Reports of Biochemistry & Molecular Biology
Vol.12, No.2, Jul 2023

Original article

www.RBMB.net

Relationship Between Red Cell Distribution Width and Oxidative Stress Indexes in Patients with Coronary Artery Disease

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Abstract

Background: Red blood cell distribution (RDW), an index of the size variability of erythrocytes, is significantly associated with coronary stenosis and can strongly predict the mortality risk in coronary artery disease (CAD). The biological mechanisms involved are not fully understood but may include oxidative stress. We sought to investigate the relationship between RDW and markers of oxidative stress in patients with CAD.

Methods: Participants were 112 consecutive patients referred to department of cardiac surgery for evaluation of chest pain. 32 patients had stable CAD, 40 patients had unstable CAD and 40 subjects were diagnosed as non-CAD. The levels of lipid peroxidation (TBARS) were measured in plasma and membrane samples by a fluorometric method. The plasma levels of glutathione (GSH) and total antioxidant capacity (TAC) were determined using spectrophotometric methods.

Results: Lipid peroxidation levels were significantly higher in the erythrocyte membrane of stable CAD patients than non-CAD patients. The levels of TAC were significantly lower in both stable and unstable groups when compared to that of the control group ($P< 0.019$ and $P< 0.001$, respectively), but did not differ between stable and unstable CAD. In addition, there was no significant difference in the serum GSH levels among the study groups. Membrane TBARS was directly associated with RDW in three groups of study.

Conclusions: We found an independent association between RDW levels and membrane lipid peroxidation in patients with CAD. This finding suggests that oxidative stress may be a potential underlying biological mechanism for increased RDW in CAD patients.

Keywords: Red blood cell distribution (RDW), Coronary stenosis, Oxidative stress, Total antioxidant capacity.

Introduction

Red blood cell distribution (RDW) is an index of the size variability of mature erythrocytes and is reported as a component of the complete blood count (CBC) (1). It is directly associated with variability in the size of circulating red blood cells (RBCs). Increased RDW is associated with the known risk factors for atherosclerosis such as hypertension, inflammatory markers, iron and **vitamin** D_3 deficiency, and indicators of oxidative stress (2, 3). The results of several studies have shown that RDW is significantly associated with coronary stenosis and can strongly predict the mortality risk in coronary artery disease (CAD) (1, 3-6). However, the underlying mechanism has not been entirely clarified.

Oxidative stress is considered to significantly contribute to the initiation and development of atherosclerosis (7). During oxidative stress, excess reactive oxygen species (ROS) can damage cells by increasing the oxidation of

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Received: 25 Apr, 2023; Accepted: 4 Sep, 2023

[DOI: 10.61186/rbmb.12.2.241]

lipids, proteins and carbohydrates (8).

Some studies suggest that oxidative stress can lead to a decrease in the half-life of circulating RBCs and may also be a possible mechanism of increased RDW in patients with CAD (9). However, to the best of our knowledge, the correlation between RDW and oxidative stress markers in stable CAD and unstable CAD patients in comparison with non-CAD subjects has not been evaluated. The aim of our study was first to evaluate if RDW levels increase in CAD patients compared with non-CAD subjects and second, to examine whether RDW is associated with oxidative stress parameters, which may clarify the possible association between RDW and oxidative stress in CAD pathogenesis.

Materials and Methods

Patient Populations

Participants were 112 consecutive patients referred to Department of cardiac surgery (Shahid Beheshti Hospital, Kashan, and Iran) for evaluation of chest pain. All subjects underwent coronary angiography and 32 patients had stable CAD (16 men and 16 women; mean age 64 ± 10 years, range 41 to 82), 40 patients had unstable CAD (23 men and 17 women; mean age 62.0 ± 13 years, range 34 to 83) and 40 subjects (18 men and 22 women; mean age 59 ± 11 years, range 41 to 81) were diagnosed as non-CAD. All groups were matched for age and sex.

Unstable CAD patients had increasing unpredictable chest pain or chest pain at rest as well as an abnormal electrocardiogram, transient ST-segment elevations, and/or inverted T waves, but they had normal serum cardiac troponin. Stable patients had chest pain with a constant pattern and frequency that triggered by physical activity or emotional stress and was relieved by rest. The patient's history was collected through a standardized questionnaire for risk factors and drugs. Patients who had a history of recently diagnosed myocardial infarction (MI), hematologic diseases, endocrine or metabolic diseases, inflammatory diseases, or cancer were not enrolled in this study.

