Int J Clin Exp Med 2016;9(7):14586-14595
www.ijcem.com /ISSN:1940-5901/IJCEM0023777

Original Article

Can serum asymmetric dimethyl-arginine and homocysteine levels be a new activity parameter of disease in patients with rheumatoid arthritis?

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Received January 11, 2016; Accepted May 18, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: In this study, we evaluated serum asymmetric dimethyl arginine (ADMA) level, homocysteine level and its clinical significance in rheumatoid arthritis (RA). The study included 40 patients with RA and 30 controls. Serum ADMA and homocysteine levels were measured using *High Performance Liquid Chromatography* system. Patients with DAS 28 scores ≤ 3.2, and > 3.2 were allocated into lower and high/moderate disease activity groups, respectively. Additionally patients were divided into 2 groups as early RA (disease duration ≤ 2 years) and established RA (duration of the disease ≥ 2 years). Functional disability was evaluated using Health Assessment Questionnaire (HAQ). Radiographs were scored using the modified Larsen score. Serum ADMA level was significantly higher than controls (P=0.008) in RA patients, while homocysteine level was comparable (P=0.059). Serum ADMA and homocysteine levels did not correlate with clinical and laboratory parameters of disease activity such as erythrocyte sedimentation rate, C-reactive protein, Disease Activity Score (DAS) 28 and Health Assessment Questionnaire (HAQ). These outcomes demonstrate that ADMA and homocysteine levels cannot be used as a parameter for disease activity in RA.

Keywords: Rheumatoid arthritis, asymmetric dimethyl arginine, homocysteine, disease activity

Introduction

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease frequently affecting peripheral joints which characterized by symmetric and erosive synovitis. Incidence of myocardial infarction, cardiovascular events and deep vein thrombosis increases 2-fold among RA patients compared to healthy population [1, 2]. Expected life span in RA patients is shortened relative to the healthy population due to increased cardiovascular events [3]. Early stage atherosclerosis develops in RA due to accelerated endothelial dysfunction [4]. Though increased atherosclerosis can be explained by traditional factors, development of atherosclerosis has been linked to inflammatory activation, duration and progression of the disease in most of RA patients [5]. Macrophage, T-cell, derangement of the Th1/Th2 ratio, increase in the acute phase reactants, adhesion and production of endothelin and activation of neoangiogenesis involve in the relationship between RA and atherosclerosis [6, 7]. Gerli et al. [8] reported an increase in CD4 + CD28 (null) cells which produces IFNγ that induces Th1 cell activation, leading to variations in cytokines and causing long-term changes in immune activation. Importance of this pathway is acknowledged in the development of atherosclerosis and unstable angina. Substantial contribution of neoangiogenesis which is an important factor in the pathogenesis of RA, has been demonstrated in another study [9].

Asymmetric dimethyl arginine (ADMA) is a catabolic end product of protein which contains denaturated arginine remnants and it metabolizes to citrulline by dimethyl arginine aminohydrolase [10]. Recently, ADMA has been indicated as a marker of cardiovascular risk factor [11, 12]. ADMA is an endogenous inhibitor of nitric

oxide synthetase (NOS) that mostly regulates the bioavailability of nitric oxide. NOS can enter into competition with substrates as endogenous L-arginine and ADMA. ADMA has an important role in the synthesis of nitric oxide (NO) [13]. ADMA, which prevents production of NO, causes endothelial dysfunction which is closely related to atherosclerosis. Higher plasma ADMA levels can impair NO production and facilitate progression of atherosclerosis [14]. Endothelial dysfunction is a starting point in atherogenesis. Vascular endothelium has a few functions which regulate vascular tonus, blood-tissue exchange and proliferation of smooth muscles. Besides, it controls blood viscosity, leukocyte adhesion and migration together with platelet aggregation [15]. Impairment of hemostasis and local blood flow is associated with disruption of the balance between NO and super oxide in endothelial cells [16]. Endothelial dysfunction and accumulation of ADMA in patients with coronary artery disease have been defined as an independent marker of potential cardiovascular events [17]. Increased ADMA levels has been detected in RA patients without cardiovascular disease [18]. In addition to inhibition of NO synthesis, ADMA can directly cause oxidative stress and cellular apoptosis and also it involves in inflammatory reactions [19, 20].

