HHS Public Access

Author manuscript

J Intern Med. Author manuscript; available in PMC 2024 August 28.

Published in final edited form as:

J Intern Med. 2023 May ; 293(5): 589–599. doi:10.1111/joim.13612.

Metabolomic markers mediate erythrocyte anisocytosis in older adults: Results from three independent aging cohorts

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Abstract

Background.—Anisocytosis reflects unequal-sized red blood cells and is quantified using red blood cell distribution width (RDW). RDW increases with age and has been consistently associated with adverse health outcomes, such as cardiovascular disease and mortality. Why RDW increases with age is not understood. We aimed to identify plasma metabolomic markers mediating anisocytosis with aging.

Methods.—We performed mediation analyses of plasma metabolomics on the association between age and RDW using resampling techniques after covariate adjustment. We analyzed data from adults aged 70 or older from the main discovery cohort of the Baltimore Longitudinal Study of Aging (BLSA, $n = 477$, 46% women) and validation cohorts of the Health, Aging and Body Composition Study (Health ABC, $n = 620$, 52% women) and Invecchiare in Chianti, Aging in the Chianti Area (InCHIANTI) study ($n = 735$, 57% women). Plasma metabolomics was assayed using the Biocrates MxP Quant 500 kit in BLSA and Health ABC and liquid chromatography with tandem mass spectrometry in InCHIANTI.

Results.—In all three cohorts, symmetric dimethylarginine (SDMA) significantly mediated the association between age and RDW. Asymmetric dimethylarginine (ADMA) and 1-methylhistidine were also significant mediators in the discovery cohort and one validation cohort. In the discovery

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Author contributions

Qu Tian and Luigi Ferrucci designed the study and acquired metabolomics data in BLSA and Health ABC. Qu Tian and Brendan A. Mitchell performed the analysis and drafted the manuscript. Ruin Moaddel, Carmine Zoccali, and Stefania Bandinelli contributed to the interpretation of the findings and critically evaluated the manuscript. All authors edited and approved the manuscript.

Conflict of interests statement

The authors declare no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

cohort, we also found choline, homoarginine, and several long-chain triglycerides significantly mediated the association between age and RDW.

Conclusions and relevance.—This metabolomics study of three independent aging cohorts identified a specific set of metabolites mediating anisocytosis with aging. Whether SDMA, ADMA, and 1-methylhistidine are released by the damaged erythrocytes with high RDW or they affect the physiology of erythrocytes causing high RDW should be further investigated.

Keywords

1-methylhistidine; ADMA; aging; metabolomics; red blood cell distribution width; SDMA

Introduction

Anisocytosis is a condition of high variation in erythrocyte volume and size. It is usually associated with low iron stores, low hemoglobin, and high erythropoietin levels despite normal ferritin levels, which may indicate impaired mobilization of iron [1]. It can trigger the risk of atherosclerosis and is also associated with the severity of atherosclerosis, perhaps due to its strong link to chronic vascular inflammation [2]. Anisocytosis can be assessed clinically using the metric of red blood cell distribution width (RDW), which provides information on the degree of heterogeneity or variation of erythrocyte volume and size. RDW over 14% is considered abnormal, and high RDW has been consistently associated with adverse outcomes, such as cardiovascular disease, metabolic syndrome, and mortality [2–8]. RDW has also been proposed as an inflammatory marker because it is associated with tumor necrosis factor-alpha and interleukin 6.

Several studies have reported that RDW increases with age even in individuals without overt disease conditions [9–12]. The median RDW of adults aged 60 or older is approximately 11% higher than those younger than 60 [9], and this increase with age appears steeper after age 70. Additional data have shown that this age-related elevation of RDW is not caused by technical issues in the assessment equipment [11].

Mechanisms underlying age-related elevation of RDW remain a mystery. It is hypothesized that the progressively higher anisocytosis in older age may contribute to the association between age and RDW, perhaps due to inflammation and deficiencies of folate and B12 vitamin [12, 13]. However, folate and B12 vitamin only explain a small proportion of the relationship. In-line with the “Geroscience Hypothesis”, we formulated the hypothesis that the mechanisms of the biology of aging cause increased anisocytosis with aging. In this study, we aimed to identify plasma metabolites that would account for the age-related elevation of RDW in older adults living in the community, and all participants were volunteers and tended to be healthier than the general population. We analyzed the mediation effects of metabolites on the association between age and RDW in 477 participants aged 70 or older in the Baltimore Longitudinal Study of Aging (BLSA) and validated the initial findings in the Health, Aging and Body Composition Study (Health ABC; $n = 620$) and also the Invecchiare in Chianti, Aging in the Chianti Area (InCHIANTI) study ($n = 735$).

