Proteins in the pathway from high red blood cell width distribution to all-cause mortality

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EBioMedicine 2022;76: 103816
Published online xxx
https://doi.org/10.1016/j.ebiom.2022.103816

Summary

Background The pathophysiological mechanisms underlying the association between red blood cell distribution width (RDW) and all-cause mortality are unknown. We conducted a data-driven discovery investigation to identify plasma proteins that mediate the association between RDW and time to death in community-dwelling adults.

Methods At baseline, 962 adults (women, 54·4%; age range, 21–98 years) participated in the InCHIANTI, "Aging in the Chianti Area" study, and proteomics data were generated from their plasma specimens. Of these, 623 participants had proteomics data available at the 9-year follow-up. For each visit, a total of 1301 plasma proteins were measured using SOMAscan technology. Complete data on vital status were available up to the 15-year follow-up period. Protein-specific exponential distribution accelerated failure time, and linear regression analyses adjusted for possible covariates were used for mortality and mediation analyses, respectively (survival data analysis).

Findings Baseline values of EGFR, GHR, NTRK3, SOD2, KLRF1, THBS2, TIMP1, IGFBP2, C9, APOB, and LRP1B mediated the association between baseline RDW and all-cause mortality. Changes in IGFBP2 and C7 over 9 years mediated the association between changes in RDW and 6-year all-cause mortality.

Interpretation Cellular senescence may contribute to the association between RDW and mortality.

Funding This study was funded by grants from the National Institutes of Health (NIH) and the National Institute on Aging (NIA) contract and was supported by the Intramural Research Program of the NIA, NIH. The InCHIANTI study was supported as a 'targeted project' by the Italian Ministry of Health and in part by the U.S. NIA.

Keywords: Aging; Proteomics; Red blood cell distribution width; Insulin-like growth factor-binding protein 2; Mortality

Introduction

Red blood cell distribution width (RDW), which is typically included in a complete blood count, represents the degree of heterogeneity of erythrocyte volume (conventionally known as anisocytosis). There is a strong association between RDW and anaemia, with RDW differentiating iron-deficient anaemia from folate-deficient anaemia.[1] In addition, a large body of evidence implicates high RDW as a strong risk factor for cardiovascular and all-cause mortality, both in healthy populations and a clinical series of patients with specific diseases.[2,3]

RDW is a powerful correlate for a long list of age-related conditions (e.g. cardiovascular disease, venous thromboembolism, cancer, diabetes, pneumonia, chronic obstructive pulmonary disease, and liver and kidney failure).[2] Excessive oxidative stress, inflammation, and cell senescence in the erythropoietic compartment have been proposed as the conditions through

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Research in context

Evidence before this study

We searched PubMed for research articles with key words "red blood cell distribution width" (RDW) and "mortality" to identify publications related to the association between RDW and all-cause mortality and its possible underlying mechanisms. We found only research articles reporting higher RDW predicts higher risk of all-cause and cause-specific mortalities. However, the pathophysiological mechanisms underlying the association are unknown.

Added value of this study

We performed two parallel analyses to examine promising plasma proteins that mediate the association between RDW and time to death in a representative sample of community-dwelling adults (in the InCHIANTI, "Aging in the Chianti Area" study). This is the first study to identify circulation level of IGFBP2 as a significant mediator of the association between RDW and all-cause mortality in community dwelling adults with or without serious comorbidities. Interestingly, both IGFBP2 measured at single time point and change over time in IGFBP2 (9-year) mediated the association between RDW and mortality.

Implications of all the available evidence

The highlights of our new findings are two points. First, plasma IGFBP2 mediated the relationship between red blood cell distribution width (RDW) and all-cause mortality. Second, senescence-associated secretory proteins may also contribute to mechanisms underlying the RDW-mortality association.

which high RDW is associated with mortality,[2,4,5] but empirical evidence supporting these mechanisms is limited. Thus, clinicians who detect high RDW in their patients have no direction on treatments that may prevent or at least delay these outcomes. Understanding the biological mechanisms by which RDW is linked to multiple health outcomes may help identify potential therapeutic targets.

Proteomic analysis has the potential to identify candidate biomarkers that connect elevated anisocytosis status with health consequences, including excess mortality. To minimise the identification of false positive findings due to the large number of proteins tested, two parallel analyses were performed. First, proteins measured at baseline were tested to examine whether they mediated the association between RDW and mortality over a 15-year follow-up. In the second analysis, to reduce the time gap between exposure and outcome events, we identified proteins for which the change between baseline and year 9 mediated an association between changes in RDW and 6-year mortality. The goal of this project was to identify common proteins that mediated an association between RDW and all-cause mortality in these two analyses and identify promising biomarkers connecting elevated anisocytosis and mortality in a representative sample of community-dwelling adults.

Methods

Study population and design

The Invecchiare in Chianti, "Aging in the Chianti Area" (InCHIANTI) study, is a population-based prospective cohort study designed to investigate the aging process and identify mechanisms underlying the decline in physical function and mobility with age.[6] The study recruited local residents living in the Chianti region in Tuscany, Italy. The details of the study have been published.[6] For the analysis presented here, we selected 962 InCHIANTI participants (21–98 y) whose plasma proteomics data and RDW with concurrent data on BMI, education level, smoking status, and haemoglobin levels at the baseline visit were available. The mean follow-up time was 12·6 ± 3·2 years.