As is known, plasma levels of homocysteine increase in patients with RA [21]. Homocysteine is derived from an essential amino acid methionine and contains a sulfydryl group. Its plasma concentrations vary with age, gender, life style (coffee consumption, smoking, physical activity, alcohol), genetic mutations which cause serious deficiency of enzymes involving in homocysteine catabolism, drugs and diseases affecting homocysteine metabolism and most important of all vitamin B intake [22]. Hyperhomocysteinemia has been accepted as a risk factor for atherosclerosis and other vascular diseases [23]. Homocysteine-induced vascular damage can be associated with indirect mechanisms as induction of a prothrombotic condition which mediates its effects on direct endothelial toxicity, coagulation factors and platelets and atherogenic modification of LDL. Besides, recent studies have demonstrated that homocysteine has properties related to both immunomodulator and proinflammatory activation. Homocysteine increases production of both monocyte chemoattractant protein-1 which is released by IL6, IL8, monocyte-macrophage and endothelial cells and also NO released from vascular smooth muscle cells, matrix metalloproteinase-9 and vascular cell adhesion molecule (VCAM-1) [24-26].

Limited number of studies have indicated increase in ADMA and homocysteine levels in patients with rheumatoid arthritis. Besides the association between disease activation and homocysteine levels is not known completely. The objective of this study was to determine plasma ADMA and homocysteine levels and investigate their relationship between disease activation parameters and radiological progression.

Material and method

Patient population

A total of 40 (32 female and 8 male) patients who were followed up at least for one year with the diagnosis of RA and age and gender-matched 30 healthy control were included in the study. All of these patients fulfilled ACR criteria for RA [27]. Patients who were using a biological agent or leflunomide were excluded from the study. As a prerequisite, any change (excl. changes in dosages) in DMARDs within 3 months was considered as an exclusion criterion. Patients with autoimmune diseases apart from RA, acute and chronic infection, malignancy, any known serious pulmonary, hepatic, renal or hormonal diseases were not included in the study. Patients with present or previous clinical cardiovascular disease, smoking history, arterial hypertension (blood pressure ≥ 140/90 or antihypertensive use), hypercholesterolemia and those aged below 20 and over 70 years of age were not enrolled in the study. The study was approved by the local ethics committee and written informed consent forms were obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Clinical evaluations

Routine clinical evaluations including global assessments of the patient and the physician concerning pain and fatigue were performed using Visual Analogue Scale (VAS) were performed. Morning stiffness was evaluated with

Table 1. Comparisons of demographic, clinical and laboratory parameters between patient and healthy control groups

	Rheumatoid arthritis (n=40)	Control (n=30)	*P* value
Age (years)	51.42±11.16	53.03±11.65	0.890
Female/Male	32/8	22/8	0.511
Duration of the disease (years)	5.52±4.56		
Morning stiffness (min)	78.37±65.89		
Pain (0-100 mm VAS)	55.12±24.08		
Global assessments of the patient (0-100 mm VAS)	51.02±24.66		
Global assessments of the physician (0-100 mm VAS)	46.70±30.56		
Number of the swollen joints (0-28)	1.17±2.24		
Number of the tender joints (0-28)	4.10±4.43		
Erythrocyte sedimentation rate (mm/h)	30.80±15.26	19.90±6.80	0.001*
C reactive protein (g/dl)	23.45±23.48	3.77±0.63	0.001*
Rheumatoid factor (IU/ml)	157.16±241.8	9.82±0.57	0.001*
Serum ADMA (μmol/L)	0.44±0.15	0.35±0.11	0.008
Serum Homocysteine (μmol/L)	13.65±3.19	12.18±1.72	0.059
Disease Activity Score 28	4.07±1.45		
Health Assessment Questionnaire 20	1.31±0.75		
Modified Larsen Score	30.25±23.55		

