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Human Aging and Longevity Are Characterized by High Levels of Mitokines

Maria Conte, PhD,[1,2,*] Rita Ostan, PhD,[1,2,*] Cristina Fabbri, PhD,[1] Aurelia Santoro, PhD,[1,2] Giulia Guidarelli, MStat,[1] Giovanni Vitale, MD,[3,4] Daniela Mari, MD,[5] Federica Sevini, MSc,[1] Miriam Capri, PhD,[1,2] Marco Sandri, MD,[6,7] Daniela Monti, PhD,[8] Claudio Franceschi, MD,[9] and Stefano Salvioli, PhD[1,2]

[1]Department of Experimental, Diagnostic and Specialty Medicine (DIMES) and [2]Interdepartmental Centre "L. Galvani" (CIG), University of Bologna. [3]Department of Clinical Sciences and Community Health, University of Milan. [4]Laboratory of Geriatric and Oncologic Neuroendocrinology Research, Istituto Auxologico Italiano IRCCS, Cusano Milanino. [5]Geriatric Unit, Fondazione Ca' Granda, IRCCS Ospedale Maggiore Policlinico, Milan. [6]Venetian Institute of Molecular Medicine, Padova. [7]Department of Biomedical Science, University of Padova, Padova. [8]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence. [9]IRCCS, Institute of Neurological Sciences of Bologna, Bologna, Italy.

Address correspondence to: Maria Conte, PhD, Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Via San Giacomo 12, 40126 Bologna, Italy. E-mail: m.conte@unibo.it

*These authors contributed equally to this work.

Abstract

Mitochondrial stress elicits the production of stress response molecules indicated as mitokines, including fibroblast growth factor 21 (FGF21), growth differentiation factor 15 (GDF15), and humanin (HN). Many diseases are characterized by progressive mitochondrial dysfunction with alterations of mitokine secretion. It is still controversial whether healthy aging and extreme longevity are accompanied by an altered production of mitokines. We analyzed FGF21, HN, and GDF15 plasma levels in 693 subjects aged from 21 to 113 years, and the association of these mitokines with parameters of health status. FGF21, HN, and GDF15 resulted increased in old age, with the highest levels found in centenarians. These molecules are associated with worsened parameters (such as handgrip strength, insulin sensitivity, triglycerides), particularly in 70-year-old persons, and their levels are inversely correlated with survival in the oldest subjects. Considering the positive biological effect of these molecules, our results can be interpreted in the framework of the hormetic paradigm as an attempt of the cells/tissues to cope with a stress that can have beneficial or detrimental effects depending on its intensity. Finally, persons with Down Syndrome (characterized by accelerated aging) have higher levels of GDF15 and HN with respect to their siblings, suggesting that these molecules, especially GDF15, could be considered markers of biological age.

Keywords: FGF21, Humanin, GDF15, Centenarians, Mortality

Over the past years, a great number of studies have been devoted to clarify the cellular and molecular mechanisms of human aging. Among these mechanisms, mitochondrial dysfunction and reactive oxygen species (ROS) production have been long considered as one of the principal causes of aging (1,2). However, more recently, this theory has been questioned by data showing that the complete elimination of oxidative stress actually does not extend life span in animal models (3,4), while a mild mitochondrial dysfunction may contribute to an increase of life span (5,6). This beneficial effect of mitochondrial dysfunction is mediated by increased mitophagy and a series of stress response mechanisms including the mitochondrial variant of unfolded protein response (UPR^{mt}). These responses can elicit protective effects not only in the affected cell but also in distant cells/tissues. As an example, in *Caenorhabditis elegans*, the induction

of UPR^{mt} in the nervous system activates the same response in the intestine, leading eventually to a positive effect on life span (7). Likewise, in *Drosophila melanogaster*, the moderate knock-down of some electron transport chain components in specific tissues, such as brain or muscle, is sufficient to promote organismal longevity (8,9). Interestingly, a threshold effect has been noticed, where moderate dysfunction resulted in life extension, while strong dysfunction was detrimental (5,8). The mitochondrial defects that can activate the retrograde response range from autophagy defects (eg, Atg7 disruption), deletion of mitofusin 1 and 2 leading to disorganised mitochondria (10), to inhibition of respiratory complex I (11). This distal response can delay the aging process and elicit life span extension in animal models, including rodents, likely by improving systemic viability through the coordination of cellular stress responses (8,12–14). Dillin and coworkers hypothesized that the spreading of this response to distal tissues occurs via soluble factors indicated as "mitokines" (7). In fact, a number of mitokines produced and secreted in response to a mitochondrial stress have been identified. Some of these mitokines are nuclear encoded and are expressed in response to UPR^{mt}. These include, among others, fibroblast growth factor 21 [FGF21; (10)] and growth differentiation factor 15 [GDF15; (15)].

