A preliminary candidate approach identifies the combination of chemerin, fetuin-A, and fibroblast growth factors 19 and 21 as a potential biomarker panel of successful aging

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Abstract Although the number of centenarians is growing worldwide, the potential factors influencing the aging process remain only partially elucidated. Researchers are increasingly focusing toward biomarkers as tools to shed more light on the pathophysiology of complex phenotypes, including the ability to

reach successful aging, i.e., free of major chronic diseases. We therefore conducted a case-control study examining the potential associations of multiple candidate biomarkers in healthy centenarians and sex-matched healthy elderly controls. Using a case-control study of 81 centenarians (aged ≥ 100 years) selected based on the fact that they were disease-free and 46 healthy elderly controls (aged 70–80 years), serum levels of 15 different candidate biomarkers involved in the regulation of metabolism, angiogenesis, inflammation, and bone formation were measured. Of the 15 biomarkers tested, four molecules (chemerin, fetuin-A, and fibroblast growth factors [FGF] 19 and 21) were found to be independently associated with successful aging regardless of sex. Logistic regression analysis confirmed that chemerin, fetuin-A, FGF19, and FGF21 were independently associated with successful aging [predicted probability (PP) = $1/[1+1/\exp(11.832 - 0.027 \times (\text{chemerin}) - 0.009 \times (\text{fetuin-A}) + 0.014 \times (\text{FGF19}) - 0.007 \times (\text{FGF21})]$]. The area under the curve (AUC) of predicted probability values for the four-biomarker panel revealed that it can discriminate between centenarians and elderly controls with excellent accuracy (AUC > 0.94, $P < 0.001$). Although preliminary in essence and limited by the low sample size and lack of replication in other independent cohorts, our data suggest an independent association between successful aging and serum chemerin, fetuin-A, FGF19, and FGF21, which may provide novel information on the mechanisms behind the human aging process. Whether the four-biomarker panel may predict successful aging deserves further scrutiny.

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Introduction

Studies of long-lived humans can give important clues to the possible factors and mechanisms that may promote healthy lifespan (Gavrilova and Gavrilov 2010; Leeson 2014). In this context, research on people living 100 years and beyond has recently gained momentum (Ailshire et al. 2014; Robert et al. 2014). Centenarians are not only the survival tail of the population but also a model of successful aging, because they have usually postponed—if not avoided—age-related diseases and their fatal consequences (Christensen et al. 2008). Moreover, they have also frequently delayed the onset of disability until they were well into their mid-nineties (Terry et al. 2008).

Although the number of centenarians is growing worldwide, the potential factors influencing successful aging (i.e., free of major chronic diseases) remain only partially elucidated (Gavrilova and Gavrilov 2010; Robert and Fulop 2014). In an effort to shed more light on the pathophysiology of complex phenotypes involved in the aging process, including the ability to reach advanced ages free of major diseases, researchers are increasingly focusing on biomarkers. In this regard, several studies have recently measured serum levels of candidate biomarkers potentially associated with the status of reaching 100+ years of age. Such molecules include adiponectin (Bik et al. 2013), soluble receptor for advanced glycation end-products (Geroldi et al. 2006), irisin (Emanuele et al. 2014a), beclin-1 (Emanuele et al. 2014b), and different inflammatory mediators (Gerli et al. 2000; Gangemi et al. 2003; Basile et al. 2012).

Knowledge derived from biomarker studies of centenarians may give new insights into the biology of the aging process and identify new targets of the anti-aging armamentarium. Herein, we performed an evaluation of 15 candidate biomarkers related to four major pathophysiological pathways (metabolism, angiogenesis, inflammation, and bone formation) that may be implicated in major human age-related diseases. All biomarkers were assayed in serum samples of healthy centenarians and younger controls under a case-control design.