*The results are statistically significant ($P < 0.05$).

respect to its duration in minutes disease activity was evaluated using DAS 28 (incl. number of tender and swollen joints, erythrocyte sedimentation rate and global health evaluation) [28]. The patients were divided as low (DAS 28 score ≤ 3.2 pts) and high/moderate (> 3.2 pts) disease activity [29]. Besides, the patients were divided into 2 groups as early onset RA (duration of the disease ≤ 2 years) and established RA (duration of the disease ≥ 2 years). Functional disability was evaluated using HAQ scale [30].

Laboratory evaluations

Routine laboratory results (erythrocyte sedimentation rate, blood biochemistry, blood counts, urinalysis) were recorded. Rheumatoid factor (RF) and C-reactive protein (CRP) levels were measured using nephelometer. Blood samples obtained from patients and controls were and collected in citrate tubes and centrifuged at 2000 rpm for 15 minutes. Serum samples were stored at -80°C.

Determination of ADMA levels

For the determination of asymmetric dimethyl arginine 20 mg 5-sulphosalicylic acid was added to 1 ml serum and the mixture was kept for 10 minutes in ice bath. The precipitated protein was separated by centrifuging the sample at 2000 rpm for 10 minutes. After filtering 10 μl of the supernatant, the supernatant was mixed with 100 μl derivatization reactive and injected into chromatographic system. For the separation of asymmetric dimethyl arginine, phenyl columns with dimensions of 250 mm x 4.6 mm were used. ADMA levels were measured using commercial kits of High Performance Liquid Chromatography (HPLC) system [(Shimadzu RF-10AxL) (Immuchrom GmbH, Germany, catalogue no; IC280-1410211)] with flow rate 1 ml/min, excitation 420, emission 483 nm wavelength. Linearity and sensitivity of HPLC was > 16.0 μmol/L and < 0.01 μmol/L respectively.

Determination of homocysteine levels

50 μl internal standard, 20 μl reduction solution and 100 μl derivatization solution were added to 50 μL of the serum sample and incubated at 60°C for 10 minutes. The samples were cooled down to 2-8°C and after adding 100 μl precipitating agent and they were centrifuged at 10,000 g for 5 minutes. For the determination of homocysteine levels, 20 μl of supernatant was analyzed using commercial kits of HPLC system [(Shimadzu RF-10AxL)

Table 2. Comparisons among patient subgroups with high/moderate (DAS 28 > 3.2) and low disease activity (DAS 28 ≤ 3.2)

	High/moderate disease activity (n=23)	Low disease activity (n=17)	*P* value
Age (years)	50.91±12.83 (24-72)	52.11±8.75 (37-67)	0.157
Female/Male	19/4	13/4	0.631
Duration of the disease (years)	5.54±3.84	5.50±5.51	
Prednisolone (mg/day)	7.71±3.60	6.47±2.50	
Methotrexate (mg/week)	11.94±2.08	10.38±2.00	
Erythrocyte sedimentation rate (mm/h)	37.13±15.58	22.23±9.91	
C reactive protein (g/dl)	35.65±24.30	6.95±5.36	
Serum ADMA (μmol/L)	0.45±0.17	0.43±0.11	0.956
Serum Homocysteine (μmol/L)	13.11±2.88	14.38±3.53	0.382

ADMA: Asymmetric Dimethyl Arginin.

(EURKA srl-Lab Division, Chiaravalle, Italy catalogue no; Z58010] with flow rate: 0.7-1.0 ml/min, excitation 385 and emission 515 nm wavelength.

Radiological evaluation

Plain hand radiograms which obtained within last six months were collected from all patients' digital data. Joint injuries were evaluated on hand radiograms by an observer blinded to clinical and laboratory data of the patients using Modified Larsen Scoring system. In this scoring system a total of 24 joints of both hands were scored between 0 and 5 points to obtain a total score (minimum score: 0 and maximum score: 120 points) [31].