Participants were considered hypertensive if they had a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg, or if they were taking antihypertensive medications. They were also considered hyperlipidemic if they had increased levels of triglycerides, total cholesterol, LDL-cholesterol, or if they were taking any antihyperlipidemic agents.

Angiographic Analyses

Coronary angiography was performed by the Judkins technique in all participants. In order to assess CAD severity, all angiograms were analysed by two cardiologists who were blinded to the patient's history and biochemical data. The stenosis severity of coronary arteries was then scored from 0 to 21 based on the results of coronary angiography (10).

Blood Specimen Collection and Processing

Ten mL of blood was collected from patients who were fasting for at least 12 hours. About 6 mL of collected blood was collected into heparin tubes (BD Vacutainer®) and used for preparation of packed RBC and plasma. The remaining 4 ml of whole blood was collected into EDTA tubes (BD Vacutainer®) and used for complete blood count (CBC) analysis. In order to prepare packed RBC and prepare plasma from whole blood samples, heparinized blood was centrifuged for 10 min at 1500 g. The plasma was carefully isolated and aliquoted into 0.3 mL aliquots and stored at -70 °C until use. Then, the buffy coat and the top layer of erythrocytes were removed and the remaining RBCs were washed two times with Tris buffered saline (Merck) (TBS; 150 mM NaCl, 50 mM Tris-HCl, and pH 7.5) (11). After last centrifugation at 1500 g for 10 min, the packed cells were used for ghost preparation.

Preparation of Erythrocyte Ghost Membrane

In this study, we used hypotonic lysis of washed and packed erythrocytes for preparation of erythrocyte ghost. Briefly,

[DOI: 10.61186/rbmb.12.2.241]

about 2 mL of washed RBCs was lysed by a 10-fold volume of ice cold 5 mM Tris buffer (pH 7.6) containing 0.1 mM Na_2EDTA (Merck). The tubes were softly shaken and kept for 20 min at 4 °C. The lysate was then centrifuged for 20 min at 20,000 g at 4 °C and the supernatant was thrown away. The precipitated membranes were washed three times with 5 mM Tris-HCl buffer, pH 7.6, containing 17 mM NaCl (Merck), and then three times with 10 mM Tris-HCl buffer, pH = 7.5. After the final centrifugation at 20,000 g, the erythrocyte ghosts were resuspended in 1 ml of 10 mM Tris-HCl buffer (12) and the protein concentration was determined by a modified method of Lowry (13). Finally, 200 ml aliquots of the ghost sample were frozen in liquid nitrogen and stored at -80 °C until further analysis.

Complete Blood Count Analysis

Hematological parameters including red and white blood cell count, hemoglobin levels, mean corpuscular volume (MCV), and RDW were determined using the KX-21N hematology analyzer (Sysmex, Japan). RDW is expressed as coefficient of red cell variation, in percent.

Biochemical Analysis

Serum fasting blood glucose (FBG) and lipid profile including total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured by determined by enzymatic methods. High-sensitivity C-reactive protein (hs-CRP) was measured by an immunoturbidometric method. All the above-mentioned biochemical variables were determined by an automated analyzer (BT-3500, Italy) and commercial kits obtained from Pishtazteb (Pishtazteb Co., Iran).

Determination of Lipid Peroxidation

The levels of thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation were measured in plasma and membrane samples according to a fluorimetric method developed by Ohkawa and colleagues (14). Briefly, 1.5 mL of 0.8% (w/v) aqueous thiobarbituric acid (Merck), 1.5 mL of acetic acid (Merck) (20%, v/v, pH 3.5), 0.2 mL of 8.1% sodium dodecyl sulfate (Sigma) and 0.6 mL of deionized water were added to 0.2 mL of ghost or plasma and mixed vigorously. Tubes were then heated for 60 min at 100 °C in a boiling water bath. After cooling to room temperature, 5.0 mL of n-butanol: pyridine (Merck) (15: 1, v/v) and 1 mL of distilled water were added, and the mixture was shaken vigorously and centrifuged at 2000 g for 10 min. The pink colored organic layer was taken, and the fluorescence was measured using PerkinElmer LS-55 spectrofluorometer (PerkinElmer, USA) at 515 nm excitation and 553 nm emission. 1,1,3,3-Tetraethoxypropane was used to construct a calibration.