Measurement of plasma proteomics

Blood samples for routine testing and proteomics were taken in the early morning after an overnight fast. In accordance with the guidelines for protein biomarker work, all samples were stored at 4 °C, centrifuged for 4 h, immediately aliquoted, and frozen at −80 °C.[7] Plasma proteins remain stable for 14–17 years in storage at −80 °C and for up to 25 freeze–thaw cycles.[8,9] The present study measured plasma proteomics at baseline and the 9-year follow-up using the 1·3k HTS SOMAscan assay (SomaLogic, Boulder, CO).[10,11] All proteomics data were expressed as abundance in relative fluorescence units and normalised according to the Trans-NIH Center for Human Immunology, Autoimmunity and Inflammation (CHI) pipeline. Data normalization procedures have been described elsewhere.[10] The overall technical variability of the assay is low (median intraplate CV in the 3% to 4% range). Among the proteomic profiles of 1322 SOMAmers in plasma, the following SOMAmers were removed: 12 hybridisation controls, four viral proteins (HPV type 16, HPV type 18, isolate BEN, and isolate LW123) and five SOMAmer reagents that have been reported to be non-specific (P05186/ALPL, P09871/C1S, Q14126/DSG2, Q93038/TNFRSF25, and Q9NQC3/RTN4). As a result, we used 1301 plasma proteins measured at both baseline and the 9-year follow-up visit for further analyses.

All-cause mortality over a 15-year follow-up period

To obtain information on vital status, the study utilised data from the Mortality General Registry operated by

the Tuscany Region and death certificates deposited immediately after death at the registry office in the municipality of residence. In the present analysis, complete data on vital status were available until the 15-year follow-up visit.

Other covariates

The other covariates included sociodemographic variables (sex, age, and years of education), smoking status (current smoker or not), and baseline haemoglobin levels. Weight was measured using a high-precision mechanical scale. Standing height was measured to the nearest 0·1 cm. Body mass index (BMI) was included in the analysis.

Ethics

The study protocols complied with the Declaration of Helsinki and were approved by the Italian National Institute of Research and Care on Aging Ethical Committee, Ancona (baseline, protocol n° 14/CE, 28 February 2000) and by the Local Ethical Committee at Azienda Sanitaria of Florence (follow-up, protocol n° 5/04, 12 May 2004). Written informed consent was obtained from all participants after an extensive description of the study.

Statistics

Descriptive data are shown as mean ± SD for continuous variables and percentage for categorical variables. We checked the assumption of normality using the mean ± SD, Q-Q plot, and Shapiro-Wilk test. If we could not assume normality, log-transformed data were used for further analysis. Differences in age and sex between censored and deceased individuals were tested using the Student's *t*-test and chi-square test, respectively. All other characteristics evaluated at baseline between the same two groups were tested using age-adjusted linear regression models for continuous variables or logistic regression models for categorical variables. Time differences in RDW and 1301 proteins were tested using an age-adjusted linear mixed-effect model. All protein data above or below 3 SD from the mean values were considered outliers and excluded from the analysis.

Figure 1 shows a hypothetical causal diagram. Because of strong evidence that higher RDW predicts a higher risk of all-cause mortality,[3] we hypothesised that higher RDW reflects the dysregulation of some of the biological pathways that are primarily responsible for high risk of mortality and that the nature of such dysregulation may be revealed by the up/downregulation of specific plasma proteins.[3] To test this hypothesis, we set RDW as the exposure, a specific protein as the mediator, and all-cause mortality as the outcome and used the mediation analysis approach proposed by Valeri and VanderWeele.[12,13] We examined (i) the direct association between RDW and all-cause mortality independent of the protein, (ii) the indirect association between RDW and all-cause mortality mediated by one specific protein, and (iii) the total association between RDW and all-cause mortality (i.e., the sum of direct and indirect associations). This formula also provides the proportion of the total association of RDW mediated by the protein examined (%). The analysis relating exposure and outcome was conducted using an exponential distribution accelerated failure time (AFT) model. The relationships between exposure and mediators were explored using linear regression models. All models were protein-specific and adjusted for sex, baseline age, BMI, education level, smoking status, and haemoglobin level.

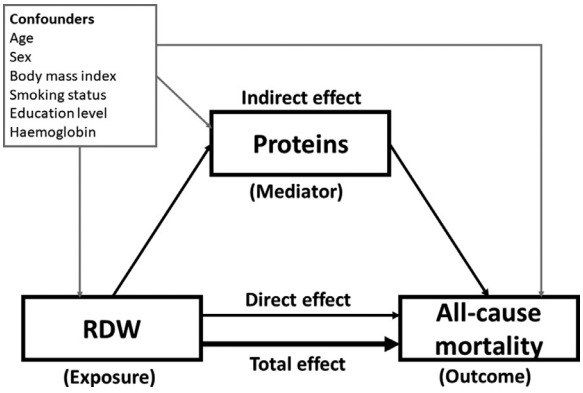

Figure 1. Hypothesised causal diagram for the mediation of proteins in the association between RDW and all-cause mortality.

To test the hypothesis that changes in RDW between baseline and 9-year follow-up visits predict all-cause mortality, and that this association is mediated by changes in a specific set of proteins, we estimated annualised changes in RDW (Δ RDW) or proteins (Δ protein) as a ratio "(9-year follow-up visit − baseline visit)/exact time interval between baseline and 9-year follow-up visit". We repeated mediation analysis using Δ RDW and Δ protein and the same set of covariates as described above. For this analysis, the time to death was calculated using the date of the 9-year follow-up visit as time 0, with the outcome as the 6-year all-cause mortality status.