Materials and methods

Participants

The group of cases and controls was composed by 81 healthy centenarians (40 men; mean age 101 ± 1 years, age range 100–104 years) and 46 healthy younger elderly people (24 men; mean age 75 ± 3 years, age range 70–80 years), respectively. The participant ages were defined by the dates of birth as stated on identity cards. All patients and controls were Caucasian whites ascertained to be of Italian descent (Northern Italy, mainly from Lombardy and Piedmont). The centenarians were ascertained mainly via general practitioners in the community; they represent a convenience sample that has been previously described (Emanuele et al. 2014a, b). The history of past and current diseases was accurately collected, checking the centenarians' medical documentation and the current drug therapy. Accordingly, all the Italian centenarians were free of major age-related diseases, i.e., severe cognitive impairment, clinically evident cancer, cardiovascular disease, renal insufficiency, or severe physical impairment. Only part of this group had decreased visual or auditory acuity. Consequently, all the centenarians were in good health relative to their very advanced age. Controls were in apparent good physical health, with exclusion criteria being as follows: presence of cardiovascular disease or cerebrovascular disease, cancer, dementia, chronic autoimmune or inflammatory disorders, renal or hepatic failure, and major psychiatric disorders. Sex-matched controls were randomly selected from elderly subjects who participated in previous research as healthy comparison subjects (Emanuele et al. 2011). The study protocol complied with the tenets of the Declaration of Helsinki and was approved by the local ethics committee. All study participants provided their written informed consent.

Biomarkers

We selected 15 candidate biomarkers related to four major pathophysiological pathways (metabolism, angiogenesis, inflammation, and bone formation) that may be thus implicated in the main age-related diseases. The details and pathophysiological significance of the selected biomarkers are summarized in the Supplementary file 1. The four biomarker clusters were as follows. The metabolism cluster included adiponin,

apelin, chemerin, fibroblast growth factor (FGF) 19, FGF21, obestatin, omentin-1, zinc- α 2-glycoprotein (ZAG), and pigment epithelium-derived factor (PEDF). The angiogenesis pathway cluster included vascular endothelial growth factor (VEGF)-A and VEGF receptor-1 (sFlt-1). The inflammation pathway included fetuin-A and progranulin. Finally, the bone formation pathway included osteocalcin (OC) and osteoprotegerin (OPG). All biomarkers were measured in venous blood samples drawn after an overnight fast. Separated serum was frozen in aliquots at -20°C . Serum levels of biomarkers were assessed using commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's protocol. Details for each ELISA are available from the authors upon request. For all assays, the intra- and inter-assay coefficients of variation were less than 7 % and less than 9 %, respectively. Each sample was analyzed in duplicate, and the mean value of the two measures was used for the analyses. Laboratory personnel were blinded with regard to case-control status.

Statistical analysis

All statistical analyses were performed with the IBM SPSS 22.0 package for MAC (SPSS, Inc., Chicago, IL) except calculation of statistical power (see below). Descriptive data are expressed as means $\pm$ SD or as medians and ranges for skewed variables. An independent sample Student's *t* test (or its nonparametric equivalent, the Mann-Whitney *U* test) was used to compare serum biomarker levels. To minimize the risk of statistical error type I, analyses were corrected for multiple comparisons using the stringent Bonferroni's method, in which the threshold *P* value is obtained by dividing 0.05 by the number of comparisons, i.e., $n=15$, corresponding to the 15 biomarkers we studied (thus, threshold *P* value <0.003). Statistical post hoc power analysis of between-group comparisons (with $\alpha=0.05$, two-tailed) was calculated using the G*Power 3 program for MAC (Faul et al. 2007). We also estimated the optimal sample size to obtain a statistical power ≥ 90 % for detecting differences between group means (with $\alpha=0.05$, two-tailed).

Receiver operating characteristic (ROC) curves were calculated to determine the optimal cutoff point for the association with the likelihood of being a centenarian. The optimal cutoff point from ROC plots was determined by the Youden index, and we calculated areas

under the curve (AUC) and 95 % confidence interval. "Perfect" classification is represented by an AUC of 1, whereas an area of 0.5 represents a complete absence of classification. AUC values of ≥ 0.90 are considered to be "excellent," whereas values between 0.80–0.90, 0.70–0.79, and <0.70 are considered to be "good," "fair," and "poor," respectively (Trost et al. 2012). Logistic regression analysis with the forward conditional selection method was conducted in order to find a combination of biomarkers that would be potentially associated with successful aging. The predicted probability (PP) value was calculated in each logistic regression analysis.

Results

Differences in serum biomarkers between centenarians and elderly controls

In univariate analyses, significant intergroup univariate differences were found for chemerin, fetuin-A, FGF19, FGF21, OC, OPG, PEDF, progranulin, and sFlt1, with centenarians presenting lower mean values of fetuin-A, FGF21, PEDF, and progranulin, and higher values of FGF19, OC, OPG, and sFlt1 (all $P<0.001$, Table 1). Of these between-group differences, all reached a statistical power ≥ 98 %, except for OC (power=52 %). The between-group differences in fetuin, FGF19, FGF21, progranulin, and sFlt1 were confirmed when both groups were compared separately by sex (Supplementary file 2).