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric Reducing Antioxidant Power (FRAP) was measured with the Benzie and Strain method (15). A working solution of FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine (Sigma-Aldrich) in 40 mM HCl (Merck), and 20 mM $FeCl_3 \cdot 6H_2O$ (Merck) in ratio of 10:1:1 (v/v/v). 750 ml of FRAP reagent was mixed with 25 ml of plasma samples and absorbance was measured at 593 nm after 5 min. The FRAP working solution was used as blank. An aqueous solution of $FeSO_4.7H_2O$ (Merck) was used for construction of a calibration curve.

Determination of GSH levels

The GSH levels were measured spectrophotometrically by the method of Beutler and Gelbart (16). The assay mixture contained 100 ml of sample, 0.5 ml of 0.3 M phosphate buffer (pH 8.0), and 0.5 ml of 5-5′-dithiobis-2-nitrobenzoic acid (40 mg in 100 mL dH_2O containing 1% sodium citrate (Sigma-Aldrich). The absorbance was read at 412 nm after incubation within 5 min.

Statistics

All the statistical analyses were conducted

[DOI: 10.61186/rbmb.12.2.241]

using the SPSS statistical software package (Version 16). Results for categorical variables were given as percentages and data for continuous variables with normal distribution were expressed as mean ± SD. If the distribution of a continuous variable was skewed, medians and interquartile ranges were expressed.

The normal distribution of data was tested by the Kolmogorov–Smirnov test and continuous variables with normal distribution were compared between groups using one-way analysis of variance (ANOVA) with Post-hoc Scheffe test. Comparison between categorical variables was performed by chi-square test. Multivariable linear regression analysis was used to correlate RDW levels with oxidative stress parameter and cardiovascular risk factors. Pearson's correlation coefficient was used to evaluate correlation between variables. P-value < 0.05 was considered statistically significant.

Results

There were no significant differences in age, sex, body mass index (BMI), waist-to-hip ratio (WHR), as well as other cardiovascular risk factors (including hypertension and smoking) among the three groups. Table 1 shows the demographic, anthropometric, biochemical, and clinical data in participants.

Table 1. Demographic, anthropometric, biochemical, and clinical data in participant.

Variables	Non-CAD (n=40)	Stable (n=32)	Unstable (n=40)	P-Value		
	Demographic data			S vs.N	U vs.N	S vs.U
Age (years)	59.3 ± 11.0	64.3 ± 10.3	62.0 ± 13.3	0.20	0.60	0.70
Men/women (n)	18/22	16/16	23/17	0.32	0.10	0.30
Body mass index (kg/m2)	27.2 ± 5.1	26.2 ± 4.1	25.6 ± 4.0	0.63	0.31	0.88
Waist hip ratio	0.89 ± 0.04	0.91 ± 0.04	0.91 ± 0.04	0.47	0.35	0.98
Systolic pressure (mm Hg)	127.9 ± 17.3	131.3 ± 14.9	126.4 ± 15.3	0.68	0.91	0.44
Diastolic pressure (mm Hg)	79.2 ± 9.8	79.6 ± 7.4	79.2 ± 12.8	0.98	1.00	0.98
Risk factors						
Hypertension, n (%)	21 (52.5)	19 (59.4)	19 (47.5)	0.53	0.74	0.35
Hyperlipidemia, n (%)	20 (50.0)	10 (31.2)	14 (35.0)	0.11	0.20	0.69
Family history of CAD, n (%)	12 (30.0)	5 (15.6)	7 (17.5)	0.16	0.21	0.96
Smoking, n (%)	3 (7.5)	4 (12.5)	3 (7.5)	0.47	0.97	0.80
Full blood count analysis						
Erythrocyte count ($\times 10^6/\mu L$)	4.88 ± 0.50	4.91 ± 0.47	4.63 ± 0.62	0.97	0.13	0.10
Hematocrit (%)	40.80 ± 4.21	42.01 ± 3.39	40.19 ± 4.27	0.46	0.80	0.18
Hemoglobin (g/dL)	13.86 ± 1.63	14.31 ± 1.29	13.45 ± 1.57	0.47	0.49	0.07
MCV (fl)	85.69 ± 4.18	86.02 ± 4.11	86.94 ± 5.18	0.95	0.48	0.70
RDW (%)	13.3 ± 0.5	13.8 ± 0.7	13.5 ± 0.5	0.001*	0.39	0.06
White blood cell count ($\times 10^3$)	6.20 ± 1.42	7.10 ± 2.10	6.72 ± 1.78	0.11	0.43	0.68
ESR (mm/h)	9 ± 1	10 ± 2	16 ± 4	0.95	0.01	0.004
Medications						
Aspirin, n (%)	32 (80.0)	22 (68.7)	24 (60.0)	0.273	0.06	0.49
Nitrates, n (%)	20 (50.0)	20 (62.5)	14 (35.0)	0.27	0.20	0.02†
ACE inhibitors	17 (45.50)	12 (37.5)	10 (25.0)	0.68	0.11	0.27
Clopidogrel, n (%)	13 (32.5)	10 (31.2)	9 (22.5)	0.92	0.35	0.43
Statins, n (%)	28 (7.0)	17 (53.8)	19 (47.5)	0.14	0.06	0.69
Biochemistry						
Fasting blood glucose (mg/dl)	94.2 ± 13.0	90.1 ± 18.4	94.3 ± 16.2	0.52	1.0	0.52
Triglycerides (mg/dl)	141.3 ± 42.2	158.4 ± 50.5	139.2 ± 63.0	0.63	1.0	0.62
Total cholesterol (mg/dl)	183.2 ± 36.2	189.1 ± 43.0	161.7 ± 40.5	0.82	0.07	0.02*
HDL cholesterol (mg/dl)	59.0 ± 11.3	60.0 ± 10.2	49.6 ± 12.1	0.93	0.002*	0.001*
LDL cholesterol (mg/dl)	97.4 ± 20.2	103.9 ± 24.3	84.9 ± 25.5	0.51	0.07	0.005*
hs-CRP mg/L	1.4 (1-3.9)	1.4 (0.9-3.6)	1 (0.4-1.7)	0.82	0.07	0.09

