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Select aging biomarkers based on telomere length and chronological age to build a biological age equation

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Abstract The purpose of this study is to build a biological age (BA) equation combining telomere length with chronological age (CA) and associated aging biomarkers. In total, 139 healthy volunteers were recruited from a Chinese Han cohort in Beijing. A genetic index, renal function indices, cardiovascular function indices, brain function indices, and oxidative stress and inflammation indices (C-reactive protein [CRP]) were measured and analyzed. A BA equation was proposed based on selected parameters, with terminal telomere restriction fragment (TRF) and CA as the two principal components. The selected aging markers included mitral annulus peak E anterior wall (MVEA), intima-media thickness (IMT), cystatin C (CYSC), D-dimer (DD), and digital symbol test (DST). The BA equation was: $BA = -2.281TRF + 26.321CYSC + 0.025DD - 104.419MVEA + 34.863IMT - 0.265DST + 0.305CA + 26.346$. To conclude, telomere length and CA as double benchmarks may be a new method to build a BA.

Keywords Telomere length · Biological age · Chronological age · Aging markers

Wei-Guang Zhang and Shu-Ying Zhu contributed equally to this work.

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Introduction

Functional decline is an inevitable consequence of aging progress (Tang et al. 2010; Mak 2013). The most common indicator to evaluate aging function is chronological age (CA). However, the aging process occurs gradually. Aging is a highly individual process and has a high degree of inter-individual and between-individual differences (AihieSayer et al. 1999). For example, some individuals at a CA of 85 years old have similar physiological conditions as younger individuals; however, physiological dysfunction in some individuals may occur before 60 years old (Gunn et al. 2009). Due to the large variation between individuals, CA does not accurately reflect the performance of the aging body (Sprott 2010).

Correct assessment of the functional status of aging is essential to study and treat aging-related diseases

(Blagosklonny 2009). Biological aging is defined as a set of processes derived from the gradual reduction in the viability of the organism and gradual increase in the vulnerability (Mitnitski et al. 2013). Since CA is not an accurate indicator of biological aging (Simm and Johnson 2010), researchers have attempted to find an alternative biological indicator to measure age. The evaluation of biological aging uses indices to measure the extent of aging and to identify different individuals that have the same CA but different biological activity.

A number of representative parameters have been chosen to reflect the extent and rate of individuals to measure the aging process (Sprott 2010; Baker and Sprott 1988; Butler et al. 2004; Majkić-Singh 2011; Bae et al. 2008). However, studies have not found a single biomarker that fully reflects the degree of aging. Therefore, statistical methods have been developed to evaluate representative system/organ markers to build functional age equations (Park et al. 2009; Jee et al. 2012), such as vascular age (Cuende et al. 2010; Lloyd-Jones et al. 2004), skin age(Guinot et al. 2002), mental test score (Hodkinson 2012), dental age (Gupta et al. 2013; Hägg and Matsson 1985), skeletal Age (Gupta et al. 2013), perceived age (Rippon et al. 2013), and cognitive age (Hong et al. 2013). Other equations have been created to evaluate the overall function of the "functional age" concept, such as the biological age (Nakamura and Miyao 2007; Rippon et al. 2013), and Frailty index (Searle et al. 2008; Sternberg et al. 2011; Jones et al. 2004).

Selecting biomarkers of aging is closely related to parameters that reflect the biological aging process, as shown in Table 1 (Bai et al. 2010; Park et al. 2009; Nakamura et al. 1998; Nakamura and Miyao 2007; Ueno et al. 2003). CA does not accurately reflect the functional state of the body. Thus, CA may not be a suitable benchmark for selecting aging biomarkers. However, many biomarkers are chosen based on CA as a benchmark (Nakamura et al. 1994; Nakamura and Miyao 2007). In this study, we tried to explore a new age benchmark to select biomarkers of aging.

Genetics play an important role in aging; however, genetic indicators are rarely used as biomarkers of aging for building BA equations (Sprott 2010). Telomere restriction fragment (TRF) is generally recognized as a genetic marker of aging at the cellular level (Mather et al. 2011; Sanders and Newman 2013; Saeed et al. 2012), appears to be associated with systemic aging (Zglinicki and Martin-Ruiz 2005; Mather et al. 2011; Bekaert et al. 2005), and reflects the dynamic aging process (Zglinicki and Martin-Ruiz 2005; Benetos et al. 2001). However, it is unclear whether TRF can serve as a biomarker of aging (Fossel 2012; Mather et al. 2011). Recent studies indicate that TRF reflects changes in functional aging (Boonekamp et al. 2013; López-Otín et al. 2013). In this study, we attempted to use TRF as a benchmark to select other biomarkers of aging to build a BA equation.