Statistical evaluation

Statistical analysis of the study was performed using SPSS Statistics for Windows version 20.0 program (IBM Corporation, Armonk, NY, USA). The results of the study were evaluated using parametric and nonparametric statistical methods for data with normal and non-normal distribution, respectively. Intergroup comparisons were performed using independent groups t test for parametric and Mann-Whitney U test for non-parametric values. Correlations between parameters were evaluated using Spearman correlation coefficient. A *p* value of < 0.05 was accepted as the level of significance in statistical evaluations. The results were expressed as mean ± standard deviation.

Results

There was no significant difference between age (p=0.89) and sex (p=0.51) of RA patients and controls (**Table 1**). Mean duration of the disease was 5.52±4.56 years. Twenty-six (65%) patients were RF positive. All patients were receiving corticosteroid treatment and mean dose of prednisolone was 7.5±3.38 (2.5-20) mg/day. Eleven patients (40.7%) were receiving prednisolone monotherapy, while the remaining 34 patients (59.3%) were receiving one or more than one DMARD (incl. methotrexate, sulphalazine and hidroxychloroquine) in addition to prednisolone.

Serum ADMA levels were significantly higher than those of the control group (p=0.008) in patients with RA. Serum homocysteine (P= 0.059) levels were comparatively higher in patients with RA without any statistically significant intergroup difference (**Table 1**). Serum ADMA (P=0.956) and homocysteine (P=0.382) levels were similar in RA subgroups with moderate/high and low disease activity levels (**Table 2**). Besides serum ADMA (P=0.839) and homocysteine (P=0.240) levels were similar in subgroups of early onset and established RA.

Serum ADMA and homocysteine levels did not correlate with any of the clinical and laboratory parameters which demonstrate disease activity (**Table 3**). Serum RF levels did not correlate with ADMA and homocysteine levels. Similarly, a marked correlation did not exist between radiological joint injury, serum ADMA and homocysteine levels (**Table 3**).

Serum ADMA (P=0.521) and homocysteine (P=0.256) levels were comparable in patients receiving prednisolone or methotrexate + prednisolone treatment (**Table 4**).

Table 3. Spearman correlation coefficients of various clinical and laboratory parameters and serum ADMA and homocysteine levels

	ADMA		Homocysteine	
	P value	r	*P* value	r
Morning stiffness (min)	0.442	-0.125	0.187	-0.213
Pain (0-100 mm VAS)	0.232	-0.193	0.280	-0.175
Fatigue (0-100 mm VAS)	0.365	-0.147	0.546	-0.098
Global assessments of the patient (0-100 mm VAS)	0.345	-0.153	0.337	-0.156
Global assessments of the physician (0-100 mm VAS)	0.604	-0.084	0.591	-0.088
Number of the swollen joints (0-28)	0.515	-0.106	0.407	-0.135
Number of the tender joints (0-28)	0.620	-0.081	0.644	-0.075
Erythrocyte sedimentation rate (mm/h)	0.080	-0.280	0.802	-0.041
C reactive protein (g/dl)	0.247	-0.187	0.593	-0.087
Rheumatoid factor (IU/ml)	0.432	-0.128	0.640	0.072
Disease Activity Score 28	0.396	-0.138	0.680	-0.067
Health Assessment Questionnaire 20	0.929	0.015	0.608	-0.084
Modified Larsen Score	0.805	0.040	0.495	-0.111

ADMA: Asymmetric Dimethyl Arginin.