[DOI: 10.61186/rbmb.12.2.241]

There was no difference between the study groups regarding the use of drugs at the time of study entry, except for nitrates. The number of patients using nitrates was significantly lower in the unstable group compared with controls.

Unstable patients had lower levels of total cholesterol, LDL cholesterol and HDL-C in comparison with stable patients. Serum levels of HDL-C were significantly lower in unstable group than those in stable group.

There was no significant difference between the groups in terms of hematologic variables, except for ESR and RDW. Unstable patients had higher ESR levels in comparison with stable patients and controls. Increased levels of RDW were found in stable patients as compared with non-CAD subjects. The level of lipid peroxidation (TBARS level) was significantly higher in stable patients (0.74 ± 0.37 nmol/mg of protein) than in controls (Fig. 1). Although, membrane TBARS levels of patients with unstable CAD were observed higher than non-CAD patients, this difference was not statistically significant. There was also no significant difference in the level of membrane lipid peroxidation between the patients with stable and unstable CAD. There was no significant difference in serum TBARS levels among the study groups. Although plasma levels of TAC were significantly lower in both stable and unstable groups when compared to that of controls, there was no significant difference in the TAC levels between stable and unstable patients (Fig. 2). In addition, there was no significant difference in the serum GSH levels among the study groups. We also analyzed the association of age and oxidative stress markers (including TAC, GSH and TBARS) in all participants by linear regression analysis and found that there is a negative association between age and TAC (r=-0.283, p=0.013).

Membrane lipid peroxidation was directly correlated with CAD severity in the CAD patients (n=72) (Fig. 3). Also, membrane lipid peroxidation was directly associated with RDW in the study groups (Fig. 4). The multivariate analysis revealed that membrane TBARS and CAD score were significantly associated with RDW (Table 2).

Table 2. Multivariate analysis between cardiovascular risk factors, markers of oxidative stress and RDW in the study participants.

Factor	β	t	P value
Age	0.056	0.588	0.558
Sex	-0.017	-0.173	0.863
BMI	-0.041	-0.4	0.69
WHR	-0.052	-0.514	0.609
Smoking	-0.019	-0.169	0.866
Hypertension	-0.027	-0.261	0.795
Triglyceride	-0.016	-0.145	0.885
Total cholesterol	0.252	0.906	0.367
LDL cholesterol	-0.457	-1.757	0.082
HDL cholesterol	0.252	2.339	0.022
Plasma TBARS	0.114	1.221	0.225
Membrane TBARS	0.441	4.171	<0.001
Plasma TAC	-0.028	-0.254	0.8
Plasma GSH	0.092	0.989	0.325
Statins	-0.041	-0.425	0.671
Angiography score	0.063	0.516	0.607
Significance (ANOVA)			0.002

ANOVA: analysis of variance; BMI: Body Mass Index; GSH: Glutathione; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; RDW: Red blood cell Distribution Width; TAC: Total Antioxidant capacity; TBARS: Thiobarbituric acid Reactive Substances. WHR: Waist-to Hip Ratio. Values are expressed as mean ± SD or median and interquartile range for continuous variables.

