Increased Levels of Asymmetric Dimethylarginine (ADMA) in Patients with Ankylosing Spondylitis

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Abstract

Objective Endothelial dysfunction is present in ankylosing spondylitis (AS). However, the etiology of events is still unclear. The aim of the present study was to investigate whether there are abnormalities in nitric oxide (NO) metabolism and endothelin-1 (ET-1) in AS patients.

Methods Subjects without any classical cardiovascular (CV) risk factors were studied. Fasting glucose, serum lipids, high sensitive CRP (hsCRP), ESR, asymmetric dimethylarginine (ADMA) and ET-1 were studied. Patients were also evaluated with the Bath Ankylosing Spondylitis Metrology Index, Bath Ankylosing Spondylitis Functional Index, and the Bath Ankylosing Spondylitis Disease Activity Index.

Results A total of 48 AS patients (38.6±10.6 years; 36M/12F) and 38 controls (36.4±11.1 years; 27M/11F) were studied. Acute phase reactants including hsCRP, and ESR were significantly increased in the patients group ($p<0.05$). Serum ADMA concentrations were also significantly higher in AS than in controls. Plasma levels of ET-1 did not differ between the groups ($p>0.05$). Comparison of three groups (conventional and anti-TNF treatment groups and controls) revealed that ADMA was significantly higher in the conventional treated AS than in controls. The levels of ADMA were not different between anti-TNF group and healthy subjects. Plasma ET-1 concentrations were similar between groups ($p>0.05$). Correlation analysis yielded significant correlations between ADMA, hsCRP, LDL cholesterol, HDL cholesterol and triglycerides ($p<0.05$).

Conclusion The increased ADMA levels obtained in a group of relatively young AS patients who did not have classical CV risk factors suggest that NO metabolism is impaired in AS. On the other hand, anti-TNF treatments may have a beneficial effect on vascular function in AS.

Key words: asymmetric dimethylarginine, endothelin-1, endothelium, ankylosing spondylitis, atherosclerosis

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Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease of the spine and sacroiliac joints of unknown etiology (1). AS may affect extraarticular tissues such as eyes, digestive tract, heart, and kidneys (2). The severity of cardiac involvement varies greatly ranging from asymptomatic to severe considerable problems such as severe heart failure, and arrhythmias (3). Some studies revealed an increased prevalence of cardiovascular (CV) mortality in AS (4-8). Re-

cently, an impaired endothelial function, by using ultrasonography, has been demonstrated in a relatively young age group of AS patients without known classical CV risk factors (9). However the etiology of events is still unclear. In this study, we aimed to determine the blood markers of asymmetric dimethylarginine (ADMA), a marker in nitric oxide (NO) metabolism, and endothelin-1 (ET-1) in AS patients, who were free of CV risk factors. We also investigated the effects of conventional and anti-tumor necrosis factor alpha (TNF- α) treatments on ADMA and ET-1 levels.

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Materials and Methods

Patients and controls

The sample size was calculated by using the results of previous studies that investigated the levels of ADMA (10-12), and ET-1 (13-15) based on $\alpha=0.05$ and a power of 80%. At least 13 patients (12 for endothelin, 13 for ADMA) were needed per group. A total of 60 AS patients, diagnosed according to the Modified New York criteria (16) (mean age 39.9 ± 10.6 years; 42 men [M], 18 women [F]), and 60 healthy control subjects (mean age 36.7 ± 10.5 years, 34M/26 F) were included in the study. Controls were recruited from the relatives of the health professionals and blood donors without any known chronic disease. Exclusion criteria were as follows: hypertension (HT), diabetes mellitus (DM), hyperlipidemia, and smoking. Patients who had been treated with systemic corticosteroids within 8 weeks before the study were also excluded. Disease duration, biological treatment usage and duration were recorded. Written informed consent was obtained from each subject, and research protocols were approved by the Ethical Committee of our institution. 12 AS patients (2 DM, 5 dyslipidemia and 5 HT) and 22 controls (3 DM, 14 dyslipidemia and 5 HT) were excluded from the study. Study was performed on the remaining 48 patients and 38 controls. This study was performed between March 2008 and October 2008.

Definition of variables

Hypertension: Average systolic BP ≥ 140 mmHg or average diastolic BP ≥ 90 mmHg, or receiving treatment for HT.

Dyslipidemia: Total cholesterol level ≥ 260 mg/dL or low density lipoprotein (LDL) cholesterol ≥ 160 mg/dL or the use of lipid lowering medication.

Diabetes: Current use of medications prescribed to treat DM or fasting serum glucose levels ≥ 126 mg/dL.

Smoking: Any tobacco use in the past 30 days.