Many methods have been used to build BA equation. Compared with multiple regression analysis, principal component analysis (PCA) or factor analysis (FA) is relatively stable and superior for building BA equations (Nakamura et al. 1988; Park et al. 2009). In this study, we used the FA method to a BA equation based on TRF and CA, together with a number of indices independently associated with either telomere length or CA.

Table 1 Criteria for biomarkers of aging (BoA)

Standards	Specific content	References
Primary standards for BoA	Should reflect the basic biological processes of aging	(Reff et al. 1982; Baker and Sprott 1988; Mooradian 1990; McClearn 1997; Hodkinson 1972; López-Otín et al. 2013)
	Should be quantitatively related to biological parameters	(Bai et al. 2010; Park et al. 2009; Nakamura et al. 1998; Nakamura and Miyao 2007; Ueno et al. 2003)
	Should be obtained in a healthy population	(Mooradian 1990; Johnson 2006; Sprott 2010;)
	Should reflect the aging rate in a short time	(Reff et al. 1982; Baker and Sprott 1988; Mitnitski et al. 2013; Mooradian 1990; Hodkinson 1972; McClearn 1997; Rattan 2013)
Secondary standards for BoA	Should be minimally traumatic	(Hodkinson 1972; Reff et al. 1982; Baker and Sprott 1988; McClearn 1997)
	Should be repeatable and reproducible	(Reff et al. 1982; Baker and Sprott 1988; Mooradian 1990; McClearn 1997; Hodkinson 1972; Nakamura et al. 1994)
	Should be representative	(Nakamura et al. 1994)

Methods

Screening of healthy volunteers

In 2011, 139 healthy volunteers, aged 35 to 91 years, were recruited from the Han population in Beijing city. The volunteers were not taking any medications and had not been hospitalized in the past 3 years. All participants signed an informed consent form. The study inclusion criteria and a flow chart for the study enrollment process are shown in Fig. 1. This research was approved by the Human Ethics Committee of Chinese PLA General Hospital in Beijing and was conducted in accordance with the Declaration of Helsinki and subsequent amendments.

Measurements of various indicators (Annex 1)

Indicators of cardiovascular, kidney, and brain functions, as well as clinical, inflammatory, oxidative stress, genetic, psychological, and lifestyle habit factors were assessed in all the volunteers. A total of 105 indicators were assessed as potential age-related indices, which are described in Appendix. Briefly, all subjects completed a life habit survey concerning their smoking and alcohol habits, dietary patterns, frequency of physical activity, and other lifestyle factors. Blood pressure and body weight measurements were obtained. Cardiovascular function was assessed through ultrasound and electrocardiogram (ECG) measurements. Blood biochemistry was examined through routine blood analysis and urinalysis procedures.

Genomic DNA was isolated using a cell tissue genomic DNA extraction kit (Beijing TIANGEN, Co., Ltd). TRF length was measured using the Telo TTAGGG telomere length assay (Roche, Germany). Briefly, genomic DNA (1.5 μg) was digested with the restriction enzymes, Rsa I and Hinf I, for 2 h at 37 °C. The resulting DNA fragments were separated in a 0.8 % agarose gel by electrophoresis, then were denatured, neutralized, and transferred to a nylon membrane and cross-linked