[DOI: 10.61186/rbmb.12.2.241]

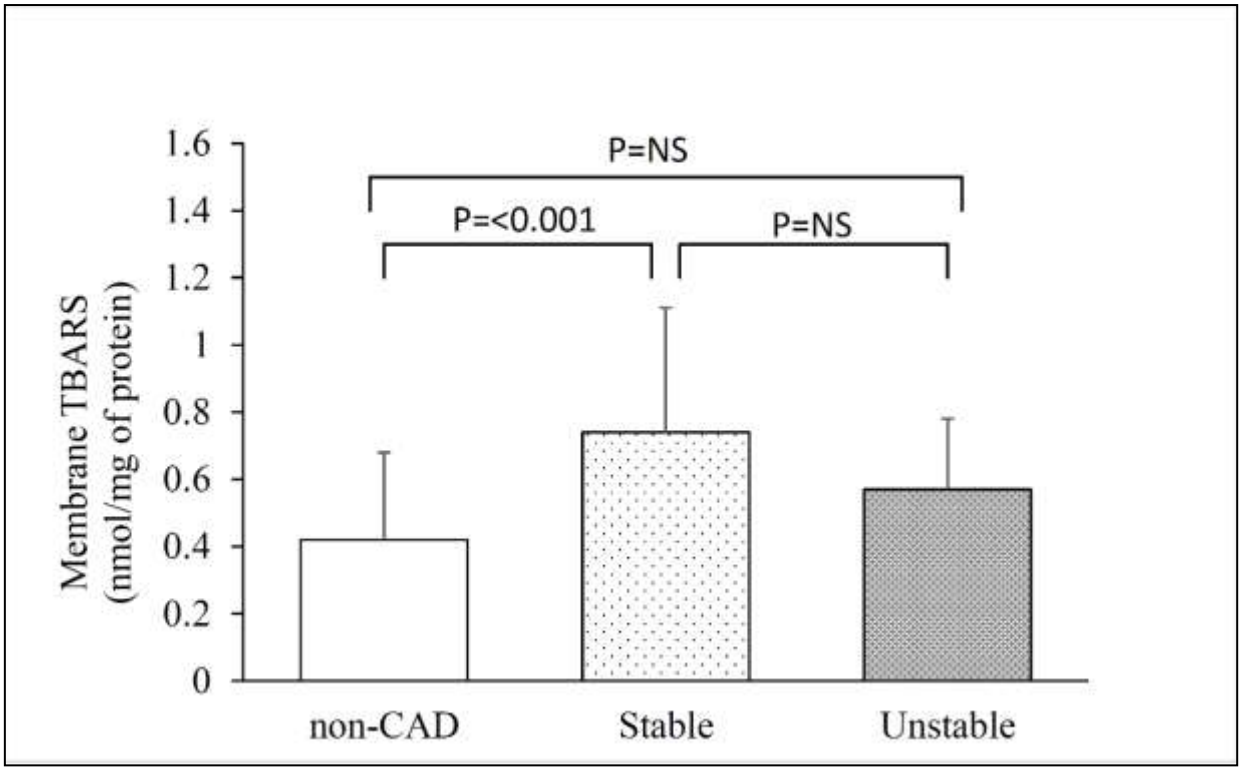

Fig. 1. Lipid peroxidation level in the erythrocyte membranes of CAD patients and non-CAD subjects. Values are mean ± SD. A P < 0.05 is statistically significant. CAD, coronary artery disease; NS, non-significant; TBARS, Thio barbituric acid-reactive substances.

