THE GERONTOLOGICAL SOCIETY OF AMERICA®

Journals of Gerontology: Biological Sciences
cite as: *J Gerontol A Biol Sci Med Sci*, 2017, Vol. 72, No. 9, 1196–1200
doi:10.1093/gerona/glx091
Advance Access publication May 12, 2017

OXFORD

Brief report

Expression and Activity of the Small RhoGTPase Cdc42 in Blood Cells of Older Adults Are Associated With Age and Cardiovascular Disease

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Received: November 16, 2016; Editorial Decision Date: May 2, 2017

Decision Editor: Rafael de Cabo, PhD

Abstract

The small RhoGTPase Cdc42 is mechanistically linked to aging of multiple tissues and to rejuvenation of hematopoietic stem cells in mice. However, data validating Cdc42 activity and expression as biomarker for aging in humans are still missing. Here, we hypothesized that Cdc42 might serve as a novel biomarker of aging in older adults and therefore we determined Cdc42 activity and expression levels in peripheral blood cells from a cohort of 196 donors. We investigated the association of these parameters with both chronological and biological aging. We also tested in this cohort of older adults a recently published algorithm determining chronological age based on DNA methylation profiles. A positive correlation with chronological age was found for both the level of Cdc42 mRNA and the level of active Cdc42 protein (the GTP bound form). Notably, the level of Cdc42 mRNA as well as total protein showed a specific strong association to cardiovascular disease and Cdc42 mRNA levels also to a history of myocardial infarction. In summary, these data validate Cdc42 as a blood biomarker of both chronological aging as well as aging-associated diseases like cardiovascular disease and myocardial infarction.

Keywords: Cdc42—CpG methylation—Aging biomarker—Cardiovascular disease—Myocardial infarction

The proportion of people aged older than 60 years is growing faster than any other age group in almost every country in the world. By 2050, the number of people over the age of 80 will triple globally (1,2). Rational approaches and therapies to allow for healthy aging are thus of high priority. The identification of valid biomarkers of aging and aging-associated diseases will support and accelerate the quest for such treatments.

Cdc42 belongs to the family of small RhoGTPases, which act as binary molecular switches that cycle between a GTP-bound active state and GDP-bound inactive state in response to a variety of extracellular stimuli. The level of activation of Cdc42 can influence cell adhesion, migration, division, and polarity (3,4).

Genome-wide association studies have previously identified expression of *Cdc42* as the gene most strongly positively associated with aging in human lymphoblastoid cell lines, and levels of expression correlated positively with both morbidity and aging (5).

Old mice present with elevated activity of Cdc42 in most tissues tested so far. Increased activity of Cdc42 is also found in undifferentiated hematopoietic cells in bone marrow of aged mice (6,7) and

elevated Cdc42 activity and signaling plays a critical role in aging of hematopoietic stem cells (HSCs) (8–10). Young mice with constitutively elevated Cdc42 activity due to a genetic deletion of the negative regulator of Cdc42 (Cdc42GAP) present with severe premature aging-like phenotypes in multiple tissues (11,12). Pharmacological inhibition of Cdc42 activity in aged HSCs rejuvenated their function (13–15). In aggregation, these data imply that Cdc42 might serve as a valid biomarker for aging, as it is causatively linked to aging in mice and associated with morbidity and aging in human lymphoblastoid cell lines. We examined in a cohort of older adults Cdc42 activity and Cdc42 expression in blood cells and correlated these to molecular and biological markers of aging, to functional markers of aging like handgrip strength and habitual walking speed as well as to morbidity.

Materials and Methods

Blood Collection, Preparation of RNA, DNA, and Protein Lysates, Cdc42 Activity and Expression

Freshly collected ethylenediaminetetraacetic acid blood samples (10 mL each donor) were transported under refrigeration and within 2 hours processed for RNA, DNA, and protein preparation. All samples were labeled with a randomly generated six-digit code and sex, age, and any other parameters of the donor were blinded to the operator. Blood samples, after having removed an aliquot (100 µL) for blood cell counts (with Hemavet 250 FS, Erba Diagnostics France, Montpellier, France), were first incubated with Red Blood Cell Lysis Buffer. The remaining white blood cells were separated into three tubes in equal amount: 1-RNA, 2-DNA, and 3-Protein. The first tube was then processed for RNA extraction (RNA extraction kit, RNAeasy Midi Kit, Qiagen GmbH, Germany) followed by cDNA retrotranscription and real-time PCR analysis (TaqMan probes #4331182, Mm01194005_g1 for human Cdc42; internal control human GAPDH #4331182, Mm99999915_g1, Thermo Fisher Scientific). The DNA was extracted with DNA Midi Kit (#13343, Qiagen GmbH). Protein were lysed with Protein Lysis Buffer MLB (Millipore) and stored at −20°C. Pulldowns were performed with Rac/cdc42 Assay Reagent (Millipore). Blood from a cohort of 12 young donors (20–30 years old) was used to control variability between blots. Actin was used to normalize Cdc42 total protein amount. Primary antibodies used were: Monoclonal Anti-β-Actin, Clone AC-15 (Sigma Aldrich) and anti-Cdc42 (Millipore).

