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AGE-RELATED DIFFERENCES IN CO-MORBIDITY NUMBER, FUNDUS ATHEROSCLEROSIS LEVEL AND THE SERUM VALUES OF GSH-PX, HS-CRP AND HDL-C IN ELDERLY CHINESE PATIENTS

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Abstract: *Background:* The ApoE genotype, atherosclerosis, status of inflammation, oxidative stress and co-morbidity may be detrimental to the elderly. *Objectives:* To identify biomarkers of aging. *Setting:* All subjects were Chinese elderly in Shanghai. *Subjects:* 549 outpatients (489 male, 60 female), divided into ≤74 year-old, 75-84 year-old and the oldest old (≥85 year-old) groups. *Methods:* A univariate analysis was used to investigate 5 age-related categorical variables and 26 continuous variables. The related variables were used to find the independent biomarkers of aging by Multivariate logistic regression analyses. *Results:* The serum values of Glutathione peroxidase, HDL-C and C reactive protein, the number of co-morbidities and fundus atherosclerosis level were the main independent age-associated factors that influenced aging. Compared with ≥85 year-old individuals, ≤74 year-old individuals had fewer co-morbidities [OR, 0.757 (95% CI, 0.636, 0.902)], lower grades of fundus atherosclerosis [Grade 0: OR, 26.059 (95% CI, 4.705, 144.324)] and [Grade I: OR, 8.539 (95% CI, 3.555, 20.513)] and lower serum levels of HDL-C [OR, 0.127 (95% CI, 0.037, 0.433)]. However, 75-84 year-old patients had significantly lower plasma levels of GSH-px [OR, 0.986, (95% CI, 0.972, 1.00)], HDL-C [OR, 0.158 (95% CI, 0.054, 0.457)] and HsCRP [Grade I: OR, 8.516 (95% CI,1.630, 44.484)], [Grade II: OR,7.699 (95% CI,1.544, 38.388)] and [Grade III: OR,7.251 (95% CI,1.346, 39.070)]. *Conclusion:* The oldest old patients had significantly high anti-oxidant capability and serum HDL-C level. However, these patients also had a significantly high systemic inflammation, number of co-morbidities and grades of fundus atherosclerosis.

Key words: Lipoprotein and apolipoprotein, antioxidant enzymes, inflammatory cytokine, fundus atherosclerosis, co-morbidity.

Introduction

Aging is characterized by a time-dependent progressive decline in organismal physiological reserves and by an increased vulnerability to age-associated diseases, which can lead to death. A number of peripheral blood proteins may involve in aging process and be potential biomarkers of aging. Centenarians were shown to have a significantly low oxidative stress grade compared with a control group of 70-80 year-old elderly persons (1). An age-related increase in enzymatic antioxidant activities was seen in subjects from less than 60 years of age to Italian nonagenarians (2). Elevated inflammatory markers, especially IL-6, have been shown to be closely associated with multiple age-related diseases and all-cause mortality (3). A high level of IL-6 long-term was associated with older age, obesity, smoking, lower physical activity and lower high-density lipoprotein cholesterol (HDL-C) (4). In aging men, systemic inflammation was associated with normal aging and age-related diseases, such as coronary atherosclerosis (AS) and lower urinary tract symptoms (5, 6). High plasma high sensitivity C reactive protein (HsCRP) concentrations, which are associated with sex, age, waist circumference and systolic blood pressure, were similar between Inuit persons and Caucasians, despite their differing lifestyles (7).

A high serum level of HDL-C is a characteristic feature of centenarians and is associated with better survival in frail, community-living elderly (8, 9). In centenarians, a progressive decline in plasma HDL was shown to be associated with cognitive dysfunction and elevated CRP and IL-6 levels (8, 10). ApoE polymorphisms are important determinants of blood lipids. ApoE ε4 allele carriers have the highest total cholesterol (TCH) and low-density lipoprotein cholesterol (LDL-C) levels and are susceptible to cardio-cerebrovascular disease, dementia and Alzheimer's disease. Multiple studies have shown that long-lived populations have lower