FGF21 is a hormone-like member of FGF family involved in metabolic processes, also described as stress hormone for its role as a potent longevity factor (16). Several findings demonstrate that the overexpression of FGF21 improves healthspan and that FGF21 is normally secreted from several tissues in response to an acute local stress. Higher levels of circulating FGF21 attenuate acute metabolic disorders and collaborate to extend life span (16,17). Despite its multiple beneficial effects, other studies indicate that circulating levels of FGF21 are elevated in several metabolic diseases, such as type 2 diabetes (18). Moreover, when chronically expressed, FGF21 increases with age in humans and contributes to precocious aging and premature death in mice (19,20). In this context, whether FGF21 is beneficial or detrimental is still debated.

GDF15 is a member of the transforming growth factor (TGF)-β family, produced by several tissues. It is considered as an indicator of mitochondrial dysfunction in muscle (21), and it has cardioprotective and neuroprotective activity. GDF15 regulates appetite and energy metabolism under both physiological and pathological conditions, possibly through a modulation of mitochondrial functions, as it can affect mitochondrial biogenesis, thermogenesis, and fatty acid metabolism (22). Interestingly, mice overexpressing human GDF15 display increased life span (23). However, it is also known that circulating levels of GDF15 are associated with cardiovascular diseases, insulin resistance and type 2 diabetes, neurodegeneration, and overall mortality (24,25). It has been therefore speculated that GDF15 can be secreted as a consequence of various cellular stresses and dysfunctions and might compensate for mitochondrial dysfunction during aging and age-related diseases, as well as in mitochondrial diseases (22). Like FGF21, also for GDF15, the biological significance of this molecule is not totally clear. Several observations, in fact, indicate that GDF15 could have a positive or a negative role depending on the state of the cell and its environment (24).

In addition to nuclear-encoded mitokines, it is known that a number of peptides encoded by mitochondrial DNA, including humanin [HN; (26,27)], mitochondrial open reading frame of the 12S rRNA-c [MOTS-c; (28)] and six additional peptides encoded in the same mitochondrial DNA region, indicated as small humanin-like peptides [SHLPs; (29)], are likely produced in response to mitochondrial stress (30) and have anti-apoptotic and neuroprotective effects. HN is the first mitochondrial-derived peptide secreted in plasma described for its cytoprotective role in several age-related diseases, such as Alzheimer's disease, diabetes, hepatic steatosis, atherosclerosis, and cardiovascular diseases. However, the role of HN in aging and age-related diseases is not completely understood.

A progressive mitochondrial dysfunction is known to occur with age (31–33). In particular, we have reported that dermal fibroblasts from centenarians but not from 70-year-old subjects display a compensated mitochondrial dysfunction, characterized by decreased complex I-driven ATP synthesis and increased H_20_2 production (34). It is presently unknown whether such a decreased functionality is sufficient to trigger a rise in the level of mitokines, and, more in general, the correlation between mitokine production, aging, and longevity in humans has not been deeply explored. We reasoned that centenarians, that is, the best example of extreme longevity and successful aging, could be characterized by a peculiar expression of mitokines. To test this hypothesis, we measured the circulating levels of the previously described mitokines, FGF21, GDF15, and HN in the plasma of about 693 volunteers of different age, from 21 to 113 years. Moreover, we evaluated the association between mitokines and a series of parameters related to health status and phenotype, and we analyzed the survival of >90-year-old persons according to their levels of mitokines.

Materials and Methods

Subjects

A total of 693 subjects in the age range from 21 to 113 years were recruited and divided into four age groups: 79 young/adult subjects (YA, age range 21–59 years), 336 elderly (EL, age range 61–79 years), 155 oldest old (OO, age range 82–99 years) and 123 centenarians (CENT, age range 100–113 years; Supplementary Table 1). All subjects were enrolled in Italy in the framework of the following projects: Italian National Project PRIN06 and PRIN09 for EL and centenarian subjects, EU Project GEHA for oldest old subjects, EU Project NUAGE for EL.

A total of 28 sibling pairs constituted by a person with Down syndrome (age range 11–43 years) and a nontrisomic sibling (age range 8–52 years) were collected in the framework of an Italian project on intellectual disability supported by CARISBO Foundation. The study protocols were approved by the Ethical Committee of Sant'Orsola-Malpighi University Hospital (Bologna, Italy). All subjects signed the informed consent before blood withdrawal, functional and anthropometric measurement, and interviews (health status, clinical anamnesis). A standard questionnaire was administered by trained physicians and nursing staff to collect demographic and lifestyle data, anthropometric measurements, functional, cognitive and health status, clinical anamnesis, and details on drug use. As for OO and CENT, in all cases where the subject was unable to respond autonomously because of hearing or sight problems, the interview was performed with a relative or a caregiver. Subjects affected by malignant neoplasia and/or those in therapy with immune suppressor drugs (like cyclosporine, methotrexate, glucocorticoids, etc.) or anticoagulant drugs were excluded from the study. As far as health status, YA, EL, and OO subjects were free of clinically evident major diseases. CENT were more heterogeneous. Supplementary Table 2 shows the prevalence of the major age-related diseases in CENT group. However, it is to note that the age at onset of such diseases is very advanced, further supporting the idea that centenarians are model of successful aging.