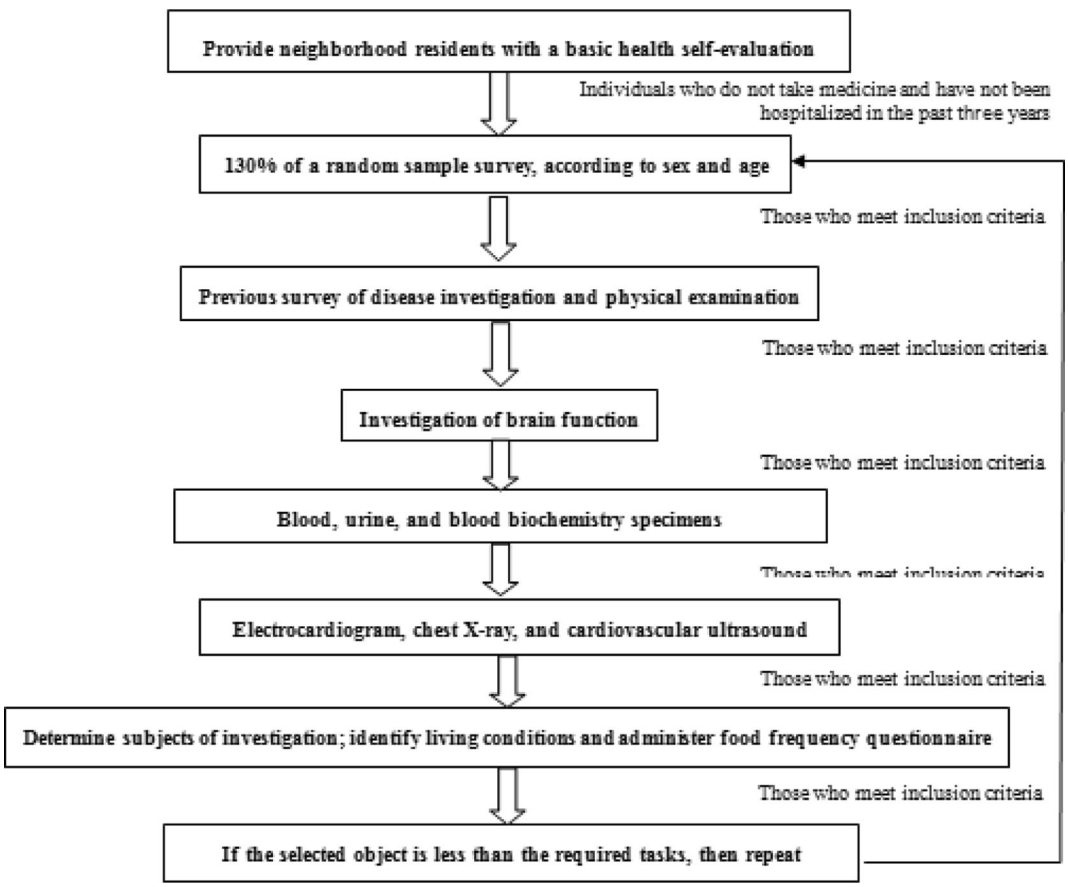

Fig. 1 Flow diagram for the screening of healthy volunteers

with UV light (UVP, USA). Blotted DNA fragments were then incubated with a telomeric probe (digoxigenin (DIG) 3′-end labeled 5′-$[CCCTAA]_3$) at 42 °C for 3 h, followed by incubation with a DIG-specific antibody covalently coupled to alkaline phosphatase. Binding sites of the telomere probe were visualized using a highly sensitive chemiluminescence substrate that metabolizes alkaline phosphatase. TRF lengths were compared with molecular weight markers (Roche, Germany) (Tsuji et al. 2002) observed on X-ray film, and mean TRF lengths were estimated using Quantity One software (BioRad) (Tsuji et al. 2002).

Selection of aging biomarkers

A pair-wise correlation analysis was performed between the candidate indicators with CA and TRF ($p<0.05$, $r>0.15$), followed by a redundancy analysis to reduce the dimensionality ($p<0.05$, $r>0.7$ or $p>0.05$). FA variables with high factor loading were selected as aging markers.

Construction of the BA formula

The marker coefficients were calculated by FA. An integral formula for the biological age score (BAS) was derived by FA. Each biomarker was subjected to data standardization using the following formula: X1 = (X − mean) / SD, where X is the original value, and SD is the standard deviation. BA was determined from the BAS equation and CA with the following formula: BA = BAS (standard CA) + mean CA. The corrected BA was determined with the following formula: Corrected BA = BA + Z (Dubina et al. 1984), where $Z = (y_i - y)(1 - b)$, y_i is the CA of an individual, y is the average CA of all samples, and b is the coefficient of the simple linear regression between BA and CA (Park et al. 2009).

Results

Study subjects

As shown in Table 2, a total of 139 healthy Han individuals (aged 35–92, mean age 60.29±14.33) were divided into five groups.