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ε4 frequencies when compared with a general or middle-aged population (11). The mechanisms of ApoE-regulated aging and age-related diseases are related to the antioxidant and anti-inflammatory properties of ApoE (12). Apolipoprotein C-III (ApoC-III) is the major component of triglyceride (TG)-rich lipoproteins. Lower serum levels of ApoC-III had a protective effect against cardiovascular disease and metabolic syndrome (13). ApoB protein level, which is mediated by the ApoE polymorphism, may be a useful predictive factor for cardio-cerebrovascular complications (10, 14). An increase in serum ApoA-I prevented AS and enhanced longevity through antioxidative and anti-inflammatory effects (15). The up regulation of ApoJ increases stress resistance and extends lifespan in Drosophila , lower in a sample of human centenarians (16, 17). Although a number of study showed many peripheral blood proteins, including inflammatory cytokines, Lipoprotein and apolipoprotein, Co-morbidity, oxidative stress and Fundus atherosclerosis grade may be potential predictors of aging. However, the study about these potential biomarkers dynamic changes in subjects with different age is little. The current study was to investigate age-associated changes of these potential aging biomarkers

Methods

Sample

The study was conducted at Huadong Hospital. Between June 1, 2012, and July 31, 2012, a total of 549 outpatients (48-103 years old) received physical examinations with complete medical histories. All the subjects had given written informed consent to participate in the study. TCH, TG, HDL-C and LDL-C levels were determined using the standard enzymatic colorimetric technique. BMI was used to indicate weight change. Subjects in the experiment had more than 12 years of education and similar life styles (a typical shanghainese diet, nearly no cigarette smoking or alcohol consumption). The categorical variant "age" was divided into three stratifications, ≤74 (66.89 ± 0.49), 75-to 84-year-old (80.12 ± 0.22) and ≥85 years (89.92 ± 0.24), and the degree of sclerotic change in fundus AS was graded as 0, I and II based on fundus photographs according to our previous study (18). Metabolic syndrome was described as "yes" or "no" according to the definition of the Joint Interim Statement (19). The Medical Ethics Committee of Huadong Hospital of Shanghai Medical College, Fudan University approved this study.

Study parameters

Co-morbidity profile

Participants in this study were asked whether they had a physician's diagnosis of the following 18 diseases that are classified as chronic based on the International Classification of Diseases (ICD): hypertension, dyslipidemia, obesity, diabetes, coronary heart disease, other heart diseases, venous insufficiency, stroke, epilepsy, hypothyroidism, hyperthyroidism, chronic renal disease, anemia, chronic pulmonary obstructive disease, liver disease, arthrosis, prostatic disease and cancer. Each recorded disease was added to generate a score ranging from 0 to 18.

Serum Cytokines

Serum from a 2-mL fasting peripheral blood sample was collected at 2000 x g for 10 minutes in the morning and rapidly stored at -80°C. The serum concentrations of cytokines were determined using Bio-Plex Human 6-Plex (IL-1β, IL-6, IL-4, IL-10, TNF-α and RANTES) kits and Bio-Plex Human 1-Plex (TGF-β) kits (Laboratories, Hercules, California, USA). To pre-wet the wells of a 96-well filter plate, coupled beads, serum samples, antibodies and streptavidin-PE (each 50 μL) were prepared and added to the wells one after another after two washes, according to the manufacturer's instructions. The samples were run in duplicate using a Bio-Plex MAGPIXTM (Bio-Rad Laboratories, Inc., US). The plate was read using Bio-Plex Manager software version 6.0. Objective concentration (pg/mL) was too low to measure, so fluorescence intensity was used as the relative concentration.

Lipid peroxidation and activity of antioxidant enzymes

The lipid peroxide (LPO) concentration in the serum was determined quantitatively by using an LPO Assay Kit (Nanjing Jianchen Bio, Jiang su, China) according to the protocol of the manufacturer. The product absorbance of a molecule of LPO and two molecules of chromogenic agent was read at a wavelength of 450 nm with a using TECAN Sunrise™ immediately after incubation at 45° C for 60 min. The concentration of LPO (μmol/L) was calculated according to a formula.

Superoxide dismutase (SOD) activity was determined using an SOD Water-Soluble Tetrazolium Salt Assay Kit (Nanjing Jianchen Bio, Jiangsu, China) according to the manufacturer's instructions. The corresponding SOD activity at 50% inhibition in the response system of 20 μL of SOD solution, 20 μL of serum samples and 200 μL of substrate was defined as one activity unit (U). The change in SOD activity was determined by measuring the absorbance at 450 nm using using TECAN Sunrise™.