Data Collection

Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters (kg/m^2). Handgrip strength test was performed to measure the maximum isometric strength of the hand and forearm muscles using a hand-held dynamometer

(SMEDLYS' dynamometer, Scandidact, Kvistgaard, Denmark) for two performances with each hand.

Blood was drawn in the morning after an overnight fast. All samples were processed immediately to collect serum and plasma. Serum was obtained after clotting and centrifugation at 760*g* for 20 minutes at 4°C, rapidly frozen and stored at −80°C. Plasma was obtained within 2 hours from venipuncture by centrifugation at 2,000*g* for 20 minutes at 4°C, rapidly frozen and stored at −80°C.

Total and HDL cholesterol, triglycerides, C-reactive protein (CRP), glycaemia, insulin, albumin, total protein, uric acid, and creatinine were measured in serum by standard biochemical assays. Insulin resistance status was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), according to the previously described formula (35): insulin (in microunits per milliliter) × glucose (in millimoles per liter)/22.5. The median value of functional, metabolic, biochemical, and inflammatory parameters for each group was reported in Supplementary Table 1.

FGF21, GDF15, and HN concentrations were determined in plasma samples by ELISA assay using commercial kits, R&D for FGF21 (DF2100; intra- and interassay CV range: 10.2%–3.0% and 10.6%–3.1%, respectively) and GDF15 (DGD150; intra- and interassay CV range: 10.9%–1.1% and 4.1%–3.0%, respectively); CUSABIO for HN (CSB-EL015084HU; intra- and interassay CV range: 5.5%–0.7% and 11.8%–3.4%, respectively), according to the manufacturer's instructions. For all the samples, FGF21, GDF15, and HN were analyzed in a blind setup.

Data on IGF-1 plasma level were available for 137 subjects (62 EL, 24 OO, and 51 CENT) from a previous study (36).

Total IGF-1 was assayed by one-step sandwich chemiluminescence immunoassay (CLIA) after prior separation of IGF-1 from binding proteins on the Liaison autoanalyzer (DiaSorin, Saluggia, Italy). Samples were acidified to separate IGF-1 from binding proteins, and excess IGF-2 was used after acidification to avoid residual interference IGF-1 with binding proteins. Intra-assay and interassay CVs were 4.4% and 5.5%, respectively. The mean recovery value was 97% of the hypothetical expected amount.

Statistical Analysis

The correlation between mitokines and age and among mitokines were calculated by Spearman rank correlation. The association between mitokines and parameters related to health status and phenotype (BMI, handgrip strength, glucose metabolism, lipid profile, and other biochemical markers) was evaluated by a multivariate general linear model adjusted for age, gender, and therapies in each age group. Gender differences were assessed by Mann–Whitney *U* test. EL, OO, and CENT subjects were clustered according to their mitokines (FGF21, HN, GDF15) levels by a two-step cluster analysis. Cumulative survival curves were employed to display 5-year all cause mortality according to clusters in nonagenarians and centenarians. The association between clusters and mortality was evaluated by a Cox regression model adjusted for age and gender in OO and CENT. All analyses were executed using SPSS 23.0 for Windows (SPSS, Inc., Chicago, IL).

Results

Plasma Levels of Mitokines Increase With Age and Are Correlated With Each Other

The plasmatic levels of FGF21, HN, and GDF15 are positively and significantly correlated with age (Figure 1A–D). Moreover, these are correlated with each other (Figure 1E–H). Slight but significant differences between men and women were present in some age groups (Supplementary Figure 1). In particular, FGF21 is higher in YA men (p = .016) and in OO women (p = .016; Supplementary Figure 1A), while GDF15 is higher in EL men (p = .001; Supplementary Figure 1B). No gender differences were observed for HN (Supplementary Figure 1C).

It is reported that the levels of HN decrease with age (37). Our results were thus in contrast with literature data. We tried to confirm our results on HN by analyzing the association with IGF-1, which is reported to suppress HN level in plasma (38). The data regarding IGF-1 plasma levels were available for 137 subjects (62 EL, 24 OO, and 51 CENT). We found a negative correlation between HN and IGF-1 (Spearman rank correlation: ρ = −0.259; p = .002) and these data indicate that the high levels of HN found in old age are paralleled, as expected, by low levels of IGF-1.

