biological age algorithms in the Rotterdam Study using the same Gompertz proportional hazards regression modeling framework, with or without incorporating the neurodegeneration markers (NfL, total-tau, amyloid beta-40 and – 42).

Validation of Levine's "phenotypic age" algorithm

We first calculated biological age based on the "phenotypic age" algorithm that Levine et al developed and validated based on the NHANES data (3). Briefly, we used chronological age and 9 biomarkers (albumin, creatinine, glucose, [log] C-reactive protein (CRP), lymphocyte percent, mean cell volume, red blood cell distribution width, alkaline phosphatase, and white blood cell count) that were selected in the "phenotypic age" algorithm. Using a parametric proportional hazard model based on the Gompertz distribution, we estimated the 10-year mortality risk of the j-th individual based on the cumulative distribution function of the model:

$$\text{MortalityScore}_j = \text{CDF}(10, X_j) = 1 - e^{-e^{xjb}(\exp(10*\gamma)-1)/\gamma}$$

Where xb represented the linear combination of biomarkers from the fitted model of phenotypic age.

To obtain the "phenotypic age", the mortality score was converted into units of years, based on parametrization of a separate Gompertz proportional hazard model fit using only chronological age.

Development and cross-validation of the new biological age algorithms

We then calculated biological age based on what we developed and cross-validated using the Rotterdam study data. We employed cross-validations with a 60:40 split of training/validation sets on the Rotterdam study cohort data (n = 1,930). In the training set, we re-fit parametric proportional hazard models based on the Gompertz distribution in the Rotterdam Study cohorts, using 1) the same set of ten predictors as in the "phenotypic age" algorithm; 2) the ten predictors as in the "phenotypic age" algorithm, as well as the addition of one of the four brain markers (plasma neurofilament level NfL, total-tau, amyloid beta-40 and − 42). In the cross-validation set, we estimated the 10-year mortality risk of the j-th individual based on the cumulative distribution function of the models obtained in 1), in accordance with the procedures conducted in validating the phenotypic age algorithm but using the linear combination of biomarkers from the newly fitted models of biological age (see Tables 3 and 4). To obtain the biological age, the mortality score was converted into units of years, based on parametrization of a separate Gompertz proportional hazard model fit using only chronological age.

Association between biological age and risk of all-cause mortality/morbidities

For validation of PhenoAge algorithm, we first examined the correlation between PhenoAge and chronological age. Next, we used Cox proportional hazard models to assess the association between Phenotypic Age and risk of all-cause mortality, with adjustment for chronological age, *APOE* status and gender. Follow-up was truncated on January 1st 2012 in the Cox-models of all-cause mortality. We used three time-intervals to examine the risks of mortality: 3-year mortality, 5-year mortality and total mortality. We cross-validated the biological age algorithms similarly. Hazard ratios were expressed as annual risk of death over the follow up period.

To assess the association between the biological age and morbidities, prevalent cases of the outcome of interest was excluded from the respective analyses. The morbidities included: diabetes mellitus, stroke, CHD, dementia, COPD and cancer (any cancer and specific cancers, such as lung cancer, breast cancer and other cancers with a significant incidence during follow-up). First morbidity was defined as first occurrence of any of these major morbidities.

Ascertainment of outcomes

The outcome measures for this analysis were all-cause mortality and morbidities. Outcome analyses included all deaths/morbidity endpoints that occurred prior to January 1st 2012. Information on vital status of participants was obtained on a weekly basis via municipal population registries and through general practitioners' and hospitals' databases. Events were coded according to the International Classification of Diseases 10th version (ICD-10) by two independent research physicians. All-cause mortality is defined as participants who died from any cause during the total follow-up period, which was completed until January 1st 2012.

MicroRNA expression profiling and normalization

Expression levels of 2083 plasma miRNAs were determined by the HTG EdgeSeq miRNA Whole Transcriptome Assay (HTG Molecular Diagnostics, Tuscon, AZ, USA) and the Illumina NextSeq 500 sequencer (Illumina, San Diego, CA, USA). MiRNA specific probes were hybridized to each miRNA.

The bioinformatics workflow consisted of two parallel paths, one for the quality control (QC) checks and one for actual results processing. For the QC checks, first the average amount of ANT probe signal was tested relative to the total signal of the sample. If the relative ANT probe signal was too high, a sign that the signal of the sample was too low, the sample was either re-tested (if re-test material was available) or otherwise rejected. For the actual results processing, we used counts per million (CPM) to quantify miRNA expression. For the purpose of standardization of adjustment for total reads per sample, Log2 transformation of CPM was used. We then applied a cut-off of 1.0 so that Log2 CPM < 1.0 were considered as non-expression in the samples. Finally, we used the lower limit of quantification (LLOQ) method for normalization to select well-expressed miRNAs, by modelling the relation between mean and standard deviation of Log2 CPM among the 1,930 participants with a monotonic smooth spline fit. The miRNAs with 50% expression values above LLOQ were defined as well-expressed in plasma. Out of the 2,083 measured miRNAs, 591 miRNAs were considered well-expressed.

Identifying differentially expressed miRNAs in relation to biological age

To identify age-related miRNA expression in plasma, we used linear regression to model individual miRNA expression as the dependent variable and biological age as an explanatory variable, with adjustment of gender. We computed false discovery rate (FDR) to account for multiple testing. We constructed a volcano plot (with $-\log10(pvalue)$ on the y-axis and fold change per 40 years on the x-axis) to display the significance and magnitude of each bivariate association. We then compared the top biological age-related miRNAs to those identified in relation to chronological age.

Declarations

Competing Interests Statement

The authors declare no competing interests.

Author contributions

JWW and AY had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: JWW, AH, AMI, MG and JG. Acquisition, analysis or interpretation of the data: All authors. First drafting of the manuscript: JWW. Critical revision of the manuscript for important intellectual content: All authors.

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