Methods

Study population and design

We used the BLSA as the main discovery cohort and the Health ABC and InCHIANTI as validation cohorts. The BLSA is a prospective study with continuous enrollment since 1958 [14, 15]. At enrollment, eligible participants must be free of major chronic conditions and cognitive impairment. Follow-up visits occur at varying intervals depending on a participant's age: every 4 years for those younger than 60, every 2 years for those aged 60–79 years, and every year for those aged 80 and older. A total of 1036 participants had concurrent data on RDW and plasma metabolomics using the Biocrates MxP Quant 500 kit at the first assessment between 2006 and 2021. To make the age range comparable to the validation cohorts of the Health ABC and InCHIANTI, we identified 477 BLSA participants aged 70 or older (46% women, 17% Black) for the analysis. The National Institutes of Health Institutional Review Board approved the BLSA protocol. All participants provided written informed consent at each BLSA visit.

The Health ABC is a longitudinal study that enrolled 3075 men and women aged 70–79 years between April 1997 and June 1998 from two sites in Memphis, Tennessee, and Pittsburgh, Pennsylvania. The aim of the study is to evaluate the impact of changes in weight and body composition on age-related physiological and functional changes in well-functioning older adults. Eligibility criteria included self-report of ability to walk one fourth of a mile, climb 10 steps, and perform daily living tasks without difficulty. Additionally, participants must be free of life-threatening illnesses and plan to remain in the geographic area for a minimum of 3 years. A total of 620 participants had concurrent data on RDW and plasma metabolomics using the Biocrates MxP Quant 500 kit at the first assessment (52% women, 42% Black). Specifically, RDW data were first collected at the year 3 visit, and plasma metabolomics by the Biocrates MxP Quant 500 kit was first available at the year 2 visit. Data collection for this analysis was between 1998 and 1999. The Health ABC study was approved by the Institutional Review Boards at the University of Tennessee Health Science Center and the University of Pittsburgh. All participants provided written informed consent.

The InCHIANTI Study is a population-based longitudinal study that began in 1998 [16]. This study was conducted by the Laboratory of Clinical Epidemiology of the Italian National Research Council on Aging in collaboration with the Laboratory of Epidemiology, Demography and Biometry at the National Institute on Aging. Eligible participants aged 65–102 were randomly selected from Greve in Chianti and Bagno a Ripoli in the Tuscany Region of Italy using a multistage stratified sampling method. Enrolled participants underwent an extensive assessment at baseline between September 1998 and March 2000 and were reevaluated every 3 years during follow-up. We selected 735 participants aged 70 or older who had concurrent data on RDW and plasma metabolite markers of interest at baseline (57% women, 0% Black). The InCHIANTI Study protocol was approved by the INRCA ethics committee. All participants in the study provided written informed consent.

Collection of plasma

In the BLSA, blood was collected at the National Institute on Aging Clinical Research Unit, MedStar Harbor Hospital in Baltimore, MD, following a standardized protocol, as described previously [17]. Participants were asked to abstain from smoking, exercising, or taking medications before the blood sample collection. Blood samples were drawn from the antecubital vein between 07:00 and 08:00 AM after an overnight fast, then stored immediately at 4°C, centrifuged within 4 h, and immediately aliquoted and frozen at −80°C. The collection of EDTA plasma in the BLSA is consistent with guidelines for biomarker studies [18].

In the Health ABC study, blood was first collected at the year 2 visit, following a standardized protocol as described previously [19]. Venous blood samples were drawn in the morning after overnight fasting and were frozen at −80°C from the time of collection until September 2020 when metabolites were measured.

In the InCHIANTI study, blood samples were collected after a 12-h fast. Serum samples were aliquoted and frozen at −80°C and were not thawed until analyzed [20].