Table 4. Comparisons between patients with rheumatoid arthritis using prednisolone alone and combination of methotrexate + prednisolone

	Prednisolone (n=11)	Methotrexate + Prednisolone (n=16)	*P* value
Age (years)	54.27±12.41	51.68±10.03	0.797
Female/Male	10/1	12/4	0.296
Duration of the disease (years)	5.18±3.75	5.46±4.85	0.980
Prednisolone (mg/day)	8.40±4.36	6.25±2.41	
Methotrexate (mg/week)		11.09±2.03	
Erythrocyte sedimentation rate (mm/h)	45.81±16.77	22.00±9.65	0.001*
C reactive protein (g/dl)	42.68±31.62	11.75±12.80	0.003*
Serum ADMA (μmol/L)	0.46±0.19	0.47±0.14	0.544
Serum Homocysteine (μmol/L)	13.38±3.34	13.69±2.59	0.256

*Statistically significant results ($P < 0.05$). ADMA: Asymmetric Dimethyl Arginin.

Discussion

Based on the data of this study three important conclusions can be drawn: Firstly, serum ADMA levels in RA patients were higher than those of the healthy controls, while serum homocysteine levels were similar. Secondly, serum ADMA and homocysteine levels did not correlate with disease activity parameters. Thirdly, methotrexate use in RA patients did not significantly affect serum ADMA and homocysteine levels.

In previous studies performed in early onset and established patients with RA, endothelial dysfunction was demonstrated as the starting phase of atherosclerosis [6, 18, 32-34]. Induction of atherosclerosis with endothelial injury is a very well-known fact, while etiology of atherosclerosis is multifactorial [15, 16, 35]. Increased inflammatory activity, corticosteroid and methotrexate use contribute to the development of atherosclerosis. Although inflammatory activity is important for the development of atherosclerosis, etiopathologic mechanism of early onset atherosclerosis has not been clearly defined yet [36]. Studies performed in animal models have demonstrated contribution of decreased NO bioavailability and oxidative stress to endothelial dysfunction [16, 37]. *In vitro* studies have demonstrated that CRP decreased expression of adhesion molecules and endothelin-1 and plasminogen activator inhibitor-1 and decrease activity of NOS and its expression from endothelial cells [38]. Most of the cardiovascular deaths occur in RA patients with higher disease activity, so pathogenetic role of CRP and a few soluble mediators in RA-related atherosclerosis has been investigated in many studies [35]. From this perspective, the presence of

a correlation between disease activity, ADMA and homocysteine levels may open new horizons in the awareness from early onset atherosclerosis in RA, its monitorization and treatment.

When compared with those of the healthy controls, our results have demonstrated significantly higher ADMA levels in patients with RA. Hypertension, cardiovascular or renal disease was not detected in any patient of our study group. In a study by Sandoo et al. [39] the authors reported increased levels of serum ADMA in RA patients which were not correlated with *in vivo* evaluation of endothelial dysfunction. In compliance with our results, Vatansev et al. [40] reported higher levels of ADMA in patients with RA. In RA patients without any clinical findings of ischemic heart disease, coronary artery abnormalities were demonstrated. A negative correlation was reported between decreased coronary flow reserve (CFR) and ADMA levels in patients with early onset RA. Therefore, authors indicated the presence of an association between increased ADMA levels and decreased CFR [41]. Although correlation between ADMA and carotid artery intima-media thickness (CIMT) was demonstrated in a study, other studies could not reveal any correlation between ADMA levels and CIMT [42, 43].

The correlation between ADMA levels and inflammation is still debatable. It has been suggested that ADMA triggers inflammatory response which might play a role in the association between inflammation and endothelial dysfunction. In our study, serum ADMA levels were higher in RA patients, however a correlation between serum ADMA levels and clinical and laboratory parameters of disease activation could not be detected. Besides, serum ADMA levels in patients with moderate/high or low- disease activation were comparable. In many studies similar outcomes have been obtained [18, 40, 42, 44, 45]. On the other hand, Kwasny et al. [46] reported correlations between RA activity and serum ADMA levels. Besides, Antoniades et al. [47] reported that low-level systemic inflammation will increase serum ADMA levels. Based on our findings, we can deduce that serum ADMA levels is not directly correlated with inflammatory activation. Controversial results reported in the literature might stem from complex mechanism of action of NO which plays a role in more than one stage of inflammation.