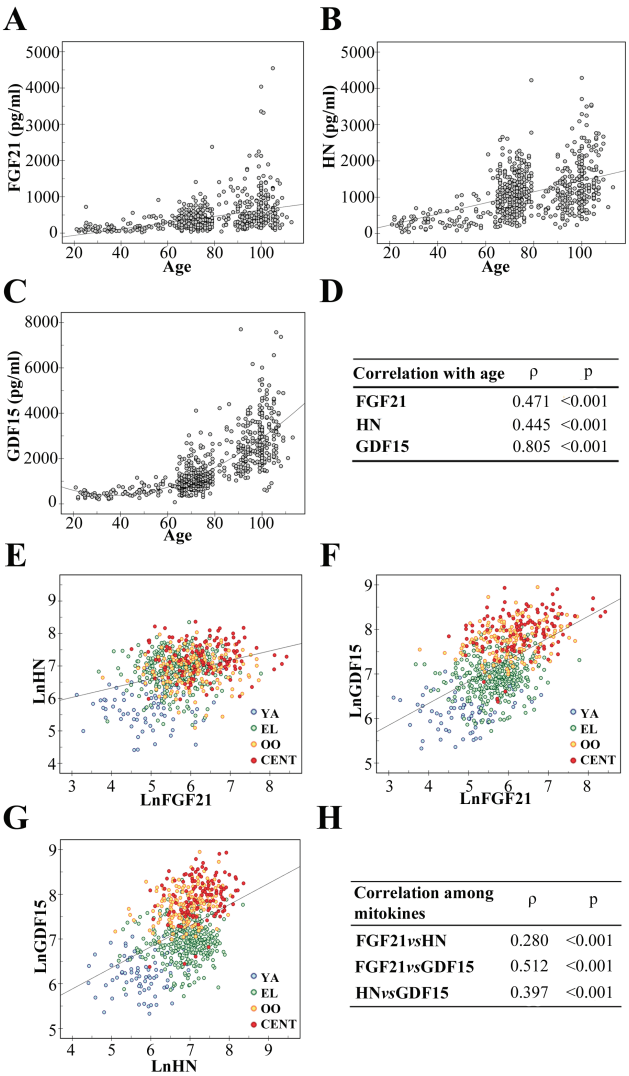


Figure 1. Correlation analysis of mitokines in young/adult (YA), elderly (EL), oldest old (OO), and centenarians (CENT). (**A–C**) Scatter plot of age vs fibroblast growth factor 21 (FGF21) (**A**), humanin (HN) (**B**), growth differentiation factor 15 (GDF15) (**C**); (**D**) Spearman rank correlation coefficients (ρ, rho) between age and FGF21, HN, and GDF15. (**E–G**) Scatter plot of FGF21 vs HN (**E**), FGF21 vs GDF15 (**F**), HN vs GDF15 (**G**); (**H**) Spearman rank correlation coefficients (ρ) among mitokines.

FGF21, HN, and GDF15 Plasma Levels Are Associated With Worsening of Functional and Biochemical Parameters, Particularly in EL Subjects

An association analysis between plasma levels of mitokines and functional, metabolic, biochemical, and inflammatory parameters (BMI, handgrip strength, HOMA-IR index, HDL cholesterol, triglycerides, CRP, albumin, total protein, uric acid, and creatinine) was performed in each age group according to a general linear model adjusted for age, gender, and therapies (Supplementary Table 3, panel A and B). The association between cognitive status (assessed by Mini-Mental State Evaluation) and mitokines was also evaluated, and no significant association was found (data not shown). Plasma FGF21 level is positively associated with BMI in EL and with triglycerides in both EL and OO (Supplementary Table 3, panel A); it is positively associated also with uric acid in EL and with CRP in OO, and negatively associated with total protein content in CENT (Supplementary Table 3, panel B). HN plasma level is associated negatively with BMI in EL and positively with triglycerides in CENT (Supplementary Table 3, panel A); it is associated negatively with albumin in EL and positively with creatinine in OO (Supplementary Table 3, panel B). Plasma GDF15 level is positively associated with BMI, HOMA-IR and triglycerides in EL, and negatively associated with handgrip strength in both EL and OO (Supplementary Table 3, panel A); it is positively associated with CRP in CENT and with creatinine in both OO and CENT (Supplementary Table 3, panel B). GDF15 and uric acid were positively associated in YA and negatively in EL, OO, and CENT (Supplementary Table 3, panel B).

These data show that increased levels of mitokines (in particular, GDF15) are associated with worsening of functional and biochemical parameters (reduction of handgrip strength, HDL cholesterol, serum albumin and total proteins, and increase of BMI, HOMA-IR index, triglycerides, and uric acid), particularly in EL subjects. In fact, among the 20 significant associations, half of them are found in EL group. For this reason, a two-step cluster analysis was performed in this group according to the plasma levels of mitokines. Two clusters of EL subjects were identified: one with high levels of mitokines (HL), and another with low levels of mitokines (LL; Table 1). No difference in gender distribution was observed between clusters. LL group has lower BMI, lower glucose metabolism and insulin resistance markers (glycaemia, insulin, HOMA-IR), a better lipid profile (higher HDL cholesterol, lower triglycerides), lower CRP and creatinine compared with HL group (Table 1).