Measurement of plasma metabolomics

In both BLSA and Health ABC, plasma metabolomics was measured using liquid chromatography with tandem mass spectrometry (LC–MS/MS) for small molecules, and lipids and hexoses were measured by flow injection analysis–tandem mass spectrometry (FIA–MS/MS). Metabolites were extracted, and concentrations were measured using the MxP Quant 500 kit (Biocrates Life Sciences, AG, Innsbruck, Austria) following the manufacturer's protocol for a 5500 QTAP (Sciex, Framingham, MA, USA), as described in detail elsewhere [21]. In the InCHIANTI study, targeted metabolomics data are not collected. Based on initial findings from the BLSA, we aimed to validate the mediation effects of a specific set of plasma metabolites that were assayed by LC–MS/MS [22], including symmetric dimethylarginine (SDMA), asymmetric dimethylarginine (ADMA), homoarginine (hArg), and arginine.

Statistical analysis

Differences in participants' characteristics between the three cohorts were tested using independent *t*-tests and chi-square tests as appropriate. In all three cohorts, we first examined the association between age and RDW using multivariate linear regression, adjusted for sex, race, and study site if applicable. We preprocessed plasma metabolomics data as follows. Metabolites with more than 30% missing values (below the limit of detection) were first excluded. For the remaining metabolites, we imputed the missing values by half of the minimum. Metabolite concentrations were then normalized using log2 transformation. Of 638 metabolites potentially assessed by the MxP Quant 500 kit, 468 metabolites in the BLSA and 496 metabolites in Health ABC were included in this analysis. 457 metabolites were in common, 11 metabolites were unique to the BLSA sample, and 39 metabolites were unique to the Health ABC sample (Fig. S1).

We used the *mediation* R package to examine the mediation effect of each metabolite on the association between age and RDW [23]. We set age as the exposure, each metabolite as the mediator, and RDW as the outcome. We examined (i) the direct effect of age (exposure) on RDW (outcome) while adjusting for each metabolite (mediator), (ii) the indirect effect that was the product between the coefficient of the effect of age on each metabolite and the coefficient of the effect of each metabolite on RDW, (iii) the total effect that was the sum of direct and indirect effects, and (iv) the proportion of the total effect of age on RDW while adjusting for the metabolite. These associations were analyzed using multivariate linear regression models, adjusted for sex, race, and study site if applicable. We then repeated the mediation analysis in a subset of the BLSA sample aged 70–85 to match the higher age limit in the Health ABC.

Because initial findings showed the mediation effects of SDMA, ADMA, and 1-methylhistidine, all of which were related to kidney disease and renal function [17, 24–26], we additionally adjusted for glomerular filtration rate (GFR) as sensitivity analyses. GFR was assessed using the creatinine Chronic Kidney Disease Epidemiology Collaboration (CKD–EPI) equation. We checked whether additional adjustments for body mass index (BMI) or hemoglobin accounted for the mediating effects of metabolites as sensitivity analyses. We also examined the proportion mediated by both ADMA and SDMA as mediators in the model using the multiple mediation analysis.

All analyses were performed using RStudio version 4.0.2 (Boston, MA). In this exploratory analysis, we set the level of statistical significance as two-sided $p < 0.05$. Because the *mediation* package uses quasi-Bayesian resampling simulations to estimate the uncertainty of the effects derived from the parametric linear regression models, we used 10,000 simulations per model to provide stable estimates.

Results

Participant characteristics of the three cohorts are presented in Table 1. Compared to the main discovery cohort, Health ABC participants were older and had higher BMI and GFR, and InCHIANTI participants had higher BMI and RDW (all $p < 0.05$). There were no significant differences in hemoglobin between the discovery and validation cohorts ($p > 0.05$). In all three studies, older age was associated with higher RDW after covariate adjustment (BLSA: $\beta = 0.025$, 95% CI: 0.0094–0.0407, $p = 0.002$; Health ABC: $\beta = 0.042$, 95% CI: 0.0104–0.0741, $p = 0.009$; InCHIANTI: $\beta = 0.059$, 95% CI: 0.0471–0.0715, $p < 0.001$). Scatter plots of RDW as a function of age in these three studies are shown in Fig. 1.

