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ORIGINAL ARTICLE

Determination of serum methylarginine levels by tandem mass spectrometric method in patients with ankylosing spondylitis

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Abstract

Our aim in this study was to measure serum levels of methylarginines and related metabolites in patients with ankylosing spondylitis (AS), moreover, to investigate the relationship between these parameters and various clinical and laboratory parameters of patients with AS. The study included 60 patients with AS and 60 healthy volunteers. Serum asymmetric dimethylarginine (ADMA), L–N monomethylarginine (L-NMMA), symmetric dimethylarginine (SDMA), arginine (Arg), homoarginine (hArg), ornithine, and citrulline concentrations were measured with tandem mass spectrometry. In addition, participants were divided into three groups according to the treatment regimen: TNF-α inhibitor group ($n=25$), conventional therapy group ($n=35$), and control group ($n=60$). These groups were compared in terms of serum levels of methylarginine pathway metabolites and various biochemical parameters. It was found that total methylated arginine load significantly increased in patients with AS ($p<0.001$), and the Arg/ADMA ratio was positively correlated with HDL levels and negatively correlated with glucose, ESR, total cholesterol, triglyceride, and LDL levels. In addition, serum ADMA, SDMA, total methylated arginine load, and CRP levels were lower ($p<0.05$) in the TNF-α group compared to the conventional treatment group. To the best of our knowledge, this is the first study to comprehensively investigate serum methylarginine levels in patients with AS. Elevated total methylated arginine load and decreased global arginine bioavailability ratio (GABR) indicate that NO metabolism is impaired in patients with AS. Therefore, the increased cardiovascular risk in patients with AS may be related to the decreased NO production or bioavailability due to the elevated total methylarginine load.

Keywords Ankylosing spondylitis · Methylarginines · Asymmetric dimethylarginine · Endothelial dysfunction

Abbreviations

ADMA	Asymmetric dimethylarginine
ARG	Arginine
AS	Ankylosing spondylitis
AS	Arterial stiffness
BASDAI	Bath Ankylosing Spondylitis Disease Activity Index
BASFI	Bath Ankylosing Spondylitis Functional Index
BASMI	Bath Ankylosing Spondylitis Metrology Index
CCA-IMT	Carotid artery intima-media thickness
DDAH	Dimethylarginine dimethylaminohydrolase
ENOS	Endothelial nitric oxide synthase
ESR	Erythrocyte sedimentation rate
FMD	Flow-mediated dilatation
GABR	Global arginine bioavailability ratio
HARG	Homoarginine
HDL	High-density lipoprotein
HLA-B27	Human leukocyte antigen-B27
HS-CRP	High sensitivity-CRP
IL-8	Interleukin 8
INOS	Inducible nitric oxide synthase
LDL	Low-density lipoprotein
L-NMMA	L-N monomethylarginine
NNOS	Neuronal nitric oxide synthase

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NO	Nitric oxide
PRMTS	Protein arginine methyltransferases
ROS	Reactive oxygen derivatives
SDMA	Symmetric dimethylarginine