In our study, any correlation did not exist between serum ADMA levels and radiologically detected joint injury. In literature review, we couldn't find a study related to this issue. Besides, we couldn't find any correlation between serum ADMA levels in patients with early onset and established RA patients.

We couldn't determine any significant difference between patients receiving only prednisolone or methotrexate + prednisolone as for serum ADMA levels. Similar to our results, Turiel et al. reported lack of any direct impact of DMARD treatment on CIMT and ADMA levels in RA patients without any cardiovascular symptoms and signs [45]. The effect of methotrexate treatment on serum ADMA levels in RA patients has not been adequately defined in the literature yet.

In RA patients, correlations among increased homocysteine concentrations, antiphospholipid antibodies and thrombotic events have been demonstrated [48]. Although pathogenesis of RA has not been elucidated fully, activation of T-lymphocytes and macrophages and production of cytokines appear to play a critical role in the onset and maintenance of the disease [49]. However, homocysteine deposits were found on stimulated peripheral leucocytes which trigger activation of the immune system which involves in hyperhomocyteinemia [50]. In our study, we investigated homocysteine as a potentially active mediator of an inflammatory process which plays a role in the activation of the disease and progression of the joint injury in patients with RA. In our literature review, we haven't encountered any study which investigated the correlation between homocysteine as an inflammatory mediator and clinical and laboratory parameters of disease activity.

In our study, serum homocysteine levels were similar both in RA patients and the control group. Besides, any correlation between serum homocysteine levels and disease activity was not found. Similar to our study outcomes, Borman et al. [51] could not detect any difference between RA and control groups as for serum homocysteine levels. However, in some articles, significantly higher serum homocyste-

ine levels have been reported in patients with RA relative to the control group [52, 53].

In our study serum homocysteine levels in patients with moderate/high or low-disease activity were comparable. Similarly any correlation did not exist between serum homocysteine levels and radiologically detected joint injury. In our literature research we haven't found any relevant study conducted in patients with RA. Besides, we haven't detected any difference between serum homocysteine levels in patients with early-onset or established RA.

Serum homocysteine levels in patients who received only prednisolone or methotrexate + prednisolone did not differ significantly. Similar to our study results, Tiftikçi et al. [54] reported that methotrexate treatment did not affect serum homocysteine levels in patients with RA. In a study with different outcomes from ours, higher serum homocysteine levels were found in patients treated with methotrexate relative to control groups [55]. However, Roubenoff et al. [21] reported that increased homocysteine levels in RA patients were not induced by methotrexate intake, but it might be caused by the direct effect of the disease itself. Since all patients included in our study were under treatment and they were receiving folic acid replacement, results may not reflect direct effect of RA on homocysteine levels. Besides, this diversity might stem from ethnically different characteristic of thymidylate synthase gene. In a study by Kim et al. [56] plasma cortisol levels of rats treated with cortisol were found to be lower when compared with those of the controls. Glucocorticoids increase the activity of betaine-homocysteine methyl transferase which converts homocysteine into methionine [57]. In addition, steroids may induce genomic inhibition of a few types of cytokines which increase bioavailability of vitamin B6. In a study by Lazzerini et al. [53] the authors reported decrease in plasma homocysteine levels in RA patients under pulse glucocorticoid therapy. In our study, serum homocysteine levels in all RA patients who were using prednisolone were similar to those of the control group. This condition may stem from methotrexate used by some of our patients or refraining from using higher doses of steroids.

The main limitation of our study is its cross-sectional design. Besides scarce number of patients and investigation for only serum ADMA and homocysteine levels are other limitations of our study.

In conclusion, our study has demonstrated that serum homocysteine levels in patients with RA do not increase irrespective of the disease activity. On the other hand, our study has revealed that serum ADMA levels increased without any correlation with the parameters of disease activity. Our findings have displayed that ADMA may play an independent role in the development of atherosclerosis in patients with RA. Serum ADMA levels can be used as an increased risk factor involving in the early phase of atherogenesis rather than disease activity and monitorization of the treatment response.

Disclosure of conflict of interest

None.

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