Laboratory evaluation

Following an overnight fasting, venous blood samples were collected for the measurement of laboratory tests in the morning (8:00-9:00 A.M). Erythrocyte sedimentation rate (ESR), fasting blood glucose, and serum lipids (total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides) were studied during the same day. Serum and plasma samples for the measurement of high sensitive C-reactive protein (hsCRP), ADMA and ET-1 were preserved at -80°C until analysis. Analyses of hsCRP, ADMA, and ET-1 were performed by using commercially available ELISA kits in accordance with the supplier's instructions. Other laboratory tests were measured according to standard procedures.

hsCRP: Serum hsCRP was determined using a commercially available sandwich ELISA kit (DRG International, USA, Cat No: EIA-3954). Diluted serum samples and a

horseradish-peroxidase labelled secondary goat anti-human CRP antibody were applied to the ELISA plates coated with a mouse monoclonal anti-human CRP antibody according to the manufacturer's instructions. Color was developed with 3,3' 5' 5-tetramethylbenzidine and absorbance was measured at 450 nm against standard curves. Mean values were reported. The sensitivity of hsCRP was 0.1 mg/L. The intra-assay and inter-assay coefficient of variations were 2.3% and 2.5%, respectively.

ADMA: Serum ADMA concentration was determined by using a competitive 96-well plate ADMA-ELISA kit (DLD, Diagnostika GMBH, Hamburg, Germany) following the supplier's protocol. This immunometric assay is based on the competition of acylated ADMA in samples with solid phase bound ADMA for a fixed number of rabbit anti-ADMA antiserum binding sites. ADMA shows negligible cross reactivity with L-arginine ($<0.02\%$) and other endogenous derivatives of L-arginine. Coefficients of variation are 8.3-10.3%; and 4.5-7.5% for inter-assay and intra-assay, respectively. The ELISA assay can accurately measure ADMA concentrations over the full range of physiologically-relevant concentrations (i.e. $0.05 \mu\text{mol/L}$ to $2 \mu\text{mol/L}$). The specificity of ADMA ELISA kit is 100% and the sensitivity is $0.05 \mu\text{mol/L}$.

Endothelin -1: Plasma endothelin was determined by using 100 μL of supernatant per well of a 96-well plate with a endothelin EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA Cat No:583151) following the supplier's protocol. This immunometric assay is based on a double-antibody 'sandwich' technique and permits endothelin measurements within the range of 0-250 pg/mL, typically with a limit of detection of 1.5 pg/mL. Monoclonal anti ET-1 antibody in this kit had a cross reactivity of 100% with ET-2 and 100% with ET-3. Samples were assessed with no prior purification. In this method monoclonal antibody specific to endothelin and acetylcholinesterase: Fab' Conjugate (AChE: Fab') bind to different epitopes on the Endothelin-1 molecule and forming sandwich. This sandwich is immobilized on the plate so the excess reagents are washed away. The concentration of analyte is detected by measuring the enzymatic activity of the AChE by adding Ellman's Reagent [5, 5'-dithiobis-(2-nitrobenzoic acid) or DTNB] which contains the substrate for AChE. The addition of Ellman's Reagent produces a yellow-colored product which can be measured spectrophotometrically. The intensity of the color is directly proportional to the amount of bound conjugate which in turn is proportional to the concentration of the endothelin. The intra- and inter-assay coefficients of variation of the method were 5 and 6%, respectively.

Other measurements

Spinal mobility was assessed by the Bath Ankylosing Spondylitis Metrology Index (17). Patients were also evaluated with the Bath Ankylosing Spondylitis Functional Index (BASFI) (18) and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (19). BMI and waist circumfer-

Table 1. Clinical and Laboratory Characteristics of AS Patients and Controls

	AS patients (n=48)	Controls (n=38)	p value
Age (yr)	38.6±10.6	36.4±11.1	0.3
Sex (M/F)	36/12	27/11	0.8
BMI (kg/m ²)	25.4±4.5	25.2±3.7	0.8
Waist circumference (cm)	85.7±11.4	83.8±10.8	0.4
Mean SBP (mmHg)	111±10.8	110±11	0.8
Mean DBP (mmHg)	68±7	69±6.6	0.6
Fasting glucose (mg/dL)	91.4±11.9	92.3±11.3	0.7
Total Cholesterol (mg/dL)	175.5±32.9	186.1±37.1	0.2
HDL Cholesterol (mg/dL)	46.6±11.1	48.8±11.8	0.4
LDL Cholesterol (mg/dL)	109.2±29	112.6±25.8	0.6
Triglyceride (mg/dL)	96.6±46	106.8±59.1	0.4
†ESR (mm/h)	11±19	3±6	<0.001
hsCRP (mg/L)	9±5.5	4.2±3.6	<0.001
ADMA (μmol/L)	1.6±1	0.9±0.9	0.003
Endotelin (pg/mL)	19±3.9	20.4±5.3	0.2

Results were presented as mean±SD, and †median±IQR. p value <0.05 accepted as statistically significant. BMI=Body mass index; SBP=Systolic blood pressure; DBP=Diastolic blood pressure; ESR=Erythrocyte sedimentation rate; hsCRP=High sensitive C-reactive protein; ADMA=Asymmetric dimethylarginine

ence were calculated.