Peroxidase (POD) activity was measured using a POD Assay Kit (Nanjing Jianchen Bio, Jiangsu, China). The corresponding POD activity of 1 μg of substrate (H_2O_2) in 1 mL of serum at 37°C for 1 min was defined as one activity unit (U). The change in POD activity was

determined by measuring the absorbance at 420 nm using using TECAN Sunrise™.

Catalase (CAT) activity was measured using a CAT Assay Kit (Nanjing Jianchen Bio, Jiangsu, China). Dissociation of 1 μmol of substrate (H2O2) in 1 mL of plasma at 370C for 1 min was defined as one activity unit (U). The chromogenic agent ammonium molybdate combined with surplus H2O2 substrate in the response system produced a yellow-colored product that was measured at 405 nm using a Hitachi F-2000 fluorescence spectrophotometer.

Glutathione peroxidase (GSH-px) activity was measured using a GSH-px Assay Kit (Nanjing Jianchen Bio, Jiangsu, China), according to the manufacturer's protocol. When 4 μL of whole blood reacted with the substrate (H2O2) at 370C for 5 min, a GSH concentration decline of 1 μmol/L in the response system was defined as one activity unit (U), after deducting the effect of the non-enzymatic response. GSH combined with the chromogenic agent dithiobisnitrobenzoic acid (DTNB) produced stable yellow five-glucosinolate two-nitro benzoic acid anions that were measured at 412 nm using a Hitachi F-2000 fluorescence spectrophotometer.

Serum apolipoproteins

The levels of different apolipoproteins were detected using an ELISA KIT (ApoE, ApoH; abcam, Cambridge, UK), an ApoJ kit (R&D) and a Milliplex 5–plex kit (ApoA I, ApoA II, ApoB, ApoC II, ApoC III) (Millipore, MA, US). For the ELISA test, serum samples were diluted into diluent at 1:400. Apolipoprotein standards or human apolipoprotein samples (50 μL) were added to each well of a microplate, and the assay was run according to the manufacturer's protocol. After finishing the experiments, the absorbance was immediately read on a microplate reader at a wavelength of 450 nm. The mean values of the duplicate or triplicate readings for each standard and sample were calculated, and a standard curve using eight standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis was generated. The best-fit line was determined by regression analysis using log-log or a four-parameter logistic curve-fit. The unknown sample concentration from the standard curve was multiplied by the dilution factor. The average intra- and inter-assay CVs were 4.6% and 7.4%, respectively. The minimum detectable dose of apolipoprotein was typically ~0.03 μg/mL, and the standard added value was 0.05-0.5 μg/mL. For the Milliplex 5–plex apolipoprotein assay, similar as for the Bio-Plex Human 6-Plex assay with Bio-Plex MAGPIXTM, the coupled beads, serum samples (5 μl) and antibodies were prepared according to the manufacturer's instructions. The samples were run in duplicate using the Luminex 200TM System (Luminex, Austin, US). The objective concentration unit was ng/mL.

Serum HsCRP

The serum HsCRP concentrations of the study subjects were determined with an Hs-CRP kit (Jun Shi Bioscientific, Shanghai, China) using an immunonephelometric assay that had been improved to provide greater sensitivity; this has been previously described in detail (20). The World Health Organization CRP reference standard was used. The intraassay and interassay coefficient of variation for this assay were ≤ 4.0% and ≤5.0%, respectively. The technicians were blinded to the case-control status of the samples. The normal value of Hs-CRP was < 0.3 mg/L. The study subjects with Hs-CRP ≥ 10 were considered to be suffering from an acute infection and were excluded from the study.

ApoE genotyping

Genomic DNA was extracted from peripheral whole blood samples using standard methods. Two single-nucleotide polymorphisms (SNPs; rs429358 and rs7412) were genotyped to identify APOE genotypes composed of the APOE ε2, ε3 and ε4 alleles using a SNaPshot mini-sequencing assay (21). As ε2 is regarded as a protective factor and ε4 is regarded as a risk factor (ε3 is regarded as neutral), persons with the ε2/4 genotype were removed from the sample as their inclusion might have weakened the contrast between ε2 and ε4, which was the primary interest of the study. For this investigation, the APOE group composition was as follows: ε2 = ε2/2 + ε2/3; ε3 = ε3/3; and ε4 = ε3/4 + ε4/4.