The determination of the DNA methylation profile was performed by Cyngenia GmbH (Ulm, Germany).

Statistical Analysis

Descriptive statistics were calculated to describe the main characteristics of the study population. Correlations between chronological age with CDC42 (total protein levels), CDC42 (mRNA levels), CDC42-GTP (active protein levels over total), and predicted age based on CpG methylation were presented as scatterplots and quantified using Spearman's correlation coefficient.

Chronological age and each biomarker for biological ageing were categorized according to the respective quartiles in two groups: "quartiles 1–3" and "quartile 4" to contrast persons in the most extreme quartiles against all others. As health outcomes the following parameters were used and dichotomized according to clinical thresholds or median values: self-rated health (fair or bad vs good, very good, or excellent), cardiovascular disease (CVD; yes vs no), cancer (yes vs no), diabetes (yes vs no), osteoporosis (yes vs no), low handgrip strength (median split: men <36 vs ≥36 kg, women <22 vs ≥22 kg), low Euro QoL VAS (Visual Analogue Scale) values (median split: <80 vs ≥80), low walking speed (median split: <1.19 vs ≥1.19 m/s).

Logistic regression models were used to estimate bivariate odds ratios (ORs) and 95% confidence intervals (CIs) for the effect of chronological age and biomarkers for biological ageing on the selected health outcomes. In each model, the group "quartiles 1–3" served as reference category.

Results

The study population consisted of 196 subjects (125 men and 71 women, mean age = 75.6 [*SD* = 6.17] years), with complete data on chronological age and at least one biological aging marker. The study cohort is part of a bigger population-based cohort study of subjects aged older than 65 years located in Ulm, Germany, that was started as the Activity and Function in the Elderly in Ulm cohort (ActiFE Ulm) (16) and partly continued as EPOSA in 2011–2012 (17). All participants gave written informed consent. Characteristics of the study population are presented in Table 1. The self-reported disease prevalence was 40.0% for hypertension, 21.9% for CVD, 17.4% for cancer, 10.2% for diabetes, and 9.2% for osteoporosis. About one forth rated their health as fair or bad.

Cdc42 expression (mRNA as well as protein) and Cdc42 activity (Cdc42-GTP protein relative to Cdc42 protein) were determined in cells from peripheral blood by quantitative RT–PCR

Table 1. Baseline Characteristics of Study Population

Characteristic	Total (*n* = 196)
Chronological age (y), mean (*SD*)	75.6 (6.17)
Women, *n* (%)	71 (36.2)
Duration of school education ≤9 y, *n* (%)	110 (56.1)
Current smoker, *n* (%)	8 (4.1)
Daily alcohol consumption, *n* (%)	65 (33.2)
BMI (kg/m^2), mean (*SD*)	26.6 (3.93)
≥30 kg/m^2, *n* (%)	36 (18.4)
Mini-Mental State Examination <25, *n* (%)	4 (2.0)
Self-reported comorbidity, *n* (%)	
Hypertension	79 (40.3)
Cardiovascular disease	43 (21.9)
Myocardial infarction	14 (7.1)
Cancer	34 (17.4)
Diabetes	20 (10.2)
Osteoporosis	18 (9.2)
Self-rated health "fair or bad"	48 (24.5)
GaitRITE habitual walking speed (m/s), mean (*SD*) (*n* = 154)	1.17 (0.22)
Handgrip strength (kg), mean (*SD*)	31.5 (10.2)
EURO-QoL VAS	68.8 (26.8)
CDC 42 total, mean (*SD*) (*n* = 182)	1.17 (0.83)
CDC 42 mRNA, mean (*SD*) (*n* = 175)	1.29 (0.70)
CDC 42 GTP, mean (*SD*) (*n* = 182)	1.17 (0.81)
Age (predicted based on CpG methylation) (y), mean (*SD*) (*n* = 172)	71.7 (16.7)
WBC count, mean (*SD*) (*n* = 193)	6.10 (1.55)
RBC count, mean (*SD*) (*n* = 193)	4.99 (0.55)
Lymphoid, mean (*SD*) (*n* = 193)	0.88 (0.43)
Myeloid, mean (*SD*) (*n* = 193)	5.20 (1.43)