Nonagenarian Subjects and Centenarians With Lower Levels of Mitokines Have a Slight Survival Advantage

As a whole, the data previously described suggest that circulating levels of mitokines are strongly associated with age and with parameters indicative of the health status. Taking advantage of the heterogeneity of the CENT group regarding morbidity, we could split this group into three subgroups (0–2 diseases; 3–4 diseases; and ≥5 diseases). No significant difference in mitokines levels was found among the three subgroups (data not shown). We then wondered whether these mitokines are also associated with survival. Taking advantage of the mortality follow-up available for the subjects over 90 years of age, we performed a survival analysis on nonagenarians (90–99 years) and CENT (100–113 years). Both nonagenarians and CENT were clustered according to the plasma levels of FGF21, HN, and GDF15 by a two-step cluster analysis. As previously described, this cluster analysis divided the subjects into two groups, one with high levels of mitokines (HL), and another with low levels of mitokines (LL).

Concerning nonagenarians, the LL group shows higher handgrip strength (p = .007) and lower levels of albumin (p = .018), uric acid (p = .000), and creatinine (p = .001) with respect to the HL group (Figure 2A). No significant differences for other metabolic and biochemical parameters were observed (data not shown). Moreover, LL group has a significantly longer estimated survival time compared with the HL group (p = 0.000). Figure 2B shows the cumulative survival curves for the two groups and the association with the mortality estimated by univariate Cox regression model adjusted for age and gender. The HL group shows an increased hazard and increased mortality rate compared the LL group used as reference (p = .000; Figure 2B).

In CENT, the LL group shows lower level of creatinine (p = .002) and higher insulin (p = .007) and HOMA-IR (p = .006) in comparison with the HL group (Figure 3A). No significant differences for other parameters (functional, biochemical, metabolic, and inflammatory markers) were observed (data not shown). Similar to what we observed in nonagenarians, also in CENT the LL group has a

Table 1. Functional and Biochemical Parameters in EL Subjects Clustered According to Plasma Levels of Mitokines

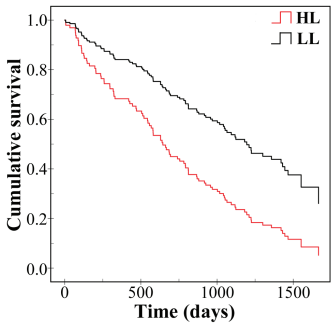
Group (*N*)	LL (273)	HL (63)	*p*
Men/women (*N*)	127/146	36/27	.130
Age, mean (*SD*)	71.0 (4.0)	72.7 (3.9)	.011
FGF21 (pg/mL)	284.1 [201.2]	431.5 [445.8]	.000
HN (pg/mL)	1086.2 [636.8]	1047.1 [800.9]	.876
GDF15 (pg/mL)	952.0 [358.4]	1835.2 [531.5]	.000
BMI (kg/m^2)	26.6 [4.6]	28.6 [5.0]	.007
Glycemia (mg/dL)	98.2 [14.7]	103.2 [21.0]	.012
Insulin (μU/mL)	8.6 [7.9]	10.0 [8.5]	.007
HOMA-IR index	2.0 [2.0]	2.6 [2.6]	.002
Total cholesterol (mg/dL)	200.0 [44.6]	186.5 [49.5]	.036
HDL cholesterol (mg/dL)	55.9 [22.0]	49.8 [25.7]	.015
Triglycerides (mg/dL)	96.3 [49.7]	107.6 [78.7]	.037
C-reactive protein (mg/L)	1.0[1.7]	1.3 [2.5]	.012
Creatinine (mg/dL)	0.8 [0.2]	0.9 [0.3]	.003

Notes: LL = subjects with low levels of mitokines; HL = subjects with high levels of mitokines. Statistical analysis was performed by Mann–Whiney *U* test. Age is reported as mean (*SD* = standard deviation), other data are shown as median [IQR = interquartile range].

A

Cluster (n)	LL (74)	HL (58)	*p*
Men/Women (n)	33/41	21/37	.331
Age (years)	94.5 (3.1)	95.7 (3.0)	.025
FGF21 (pg/mL)	350.6 [279.0]	550.0 [631.9]	<.001
HN (pg/mL)	893.0 [436.1]	1513.6 [729.7]	<.001
GDF15 (pg/mL)	2012.9 [788.3]	3192.5 [1063.4]	<.001
BMI (kg/m^2)	26.1 [5.6]	24.0 [5.9]	.060
Handgrip strength (kg)	18.2 [10.9]	15.0 [9.3]	.007
Albumin (g/dL)	4.6 [1.7]	5.5 [3.4]	.018
Uric acid (mg/dL)	4.9 [1.4]	6.3 [2.5]	<.001
Creatinine (mg/dL)	1.3 [0.6]	1.0 [0.4]	.001
Estimated survival time (days)	1142 (1010-1275)	691 (556-826)	<.001