After covariate adjustment, of the 468 metabolites examined in the BLSA, 10 metabolites showed significant mediation effects on the association between age and RDW, including choline, SDMA, ADMA, 1-methylhistidine, hArg, and several long-chain triglycerides (C18:2, C18:1, C18:0, C17:0, C16:0) (Table 2). The proportion mediated by these metabolites ranged between 7.3% and 17.8%. When both ADMA and SDMA were included as multiple mediators, the proportion mediated by both markers increased to 25%. In a subset of BLSA participants aged 70–85 years, three metabolites (choline, SDMA, and ADMA) remained significant mediators (Table S1). Of the 496 metabolites examined in the

Health ABC, 7 metabolites significantly mediated the association between age and RDW, including SDMA, 1-methylhistidine, homocysteine, glutamic acid, phosphatidylcholine aa C38:4, hydroxysphingomyelin C22:1, and valine. The proportion mediated by these metabolites ranged between 8.2% and 12.4% (Table 2). The proportion mediated by ADMA and SDMA as multiple mediators was 15%. In the InCHIANTI study, SDMA and ADMA significantly mediated the association between age and RDW (Table 2). The proportion mediated by SDMA was 12.5%, and the proportion mediated by ADMA was 5.5%. The mediation effects of hArg and arginine were not significant. The proportion mediated by both ADMA and SDMA as multiple mediators increased to 27%. Scatter plots of SDMA, ADMA, and 1-methylhistidine as a function of age are shown in Fig. 1.

The mediation effects of SDMA and ADMA were attenuated after additional adjustment for GFR in the BLSA but remained significant in InCHIANTI, and the mediation effect of SDMA remained significant in the Health ABC (data not shown). The mediation effect of 1-methylhistidine was attenuated after GFR adjustment in the BLSA but remained a marginal significance in the Health ABC (data not shown). After adjustment for BMI or hemoglobin, some mediation effects of ADMA and SDMA remained in either the discovery cohort or the validation cohort but the mediation effect of 1-methylhistidine was attenuated (data not shown).

Discussion

In a sample of community-dwelling older adults aged 70 or older, we found that a specific set of metabolites mediated the association between age and RDW, including SDMA, ADMA, choline, hArg, 1-methylhistidine, and several long-chain triglycerides. Of these, SDMA, ADMA, and 1-methylhistidine were also found to mediate the relationship between age and RDW in different aging cohorts aged 70 or older. These findings from three independent cohorts of community-dwelling older adults provide for the first time novel insights into the mechanisms underlying the age-related increase in RDW.

Findings of ADMA and SDMA in the main discovery and validation cohorts are scientifically important, which may suggest that ADMA and SDMA are released from erythrocytes with elevated RDW in aging. It is also possible that arginine metabolism affects the physiology of erythrocytes and causes anisocytosis. Both ADMA and SDMA are nonproteinogenic amino acids and posttranslationally modified forms of arginine, and after proteolysis, both are released into the cytoplasm [27]. In middle-aged and older adults, both markers are associated with a wide range of adverse outcomes, particularly cardiovascular disease, kidney disease, sepsis, and mortality [24, 28, 29]. The mechanism underlying ADMA and SDMA with cardiovascular disease is thought to be their inhibiting roles in nitric oxide synthase (NOS). ADMA is an endogenous inhibitor of NOS, and SDMA may indirectly inhibit NOS by inducing deficiency in intracellular arginine. Because NOS mediates vascular tone, decreased NO production contributes to impaired endothelial function and the development of atherosclerosis. Importantly, cellular levels of ADMA can be 5- to 20-fold greater than plasma levels and the range at cellular levels can tonically inhibit NOS [30]. Relatively recent data using both human and animal models have shown that anisocytosis disrupts intravascular hemodynamics and leads to increased

interactions between circulating cells and vessel walls, which contribute to subsequent vascular pathology [31]. It is worth noting that the erythrocyte is a storage site for ADMA [32]. In acute conditions, the discrepancy between intraerythrocyte and plasma ADMA increases, probably due to the higher turnover of erythrocyte precursor cells in the bone marrow. In acute conditions, there is an increase in anisocytosis (or high RDW). Thus, ADMA may be a marker of enhanced turnover of erythrocyte precursors [32].