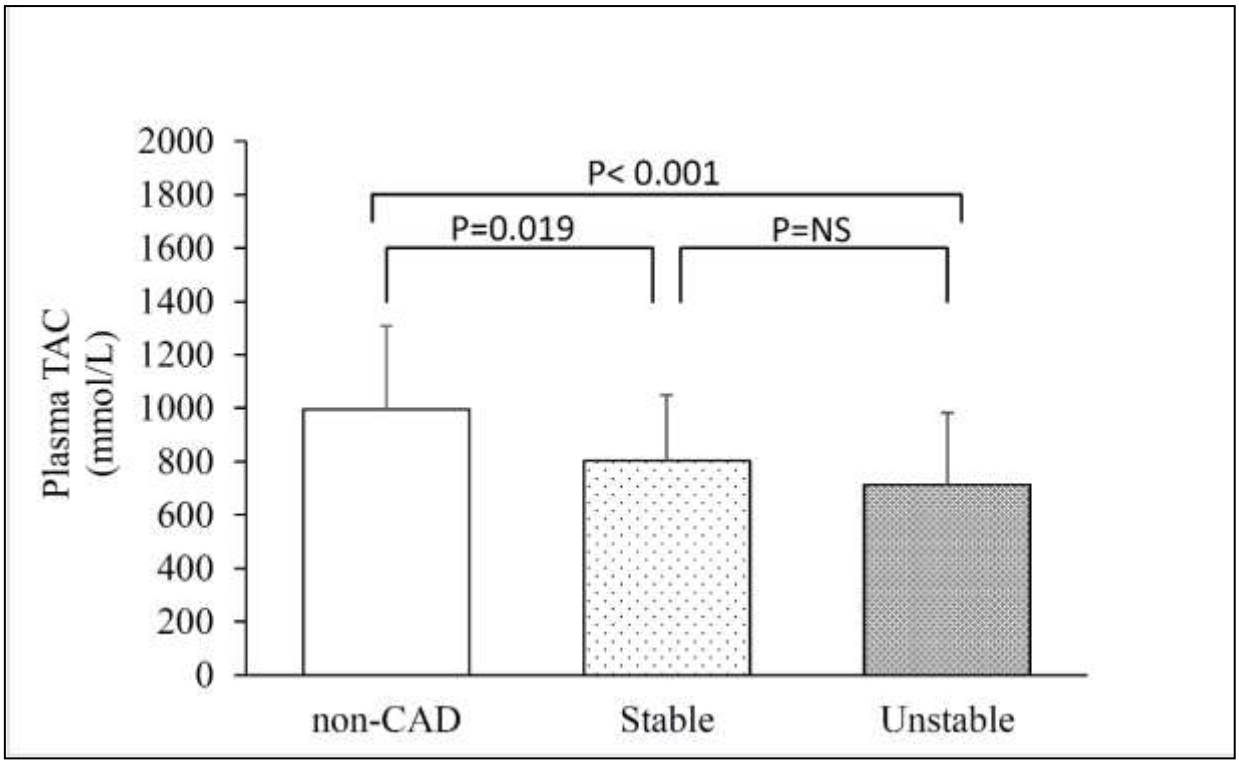

Fig. 2. Plasma TAC levels in the study groups. Values are mean ± SD. A P < 0.05 is statistically significant. CAD, coronary artery disease; NS, nonsignificant; TAC, total antioxidant capacity.

[DOI: 10.61186/rbmb.12.2.241]