Note: BMI = body mass index; RBC = red blood cell; QoL = quality of life; VAS = Visual Analogue Scale; WBC = white blood cell.

and quantitative western "pull-down" blots. We also obtained white blood, lymphocyte, myeloid, and red blood cell counts from these blood samples. Additionally, from these same samples, we determined a preselected CpG methylation profile pattern on DNA, validated for the prediction of the chronological age of the donor (18). Correlation analyses between age and age-related markers were performed using Spearman's correlation coefficient. Furthermore, the relationship with clinical outcomes was investigated by contrasting persons in the highest quartile against all other persons. For this purpose, logistic regression models were used to estimate ORs for the association of the dichotomized parameters of chronological age and biomarkers for biological aging ("quartile 4" vs "quartiles 1–3") with selected dichotomized comorbidities such as hypertension, CVD (myocardial infarction [MI], angina pectoris, heart failure, arrhythmia), cancer, diabetes, and osteoporosis or other functional parameters (such as handgrip strength, habitual walking speed, quality of life) and other health parameters (alcohol consumption, smoking, body mass index, mental state, education).

Both the level of Cdc42 mRNA expression (mean ± *SD* of 1.29 ± 0.70) and the level of activity of Cdc42-GTP (mean ± *SD* of 1.17 ± 0.81) showed a significant linear association with chronological age (correlation coefficient of $r = .15$, $p < .04$, Figure 1A and correlation coefficient of $r = .16$, $p < .04$, Figure 1B and Table 2), while the amount of Cdc42 protein (mean ± *SD* of 1.17 ± 0.83) did not (correlation coefficient of $r = .06$, $p = .42$, Figure 1C and Table 2). Chronological age predicted by the CpG methylation pattern (mean ± *SD* of 71.7 ± 16.7 years) correlated with the chronological age of the donor (correlation coefficient of $r = .21$, $p < .001$, Figure 1D and Table 2). No significant correlation was detected between Cdc42 mRNA, total amount of Cdc42 protein, and GTP level and age predicted by CpG methylation pattern (Supplementary Table S2). The white blood cell count and the myeloid cells count presented with a trend towards an increase with age. The red blood cell count and the lymphoid cell count presented with a trend towards a decrease upon aging (Table 2). These changes in blood cell counts upon aging are in agreement with expected aging-associated hematopoietic alterations in humans (19). None of these blood cell count parameters showed an association with chronological age, Cdc42 total protein levels, Cdc42 mRNA, Cdc42 GTP levels, and CpG methylation predicted age, except the lymphoid count, which was inversely associated with Cdc42 mRNA levels (correlation coefficient of $r = -.17$, $p < .03$, Table 2).

We also tested for associations between dichotomized parameters of aging and selected dichotomized comorbidity phenotypes and health-related outcomes. For each dichotomized parameter of aging, the group "quartiles 1–3" served as reference category. Chronological age was significantly associated with diabetes, low handgrip strength, low quality of life, and low walking speed, but not with CVD, MI, cancer, and osteoporosis (Table 3). The strongest OR (95% CI) of 9.35 (3.63; 24.11) was found for the association between chronological age (quartile 4) and low walking speed (Table 3). When looking at our Cdc42 markers, the fourth quartiles of the amount of Cdc42 protein and the level of Cdc42 mRNA presented with a strong association with CVD (observed ORs were 2.49 [95% CI: 1.18; 5.27] and 3.41 [95% CI: 1.59; 7.30]; Table 3). Even after adjustment for chronological age, the ORs remained statistically significant with 2.57 (95% CI: 1.20; 5.48) for Cdc42 total and 3.32 (95% CI: 1.54; 7.13) for Cdc42 mRNA (data not shown). The forth quartile of the level of Cdc42 mRNA expression showed also a strong association with MI (observed OR was 3.44 [95% CI: 1.09; 10.84]; Table 3). Age