Table 2 Group characteristics

Age group	Male	Female	Total
30–44	14	13	27
45–54	13	13	26
55–64	13	14	27
65–74	15	16	31
>75	14	14	28
Total	69	70	139

Selected aging markers

A positive correlation was found between TRF and CA ($r=0.314$, $p<0.01$). The correlation analysis between each index and TRF or CA was performed, and the results are shown in Tables 3 and 4, respectively. The indices, which were independently related to telomere length and CA, were considered as aging biomarkers. $R>0.7$ ($p<0.05$) was used as the standard threshold to exclude the redundant indices. The remaining indicators were mitral annulus peak E anterior wall (MVEA), intima-media thickness (IMT), Cystatin C (CYSC), D-dimer (DD), and digital symbol test. A summary of the selected markers is shown in Table 5.

Construction of the BAS formula

TRF, MVEA, IMT, CYSC, DD, and DST underwent FA. The results are shown in Table 6. The proportion of each index was greater than 0.5 (0.540–0.682).

The score coefficient of each biomarker is shown in Table 6. BAS was derived via FA and was as follows: $BAS = -0.240TRF + 0.265CYSC - 0.241DST + 0.282IMT - 0.303MVEA + 0.295DD$. The calculated BA using the BAS formula and CA was as follows: $BA = -2.281TRF + 26.321CYSC + 0.0256DD - 104.419MVEA + 34.863IMT - 0.265DST + 44.734$. The final corrected BA formula was as follows: Corrected $BA = -2.281TRF + 26.321CYSC + 0.0256DD - 104.419MVEA + 34.863IMT - 0.265\ DST + 0.305CA + 26.346$ (Table 7). The scatter plot of the corrected BA formula with CA is shown in Fig. 2.

Discussion

The base of building a BA equation is the selection of biomarkers of aging, which are closely related with

Table 3 Correlation of each index with telomere length (TRF)

Index		AID	MVEI	MVEA	TMD	IMD	IMT	EDVmax	CYSC	DD	DST
TRF	R	−0.203	0.225	0.199	−0.153	−0.220	−0.249	−0.182	−0.193	−0.187	0.252
	P	0.017	0.008	0.019	0.071	0.012	0.004	0.040	0.023	0.036	0.005
AID	R		−0309	−0.336	0.330	0.189	0.121	0.011	0.341	0.138	−0.178
	P		0.001	0.001	0.001	0.031	0.170	0.905	0.001	0.124	0.049
MVEI	R			0.654	−0.278	−0.198	−0.406	−0.136	−0.322	−0.160	0.223
	P			0.001	0.001	0.024	0.001	0.125	0.001	0.073	0.014
MVEA	R				−0.244	−0.250	−0.326	−0.086	−0.304	−0.225	0.208
	P				0.004	0.004	0.001	0.333	0.001	0.011	0.022
TMD	R					0.506	0.194	0.001	0.261	0.094	−0.058
	P					0.001	0.026	0.990	0.002	0.298	0.522
IMD	R						0.381	−0.033	0.208	0.099	−0.027
	P						0.001	0.714	0.017	0.283	0.773
IMT	R							0.091	0.291	0.225	−0.076
	P							0.309	0.001	0.014	0.419
EDVmax	R								0.175	0.190	−0.117
	P								0.049	0.041	0.213
CYSC	R									0.263	−0.108
	P									0.003	0.234
DD	R										−0.192
	P										0.038

biological aging parameters. In the past, CA has served as a benchmark for selecting aging biomarkers; however, CA does not accurately reflect aging conditions. Thus, in this study, we used an alternative benchmark to select the aging biomarkers.

Genetic components play an important role in aging (Guarente and Kenyon 2000), and 20–50 % of the variation in lifespan is caused by genetic differences (Yashin et al. 1999; Mitchell et al. 2001; Skytthe et al. 2003). Telomeres are the cap structure of eukaryotic chromosome ends and play an essential role in preventing chromosome degradation, fusion, or recombination, in maintaining the stability and integrity of the chromosome and to ensure a complete copy of the genetic information (Blackburn 1991). Telomeres shorten with each cell division, ultimately leading to critically short telomeres in normal somatic cells. Telomere shortening is an inherent mechanism of cellular senescence and reflects the changes in functional aging. Thus, telomere length is considered to be a marker of biological age (Sanders and Newman 2013; Boonekamp et al. 2013; López-Otín et al. 2013). In this study, we attempted to use TRF as a benchmark to select other biomarkers of aging to build a BA equation. Considering that this is the first study to use the TRF as a benchmark, we also included CA as the conventional benchmark to maintain consistency with previous studies. We found that all the indices related to CA were related to TRF. Surprisingly, the indices correlated better with TRF than with CA, suggesting that TRF is a more rigorous benchmark than CA. The aging markers in this study were MVEA, IMT, CYSC, DD, and DST.