Statistical Analysis

To investigate the association between single categorical variables, such as sex, ApoE genotype, metabolic syndrome, the grade of fundus atherosclerosis and Hs-CRP, with age, a chi-squared test was used. Hs-CRP levels were stratified into <0.3, 0.3~1, 1-3 and ≥3. To investigate the association between single continuous variables, such as BMI, LPO or anti-oxidant enzymes, inflammatory cytokines, lipoproteins, apolipoproteins and number of co-morbidities, with age, an ANOVA (for Gaussian distributions) or Kruskal-Wallis test (for non-Gaussian distributions) was used. The most commonly missing values were those for variable SOD and accounted for 12.02% of the subjects. All significant associations of categorical and continuous variables with age were further analyzed using multivariate logistic regression analyses; these studied the same effects after adjusting for multiple covariates. A p value < 0.05 was considered statistically significant. All analyses were performed using SPSS 18.0 software.

Table 1
The associations between single categorical variables and age by univariate analysis (n = sample number)

		All n = 549	≤74 years old n = 176 (32.06%)	75- to 84-year-old n = 203 (36.98%)	≥85 years old n = 170 (30.97%)	p-value[a]
Sex	Male	489	162(33.1%)	182(37.2%)	145(29.7%)	0.125
	Female	60	14(23.3%)	21(35.0%)	25(41.7%)	
ApoE genotype	ε2	69	19(27.5%)	33(47.8%)	17(24.6%)	0.287
	ε3	380	124(32.6%)	138(36.3%)	118(31.1%)	
	ε4	71	28(39.4%)	25(35.2%)	18(25.4%)	
AS level	0	30	22(73.3%)	5(16.7%)	3(10.0%)	<0.001
	I	365	140(38.4%)	135(37.0%)	90(24.7%)	
	II	150	12(8.0%)	62(41.3%)	76(50.7%)	
Metabolic syndrome	No	365	125(34.2%)	118(32.3%)	122(33.4%)	0.004
	yes	179	49(27.4%)	84(46.9%)	46(25.7%)	
Hs-CRP	<0.3	157	59(37.6%)	56(35.7%)	42(26.8%)	0.043
	0.3~1	217	66(30.4%)	90(41.5%)	61(28.1%)	
	1~3	76	19(25.0%)	33(43.4%)	24(31.6%)	
	3~10	22	7(31.8%)	3(13.6%)	11(54.5%)	

a. Pearson chi-squared test

Results

Table 1 shows the demographic characteristics and percentages of three age stratifications. A univariate analysis of five categorical variables showed that metabolic syndrome ($p = 0.004$), the grade of fundus AS ($p < 0.001$) and the stratification of HsCRP ($p = 0.043$) had significant associations with age (Table 1). The 75- to 84-year-old group had a higher percentage of metabolic syndrome. The older individuals had higher grades of fundus AS and higher values of HsCRP. However, sex and ApoE genotype were not significantly associated with age (Table 1).

The continuous variables analyzed included five redox homeostasis, seven inflammatory, four lipoprotein and eight apolipoprotein parameters as well as the number of co-morbidities and BMI (Table 2). For these continuous variables, a univariate analysis showed that levels of the anti-oxidant enzymes POD ($p = 0.004$), SOD ($p = 0.003$) and GSH-px ($p = 0.018$) and the levels of the pro-inflammatory cytokine IL-6 ($p < 0.001$), apolipoprotein AII ($p < 0.001$), B ($p = 0.039$), CII ($p < 0.001$), CIII ($p = 0.002$) and H ($p = 0.008$), lipoprotein HDL-C ($p < 0.001$) and LDL-C ($p = 0.004$) and the number of co-morbidities ($p < 0.001$) were significantly associated with age (Table 2). Higher activities of anti-oxidant enzymes, including POD, SOD, lower activity of GSH-px, higher levels of the inflammatory cytokine IL-6, lower levels of ApoAII, B, CII and CIII, higher levels of ApoH, HDL-C and LDL-C and a greater number of co-morbidities were found in the older patients (Table 2). Other continuous variables, including the levels of LPO, CAT, IL-1beta, IL-4, IL-10, TNFalpha, TGFbeta, RENTS, ApoAI, ApoE, ApoJ, TCH, TG and BMI were not associated with age.