B



Hazard ratio			
	B coefficient	Exp(B) (95% C.I.)	*p*
LL	-	-	-
HL	0.789	2.2 (1.4-3.4)	<.001

Figure 2. Functional parameters and survival analysis in nonagenarians clustered according to the plasma levels of mitokines. (**A**) Mitokines, body mass index (BMI), handgrip strength, albumin, uric acid and creatinine levels, and estimated survival time in nonagenarians clustered according to their fibroblast growth factor 21 (FGF21), humanin (HN), and growth differentiation factor 15 (GDF15) plasma levels (LL = low levels; HL = high levels). Age is reported as mean (*SD* = standard deviation), other data are shown as median [IQR = interquartile range]. Statistical analysis was performed by logistic regression for gender, Mann–Whiney *U* test for age, mitokines, BMI, handgrip strength, albumin, uric acid and creatinine levels, and logrank (Mantel–Cox) for estimated survival time. (**B**) Cumulative survival curves in nonagenarians clustered according to their FGF21, HN, GDF15 plasma levels, and mortality estimated by Cox regression model adjusted for age and gender. Nonagenarians belonging to LL cluster were considered the reference for the regression analysis.

significantly longer estimated survival time compared with HL group (p = .039; Supplementary Table 5). Finally, HL group shows an increased hazard and increased mortality rate compared to LL group used as reference (p = .018; Figure 3B).

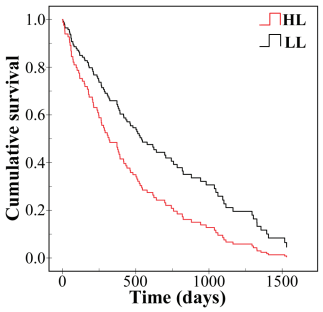
To understand which mitokine, among FGF21, HN, and GDF15, has the greater influence on mortality, a further association analysis was performed. Supplementary Table 4 shows the association of every single mitokine with mortality in nonagenarians and CENT estimated by a Cox regression model with log-transformed variables adjusted for age and gender. According to this analysis, GDF15 is the most associated mitokine (p = .006; Supplementary Table 4).

It is well known that handgrip strength is a strong predictor of mortality. To further investigate whether these mitokines synergize with handgrip strength in predicting mortality, we performed

A

Cluster (n)	LL (87)	HL (30)	*p*
Men/Women (n)	22/65	8/22	.881
Age (years)	103.0 (3.0)	102.7 (2.4)	.772
FGF21 (pg/mL)	492.3 [487.5]	1031.6 [917.5]	<.001
HN (pg/mL)	1310.0 [999.5]	1502.6 [1479.1]	.058
GDF15 (pg/mL)	2466.1 [1180.0]	4599.0 [942.1]	<.001
Insulin (μU/mL)	5.1 [5.7]	3.3 [2.3]	.007
HOMA-IR index	1.0 [1.3]	0.7 [0.4]	.006
Creatinine (mg/dL)	1.0 [0.4]	1.4 [0.9]	.002
Estimated survival time (days)	530 (317-743)	386 (222-549)	.039

B



Hazard ratio			
	B coefficient	Exp(B) (95% C.I.)	*p*
LL	-	-	-
HL	0.554	1.7 (1.1-2.7)	.018

Figure 3. Functional parameters and survival analysis in centenarians (CENT) clustered according to the plasma levels of mitokines. (**A**) Mitokines, insulin resistance markers, creatinine levels, and estimated survival time in CENT clustered according to their fibroblast growth factor 21 (FGF21), humanin (HN), growth differentiation factor 15 (GDF15) plasma levels (LL = low levels; HL = high levels). Age is reported as mean (*SD* = standard deviation), other data are shown as median [IQR = interquartile range]. Statistical analysis was performed by logistic regression for gender, Mann–Whiney *U* test for age, mitokines, insulin resistance markers, creatinine levels, and logrank (Mantel–Cox) for estimated survival time. (**B**) Cumulative survival curves in CENT clustered according to their FGF21, HN, GDF15 plasma levels, and mortality estimated by Cox regression model adjusted for age and gender. CENT belonging to LL cluster were considered the reference for the regression analysis.

a multivariate Cox regression with forward stepwise selection. We confirmed that handgrip strength is associated with mortality (Supplementary Table 5, Model 1). Among the mitokines, only GDF15 was able to improve the prediction capability of handgrip strength (Supplementary Table 5, Model 2).