Both ADMA and SDMA are related to kidney disease and renal function, and their mechanisms appear different. ADMA, but not SDMA, is mainly metabolized by dimethylarginine dimethylaminohydrolase 1 and 2 to citrulline and dimethylamine [33]. Some data suggest that elevated ADMA is associated with renal fibrosis through collagen and TGF-beta1 synthesis [25]. SDMA is produced by the protein–arginine methyltransferase 5 (PRMT 5) and PRMT 7, both of which are type II methyltransferases [34, 35]. SDMA is almost exclusively eliminated by renal excretion, and it is considered a novel marker of GFR and renal dysfunction [36]. Recent data suggest that SDMA is more sensitive in predicting renal function compared to ADMA and other methylarginines [24, 37]. In our study, the attenuated associations after additional adjustment for renal function may suggest that compromised renal function, indicated by ADMA and SDMA, explains at least in part the age-related increase in RDW.

Homoarginine (hArg), another nonproteinogenic amino acid, also significantly mediated the association between age and RDW in the main discovery cohort. Note that the mediation effect of hArg was not significant in the Health ABC or InCHIANTI studies. Because hArg has a low affinity to NO, it is not considered an important NOS inhibitor. Studies have shown that elevated ADMA and SDMA and lower hArg are associated with cardiovascular disease and renal function [26, 28, 38, 39]. Mechanisms underlying these amino acids with cardiovascular disease are thought to be impaired endothelial function due to inhibited NOS and increased oxidative stress. This long-regarded view has become controversial because ADMA and SDMA show weak inhibitor potency, and hArg shows a low affinity to endothelial NOS. Moreover, these three amino acids are not associated with oxidative stress. Thus, it has been hypothesized that their precursor proteins may be the underlying causes of pathophysiological changes in the development of cardiovascular disease, particularly the antagonistic actions of N^G-methylated and guanidinated proteins [40].

Other metabolites, such as 1-methylhistidine, choline, and several long-chain triglycerides, were also found to mediate the age-related elevation of RDW in the main discovery cohort of BLSA. The mediation effect of 1-methylhistidine was replicated in the validation cohort of the Health ABC. 1-Methylhistidine is a major methyl-containing derivative of histidine and an indicator of meat dietary intake because humans cannot synthesize it. Because 1-methylhistidine has a relatively long elimination half-life among healthy individuals (i.e., 17 h), elevated levels of 1-methylhistidine can indicate the amount of meat intake [41, 42]. Previous studies have shown that high red meat consumption is associated with an elevated risk of developing CKD [43], and individuals with CKD had higher levels of 1-methylhistidine than those without CKD [17]. In the current study of both discovery and validation cohorts, additional adjustment for renal function attenuated the mediation effect of 1-methylhistidine. The increase in RDW may be due to impaired

renal function as indicated by 1-methylhistidine. 1-methylhistidine may also indicate decreased digestive activity and increased gut permeability. Some studies suggest that 1-methylhistidine is affected by muscle mass and endogenous muscle catabolism and others suggest it is independent of and does not indicate muscle catabolism [44–47]. A release of 1-methylhistidine from erythrocytes with high RDW in aging is perhaps due to impaired renal function or increased gut permeability.

This study has several strengths. First, using data from three independent aging cohorts allowed us to validate findings through replication. Second, participants from all three cohorts are community-dwelling, allowing us to study the age effect on RDW. Third, both BLSA and Health ABC studies used the same targeted metabolomics platform by the same company, which minimized the effects of different assays on the results and allowed for robust replication of findings. Fourth, findings from the sensitivity analysis in a smaller subset of the discovery cohort matching the exact age range of the validation cohort strengthened our findings and validation. This study has limitations. The sample size is modest. Participants from both cohorts are volunteers from the community and tend to be healthier than the general population. Due to the inclusion criteria regarding health conditions and functions, particularly participants in the Health ABC study, are well-functioning in their 70s–80s, which may lead to a survival bias. Our study is cross-sectional, and the possibility of reversed causality, namely, that high RDW causes changes in specific metabolites cannot be ruled out. Future longitudinal studies of repeated measures of RDW and metabolites can help confirm the directionality of the mediation effect.