In current and our previous studies, IMT was selected as a biomarker of aging in healthy individuals. Carotid IMT has been associated with many cardiovascular outcomes such as systemic endothelial function (Halcox et al. 2009). It was proposed as a predictor of vascular events in the IMPROVE study (Baldassarre et al. 2013) and as a predictor of stroke in a multiethnic study of atherosclerosis (Polak et al. 2011). D-dimer levels increase with age (Tita-Nwa et al. 2010) and have served as a marker of atherothrombotic risk in healthy postmenopausal women (Pradhan et al. 2004). In addition, fibrin D-dimer associated with increased risk of stroke in aged males may be a useful marker to identify hypertensive patients with a high risk for stroke

Table 4 Correlation of each index and CA

Index		AID	MVEI	MVEA	TMD	IMD	IMT	EDVmax	CYSC	DD	DST
CA	R	0.421	−0.630	−0.670	0.335	0.335	0.464	0.230	0.405	0.326	−0.356
	P	0.001	0.001	0.001	0.001	0.001	0.001	0.009	0.001	0.001	0.001
AID	R		−0.309	−0.336	0.330	0.189	0.121	0.011	0.341	0.138	−0.178
	P		0.001	0.001	0.001	0.031	0.170	0.905	0.001	0.124	0.049
MVEI	R			0.654	−0.278	−0.198	−0.406	−0.136	−0.322	−0.160	0.223
	P			0.001	0.001	0.024	0.001	0.125	0.001	0.073	0.014
MVEA	R				−0.244	−0.250	−0.326	−0.086	−0.304	−0.225	0.208
	P				0.004	0.004	0.001	0.333	0.001	0.011	0.022
TMD	R					0.506	0.194	0.001	0.261	0.094	−0.058
	P					0.001	0.026	0.990	0.002	0.298	0.522
IMD	R		–	–			0.381	−0.033	0.208	0.099	−0.027
	P						0.001	0.714	0.017	0.283	0.773
IMT	R							0.091	0.291	0.225	−0.076
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EDVmax	R					–			0.175	0.190	−0.117
	P								0.049	0.041	0.213
CYSC	R									0.263	−0.108
	P									0.003	0.234
DD	R										−0.192
	P										0.038

(Wannamethee et al. 2012). Increased levels of D-dimer in endothelial dysfunction have also been identified (Quinn et al. 2011). Serum cystatin C is a marker of glomerular filtration rate (GFR) and serves as a more sensitive marker of kidney function than serum creatinine in measuring normal kidney function (Dharnidharka et al. 2002). In addition, cystatin C can predict all-cause mortality and cardiovascular disease (Emberson et al. 2010; Lee et al. 2010; Bansal et al. 2012) as well as acute kidney injury (Zhang et al. 2011).

Our study found that the correlation coefficient of TRF and CA was −0.314, similar to the average level of the 124 cross-sectional studies reviewed by Müezzinler et al. (2013).

In this study, we found a correlation between telomere length and the indicators of cardiovascular function, including MVEA and IMT, but correlations were not found between telomere length and ECG parameters. This is consistent with previous studies (De Meyer et al. 2009; O'Donnell et al. 2008; Fitzpatrick et al. 2007). The reason may be that ECG is an indirect indicator for heart diseases but is not age dependent. Interestingly, in previous studies, blood pressure (BP)

Table 5 General summary of selected biomarkers of age (BoA)

BoA	Mean	Std. deviation
TRF	6.272	1.507
CYSC	0.769	0.144
DD	426.786	165.420
MVEA	0.135	0.0415
IMT	0.641	0.115
DST	35.670	12.978

Table 6 Component matrix and component score coefficient matrix of biomarkers of age (BoA)

BoA	Component matrix	Component score coefficient matrix
TRF	−0.540	−0.240
CYSC	0.596	0.265
DD	0.665	0.295
MVEA	−0.682	−0.303
IMT	0.635	0.282
DST	−0.542	−0.241