Because anaemia status and specific medical conditions can affect the association between RDW, proteins, and all-cause mortality,[3] we repeated the analyses limited to participants who were non-anaemic (n = 920 for the cross-sectional analysis; n = 601 for the longitudinal analysis) and free of chronic diseases (cancer, angina pectoris, myocardial infarction, congestive heart failure, stroke, and diabetes; n = 786 for the cross-sectional analysis; n = 529 for the longitudinal analysis) at baseline. anaemia was defined using the World Health organisation values of < 13·0 g/dL haemoglobin in men and < 12·0 g/dL in women.

SAS software version 9·4 for Windows (SAS Institute, Inc., Cary, NC) was used for all data processing

	Overall		Censored		Deceased	
	(*n* = 962)		(*n* = 595)		(*n* = 367)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	66.1	15.4	60.1	15.9	75.9	7.7
Women (n,%)	523 (54.4)		341 (57.3)		182 (49.6)	
Body mass index (kg/m^2)	27.1	4.1	27	3.9	27.4	4.5
Education level (6 years or higher,%)	41.7		52.4		24.3	
current smoker (%)	19.2		21		16.4	
haemoglobin (g/dl)	13.9	1.3	13.9	1.2	13.8	1.4
anaemia (%, n)	42 (4.4)		23 (3.9)		19 (5.2)	
Red cell distribution width (%)	13.5	0.9	13.3	0.7	13.8	1.1
Follow-up time (years)[†]	12.6	3.2	14.4	0.2	9.6	3.6

***Table 1*: Participant characteristics at enrollment.**
[†] Defined as the time between the date of baseline visit and the date of either death or 15-year follow-up visit.

and statistical analyses. We set the level of statistical significance as $p < 0{\cdot}05$ (two-sided) to discover novel biomarkers as mediators without adjustment for multiplicity of testing. Thus, we did not report p-values for outcomes other than the primary analysis. Confidence intervals were two-sided with a 95% confidence level and were not adjusted for multiplicity of testing.

Role of funding source

This study was funded by grants from the National Institutes of Health (R01AG027012, R01AG057723, R01HL111271, and R21HL112662) and National Institute on Aging contract 263MD9164 and was supported by the Intramural Research Program of the National Institute on Aging, National Institutes of Health. The InCHIANTI study baseline (1998–2000) was supported as a 'targeted project' ICS110.1/RF97.71) by the Italian Ministry of Health and in part by the U.S. National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821,336); the InCHIANTI Follow-up 1 (2001–2003) was funded by the U.S. National Institute on Aging (Contracts: N.1-AG-1–1 and N.1-AG-1–2111); and the InCHIANTI Follow-ups 2 and 3 studies (2004–2010) were financed by the U.S. National Institute on Aging (Contract: N01-AG-5–0002) and supported in part by the Intramural Research Program of the National Institute on Aging, National Institutes of Health, Baltimore, Maryland. The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

Results

Baseline characteristics

Table 1 shows the baseline characteristics of the study participants. Among the 962 participants examined in the cross-sectional analysis, 367 (38·1%) died during the 15-year follow-up period. Among the 623 participants who had RDW and proteomics both at baseline and at the 9-year follow-up visits, 137 participants (22·0%) died during the following 6-year period (Appendix Table 1). The number of outliers varied across 1301 proteins (cross-sectional analysis, 9·0 [interquartile, IQR, 5–12); longitudinal analysis, 6·0 (IQR 4–8)). The RDW increased over time (β = 0·06, SE = 0·004). Among 1301 proteins, while circulation levels of 336 proteins increased, those of 186 proteins decreased (Appendix Table 2).

Mediation analysis

In the simple model that did not include proteins as independent variables, both baseline RDW and Δ RDW were associated with mortality: $\beta = -0{\cdot}08$, 95% CI = (−0·08, −0·04) for baseline RDW; and $\beta = -0{\cdot}08$, 95% CI = (−0·13, −0·03) for Δ RDW. Tables 2 and 3 show a list of proteins that mediated the association between RDW at baseline and 15-year all-cause mortality, as well as between changes in RDW and 6-year all-cause mortality (a full list of proteins is shown in Appendix Tables 3 and 4). Among the 1301 proteins measured at baseline, 11 proteins (epidermal growth factor receptor [EGFR], growth hormone receptor [GHR], NT-3 growth factor receptor [NTRK3], superoxide dismutase mitochondrial [SOD2], killer cell lectin-line receptor subfamily F member 1 [KLRF1], thrombospondin-2 [THBS2], metalloproteinase inhibitor 1 [TIMP1], insulin-like growth factor-binding protein 2 (IGFBP2), complement component C9 (C9), apolipoprotein B-100 (APOB), and low-density lipoprotein receptor-related protein 1 B (LRP1B)) mediated the association between RDW at baseline and 15-year all-cause mortality. These proteins mediated 47·9–53·0% of the association between baseline RDW and 15-year all-cause mortality. Meanwhile, changes in two proteins, IGFBP2 and complement component C7, mediated the association