In conclusion, during aging, specific metabolites may be released from abnormal erythrocytes with high RDW, including SDMA, ADMA, and 1-methylhistidine. These metabolites are important markers for vascular pathology, renal function, and inflammation. These findings provide new insights into anisocytosis (or high RDW) with adverse outcomes, including cardiovascular disease, CKD, and mortality. Whether SDMA, ADMA, and 1-methylhistidine are released by the damaged erythrocytes with high RDW or they affect the physiology of erythrocytes causing high RDW should be further investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The BLSA was supported by the Intramural Research Program of the National Institutes of Health, National Institute on Aging. The Health ABC study was supported by National Institute on Aging contracts N01-AG-6-2101, N01-AG-6-2103, and N01-AG-6-2106; National Institute on Aging grant R01-AG028050; and National Institute of Nursing Research grant R01-NR012459, and was funded in part by the Intramural Research Program of the National Institutes of Health, National Institute on Aging. The InCHIANTI study was supported by National Institute on Aging contracts 263MD9164 (Dr Ferrucci) and 263MD 821336, N01-AG-1-1, N01-AG-10211, and N01-AG-5-0002 (Dr Bandinelli). We would like to thank Dr. Luke Pilling for providing statistical assistance in the mediation analysis.

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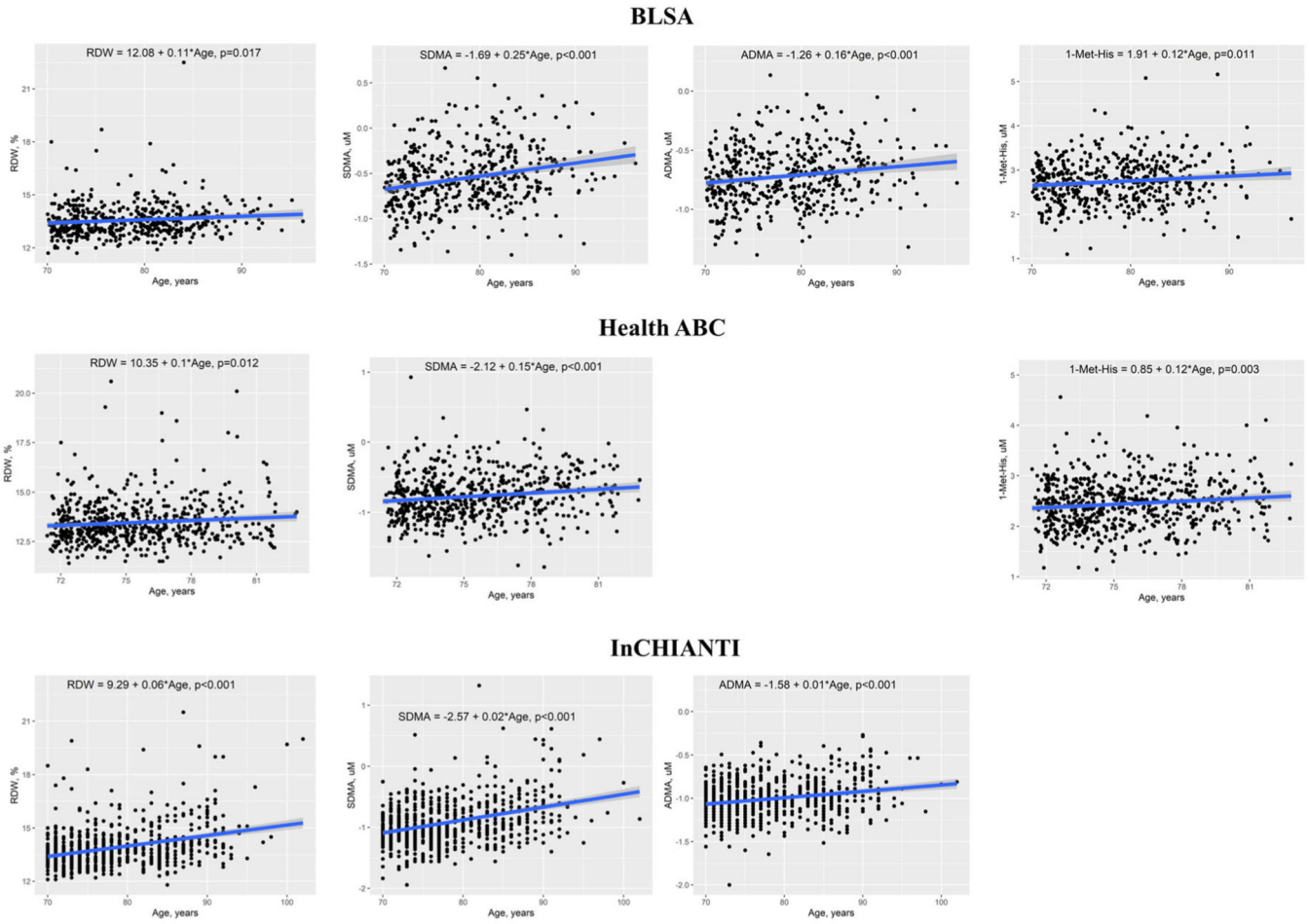

Fig. 1.