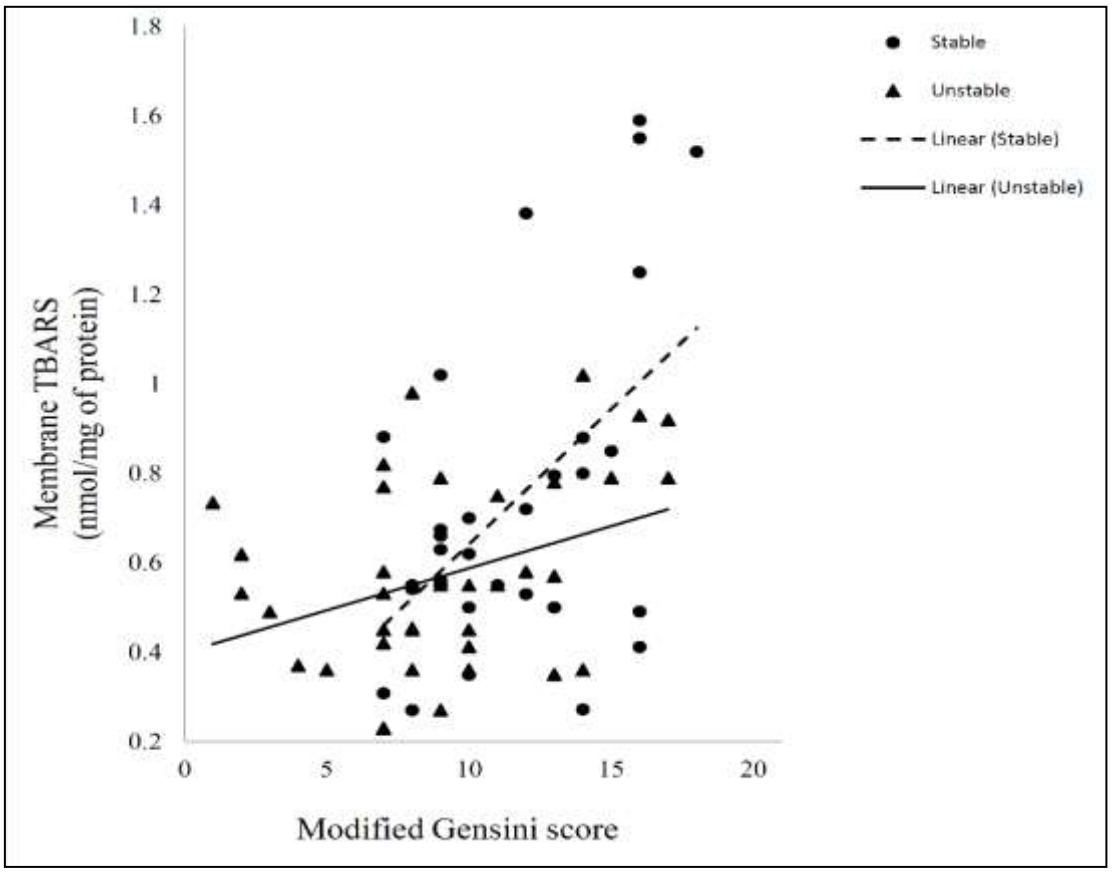

Fig. 3. Correlation of coronary stenosis scores with membrane TBARS in CAD patients .Unstable: r=0.344, P=0.035; Stable: r=0.373, P=0.039.

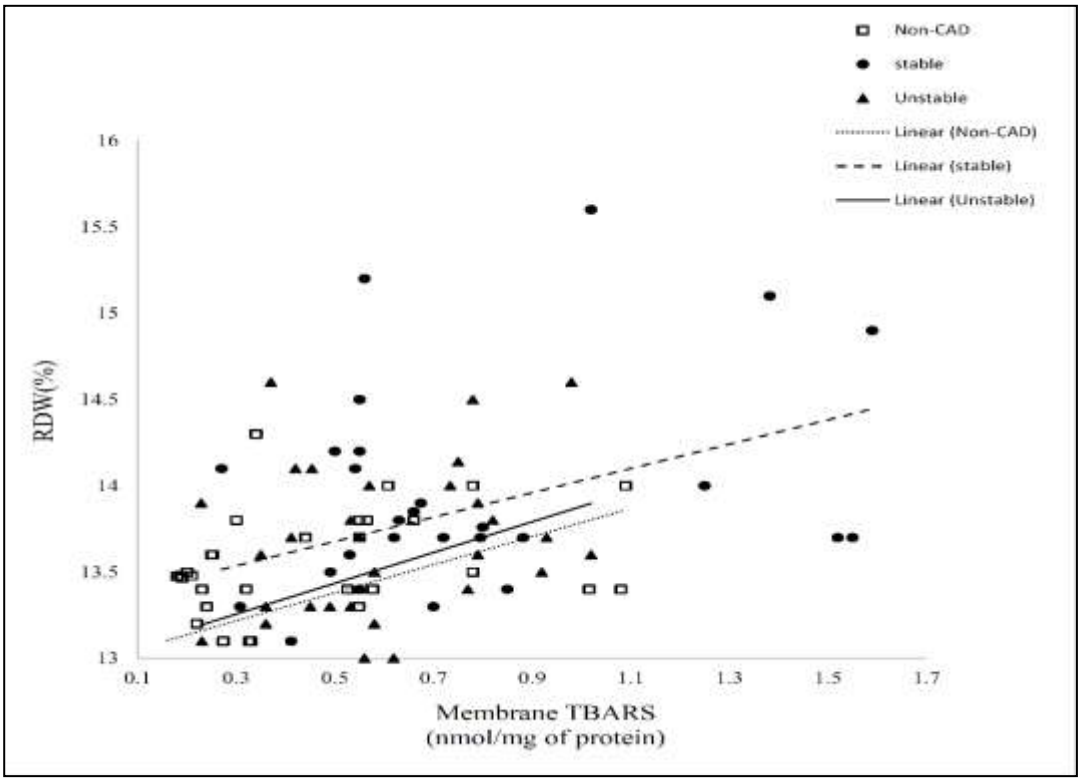

Fig. 4. Correlation of membrane lipid peroxidation with RDW in the study groups. Unstable: r=0.0.344, P=0.035; Stable: r=0.373, P=0.039; Non-CAD: r=0.408, P=0.01.