Rank	Mediator		Total association of RDW with all-cause mortality				Direct association of RDW with all-cause mortality				Indirect association of RDW with all-cause mortality through mediator				Proportion mediated
	Target	EntrezGene Symbol	β	95%CI		p-value	β	95%CI		p-value	β	95%CI		p-value	
1	ERBB1	EGFR	0.85	0.77	0.94	0.002	0.88	0.8	0.98	0.015	0.96	0.94	0.99	0.006	52.8%
2	Growth hormone receptor	GHR	0.84	0.76	0.93	0.001	0.88	0.79	0.97	0.011	0.96	0.93	0.99	0.008	53.0%
3	TrkC	NTRK3	0.85	0.77	0.94	0.002	0.88	0.79	0.97	0.011	0.97	0.95	0.99	0.011	51.1%
4	Mn SOD	SOD2	0.86	0.78	0.96	0.005	0.89	0.8	0.98	0.022	0.97	0.95	0.99	0.014	51.9%
5	KLRF1	KLRF1	0.85	0.77	0.94	0.001	0.87	0.78	0.96	0.007	0.98	0.96	0.997	0.02	49.6%
6	TSP2	THBS2	0.85	0.77	0.94	0.002	0.86	0.78	0.96	0.005	0.99	0.97	0.998	0.024	48.6%
7	TIMP-1	TIMP1	0.85	0.77	0.94	0.002	0.86	0.78	0.95	0.004	0.99	0.98	0.999	0.035	47.9%
8	IGFBP-2	IGFBP2	0.85	0.77	0.94	0.002	0.86	0.78	0.96	0.005	0.99	0.97	0.999	0.035	48.4%
9	C9	C9	0.85	0.77	0.94	0.002	0.86	0.78	0.96	0.005	0.98	0.97	0.999	0.035	49.0%
10	Apo B	APOB	0.85	0.77	0.95	0.003	0.87	0.79	0.97	0.009	0.98	0.96	0.999	0.039	49.7%
11	LRP1B	LRP1B	0.86	0.78	0.96	0.005	0.88	0.79	0.97	0.013	0.98	0.97	0.9996	0.044	49.7%

***Table 2*: Mediation of baseline proteins on the association between RDW and all-cause mortality (sorted by p-value in indirect association).**
Mediation analysis was performed using SAS macro (%macro mediation for survival data, Valeri & VanderWeele, 2015). Exposure-outcome association was examined by running protein-specific accelerated failure time model with exponential estimation. RDW-mediator (protein) associations were modeled with multivariable linear regression model. All models were adjusted for age, sex, body mass index, smoking status (current smoker or not), education level (6-year education or higher education or not), and haemoglobin. RDW and proteins were measured at baseline. Outcome was 15-year all-cause mortality.

Rank	Mediator		Total association of RDW with all-cause mortality				Direct association of RDW with all-cause mortality				Indirect association of RDW with all-cause mortality through mediator				Proportion mediated
	Target	EntrezGene Symbol	β	95%CI		p-value	β	95%CI		p-value	β	95%CI		p-value	
1	IGFBP-2	IGFBP2	0.22	0.05	0.86	0.03	0.28	0.07	1.12	0.072	0.77	0.6	0.99	0.038	23.5%
2	C7	C7	0.23	0.06	0.94	0.041	0.31	0.08	1.28	0.106	0.75	0.56	0.998	0.048	25.8%

***Table 3*: Mediation of change in proteins on the association between change in RDW and all-cause mortality (sorted by p-value in indirect association).**
Mediation analysis was performed using SAS macro (%macro mediation for survival data, Valeri & VanderWeele, 2015). Exposure-outcome association was examined by running protein-specific accelerated failure time model with exponential estimation. RDW-mediator (protein) associations were modeled with multivariable linear regression model. All models were adjusted for age, sex, body mass index, smoking status (current smoker or not), education level (6-year education or higher education or not), and haemoglobin. RDW and proteins were measured at baseline. Outcome was 15-year all-cause mortality.

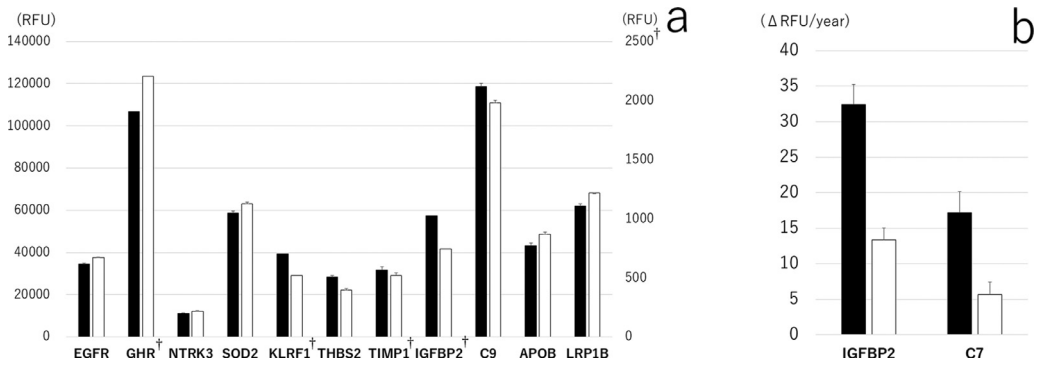

Figure 2. **A**. Abundances in significant plasma proteins at baseline that mediated the association between baseline RDW and 15-year all-cause mortality. **B**. Abundances in significant changes in plasma proteins that mediated the association between change in RDW and 6-year all-cause mortality. Bar graphs represent least-squares means and standard errors from a linear regression model adjusted for age, sex, body mass index, smoking status, years of education, and haemoglobin level (black bars, deceased; white bars, censored). † secondary axis.

between changes in RDW and 6-year all-cause mortality. Δ IGFBP2 mediated 23·5% and Δ C7 mediated 25·8% of the association between Δ RDW and 6-year all-cause mortality, respectively. Of note, there were differences between censored and deceased individuals in protein abundance or changes in protein abundance (Figure 2).