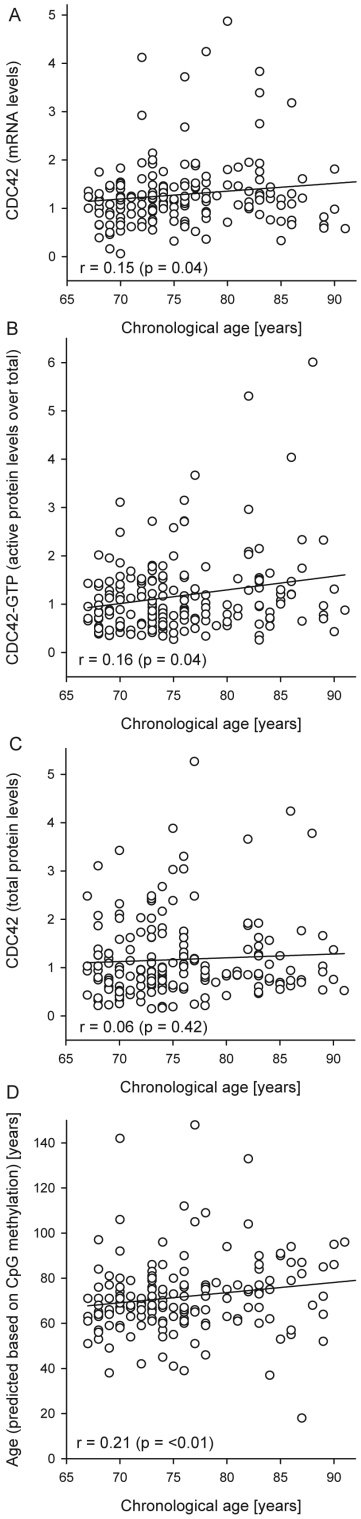

Figure 1. Relationship between chronological age and the biomarkers for biological ageing. Scatterplots and Spearman's correlation coefficient for chronological age with CDC42 (total protein levels) (A), CDC42 (mRNA levels) (B), CDC42-GTP (active protein levels over total) (C), and predicted age based on CpG methylation (D). Cdc42 mRNA (A) and Cdc42 GTP (B) levels showed statistically significant linear associations with chronological age, while Cdc42 total protein (C) levels were found not significantly correlated with chronological age. A strong correlation was found also between chronological age and predicted age based on CpG methylation (D).

Table 2. Correlation Matrix

	CDC42 Total	CDC42 mRNA	CDC42 GTP	meCpG Age	WBC	RBC	Lymphoid	Myeloid
Age	.06 (.42)	**.15 (.04)**	**.16 (.04)**	**.21 (<.01)**	.08 (.25)	−.02 (.80)	−.08 (.26)	.13 (.08)
CDC42 total		.08 (.33)	**.50 (<.01)**	.15 (.06)	−.05 (.47)	−.01 (.92)	−.07 (.36)	−.05 (.53)
CDC42 mRNA			.05 (.52)	.03 (.75)	.01 (.90)	.05 (.55)	**−.17 (.03)**	.06 (.44)
CDC42 GTP				.03 (.71)	−.11 (.14)	.07 (.38)	−.08 (.27)	−.09 (.22)
meCpG age					−.00 (.99)	.11 (.14)	−.03 (.72)	.00 (.95)
WBC						**.21 (<.01)**	**.34 (<.01)**	**.95 (<.01)**
RBC							**−.25 (<.01)**	**.31 (<.01)**
Lymphoid								.09 (.22)

Note: RBC = red blood cell; WBC = white blood cell. Values are Spearman's partial correlation coefficients (*p* values). Significant values are indicated in bold.

Table 3. Odd Ratios With 95% Confidence Intervals for Associations Between Chronological Age and Markers for Biological Ageing (4th quartile vs 1st to 3rd quartile) With Selected Dichotomized Health Outcomes