Multivariate analysis

Of the 15 molecules tested, the results of the logistic regression analysis identified the combination of four biomarkers (chemerin, fetuin-A, FGF19, and FGF21) as a significant predictor variable associated with successful aging regardless of sex (Table 2). The equation defined by the analysis was as follows: predicted probability (PP) = $1/[1 + \exp(11.832 - 0.027 \times (\text{chemerin}) - 0.009 \times (\text{fetuin-A}) + 0.014 \times (\text{FGF19}) - 0.007 \times (\text{FGF21})]$.

ROC curve analysis

The optimal cutoff values, AUC, sensitivity, and specificity of the four biomarkers independently associated with successful aging (either alone or in combination) are shown in Table 3 and Fig. 1. The best individual

Table 1 Serum levels of biomarkers (mean±SD; median and range) by group

	Biomarker	Centenarians (n=81)	Controls (n=46)	P-value	Statistical power*	Optimal total sample size (n) **
Metabolism	Adipsin (ng/mL)	5329.5±1333.9 <i>5182.0 (3116, 8675)</i>	5660.6±1609.3 <i>5443.5 (3352, 10043)</i>	0.223	22%	952
	APLN (ng/mL)	1.3±0.7 <i>1.2 (0.0, 3.7)</i>	1.4±0.7 <i>1.12 (0.6, 3.3)</i>	0.266	12%	2060
	Chemerin (ng/mL)	159.4±43.0 <i>154.7 (95.3–334.4)</i>	219±65.6 <i>204.8 (119.4, 445)</i>	<0.003	+99%	44
	FGF19 (pg/mL)	276.2±187.7 <i>212.2 (70.4, 920.8)</i>	150.9±74.2 <i>126.4 (73.9, 369.0)</i>	<0.003	+99%	94
	FGF21 (pg/mL)	138.6±101.2 <i>93.4 (59.5, 482.7)</i>	305.0±237.1 <i>236.8 (59.5, 947.7)</i>	<0.003	99%	16
	Obestatin (ng/mL)	4.0±1.7 <i>3.6 (0.1, 11.6)</i>	4.4±3.2 <i>3.5 (2.1, 19.5)</i>	0.417	12%	760
	Omentin (ng/mL)	376.2±196.4 <i>322.8 (66.1, 1156.7)</i>	459.2±151.4 <i>426.7 (122.6, 804.5)</i>	0.016	70%	216
	PEDF (µg/mL)	1.5±0.5 <i>1.5 (0.5, 3.3)</i>	2.0±0.5 <i>2.1 (1.1, 3.0)</i>	<0.003	+99%	50
	ZAG (µg/mL)	75.2±48.9 <i>66 (36.8, 367.5)</i>	67.2±26.3 <i>61.1 (38.9, 218.8)</i>	0.308	22%	1570
Angio- genesis	sFlt1 (ng/mL)	1.6±0.6 <i>1.5 (0.7–4.1)</i>	1.2±0.3 <i>1.2 (0.7, 2.2)</i>	<0.003	+99%	70
	VEGFA (pg/mL)	1984.6±945.1 <i>1873.9 (379–4825)</i>	1926.3±903.1 <i>1784.8 (567, 4687)</i>	0.738	5%	11160
Infla- mmation	Fetuin-A (µg/mL)	800.5±132.4 <i>814.6 (504.3, 1081.1)</i>	939.9±126.0 <i>941.5 (687.3, 1232.2)</i>	<0.003	+99%	44
	Progranulin (ng/mL)	27.9±6.5 <i>25.6 (16.7, 46.9)</i>	35.9±12.2 <i>34.7 (9.1, 63.4)</i>	<0.003	99%	74
Bone formation						
	OC (ng/mL)	2.4±0.2 <i>2.3 (2.0, 2.9)</i>	2.3±0.3 <i>2.2 (1.9, 4.1)</i>	<0.003	52%	168
	OPG (pg/mL)	114.8±38.2 <i>116.4 (39.5, 209.2)</i>	81.3±47.4 <i>80.1 (2.9, 205.2)</i>	<0.003	98%	40

Data following a normal distribution are in italics. Gray background denotes combination of statistical significance + high statistical power (i.e., >90 %). P values below the threshold (0.003) as well as statistical power values >90 % are in bold