Appendix Table 5 shows the results of exposure-outcome (tested by the AFT model) and exposure-mediator (tested by linear regression model) analyses. RDW and protein that were measured at baseline independently predicted 15-year all-cause mortality in the AFT model, and RDW at baseline was associated with each protein. Meanwhile, in the AFT models for longitudinal analysis, Δ RDW did not predict 6-year all-cause mortality, and Δ IGFBP2 and Δ C7 predicted 6-year all-cause mortality.

Senescence-Associated secretory phenotype (SASP)

To better understand the candidate proteins observed to mediate the association between RDW and all-cause mortality, we checked whether those proteins have been reported as SASP factors.[14] Of the observed proteins that mediated the association between baseline RDW and 15-year all-cause mortality, five have been reported as SASP and both proteins were observed to mediate the association between changes in RDW and 6-year all-cause mortality were reported as SASP (Figure 3).

In anaemia-free individuals at baseline, protein-specific survival analysis identified 10 proteins that mediated the association between RDW at baseline and all-cause mortality (Table 4). Of the 11 proteins identified in the full sample, nine proteins remained significant in anaemia-free individuals, while two proteins, APOB

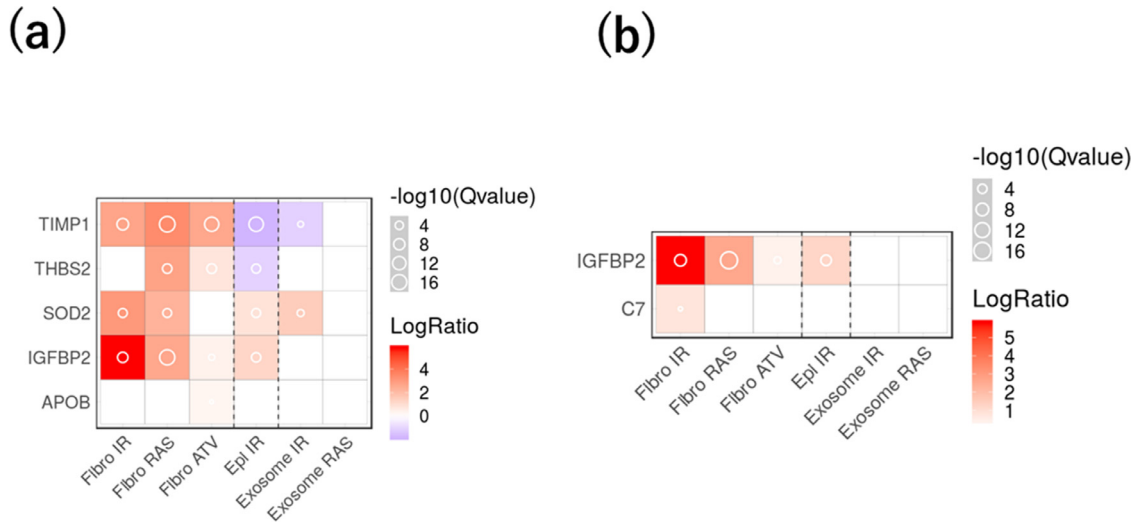

Figure 3. Senescence-Associated Secretory Phenotype (SASP) among significant proteins. a. SASP in proteins that mediated the association between baseline RDW and 15-year all-cause mortality. b. SASP in changes in protein that mediated the association between change in RDW and 6-year all-cause mortality.

Rank	Mediator		Total association of RDW with all-cause mortality				Direct association of RDW with all-cause mortality				Indirect association of RDW with all-cause mortality through mediator				Proportion mediated
	Target	Entrez Gene	β	95%CI		p-value	β	95%CI		p-value	β	95%CI		p-value	
Cross-sectional analysis[†]															
1	ERBB1	EGFR	0.85	0.76	0.94	0.002	0.88	0.79	0.98	0.02	0.96	0.94	0.99	0.01	52.8%
2	Growth hormone receptor	GHR	0.84	0.75	0.93	0.002	0.87	0.79	0.97	0.01	0.96	0.93	0.99	0.01	53.0%
3	TrkC	NTRK3	0.85	0.76	0.94	0.003	0.87	0.78	0.97	0.01	0.97	0.95	0.99	0.02	51.0%
4	IGFBP-2	IGFBP2	0.85	0.76	0.94	0.002	0.86	0.77	0.96	0.01	0.98	0.97	0.997	0.02	48.9%
5	Mn SOD	SOD2	0.86	0.77	0.95	0.004	0.88	0.79	0.98	0.02	0.97	0.95	0.996	0.02	51.2%
6	KLRF1	KLRF1	0.84	0.76	0.94	0.001	0.86	0.78	0.96	0.01	0.98	0.96	0.997	0.02	49.7%
7	TSP2	THBS2	0.85	0.76	0.94	0.002	0.86	0.77	0.96	0.01	0.98	0.97	0.998	0.03	48.4%
8	TIMP-1	TIMP1	0.85	0.76	0.94	0.002	0.86	0.77	0.95	0.004	0.99	0.98	0.999	0.03	48.0%
9	N-terminal pro-BNP	NPPB	0.85	0.77	0.95	0.003	0.86	0.78	0.96	0.01	0.99	0.97	0.999	0.03	48.4%
10	C9	C9	0.85	0.76	0.94	0.002	0.86	0.77	0.96	0.01	0.98	0.96	0.999	0.04	49.0%
Longitudinal analysis[‡]															
1	C7	C7	0.26	0.06	1.18	0.08	0.36	0.08	1.70	0.20	0.71	0.51	0.997	0.048	29.4%
2	IGFBP-2	IGFBP2	0.22	0.05	0.96	0.04	0.28	0.06	1.24	0.09	0.78	0.61	1.01	0.055	23.2%