Table 7 The linear correlation results of chronological age and biological age

Model	Unstandardized coefficients		Standardized coefficients	*t*	*p* value
	B	Std. error	Beta		
Constant	18.58	3.764		4.937	0.001
Age	0.695	0.061	0.699	11.442	0.001

indices (MacDonald et al. 2004) such as systolic BP (Nakamura and Miyao 2007, 2008; Park et al. 2009; Hollingsworth et al. 1965; Bae et al. 2008), diastolic BP (Bae et al. 2008; Yashin et al. 2010), and pulse pressure (Zhang et al. 2014; Bai et al. 2010; Yashin et al. 2010) were selected as biomarkers of aging. Telomere length is closely associated with BP and may replace BP indices in building BA equations (Benetos et al. 2001).

A study including 38 female patients of dementia or dysfunction showed that TRF shortening was independently associated with declining episodic memory and learning (p=0.032), non-verbal recognition memory (p=0.007), and working memory capacity (p=0.003) (Valdes et al. 2010). In this study, we found that DST had a high correlation coefficient with CA and TRF as compared to the trail making test (TMT) (Zhang et al. 2014), which reflects brain function. Previous studies have shown that TRF does not correlate with age-related decreases of lung function (Harris et al. 2006; Mather et al. 2010) or bone mineral density (Sanders et al. 2009; Tang et al. 2010; Valdes et al. 2007). Thus, in this study, we did not select lung or bone indicators.

Both diseases and aging can lead to TRF shortening (Sanders and Newman 2013). Thus, we selected healthy subjects in this study to avoid the interference of disease factors (Rattan 2013). In order to compare with previous studies, we used both TRF and CA as benchmarks. Interestingly, we found that all TRF-associated markers also correlated with CA, but not vice versa, suggesting that TRF is a more rigorous benchmark than CA. Note that all the aging markers are disease markers; however, not all disease markers are aging markers, indicating an intrinsic link between age and disease processes.