Scatter plots of red blood cell distribution width (RDW) and specific mediating metabolites as a function of age. Values of metabolite concentrations are log2 transformed.

Table 1.

Participants' characteristics in the discovery cohort of Baltimore Longitudinal Study of Aging (BLSA) and validation cohorts of Health ABC and InCHIANTI.

	BLSA (*n* = 477)		**Health ABC (*n* = 620)**		**InCHIANTI (*n* = 735)**	
	***N* (%) or mean ± SD**	**Range**	***N* (%) or mean (SD)**	**Range**	***N* (%) or mean (SD)**	**Range**
Age (years)	78.5 ± 5.6	70.0–96.3	75.5 ± 2.7	71.4–82.8	78.1 ± 6.4	70–102
70–80 age category	74.6 ± 2.9 (*n* = 283)	70.0–79.9	75.1 ± 2.3 (*n* = 575)	71.4–79.9	74.1 ± 2.7 (*n* = 485)	70–79
80+ age category	84.1 ± 3.4 (*n* = 194)	80.1–96.3	81.1 ± 0.7 (*n* = 45)	80.1–82.8	85.8 ± 4.1 (*n* = 250)	80–102
Women	218 (46)	–	321 (52)	–	419 (57)	–
Black	80 (17)	–	262 (42)	–	0	–
Body mass index (kg/m^2)	26.3 ± 4.0	17.8–46.8	27.0 ± 4.5	15.9–46.4	27.3 ± 4.1 (*n* = 673)	18.0–42.0
Red blood cell distribution width (%)	13.6 ± 1.0	11.7–22.5	13.5 ± 1.1	11.4–20.6	13.9 ± 1.1	11.8–21.5
Hemoglobin (g/dL)	13.6 ± 1.3	10.0–17.8	13.8 ± 1.2	9.3–17.7	13.5 ± 1.5	7.8–18.4
Glomerular filtration rate	67.5 ± 14.4	13.9–104.0	79.3 ± 16.2 (*n* = 579)	8.1–116.4	68.1 ± 14.3	8.8–98.5

Table 2.

Significant mediation effects of metabolites that account for age-related increase in red blood cell distribution width (RDW) in the discovery cohort of Baltimore Longitudinal Study of Aging (BLSA) and validation cohorts of Health ABC and InCHIANTI.