	Chronological Age	CDC42 (total protein levels)	CDC42 (mRNA levels)	CDC42-GTP (active protein levels over total)	Predicted Age Based on CpG Methylation
	n = 196	*n* = 182	*n* = 175	*n* = 182	*n* = 172
Self-rated health "fair or bad"	**2.47 (1.23; 4.98)**	1.79 (0.85; 3.75)	1.53 (0.72; 3.24)	1.15 (0.53; 2.47)	1.05 (0.46; 2.37)
CVD	1.57 (0.75; 3.29)	**2.49 (1.18; 5.27)**	**3.41 (1.59; 7.30)**	1.85 (0.87; 3.96)	1.24 (0.56; 2.77)
Myocardial infarction	1.69 (0.54; 5.31)	1.20 (0.36; 4.03)	**3.44 (1.09; 10.84)**	0.79 (0.21; 2.98)	1.99 (0.61; 6.45)
Cancer	1.27 (0.56; 2.88)	0.84 (0.34; 2.10)	1.33 (0.57; 3.08)	1.04 (0.43; 2.51)	2.00 (0.84; 4.78)
Diabetes	**2.69 (1.04; 6.95)**	0.77 (0.24; 2.45)	1.25 (0.45; 3.51)	1.06 (0.36; 3.13)	0.62 (0.17; 2.26)
Osteoporosis	2.59 (0.96; 6.99)	0.61 (0.17; 2.22)	1.23 (0.40; 3.73)	0.37 (0.08; 1.67)	0.40 (0.09; 1.84)
Low handgrip strength[a]	**3.62 (1.84; 7.12)**	1.04 (0.53; 2.03)	1.11 (0.57; 2.16)	0.92 (0.47; 1.81)	**2.42 (1.19; 4.90)**
Low Euro QoL VAS values[a]	**1.97 (1.02; 3.81)**	1.72 (0.88; 3.40)	1.09 (0.56; 2.14)	1.21 (0.62; 2.37)	1.35 (0.67; 2.69)
Low walking speed (*n* = 154)[a]	**9.35 (3.63; 24.11)**	0.69 (0.33; 1.45)	0.89 (0.42; 1.90)	1.06 (0.49; 2.28)	1.53 (0.69; 3.39)

Note: CVD = cardiovascular disease; VAS = Visual Analogue Scale. Significant values are indicated in bold.
[a]Median split.

predicted on the CpG methylation pattern showed only a statistically significant association for people in the fourth quartile with low hand grip strength (OR: 2.42 [95% CI: 1.19; 4.90]; Table 3).

Discussion

Our results demonstrate a correlation between Cdc42 expression in hematopoietic cells from blood of older adults and chronological aging and discover a novel correlation between Cdc42 expression/activity and CVD and MI. Interestingly, the incidence of CVD has been recently tightly linked to aging-associated low levels of HSC clonality in human hematopoiesis (20). In mice, elevated Cdc42 activity is causatively linked to aging and rejuvenation of HSCs (13,14). It is thus possible that Cdc42 levels in peripheral blood do not only serve as valid biomarkers, but also that changes in Cdc42 activity and/or expression might causally contribute to aging-associated changes in human organs and tissues. To note, Cdc42 is a small RhoGTPase and its activity is considered the critical contributor in most biological processes (21). In our cohort, the amount of protein itself is not directly linked to the activity level. This implies that also in this case mechanistically the amount of protein and the activity are separate entities. Future follow-up studies have to investigate the relationship between Cdc42 parameters and established clinical assessments for frailty such as the Fried frailty criteria (22).

The successful development and implementation of interventions to expand the health span will depend on the successful use of aging biomarkers (23). Our data, in combination with the previously published reports in which Cdc42 expression in lymphoblastoid cell lines was associated to mortality and morbidity and in which Cdc42 activity was causatively linked to aging and rejuvenation of HSCs (5,13,14), therefore, support Cdc42 expression and activity in blood cells as a novel biomarker of aging as well as aging-associated heart diseases like CVD and MI.

Funding

This work was supported by an Emmy Noether Grant (DFG) to M.C.F. Work in the laboratory of H.G. was supported by the Deutsche Forschungsgemeinschaft SFB 1074, SFB1149, and the KFO 142, the Excellence-Initiative of the Baden-Württemberg-Foundation, the BMBF-funded Program SyStaR, the Edward P. Evans Foundation, and the National Institute of Health, HL134617, AG040118, DK104814, and AG05065.

Acknowledgments

M.C.F., G.M., K.S., and H.K. collected blood samples and processed them for RNA, DNA, and protein analysis and performed PCRs, DNA extraction, and protein (western blots and pulldowns) assays. J.K. and D.R. performed statistical analysis and epidemiological studies. F.H. and R.P. coordinated

obtaining written consent and blood sample collection and organized and maintained data storage. M.D. coordinated clinical support and sample collection. M.C.F. and H.G. conceived the work, coordinated the analyses, interpreted the data, and wrote the manuscript. All authors have read, revised, and approved the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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