***Table 4*: Mediation of proteins on the association between RDW and all-cause mortality in anemia-free adults (sorted by p-value in indirect association).**

Protein-specific accelerated failure time model with exponetial estimation. RDW-mediator (one protein) associations were modeled with multivariable linear regression model. All models were adjusted for age, sex, body mass index, smoking status (current smoker or not), education level (6-year education or higher education or not), and haemoglobin.

[†] RDW and proteins were measured at baseline. Outcome was 15-year all-cause mortality.

[‡] Longitudinal changes in RDW and proteins were calculated from {(9-year follow-up visit - baseline visit)/exact time intervals in each participant}. Outcome was 6-year all-cause mortality from 9-year follow-up visit.

and LRP1B, did not, and one new protein, NTproBNP, became a mediator. In longitudinal analysis, while Δ C7 remained a mediator in the association between Δ RDW and 6-year all-cause mortality, Δ IGFBP2 was not a mediator in the association.

Table 5 shows the results of the mediation analyses in those with no major chronic diseases. Seven proteins (NT-2 growth factor receptor [NTRK2], KLRF1, EGFR, NTRK3, GHR, LRP1B, and IGFBP2) mediated the association between RDW at baseline and 15-year all-cause mortality. In longitudinal analysis, two proteins (Δ TFF3 and Δ IGFBP2) mediated the association between Δ RDW and 6-year all-cause mortality (indirect effect, TFF3; IGFBP2), but not Δ C7 (indirect effect).

Discussion

To the best of our knowledge, this is the first study to identify IGFBP2 as a mediator of the association between RDW and all-cause mortality in community-dwelling adults with or without serious comorbidities. Remarkably, from two parallel analyses, we found that both IGFBP2 and change over time in IGFBP2 mediated the association between RDW and mortality. Of note, from the results of the cross-sectional analysis, several proteins found to mediate the association between RDW and mortality have been described as SASP members, suggesting that cellular senescence, possibly at the level of haematopoietic cells, may be implicated in this association.

RDW has been suggested as an integrative biomarker for a multidimensional dysfunctional physiological status that is clinically revealed by anisocytosis.[15] Major proposed mechanisms of anisocytosis are oxidative stress, inflammation, and senescence of erythropoietic cells.[2,5] RDW-related genetic variants are enriched in telomere length, ribosomal RNA, and apoptosis but the role of these mechanisms has not been confirmed.[16] In this study, we found that baseline RDW predicted all-cause mortality in our population similar to previous studies.[3] A novel finding of this study is that although on average RDW increases with aging,[17] those individuals who show a steeper increase in RDW over time also have an increased risk of mortality. This finding suggests that RDW should be monitored over time, as these changes, including the rate of change, may signal an impending deterioration of health status.

Our major finding is that circulating levels of IGFBP2 mediate the association between RDW and all-cause mortality in both cross-sectional and longitudinal analyses. IGFBP2 is a member of the IGF-binding protein family (IGFBP1–6). IGFBP2 regulates the ability of IGF to activate IGF-I receptors. IGFBP2 binds IGF1, IGF2, and insulin, with the greatest affinity for IGF2.[18] IGFBP2 is an IGF system regulator, whose major functions are to promote proliferation, survival, differentiation, and motility in various cell types.[19,20] It is the second most abundant IGF-binding protein and is negatively regulated by growth hormones. In the haematopoietic system, IGFBP2 supports survival and cycling of haematopoietic stem cells.[21] Previous studies have shown that elevated IGFBP2 is associated with a higher risk of adverse changes in body composition and physical function.[22] Several lines of evidence suggest that elevated IGFBPs, including IGFBP2 in senescent endothelial and epithelial cells, are implicated in the regulation of signal pathways for cellular senescence in age-related diseases.[19,23,24] Epidemiological studies have found that IGFBP2 increases with age and is associated with age-related diseases such as cancer, dementia, and mortality in older adults.[19,22,25–28] Consistent with the literature, we found higher circulating levels of IGFBP2 or larger increases in IGFBP2 in individuals closer to death.[29] Interestingly, these results hold when we restricted our analyses to disease-free individuals, while the mediating effect of change in IGFBP2 was just shy of significance when the analysis was restricted to anaemia-free individuals at baseline (p = 0·055). A possible explanation is that the same biological pathway that connects RDW with higher mortality is also likely to cause anaemia. Future studies should test the hypothesis that higher plasma IGFBP2 levels are associated with pathological changes in the haematopoietic system that eventually cause elevated RDW.