GDF15 and HN Are Higher in Subjects With Down Syndrome With Respect to Their Nontrisomic Siblings

As a whole, these data suggest that mitokines could be considered a sort of marker for biological age. To find support to this idea, we investigated the plasma levels of mitokines in subjects with Down syndrome (DS), a condition characterized by segmental accelerated aging. We measured mitokines plasma levels in 28 adults with DS and their nontrisomic siblings. Although no difference is observed in mean age (p = .436), DS subjects have higher plasma levels of HN (p = .014) and GDF15 (p = .001) in comparison to their siblings (Supplementary Table 6).

Discussion

Dillin and coworkers hypothesized some years ago that a mitochondrial stress could elicit beneficial effects on health and life span spreading at organismal level through soluble elements putatively indicated as mitokines (7), and many data indicate indeed that a mild mitochondrial dysfunction actually promotes longevity (5,6). Since then, a number of molecules that are synthesized in response to mitochondrial stress have been identified, including FGF21 (10) and GDF15 (15). The previously discovered 24-aa peptide encoded by mitochondrial DNA indicated as HN also fulfilled the features of a mitochondrial retrograde response molecule (39). We have recently proposed the existence of a sort of "mitochondrial hormesis" (40). According to this idea, the presence of a mild mitochondrial dysfunction that can elicit the production of effective stress responses and metabolic adaptations (including mitokines production) would be more beneficial for health and longevity than a network of perfectly functioning mitochondria (38). In agreement with this hypothesis, we have demonstrated that dermal fibroblasts from centenarians have partially impaired mitochondria (34). However, mitochondrial dysfunction has been recently shown to be sufficient to accelerate aging process and cause premature death (20).

With this in mind, in this study, we analyzed the levels of FGF21, HN, and GDF15 in a cohort of subjects whose age spans from 21 to 113 years. We also evaluated the potential association of these mitokines with functional, metabolic, and biochemical parameters as well as survival (where available). Here, we report that the circulating levels of these mitokines increase with age. However, it is to note that the correlation with age of GDF15 is much higher than that of FGF21 and HN. Although the nature of the interaction among these mitokines is not yet totally clear, these data actually indicate that GDF15 is more affected by aging and it could be considered a potential marker of age. The levels of these mitokines become maximal in centenarians, indicating that the biological mechanisms that trigger their increase are still active in extreme old age.

These data are in agreement with previous literature as far as FGF21 and GDF15, which are reported to increase with age in both animal models and humans (19,20,22). On the contrary, a study by Muzumdar and coworkers (37) reported a decrease of HN levels with age in humans (37). It is to note that as we used a commercial kit for HN, while Muzumdar and coworkers as well as other groups used an antibody developed in-house. However, the commercial antibody that we used is raised against the mitochondrial peptide of HN (http://www.uniprot.org/uniprot/Q8IVG9#sequences) and no significant cross-reactivity or interference with the analogues of HN tested so far has been observed. Moreover, in the article by Muzumdar and coworkers reporting such a decrease, no description of the human samples analyzed is provided, so a comparison with our data is not possible (37). Our data are further supported by the negative correlation found between the plasma levels of HN and IGF-1. It is reported in fact that IGF-1 levels and bioavailability decrease with age (36,41), and that IGF-1 inhibits the production of HN (38).

The fact that the circulating levels of the three mitokines considered in this study increase with age fit with the idea that aging is characterized by progressive mitochondrial stress. As stress response molecules, these mitokines are reported to have many beneficial effects for the cells and the whole organism. In particular, FGF21 has cardioprotective effects (42), positive metabolic effects, including weight loss, improved glycemia, and decreased inflammation (43), it delays immunosenescence (44), and increases the life span of animals in experimental models (16); HN is a neuroprotective factor that negatively regulates apoptosis, insulin resistance, and inflammation (27,29) and it plays a protective role in many pathologies such as Alzheimer's disease, cardiovascular diseases, type 2 diabetes, inflammation, as reviewed by Gong and coworkers (45); GDF15 has reported cardioprotective and neuroprotective activity, and it can affect mitochondrial biogenesis and fatty acid metabolism (22). Moreover, mice overexpressing human GDF15 display increased life span (23). Therefore, given these premises, we would have expected that, within each age group, the subjects characterized by higher levels of mitokines would be healthier. To our surprise, this was not the case. In fact, we found that the levels of these mitokines are associated with worsened functional and biochemical parameters (higher BMI and insulin resistance, low handgrip strength, higher CRP, triglycerides, uric acid, and creatinine) in EL and decreased survival in the oldest groups (nonagenarians and CENT). GDF15 resulted the most powerful predictor of mortality among the three mitokines, being also able to improve the predictive power of mortality reported for handgrip strength. At variance, FGF21 and HN were less strongly associated with mortality.