Statistical analysis

The Kolmogorov-Smirnov test was used to determine if continuous variables were normally distributed. For normally distributed data, results were presented as mean ± standard deviation (SD) and comparison between groups of continuous variables was performed by using the Student's t test. When the variables were found not to be normally distributed (ESR, and BASMI), results were given as median ± interquartile range (IQR) comparisons between groups of data were made using Mann-Whitney U test. Differences between categorical variables were analyzed by Fisher's exact test. One-way ANOVA in conjunction with Tukey's HSD test or Kruskal-Wallis tests (ESR, and BASMI) were applied when comparing multiple groups. Relationships between variables were analyzed using Pearson or Spearman's rank correlation coefficients (ESR, and BASMI). The statistical analysis was carried out by using Statistical Package of Social Science (SPSS), version 13.0 (SPSS Inc., Chicago, IL). A p value of <0.05 was considered as statistically significant.

Results

The study group consisted of 48 AS patients (38.6±10.6 years; 36M/12F) and 38 healthy controls (36.4±11.1 years; 27M/11F). There were no differences with respect to the age and sex distribution (p>0.05). Anthropometrical measures such as BMI and waist circumference, blood pressure levels,

serum glucose and lipid concentrations of the patients and controls were also similar between groups (p>0.05). None of the subjects had renal impairment. The clinical and laboratory parameters of the study group are summarized in the Table 1. Disease duration of the patients was 13±7.5 years. BASDAI, BASFI, and BASMI were 3.6±2.3, 2.9±2.5, and 1.9±2.6, respectively. Acute phase reactants including hsCRP, and ESR were significantly increased in the patients group (p<0.001; 8.9±5.6 vs. 4.18±3.71 mg/L, and 11±19 vs. 3±6 mm/h respectively). Serum ADMA concentrations were also significantly higher in AS patients than in controls (1.6±1 vs. 0.9±0.9 μmol/L; p=0.003). However, plasma levels of ET-1 were not different between groups (19±3.9 vs. 20.4±5.3 pg/mL; p=0.2).

There were 18 patients (14M/4F; 42.3±10.1 years) treated with TNF-α blocking agents (9 etanercept, and 9 infliximab). The mean treatment duration for biological drugs was 27.7±25.8 months. 30 patients (22M/8F; 36.5±10 years) were receiving conventional treatments (non-steroidal anti-inflammatory drugs and/or sulfasalazine) and none of these subjects had been previously treated with anti-TNF-α agents. Age, sex distribution, BMI, waist circumference, disease duration, acute phase reactants, disease activity indices were similar between anti-TNF-α and conventional treatment groups (p>0.05). ADMA and ET-1 concentrations were lower in the anti-TNF-α treated group but these values were not statistically significant (1.7±1 vs. 1.3±1 μmol/L, and 19.3±4.5 vs. 18.6±2.7 pg/mL respectively). Table 2 summarizes the laboratory and clinical parameters of the groups.

Comparison of three groups (conventional and anti-TNF-

Table 2. Comparison of Subjects Receiving Conventional Treatment or Anti-TNF- α Drugs and Healthy Controls

	AS with conventional treatment (n=30)	AS with anti-TNF treatment (n=18)	Healthy controls (n=38)
Age (yr)	36.5 $\pm$ 10	42.3 $\pm$ 10.1	36.4 $\pm$ 11.1
Sex (M/F)	22/8	14/4	27/11
Disease duration (years)	11.4 $\pm$ 7.7	15.6 $\pm$ 6.7	
BASFI	3.1 $\pm$ 2.5	2.7 $\pm$ 2.6	
BASDAI	4 $\pm$ 2.4	2.9 $\pm$ 2	
[†] BASMI	0.001 $\pm$ 3	1 $\pm$ 5	
BMI (kg/m ²)	25.3 $\pm$ 4.6	25.8 $\pm$ 4.3	25.2 $\pm$ 3.7
Waist circumference (cm)	83.5 $\pm$ 12.2	89.3 $\pm$ 9	83.8 $\pm$ 10.8
[†] ESR (mm/h)	11 $\pm$ 19*	9.5 $\pm$ 17	3 $\pm$ 6
hsCRP (mg/L)	9.6 $\pm$ 5.6*	8 $\pm$ 5.3**	4.2 $\pm$ 3.6
ADMA (μ mol/L)	1.7 $\pm$ 1*	1.3 $\pm$ 1	0.9 $\pm$ 0.9
Endothelin (pg/mL)	19.3 $\pm$ 4.5	18.6 $\pm$ 2.7	20.4 $\pm$ 5.3

Results were presented as mean $\pm$ SD, and [†]median $\pm$ IQR. p values <0.05 accepted as statistically significant. *Indicates a significant difference between AS patients with conventional treatment and healthy controls. **Denotes a significant difference between AS patients with anti-TNF treatment and healthy subjects.