Fifteen variables significantly associated with age were further analyzed by multivariate logistic regression analyses (forward stepwise) to determine the main factors associated with age. The results indicated that grade of fundus atherosclerosis, number of co-morbidities, serum levels of HDL-C and GSH-px and grade of HsCRP were significantly associated with age (Table 3). Compared with individuals ≥85 years of age, individuals ≤74 years of age had significantly fewer co-morbidities [OR, 0.757 (95% CI, 0.636, 0.902)], lower grades of fundus AS [Grade 0: OR, 26.059 (95% CI, 4.705, 144.324)] and [Grade I: OR, 8.539 (95% CI, 3.555, 20.513)] and lower serum levels of HDL-C [OR, 0.127 (95% CI, 0.037, 0.433)] (Table 3). However, there were no significant differences in serum HDL-C levels and HsCRP grades between the two age groups. Compared with individuals ≥85 years of age, 75- to 84-year-old individuals had similar numbers of co-morbidities and similar grades of fundus AS, but 75- to 84-year-old patients had significantly lower serum levels of GSH-px [OR, 0.986, (95% CI, 0.972, 1.00)], HDL-C [OR, 0.158 (95% CI, 0.054, 0.457)] and HsCRP [Grade I: OR, 8.516 (95% CI,1.630, 44.484)], [Grade II: OR,7.699 (95% CI,1.544, 38.388)] and [Grade III: OR,7.251 (95% CI,1.346, 39.070)] (Table 3).

Table 2
The associations between single continuous variables and age by univariate analysis (n = sample number)

	All	≤74 years old		75- to 84-year-old		≥85 years old		p-value
	n	n	Median[b]	n	Medianb	n	Median[b]	
LPO	514	163	0.701(0.449, 0.977)	190	0.667(0.475, 0.928)	161	0.712(0.468, 1.088)	0.565
POD	521	166	7.716(5.525, 11.921)	194	7.716(5.648, 11.289)	161	9.074(6.358, 15.787)	0.004
SOD	483	154	10.071(8.787, 11.342)	186	10.573(8.780, 11.986)	143	10.785(9.600, 12.156)	0.003
CATa	505	163	52.119±0.980	193	51.731±0.774	149	52.066±1.084	0.947
GSH-px	494	158	72.922(62.277, 80.433)	190	68.648(42.239, 77.294)	146	69.623(55.601, 81.484)	0.018
IL-1beta	519	166	9.000(8.000, 10.000)	192	9.000(8.000, 10.000)	161	9.000(8.000, 10.000)	0.723
IL-4	518	166	9.000(8.000, 9.000)	192	8.000(8.000, 9.000)	160	8.000(8.000, 9.000)	0.063
IL-6	520	166	20.000(15.000, 27.625)	194	21.000(17.000, 29.000)	160	26.250(19.000, 40.750)	<0.001
Il-10	517	164	20.000(16.250; 25.000)	193	20.000(16.000, 24.750)	160	22.000(17.000, 26.000)	0.334
TNFalpha	520	166	8.000(7.000, 9.000)	193	8.000(7.000, 9.000)	161	8.000(7.000, 9.000)	0.444
TGFbeta	521	166	8478.75(539, 15721.125)	194	7623.25(453.5, 15302.13)	161	7877.0(2252.0, 13813.0)	0.664
RENTS	519	165	575(396, 847.25)	194	586.5(446.250, 804.750)	160	642.25(474.125, 877.00)	0.232
ApoAI	494	159	1.153e6 (9.857e5, 1.33e6)	182	1.12e6(9.531e5, 1 .288e6)	153	1.114e6(9.601e5, 1.31e6)	0.272
ApoAII	504	161	2.953e5(2.48e5, 3.768e5)	188	2.92e5(2.395e5, 3.487e5)	155	2.564e5(1.996e5, 3.19e5)	<0.001
ApoB	504	163	6.490e4(4.750e4, 8.32e4)	186	5.73e4(3.926e4, 8.160e4)	155	5.73e4(3.79e4, 7.570e4)	0.039
ApoCII	507	162	5.055e4(3.58e4, 7.393e4)	192	4.435e4(2.970e4, 6.92e4)	153	3.45e4(2.584e4, 5.508e4)	<0.001
ApoCIII	508	163	1.66e5(1.128e5, 2.392e5)	190	1.574e5(1.028e4, 2.27e5)	155	1.32e5(9.12e4, 1.926e5)	0.002
ApoE	525	168	51.79(38.962, 73.024)	196	56.214(40.807, 76.496)	161	56.696(42.598, 80.414)	0.196
ApoJ	525	168	140925(2568.2, 202690)	196	163699(2560.30, 206595)	161	159364(107898, 188486)	0.363
ApoH	526	168	362980(302895, 434305)	196	393830(328020, 464085)	162	386970(343345, 453390)	0.008
TCH[a]	548	176	4.783±0.062	203	4.574±0.064	169	4.643±0.071	0.071
TG	548	176	1.400(1.000, 1.900)	203	1.500(1.100, 1.900)	169	1.300(1.000, 1.750)	0.093
HDL-C	548	176	0.970(0.830, 1.158)	203	0.960(0.840, 1.110)	169	1.050(0.900, 1.315)	<0.001
LDL-C	548	176	2.990(2.513, 3.518)	203	2.740(2.270, 3.320)	169	2.750(2.145, 3.265)	0.004
BMI	547	176	24.300(22.800, 26.300)	202	24.500(22.500, 26.925)	169	23.500 (21.600, 26.600)	0.089
Number comorbidity	547	176	6.000(4.250, 7.000)	202	7.000(6.000, 8.000)	169	7.000(6.000, 8.000)	<0.001