It is, however, to note that a great debate exists on the precise role of these mitokines in aging and diseases. In particular, the role of FGF21 has been questioned, as it appears possibly dispensable for the metabolic improvements evoked by compromised mitochondrial function in skeletal muscle (46). Moreover, FGF21 appears also to be responsible for the accelerated aging phenotype when mitochondrial dysfunction is induced by acute inhibition of the fusion protein OPA1 (20). These data suggest that chronically high blood levels of FGF21 contribute to unhealthy aging. On the same vein, circulating levels of GDF15 have been found associated with cardiovascular diseases, insulin resistance and type 2 diabetes, neurodegeneration and overall mortality [reviewed in Fujita and coworkers (22)]. However, it is unlikely that these mitokines are detrimental *per se*. Rather, they may represent the attempt of the cell/tissue to cope with an ongoing stress. According to the concept of hormesis, whether this coping will be successful strictly depends on the intensity of the stress (47). If the stress is mild and possibly transient, the mitokines can efficiently cope with it and strengthen the organism; on the contrary, a stronger and possibly chronic stress can have detrimental effects, either because these effects are too intense and mitokines are not able anymore to counteract them, or mitokines may cause themselves a metabolic stress condition when secreted at excessive concentration. Whatever could be the reason, the net result is that a correlation emerges between mitokines levels and worsened health status. This hypothesis is depicted in Figure 4, where we speculated that mitokines production turns from protective to detrimental adaptive response, as the level of mitokines increases as it occurs during aging.

As a whole, it seems that these mitokines are associated with biological age. The quest for markers of biological age is a hot topic of modern Geroscience. In recent years, we and others have identified some powerful markers of biological age, such as methylation of specific CpG islands of ELOVL2 and FHL2 genes, and circulating N-glycan profiles. Accordingly, these biomarkers resulted significantly elevated in DS persons, indicating that, actually, these subjects experience an accelerated aging (48,49). To verify if these mitokines could be provisionally added to the list of putative markers of biological age, we studied their levels in a group of DS persons. We have found a higher level of HN and GDF 15 in DS subjects, in comparison to their

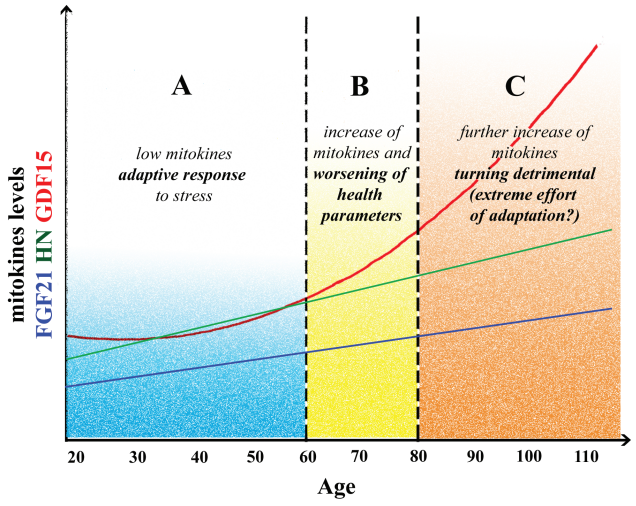


Figure 4. Hypothetical scheme of mitokine effects during age that can be interpreted in the framework of the hormetic paradigm. Blue line: fibroblast growth factor 21 (FGF21); Green line: humanin (HN); Red line: growth differentiation factor 15 (GDF15). (**A**) At young/adult age, mild (and likely transient) mitochondrial stresses may trigger the production of low levels of mitokines as an adaptive response. (**B**) With aging, stronger (and probably long-lasting or chronic) stresses may induce an increased production of mitokines and higher levels of these molecules are associated with a worsening of functional parameters. (**C**) In long-lived subjects, the lifelong accumulation of mitochondrial stress may induce a further increase of mitokine levels which is associated with increased mortality; this response may represent an extreme adaptive effort turning to be eventually detrimental.

sibling. In addition, GDF15 has been shown to be a predictor of physical decline in old subjects (50), therefore, it seems that (at least some of) these mitokines could be added to the list of the markers of biological age. Further studies are needed to fully confirm this hypothesis.

Conclusions

As a whole, our results show for the first time that plasma levels of three representative mitokines, FGF21, GDF15, and HN, are positively correlated with age in humans, and become maximal in centenarians. This correlation is particularly marked for GDF15.

The physiological *in vivo* effects of mitokines are apparently not straightforward. Studies in the literature report both positive and negative effects of these molecules. Our data indicate that the circulating levels of FGF21, GDF15, and HN are associated with worsened health parameters and with mortality in old age, and the predominant association is observed for GDF15. Although it can only be speculated that the role of mitokines turns detrimental as they increase in concentration, our data are consistent with the idea that mitokines (in particular, GDF15) can be considered as markers of biological age. This hypothesis is strongly supported by the analysis of mitokines in DS persons, characterized by segmental accelerated aging. These persons show higher levels of HN and GDF15 with respect to their siblings. Further studies are granted to better clarify the biological significance of these findings.

Supplementary Material

Supplementary data is available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of interest statement

None declared.

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