BMI=Body mass index; BASFI=Bath ankylosing spondylitis functional index; BASDAI=Bath ankylosing spondylitis disease activity index; BASMI=Bath ankylosing spondylitis metrology index;

Figure 1. Comparison of serum asymmetric dimethylarginine (ADMA) levels between groups.

α treatment groups and healthy subjects) revealed that ADMA was significantly increased in the conventionally treated AS patients compared to controls (1.7 $\pm$ 1 vs. 0.9 $\pm$ 0.9 μ mol/L; p=0.004). The levels of ADMA did not differ between anti-TNF group and healthy subjects (1.3 $\pm$ 1 vs. 0.9 $\pm$ 0.9 μ mol/L; p>0.05). Plasma ET-1 concentrations were simi-

lar between groups ($p>0.05$). Figure 1 represents the dot graphic comparison of ADMA between groups.

Correlation analysis yielded significant correlations between ADMA, hsCRP, LDL cholesterol, HDL cholesterol and triglycerides ($p<0.05$; $r=0.2, 0.3, -0.2$, and 0.2 , respectively.) On the other hand, disease activity indices, ESR, and ET-1 levels were not correlated with serum ADMA concentrations ($p>0.05$).

Discussion

In this study we demonstrated that serum ADMA levels, reflecting NO metabolism, were increased in AS patients who were free of CV risk factors. It is noteworthy that this finding was obtained in a group of relatively young patients. The other parameter, ET-1, did not differ between patients and controls. To the best of our knowledge, this is the first study investigating ADMA and ET-1 levels in AS patients.

ADMA is an endogenous inhibitor of nitric oxide (NO) and increased levels of this molecule are associated with reduced NO synthesis and vascular dysfunction (20, 21). Elevated ADMA accompanies several CV risk factors including hypertension, dyslipidemia, smoking, insulin resistance and diabetes (20, 21). In addition to accompanying the traditional risk factors, it is also recognized as a novel CV risk parameter, as prospective studies have shown that increased ADMA is associated with a 3- to 5-fold increase in the risk of experiencing serious CV events even in clinically healthy non smoking subjects (20, 22). A high ADMA concentration leads to vascular damage by counteracting the physiological effects of NO (such as vasodilatation, inhibition of adhesion molecule expression, inhibition of platelet adhesion, prevention of lipid oxidation), and it thereby promotes atherosclerosis (20, 22). In the literature, increased ADMA concentrations have been reported in various inflammatory rheumatic diseases including rheumatoid arthritis (RA) (10), Behcet's disease (12), familial Mediterranean fever (FMF) (11), systemic lupus erythematosus (SLE) (23) and scleroderma (24).

In the present study, there were no subjects with classical CV risk factors as well as active infection in both groups which might affect the results. On the other hand, it is noteworthy that our ADMA levels were significantly higher in the AS patients compared to controls even in the presence of relatively high hsCRP levels in the control group. In addition, ADMA levels showed a positive and significant correlation with hsCRP. Subgroup analysis of the current study showed that ADMA levels were significantly higher in conventionally treated AS patients than in healthy controls. In contrast, anti-TNF- α treated AS patients had comparable ADMA concentrations compared to healthy subjects, suggesting a beneficial effect of anti-TNF- α treatment on vascular functions in AS. This finding is in accordance with the results of a former study which showed an improvement in endothelial function, by using ultrasonography, after anti-TNF- α treatment (25).

Endothelin-1 is a peptide, secreted mostly by vascular endothelial cells, that possesses vasoconstrictor, proinflammatory, mitogenic and proliferative properties (26). Endothelin production is stimulated by mechanical factors such as vascular injury, lipids and cytokines including IL-1 and IL-6 (27). Elevated ET-1 levels have been reported in inflammatory rheumatic diseases particularly in scleroderma (28), SLE (14), Behcet's disease (15) and RA (13). There is no report in the literature with respect to ET-1 levels and AS. In the present study, ET-1 levels were similar between AS patients and controls. As Behcet's disease, scleroderma and SLE may cause direct vascular injury, the presence of higher ET-1 levels in these diseases might not be an unexpected finding.

In conclusion, increased ADMA levels obtained in a group of relatively young AS patients who did not have classical CV risk factors suggests that NO metabolism is impaired in AS. In addition, unchanged ADMA levels in patients treated with TNF blocking agents suggests that anti-TNF- α treatment may have a beneficial effect on vascular function in AS.

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