APLN apelin, FGF21 fibroblast growth factor 21, FGF19 fibroblast growth factor 19, OC osteocalcin, OPG osteoprotegerin, PEDF pigment epithelium-derived factor, sFlt-1 vascular endothelial growth factor receptor 1, VEGF-A vascular endothelial growth factor A

^a Statistical post hoc power analysis to detect differences between group means with a significance level (α) of 0.05 (two-tailed)

^b Estimated optimal sample size to obtain a statistical power ≥ 90 % to detect difference between group means with a significance level (α) of 0.05 (two-tailed)

serum biomarker of successful aging among the 15 tested molecules was FGF19. At an optimal cutoff value of 178.2 pg/mL, FGF19 displayed an AUC of 0.73, with a sensitivity of 62 % and a specificity of 83 %, being a “fair” predictor of EL. Notably, the combination of the four independent biomarkers (chemerin, fetuin-A, FGF19, and FGF21) performed significantly better than each biomarker alone ($P<0.001$) and had excellent

discriminatory power for successful aging (AUC=0.95, sensitivity=95 %, specificity=80 %; Table 3).

Discussion

In this study, we evaluated the potential association between several candidate biomarkers and successful

Table 2 Logistic regression analysis for biomarkers independently associated with successful aging regardless of sex

	Coefficient (β)	<i>P</i> value
Chemerin (ng/mL)	-0.027	<0.001
Fetuin-A (μ g/mL)	-0.009	<0.001
FGF19 (pg/mL)	0.014	<0.001
FGF21 (pg/mL)	-0.007	<0.001
Constant	11.832	<0.001

FGF19 fibroblast growth factor 19, *FGF21* fibroblast growth factor 21

aging. Because biomarkers frequently show an insufficient discrimination power when analyzed alone (Nolen et al. 2010), we also hypothesized that a combination of biomarkers could increase the ability to discriminate between centenarians and elderly controls. Among the 15 biomarkers tested in this study, the best individual biomarker of successful aging was FGF19. Notably, we also demonstrated that a combination of chemerin, fetuin-A, FGF19, and FGF21 performed significantly better than individual markers alone and had a very high discriminatory power for distinguishing centenarians from controls. The innovative nature of the present study conducted in centenarians is the use of a targeted biomarker approach focusing on a set of serum biomarkers which allowed a multimarker-based discrimination of subjects who reach successful aging from elderly controls.

Herein, two members of the FGF family, FGF19 and FGF21, were found to be associated with human successful aging. Specifically, when considered at an individual level, FGF19 was the most significant serum marker of successful aging (AUC=0.73, sensitivity=62 %, specificity=83 %), with its levels being significantly higher in centenarians than in the controls

(optimal cutoff value=178.2 pg/mL). Interestingly, the mean levels of this biomarker in our centenarians (276.2 ± 187.7 pg/mL) were also higher than those reported with ELISA methodology in the serum of old Chinese controls (aged 66.4 ± 10.1 years, free of coronary artery disease), i.e., 188.0 pg/mL (105.1–284.7) (Hao et al. 2013) but comparable to those reported under the abovementioned conditions in younger healthy people from China (aged 40.8 ± 8.8 years), i.e., 289 pg/mL (224–393) (Fang et al. 2013) or Turkey (aged 49.5 years [38–54]), i.e., 293.5 pg/mL (153.6–370.3) (Barutcuoglu et al. 2011). FGF19 is mainly produced by the ileum and has recently emerged as a master regulator of several different metabolic activities (including glucose and lipid metabolism) via activation of downstream signaling pathways through its binding with FGF receptors (Kir et al. 2011; Wu et al. 2011). Because low FGF19 levels have been described in several metabolic disorders (Wojcik et al. 2012; Wang et al. 2013), one possibility to explain the link between this molecule and aging may be via its role in metabolic regulation and its interaction with central signaling systems that respond to nutritional inputs. Notably, FGF19 signaling activity strictly requires beta-Klotho (Tomiya et al. 2010), a member of the Klotho family proteins which has been shown to play a paramount role in regulating aging and lifespan (Kuro-o 2010). Beta-Klotho may also participate in the control of aging by acting as a cofactor to mediate the key effects of FGF21 on metabolic changes occurring during calorie restriction, a phenomenon known to prolong lifespan (Adams et al. 2012). Accordingly, in our study FGF21 was identified as another independent biomarker of successful aging. On the other hand, levels of FGF21 were found to be significantly lower in our centenarians, a finding that may seem paradoxical given previous experimental observations suggesting that transgenic overexpression of