Notably, in addition to IGFBP2, we found that the plasma levels of several SASP proteins mediated the association between RDW and all-cause mortality.[14] In particular, four proteins (TIMP1, THBS2, SOD2, and APOB) showed mediation in cross-sectional analyses and one protein (C7) in longitudinal analysis. Anisocytosis has been related to parenchymal hypoxia and chronic inflammatory status, both of which have been shown to precipitate cellular senescence.[30–34] TIMP1 is a member of the TIMP family that inhibits matrix metalloproteinase enzymes involved in extracellular matrix maintenance and remodelling. TIMP1, which regulates the survival and proliferation of normal hematopoietic progenitor cells and increases expression level in response to inflammation, predicts cardiovascular-specific or all-cause mortality.[35–37] THBS2 belongs to a group of matricellular proteins that are components of the extracellular matrix and is overexpressed in response to damage or during remodelling in cardiovascular diseases.[38] Epidemiological studies have found elevated THBS2 level to predict incident mobility disability and all-cause mortality.[39,40] SOD2 plays a role in one of the primary defence mechanisms for the buffering of reactive oxygen species and is involved in the oxidative stress pathway.[41] SODs catalyse the dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2).[42] Using a mouse model, it was demonstrated that failure to express SOD2 impaired mitochondrial complex II activity and induced cellular senescence.[43] SOD2 is a key protein that controls the mitochondrial

Rank	Mediator		Total association of RDW with all-cause mortality				Direct association of RDW with all-cause mortality				Indirect association of RDW with all-cause mortality through mediator				Proportion mediated
	Target	EntrezGene Symbol	β	95%CI		p-value	β	95%CI		p-value	β	95%CI		p-value	
Cross-sectional analysis[†]															
1	TrkB	NTRK2	0.85	0.76	0.96	0.01	0.89	0.79	1.005	0.06	0.96	0.93	0.99	0.01	54.5%
2	KLRF1	KLRF1	0.86	0.76	0.96	0.01	0.88	0.78	0.997	0.04	0.97	0.95	0.99	0.02	51.7%
3	ERBB1	EGFR	0.87	0.77	0.98	0.02	0.89	0.79	1.01	0.06	0.97	0.94	0.99	0.02	53.3%
4	TrkC	NTRK3	0.86	0.77	0.97	0.02	0.89	0.79	1.003	0.06	0.97	0.95	0.996	0.02	52.3%
5	Growth hormone receptor	GHR	0.85	0.75	0.96	0.01	0.88	0.78	0.99	0.04	0.96	0.93	0.996	0.03	52.7%
6	LRP1B	LRP1B	0.87	0.77	0.99	0.03	0.89	0.79	1.01	0.07	0.98	0.96	0.999	0.04	51.6%
7	IGFBP-2	IGFBP2	0.87	0.77	0.98	0.02	0.88	0.78	0.99	0.04	0.99	0.97	0.9998	0.05	49.3%
Longitudinal analysis[‡]															
1	TFF3	TFF3	0.36	0.08	1.66	0.19	0.55	0.11	2.78	0.47	0.66	0.44	0.98	0.04	43.9%
2	IGFBP-2	IGFBP2	0.31	0.06	1.52	0.15	0.42	0.08	2.09	0.29	0.74	0.55	0.99	0.04	33.0%

***Table 5*: Mediation of proteins on the association between RDW and all-cause mortality in adults without medical conditions (sorted by p-values in indirect association).**

Protein-specific accelerated failure time model with exponetial estimation. RDW-mediator (one protein) associations were modeled with multivariable linear regression model. All models were adjusted for age, sex, body mass index, smoking status (current smoker or not), education level (6-year education or higher education or not), and haemoglobin.

[†] RDW and proteins were measured at baseline. Outcome was 15-year all-cause mortality.

[‡] Longitudinal changes in RDW and proteins were calculated from {(9-year follow-up visit - baseline visit)/exact time intervals in each participant}. Outcome was 6-year all-cause mortality from 9-year follow-up visit.

redox state and lower circulating levels of SOD2 with aging is associated with mitochondria dysfunction and age-related degeneration in the nervous system, and all-cause mortality.[42,44,45] APOB is implicated in lipid metabolism, and is upregulated with oxidative stress and systemic inflammation.[46] It has been shown that APOB predicts coronary heart disease-specific mortality.[47] Thus, one working hypothesis suggested by these findings is that oxidative stress and inflammation may contribute to induce cellular senescence and subsequent production of SASP proteins that circulate in blood and both locally and systemically have negative effects on the hematopoietic system leading to impaired erythropoiesis and high RDW. Future studies should test the hypothesis that RDW is a biomarker of senescent cell accumulation in different organs.

Two complement components (C7 and C9) were found to be mediators of RDW and all-cause mortality. Working with other immune or defence systems, the complement system is an arm of innate immunity that removes pathogens, necrotic, and apoptotic cells.[48] The complement system mediates inflammatory status and cell lysis.[49] Both over-expression and under-expression of the complement system have been implicated in complement-mediated inflammatory status and chronic disease risk, especially Alzheimer's disease (AD).[50–53] While the SASP Atlas shows C7 as SASP but not C9, C7, and C9 have similar structures and both proteins form the membrane attack complex (MAC).[14,54] Under oxidative stress in endothelial cells, such as brain neurons and kidneys, abnormally activated MAC is implicated in tissue injury due to downregulation of complementary complement inhibitors such as complement factor H.[52,55] The observations that C9 measured at a single time point and longitudinal change in C7 mediate RDW and all-cause mortality suggest that even in the complement system involved in the MAC, the trajectory changes in protein abundance and the role in mortality may differ by protein.