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Discussion

In the present study, we found that the level of lipid peroxidation was increased significantly in erythrocytes of CAD patients, and it was positively correlated with the RDW level. This association persisted after multivariate adjustment for potential confounding variables.

To the best of our knowledge, the association between erythrocyte membrane lipid peroxidation and RDW in CAD patients has not studied before.

We also found that the RDW levels were higher in stable CAD patients than non-CAD subjects. This is consistent with the findings of Zalawadiya et al.'s study who reported that higher level of RDW was independently correlated with a higher risk of peripheral artery disease (PAD) (19). Another study by Lippi et al. also showed that patients with acute coronary syndrome (ACS) had higher RDW than non-ACS subjects (20).

Although the principal mechanism for the increase of RDW in cardiovascular disease remains unclear, oxidative stress, inflammation, and prolonged stimulation of the neuroendocrine system are thought to be involved. It is believed that oxidative stress increases anisocytosis by disrupting erythropoiesis and thereby change the size and shape of RBCs as well as their circulation half-life, which finally leads to increased RDW (17-19). In line with our finding, Joosse et al. showed in a recently published study that an in vitro induction of oxidative stress could increase RDW and decrease erythrocyte volume. Therefore, by changing the size of the erythrocytes, increased oxidative stress might have a role in the correlation of RDW with clinical outcomes in CAD.

In the present study, we found a significant direct association between serum TAC and RDW. Semba and colleagues showed that serum antioxidants are independent predictors of RDW in older women (20). Abdel- Moneim and colleagues have also reported that serum NO and GSH levels are negatively associated with the inflammation in diabetic nephropathy which reflected by elevation in RDW(21). In another study, Zhao et al. (22) showed that RDW was associated with several inflammatory and oxidative stress markers in a canine model of rapid atrial pacing (RAP). Theoretically, higher levels of antioxidant in serum could inhibit the increase of RDW in erythrocytes by protecting them from oxidative damage.

In this study, we also found that unstable patients had significantly higher ESR than in controls and stable patients. The ESR is a powerful predictor of CAD mortality, and appears to be an independent marker of aggressive forms of cardiovascular disease (23). Andresdottir and colleagues showed in a cohort of 7988 men and 8658 women (20 years of follow-up) that ESR is a long-term independent predictor of CAD in both men and women(24). These findings support the evidence of an inflammatory process in CAD, and suggest that ESR may be an additional diagnostic marker for CAD (24-26).

Potentially, this study has some limitations that should be mentioned. First, our study is limited to Iranian patients and consequently the findings should be generalized with caution. Second, our study has a relatively small sample size, and thus, larger studies are needed to confirm our findings. Finally, although intravascular ultrasound may give more accurate data for atherosclerotic plaques than angiography, this imaging technique is too expensive, and so it was not used in the current study.

Data from the present study showed a significant association between lipid peroxidation of erythrocyte membranes and RDW in CAD patients. This finding suggests that oxidative stress may be a potential underlying biological mechanism for increased RDW in CAD patients, which in turn is related to higher mortality and worse clinical outcomes in CAD patients.

[DOI: 10.61186/rbmb.12.2.241]

Ethics
The study was conducted according to the Declaration of Helsinki and its protocol was approved by the ethics committee of our institute (Ethics code: IR.KAUMS.MEDNT.REC.1396.74). All participants signed written informed consent.

Funding
This research was funded by Kashan university of medical sciences (Grant ID: 96143).

Conflict of Interest
The authors declare there is no conflict of interest.

Acknowledgment
The authors thank the personnel of the Angiography Unit at Kashan Beheshti Hospital for their assistance. They also thank laboratory members of the Department of Clinical Biochemistry at Kashan University of medical sciences.

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[DOI: 10.61186/rbmb.12.2.241]