a. For Gaussian distribution continuous data are expressed as mean±SEM, ANOVA was used; b. For non-Gaussian distribution continuous data are expressed as medians with 25% and 75% values in interquartile range. Kruskal-Wallis test was used.

Discussion

The present study demonstrates that age-dependent alterations in the number of co-morbidities, grade of fundus AS and the profile of systemic inflammation, inflammatory cytokines, oxidative products and anti-oxidative enzyme activities is a dynamic process. Compared with middle-aged patients (≤74 years old), long-living patients (≥85 years old) had high risks for a great number of co-morbidities and a higher grade of fundus AS. However, the oldest old patients had favorable lipid profiles, indicated by significantly high serum levels of HDL-C. Moreover, two populations had similar anti-oxidative enzyme activities, systemic inflammatory profiles and inflammatory cytokine levels (Table 3). Compared with older patients (75- to 84 -year-old), the oldest old patients had similar risks for the number of co-morbidities and the grade of fundus AS. However, the oldest old patients had significantly high serum levels of HDL-C and anti-oxidative enzyme activities. Meanwhile, the oldest old patients also had a significantly high risk for systemic inflammation (Table 3).

Our results confirm that the oldest old individuals have high anti-oxidative enzyme activities and high serum levels of HDL-C after adjusting for other parameters. The high serum anti-oxidative enzyme activities observed in the oldest old individuals were in agreement with the results of previous studies (2). In the elderly, the imbalance between pro-oxidants and enzymatic anti-oxidant systems due to antioxidant deficiencies increases their susceptibility to oxidative damage, which accelerates aging and age-related diseases. Genetic polymorphisms in the pro-/anti-oxidant pathway can influence physical and cognitive performance and the survival of nonagenarians (22). Dietary restriction and redox-based therapeutic intervention to reduce mitochondrial reactive oxygen species production and the global network

Table 3
Results from multivariate regression analyses (forward stepwise) predicting age-related alteration (adjusting for single categorical variables, sex, ApoE genotype, metabolic syndrome; single continuous variables BMI, LPO other anti-oxidant enzymes, inflammatory cytokines, lipoproteins apolipoproteins)