Five additional proteins measured at baseline that were not identified in the SASP included EGFR, GHR, NTRK3, KLRF1, and LRP1B, to illuminate the underlying mechanisms between RDW at baseline and 15-year all-cause mortality. Of these proteins, EGFR and GHR, which were not identified in the SASP, have been associated with cellular senescence. For example, it was previously found that five cytokines (IL-1β, IL-13, MCP-2, MIP-3α, and SDF-1α) induced cellular senescence through EGFR activation in an EGF-independent pathway, suggesting that cellular senescence can be induced by proinflammatory cytokines via EGFR signalling.[56] Further, in another study, GHR deficiency was demonstrated to protect against age–related NLRP3 inflammasome activation and immune senescence.[57] In addition, EGFR, LRP1B, NTRK3, and GHR have been implicated in nervous system development, neurodegeneration, and development of dementia/AD.[15,58–65] Elevated RDW is associated with poor cognitive performance and RDW predicts cardiovascular diseases, stroke, and dementia, suggesting that RDW may also reflect cerebrovascular pathology.[2,15] Accordingly, LRP-1 mediates transport of β-amyloid out of the brain and is expressed in endothelial cells and pericytes of the blood brain barrier.[66] Dysfunction of LRP1 significantly exacerbates accumulation of Aβ in the brain.[67] Another protein, NKp80/KLRF1, is expressed on the surface of NK cells and indicates NK cell maturity as KLRF1 acquisition is associated with a decline in cytokine production potential.[68,69] It has been demonstrated that these proteins are up/down regulated in response to oxidative stress or inflammatory status.[70–75] Since our study examined the association among RDW, protein expression, and all-cause mortality, further studies are needed to examine cause-specific mortality to identify the possible physiopathology in which these proteins play key roles.

Our study has several strengths. An important strength is the longitudinal design with highly representative population-based samples with demographic diversity and low attrition during follow-up. We examined a wide range of proteins to explore promising candidate proteins that might elucidate the mechanisms underlying the connection between the diversity of red blood cell size and all-cause mortality. Furthermore, in addition to examining proteins measured at one time point and their mediation effects on the association between RDW at baseline and 15-year all-cause mortality, we performed longitudinal analyses using annual changes in RDW and proteins using baseline and 9-year follow-up visits. The limitations of our study include the use of an aptamer-based platform for proteomics that provides relative quantification; therefore, replication using antibody-based arrays and/or targeted LC-MS/MS methods is needed, in addition to repeating our findings in other independent populations. Second, a conservative approach to identify promising proteins by using multi-test adjustment was not used, as this study used a hypothesis-free discovery approach, and our interest was not to increase a type II error, but to interpret promising proteins identified by using nominal p-values.[76,77] Third, because protein-protein interactions can occur, a protein-specific analysis should not be independent. However, significant proteins were consistent in the sensitivity analyses, suggesting that our main results were robust. Fourth, while we accounted for possible comorbidity status including anaemia, other haematological status such as transfusion and sepsis may affect the association among RDW, proteins, and mortality; however, the InCHIANTI study did not evaluate these haematological statuses. Finally, causes of death were not available for all participants who died because cause-specific data have not yet been released by the Tuscany regional authorities.

Conclusion

The present study demonstrates that plasma IGFBP2 is a promising candidate protein that mediates the association between RDW and all-cause mortality in community-dwelling adults. We also found other promising proteins associated with cellular senescence that could play a mediating role in the observed associations. Future investigations are needed to examine the roles of these proteins in biological pathways that lead to premature death.

Contributors

LF directed and supervised the project. The SOMAscan assay was run by GF, JC, and MR conducted proteomic data normalisation and cleaning. YO and LF configured the concept and design of the study and manuscript preparation. YO and TT verified underlying data and conducted statistical analyses. YO, LF, TT, RDS, and EMS contributed to the data interpretation. YO prepared the manuscript, and all authors have contributed to and approved the final version of the manuscript.

Declaration of interests

All authors declare no conflicts of interest.

Acknowledgements

This study was funded by grants from the National Institutes of Health (R01AG027012 and R01AG057723 to Dr. Semba, and R01HL111271 and R21HL112662 to Dr. Ferrucci) and National Institute on Aging contract 263MD9164 (to Dr. Ferrucci) and was supported by the Intramural Research Program of the National Institute on Aging, National Institutes of Health. The InCHIANTI study baseline (1998–2000) was supported as a 'targeted project' ICS110.1/RF97.71) by the Italian Ministry of Health and in part by the U.S. National Institute on Aging (Contracts: 263 MD 9164 and 263 MD 821336); the InCHIANTI Follow-up 1 (2001–2003) was funded by the U.S. National Institute on Aging (Contracts: N.1-AG-1–1 and N.1-AG-1–2111); and the InCHIANTI Follow-ups 2 and 3 studies (2004–2010) were financed by the U.S. National Institute on Aging (Contract: No1-AG-5–0002) and supported in part by the Intramural Research Program of the National Institute on Aging, National Institutes of Health, Baltimore, Maryland.

Data sharing statement

Proteomics data are not publicly accessible due to the Italian law. However, the proteomic data generated from this study are available upon request through the submission of proposals at the InCHIANTI study website http://inchiantistudy.net/wp/. SAS programs are available as deposited in Zenodo (DOI: 10.5281/zenodo.5795636).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2022.103816.

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