	Total association of age with RDW $(c = a \times b + c')$			**Direct association of age with RDW** (c')			**Indirect association of age with RDW through a mediator** $(a \times b)$				
BLSA	β	**95% CI**	***p*-Value**	β	**95% CI**	***p*-Value**	β	**95% CI**	***p*-Value**	**Prop. mediated**	**%**
Choline	0.0251	0.0097, 0.0407	0.0014	0.0221	0.0066, 0.0375	0.0050	0.0030	0.0005, 0.0064	0.0078	0.1171	11.7
Triglyceride (18:2_36:0)	0.0252	0.0098, 0.0405	0.0016	0.0281	0.0124, 0.0435	0.0004	−0.0029	−0.0064, −0.0004	0.0162	−0.1094	−10.9
Symmetric Dimethylarginine[a]	0.0251	0.0095, 0.0408	0.0020	0.0206	0.0047, 0.0366	0.0114	0.0045	0.0007, 0.0091	0.0188	0.1776	17.8
Homoarginine	0.0251	0.0094, 0.0406	0.0018	0.0222	0.0065, 0.0378	0.0060	0.0030	0.0004, 0.0065	0.0200	0.1140	11.4
Triglyceride (18:0_38:6)	0.0250	0.0097, 0.0407	0.0010	0.0280	0.0126, 0.0438	0.0006	−0.0029	−0.0064, −0.0003	0.0256	−0.1127	−11.3
Asymmetric Dimethylarginine[a]	0.0253	0.0098, 0.0407	0.0004	0.0231	0.0074, 0.0385	0.0030	0.0022	0.0001, 0.0053	0.0320	0.0817	8.2
1—Methylhistidine[a]	0.0252	0.0099, 0.0408	0.0022	0.0231	0.0079, 0.0386	0.0036	0.0022	0.0001, 0.0052	0.0364	0.0809	8.1
Triglyceride (18:0_36:3)	0.0252	0.0099, 0.0410	0.0010	0.0272	0.0118, 0.0431	0.0006	−0.0021	−0.0050, −0.0001	0.0392	−0.0753	−7.5
Triglyceride (18:1_38:6)	0.0251	0.0096, 0.0411	0.0012	0.0277	0.0121, 0.0438	0.0004	−0.0026	−0.0060, −0.0001	0.0432	−0.1007	−10.1
Triglyceride (16:0_40:8)	0.0250	0.0095, 0.0406	0.0020	0.0274	0.0118, 0.0431	0.0008	−0.0024	−0.0057, −0.00003	0.0454	−0.0907	−9.1
Triglyceride (17:0_36:4)	0.0250	0.0094, 0.0409	0.0008	0.0270	0.0113, 0.0428	<0.0001	−0.0020	−0.0050, −0.00001	0.0476	−0.0731	−7.3
Health ABC											
Homocysteine	0.0421	0.0103, 0.0742	0.0106	0.0367	0.0049, 0.0687	0.0242	0.0055	0.0010, 0.0117	0.0064	0.1243	12.4
Glutamic Acid	0.0422	0.0106, 0.0746	0.0088	0.0476	0.0155, 0.0799	0.0024	−0.0054	−0.0115, −0.0010	0.0122	−0.1209	−12.1
Symmetric Dimethylarginine[a]	0.0423	0.0112, 0.0735	0.0106	0.0371	0.0054, 0.0690	0.0216	0.0052	0.0006, 0.0114	0.0202	0.1196	12.0
Phosphatidylcholine aa C38:4	0.0433	0.0100, 0.0744	0.0126	0.0383	0.0056, 0.0704	0.0228	0.0041	0.0003, 0.0096	0.0306	0.0900	9.0
Hydroxysphin-gomyelin C22:1	0.0423	0.0103, 0.0730	0.0094	0.0382	0.0064, 0.0691	0.0182	0.0040	0.0002, 0.0095	0.0374	0.0895	9.0
Valine	0.0424	0.0104, 0.0740	0.0092	0.0462	0.0146, 0.0784	0.0042	−0.0038	−0.0093, −0.0001	0.0396	−0.0834	−8.3
1-Methylhistidine[a]	0.0422	0.0097, 0.0741	0.0126	0.0385	0.0064, 0.0705	0.0210	0.0037	0.0001, 0.0091	0.0412	0.0816	8.2
InCHIANTI											

BLSA	Total association of age with RDW ($c = a \times b + c'$)			Direct association of age with RDW (c')			Indirect association of age with RDW through a mediator ($a \times b$)			Prop. mediated	%
	β	95% CI	*p*-Value	β	95% CI	*p*-Value	β	95% CI	*p*-Value		
Symmetric dimethylarginine[a]	0.0593	0.0469, 0.0717	<0.0001	0.0518	0.0386, 0.0649	<0.0001	0.0075	0.0022, 0.0130	0.0034	0.1258	12.6
Asymmetric dimethylarginine[a]	0.0593	0.0472, 0.0718	<0.0001	0.0560	0.0434, 0.0687	<0.0001	0.0033	0.0005, 0.0066	0.0158	0.0548	5.5
Homoarginine	0.0593	0.0473, 0.0716	<0.0001	0.0578	0.0456, 0.0701	<0.0001	0.0015	−0.0004, 0.0038	0.1298	0.0241	2.4
Arginine	0.0594	0.0472, 0.0716	<0.0001	0.0598	0.0477, 0.0721	<0.0001	−0.0004	−0.0016, 0.0003	0.3406	−0.0051	−0.5

Note: The order of metabolites listed was ranked based on the significance of the mediation effects. a is the regression coefficient of age on the metabolite.

b is the regression coefficient of the metabolite on RDW. c' is the regression coefficient of age on RDW (without a mediator in the model).

[a]The mediation effects of metabolites were replicated in at least two cohorts.