	≤74 years old[a]		75- to 84-year-old[a]	
	OR (95% CI) P-Value	Global P-Value	OR (95% CI) P-Value	Global P-Value
GSHpx	1.002(0.985, 1.019)	0.813	0.986(0.972, 1.000)	0.043
Hs CRP I	4.005 (1.019, 15.736)	0.047	8.516 (1.630, 44.484)	0.011
Hs CRP II	1.654(0.441, 6.200)	0.456	7.699 (1.544, 38.388)	0.013
Hs CRP III	1.300(0.295, 5.724)	0.729	7.251 (1.346, 39.070)	0.021
Hs CRP IV	0[b]	-	0[b]	-
HDL-C	0.127 (0.037, 0.433)	0.001	0.158 (0.054, 0.457)	0.001
Number of co-morbidity	0.757 (0.636, 0.902)	0.002	0.939 (0.802, 1.099)	0.434
AS level 0	26.059(4.705, 144.324)	<0.001	1.472 (0.221, 9.787)	0.689
AS level I	8.539 (3.555, 20.513)	<0.001	2.232 (1.226, 4.062)	0.009
AS level II	0[b]	-	0[b]	-

a. Reference class: ≥ 85 years old; b.Parameter redundancy and setting is 0.

of oxidative stress can counteract the progression of aging and extend the life span of animals (23, 24). A recent German population-based cohort study showed that reactive oxygen species concentration and redox control status were associated with all-cause mortality adjusted for age, sex, education, smoking, physical activity and alcohol consumption (hazard ratios and 95% confidence intervals: 1.63 [1.01; 2.63] and 0.68 [0.53; 0.87], respectively). The researchers supposed that systemic inflammation and higher co-morbidity could be intermediate states on the pathway from high reactive oxygen species concentration to mortality (25). A high serum level of HDL-C was a characteristic feature of longevity and was associated with better survival in frail, community-living elderly (9, 13). A progressive decline in plasma HDL was associated with elevated CRP and IL-6 levels (10). The high plasma levels of HDL-C in the oldest old individuals in our study were also consistent with the results of previous studies (9, 13).

We also demonstrated that the oldest old individuals have high risks for a greater number of co-morbidities, higher grade of fundus AS and systemic inflammation, after adjusting for other parameters. Because the number of abnormal systems has been shown previously to be more predictive of pathological aging than abnormalities in any particular system (26), our study included 18 chronic diseases in more than eight systems. Thus, the greater the number of co-morbidities could reflect a greater degree of aging. Fundus microcirculation changes associated with hypertension, atherosclerosis and increased risk of stroke and myocardial infarction (27). Systemic inflammation was associated with normal aging and coronary atherosclerosis (5). Oxidative stress and chronic inflammation were positively correlated with the grade of sclerotic change in the fundus oculi, promoting atherosclerosis in the retinal arteries (28). In our study, the risk of metabolic syndrome increased with age, but metabolic syndrome was not significantly association with age after adjusting for other factors (Table 1). Chronic subclinical inflammation was associated with poorer physical function in older adults with various co-morbidities (29). Therefore, the oldest old patients showed an increase in age-associated diseases and adverse clinical prognoses.

ApoE genotype, plasma levels of inflammatory cytokines and apolipoprotein, may also play important roles in aging. Our results showed that the older patients had a higher level of inflammatory cytokine IL-6, lower levels of ApoAII, B, CII and CIII, and higher levels of ApoH and LDL-C (Table 2). However, after adjusting for other factors, these age-related differences were not significant. Although the ApoE polymorphism is involved in lipid metabolism regulation, and ApoE ε4 allele frequencies were high in patients with several age-related illnesses (10, 14), ApoE ε4 allele frequencies were low in the the oldest old population (11). Our study showed that there was no significant difference in the age-related distribution of ApoE alleles.

The study provides valuable information to develop biomarkers of aging. The missing values of the study parameters accounted for less than 12% of the total study subjects. The main limitation of our study was its relatively small sample size, and particularly the low number of females, which weakens the validity of our results. The ruaral citizens is scarce in our sample was another limitation. Furthermore, as this was a cross-

sectional study, the associations of adverse prognoses, such as disability and death, with changes in the study parameters could not be determined.

In conclusion, the oldest old patients had significantly high anti-oxidative enzyme activities and serum HDL-C levels. Meanwhile, these patients also had significantly high numbers of co-morbidities, high grades of fundus AS and high systemic inflammation, which might predict the degree of aging.

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Ethical standards: The procedures followed in the manuscript were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

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