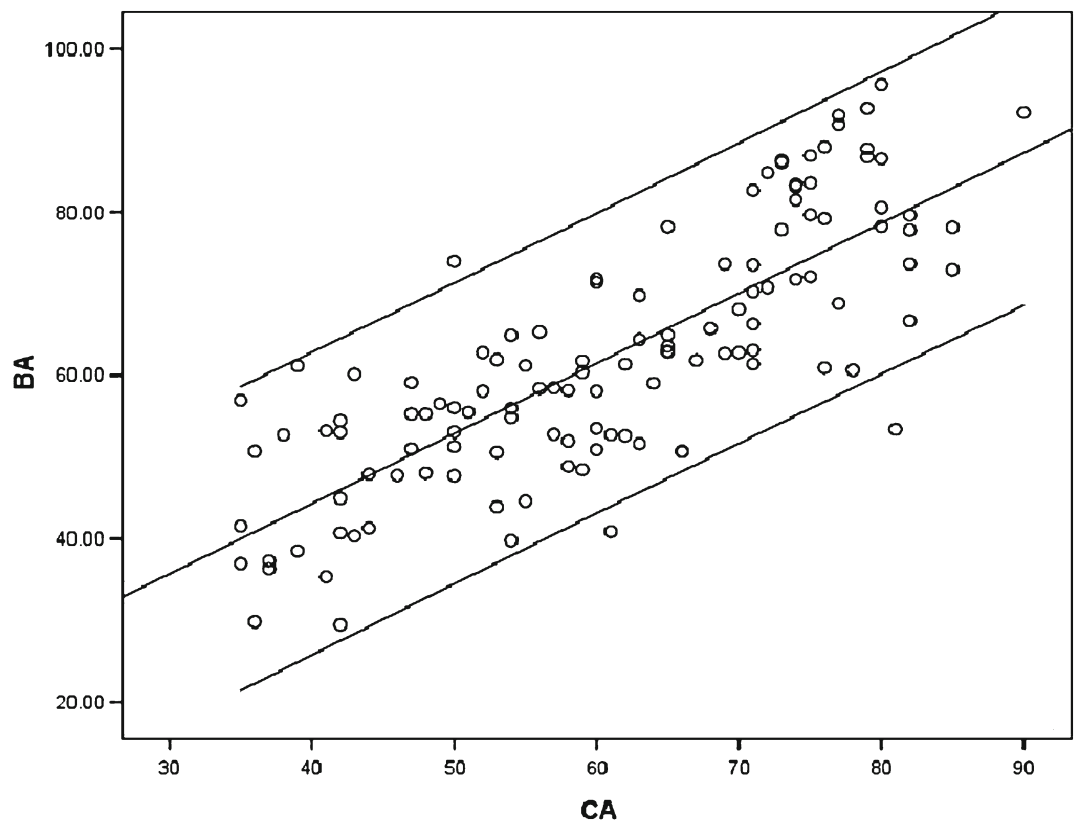

Fig. 2 Scatter plot of the corrected BA formula with CA

The number of samples analyzed in the present study was limited. This cross-sectional study needs to be validated by longitudinal studies. Nevertheless, the proposed BA equation, which uses both CA and TRF as benchmarks, consists of both genetic and physiological characteristics of aging and may serve as a better tool to assess biological age than previous biological age which only consist by vital organ function indexes.

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Appendix All indices (total 105)

Life habits survey (20)

All subjects received a survey concerning their smoking and alcohol habits, dietary patterns, frequency of physical activity, and other lifestyle factors. Daily living conditions were assessed with the following general indicators: educational extent, marital status, occupation, number of family members, relationship between family members, housing situation, annual income, self-assessment of economic status, method of staying healthy, frequency of performing daily activities, participation in interest groups, daily living conditions, smoking status, smoking age, smoking amount, smoking cessation time, number of smokers in the household, number of smokers in the workplace, frequency of exercise for >30 min, and overall mental state over the last year.

Blood pressure and body weight measurements (5)

Measurements were made in a quiet environment after the subject had rested for >15 min. Measurements were made according to the Krotkoff 5 method. The pulse pressure was calculated as PP = systolic blood pressure (SBP) − diastolic blood pressure (DBP). The body mass index (BMI) and waist-to-hip ratio (WHR) were measured simultaneously.

Cardiovascular ultrasound measurements (25)

Cardiovascular ultrasound measurements included the following parameters: left ventricular ejection fraction (LVEF); mitral early and mitral late diastolic peak flow velocity (MVE and MVA, respectively); ratio of the peak velocity of early filling to the peak velocity of atrial filling (E/A); mitral valve annulus lateral wall, anterior wall, inferior wall, and ventricular septum of the peak velocity of early filling (MVEL, MVEA, MVEI, and MVES, respectively); mitral valve annulus lateral wall, anterior wall, inferior wall, and ventricular septum of the peak velocity of atrial filling (MVAL, MVAA, MVAI, and MVAS, respectively); maximum and minimum peak systolic velocity (SPVmax and SPVmin, respectively); maximum and minimum carotid artery end-diastolic velocity (EDVmax and EDVmin, respectively); maximum and minimum internal diameter of the carotid artery (Dmax and Dmin, respectively); maximum and minimum carotid artery intimal-medial thickness (IMTmax and IMTmin, respectively); and heart rate (HR).

Blood biochemistry measurements (13)

Blood biochemistry was examined through routine blood analysis and urinalysis procedures. Serum or urine levels of urea (UR), creatinine (Cr), triglyceride, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), alanine aminotransferase, alanine transaminase, total protein (TP), albumin (ALB), total bilirubin (TBIL), direct bilirubin (DBIL), and glucose (Glu) were measured.

Brain function measurement (7)

To assess brain function, the following factors were assessed: clock drawing test (CDT); stroop response time; stroop mistake number; trail making test (TMT), forward and backward digit span tasks (FDST and BDST, respectively); and mini-mental state examination (MMSE).

Genetics indicators (1)

Terminal telomere restriction fragment (TRF).

Urine (4)

The pH, specific gravity, and conductivity of urine were analyzed.

Routine blood (18)

Routine blood analyses included measurements of the following parameters: white blood cell count (WBC), lymphocytes, granulocytes (GRAN), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell volume distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), platelet hematocrit (PCT), monocytes (MON), and the relative percentage of lymphocytes (LRR%), granulocytes (RPR%), and monocytes (MPR%).

Special index (6)

Other tested indices included Cystatin C (CysC), interleukin 6 (IL-6), C-reactive protein (CRP), D-dimer (DD), fibrinogen (Fib), and GFR (dual GFR).

ECG index (7)

ST, T, QRS, QT, QTC, PR, and heart rate.

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