Table 3 Receiver operating characteristic (ROC) results for biomarkers independently associated with successful aging

Biomarker	Optimal cutoff	AUC	95 % CI	<i>P</i> value	Standard error	Sensitivity (%)	Specificity (%)
Chemerin	321.37 ng/mL	0.19	0.11–0.27	<0.001	0.04	1	93
Fetuin-A	1042.41 μ g/mL	0.23	0.15–0.32	<0.001	0.04	1	85
FGF19	178.2 pg/mL	0.73	0.63–0.82	<0.001	0.05	62	83
FGF21	65.14 pg/mL	0.28	0.18–0.38	<0.001	0.05	93	11
Four-biomarker panel	0.43	0.95	0.90–0.98	<0.001	0.02	95	80

CI confidence interval, *AUC* area under the curve, *FGF19* fibroblast growth factor 19, *FGF21* fibroblast growth factor 21

Fig. 1 Comparison of results of receiver operating characteristic (ROC) curves for the combination of biomarkers associated with successful aging in both sexes (chemerin, fetuin-A, FGF19, and FGF21) versus individual serum markers. *FGF21* fibroblast growth factor 21, *FGF19* fibroblast growth factor 19

FGF21 markedly extended lifespan in mice and significantly delayed age-related mortality even without reducing food intake (Zhang et al. 2012). However, emerging evidence in humans indicates that FGF21 levels increase with a counter-regulatory mechanism in compensation to metabolic stress, including states of obesity and insulin resistance (Woo et al. 2013). It may be hypothesized that centenarians could have low basal levels of metabolic stress, being therefore reflected by reduced FGF21 concentrations. Another possibility is that people who reach successful aging could be highly sensitive and respond in a markedly efficient manner to endogenous FGF21. Further studies are needed to investigate this possibility.

Chemerin—an adipokine that induces insulin resistance, exacerbates glucose intolerance, and decreases glucose uptake in skeletal muscle (Fatima et al. 2014)—was another molecule whose levels were found to be lower in centenarians compared with their younger controls. Recently, exercise-induced lowering of chemerin has been associated with reduced cardiometabolic risk and glucose-stimulated insulin secretion in older adults (Malin et al. 2014). Similarly, circulating chemerin has been shown to decrease in response to a combined strength and endurance training (Stefanov et al. 2014). Because physical activity may increase life expectancy (Gremeaux et al. 2012), further studies are needed to investigate the relationships between exercise, serum chemerin levels, and the aging process. Finally, levels of fetuin-A, a negative acute phase reactant

(Heinrichsdorff and Olefsky 2012), were found to be lower in the sera of centenarians compared with controls. Notably, high fetuin-A has been associated with bone mineral density, metabolic syndrome, obesity, insulin resistance, and mortality in elderly populations (Mori et al. 2012; Rasul et al. 2012). Moreover, it has been demonstrated that fetuin-A knockout mice are protected against age-associated obesity and insulin resistance (Mathews et al. 2006). Mechanistic research is needed to gain insights into the potential benefits of lower fetuin-A levels in the longest-lived humans.

Several limitations inherent in the current study merit comments. Based on our study design, serum levels of chemerin, fetuin-A, FGF19, and FGF21 can be only preliminarily defined as potential biomarkers of successful aging. Evidence for a causal relationship and clarification of the mechanism of the associations between these molecules and the human aging process will require longitudinal follow-up studies and replication in independent, large cohorts. Our study is preliminary because the sample size was small, and the results cannot be generalized beyond Italian centenarians; nonetheless, the findings are provocative and suggest that further assessment of the relationship between serum chemerin, fetuin-A, FGF19, and FGF21 levels and the human aging process is needed, ideally using longitudinal follow-up designs. Conversely, the main strength of our study is the inclusion of centenarians without major comorbidities, who represent an ideal model of successful human aging.

In summary, our preliminary findings—which suggest an independent association between successful aging and serum chemerin, fetuin-A, FGF19, and FGF21—may provide novel information on the mechanisms behind the human aging process. Specifically, the present report suggests that a targeted biomarker profiling may potentially allow identifying people who reach successful aging. The current findings may pave the way for larger studies testing the performance of the identified biomarker panel to predict the likelihood of reaching 100+ years of age. An investigation aimed at establishing whether the identified biomarkers may be associated with mortality in elderly control subjects enrolled in this study is currently underway.

Conflicts of interest None declared. All listed authors have made a significant research contribution to the study and approved the submission.

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