Introduction

Ankylosing spondylitis (AS) is a chronic, inflammatory rheumatic disease characterized by back pain, restricted spine movement, and decreased quality of life (Allouch et al. 2017). The pathogenesis of AS is multifactorial and has not been fully elucidated yet. However, it has been suggested that human leukocyte antigen-B27 (HLA-B27) has an important role in the pathogenesis of AS and contributing to ~ 20% of AS heritability (Chen et al. 2017; Simone et al. 2018). The prevalence of AS reaches a peak at the age of 30–40 years, and it is more common in men than women (4:1) (Park et al. 2018). AS affects the sacroiliac joints and axial spine, as well as extra-articular organs such as the eye, bone, lung, and heart (Korkmaz et al. 2017). Cardiovascular diseases are the leading cause of death worldwide. Increasing evidence indicates that inflammatory rheumatic diseases are important risk factors for cardiovascular disease (Atzeni et al. 2017). Considering its extra-articular features, AS is associated with increased cardiovascular morbidity and mortality compared to the general population (Łosińska et al. 2019). The cardiovascular effects of AS are very heterogeneous, and cardiac structures such as pericardium, endocardium, coronary arteries, and heart muscle may be affected during AS (Sarzi-Puttini et al. 2010). Various studies have reported that patients with AS have an approximately 30–50% higher risk of cardiovascular morbidity and mortality compared to the general population (Łosińska et al. 2019; Haroon et al. 2015). Nitric oxide (NO) is a key signaling molecule that has biologically important pleiotropic effects and plays a critical role in cardiovascular homeostasis (Farah et al. 2018). NO and citrulline are synthesized from L-arginine via isoforms of nitric oxide synthases (NOS) including endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS) (Förstermann and Sessa 2012). NO acts as an endothelium-derived relaxing factor and additionally has many important functions related to the cardiovascular system (Kang 2014). Recently, an increasing number of studies have shown that dysregulation in NO metabolism is directly related to various cardiovascular diseases such as atherosclerosis, hypertension, stroke, and chronic heart failure (Rajendran et al. 2013). Methylarginine derivatives, such as asymmetric dimethylarginine (ADMA), L-N monomethyl arginine (L-NMMA), and symmetric dimethylarginine (SDMA), reduce NO synthesis directly or indirectly (Bełtowski and Kedra 2006). ADMA, SDMA, and L-NMMA are produced by post-translational methylation of arginine residues in proteins by enzymes called protein arginine methyltransferases (PRMTs) and are released into the cytosol by proteolysis. ADMA and L-NMMA, endogenous competitive inhibitors of the NOS enzyme, are structurally similar to arginine. SDMA indirectly reduces NO levels by inhibiting the uptake of arginine into cells (Caplin and Leiper 2012). Numerous studies have been conducted to evaluate the relationship of methylarginine pathway metabolites with cardiovascular diseases. These studies revealed that increased serum ADMA, SDMA, L-NMMA, citrulline, and ornithine levels and decreased serum homoarginine (hArg) and arginine (Arg) levels may be a risk factor for cardiovascular diseases (Karetnikova et al. 2019). This study was aimed to measure serum ADMA, SDMA, L-NMMA, Arg, hArg, citrulline, and ornithine levels in patients with AS and to calculate hArg/ADMA, SDMA/ADMA, Arg/ADMA ratios, global arginine bioavailability ratio (GABR), and total methylated arginine load, moreover, to investigate the relationship between these parameters and various clinical and laboratory parameters of patients with AS.

Materials and methods

Study design

Patients

The study included 60 healthy volunteers and 60 patients with AS who were regularly followed up in Selcuk University Faculty of Medicine Physical Therapy and Rehabilitation outpatient clinic and were diagnosed according to Modified New York Criteria (van der Linden et al. 1984). The exclusion criteria were as follows: history of cardiovascular or cerebrovascular disease, diabetes mellitus, chronic kidney diseases, other inflammatory and rheumatic diseases, hepatic failure, malignancy, smoking, use of alcohol or drugs such as systemic corticosteroids, antihypertensive, antihyperlipidemic, and antidiabetic.

8 cc of blood samples from the participants were taken into serum separator gel tubes and centrifuged at 3500 rpm for 15 min. Separated serum samples were aliquoted and stored at -80 °C until analysis. This study was approved by the Selcuk University Faculty of Medicine Ethics Committee.

Analysis of arginine derivatives

Chemicals

ADMA (CAS Number 220805-22-1), SDMA (CAS Number: 1266235-58-8), L-NMMA (CAS Number: 53308-83-1), Arg (CAS Number: 202468-25-5), ornithine (CAS Number: 3184-13-2), citrulline (CAS Number: 372-75-8), hArg (CAS Number: 1483-01-8), methanol (CAS Number: 67-56-1), HPLC grade water (CAS Number: 7732-18-5), n-butanol (CAS Number:71-36-3), acetyl chloride (CAS Number: 75-36-5), and formic acid (CAS Number: 64-18-6) were obtained from Sigma-Aldrich (St. Louis, MO, USA). d7-ADMA (Catalog No: DLM-7476-5) was obtained from Cambridge Isotope Laboratories.

Instrumentation

Chromatographic separation was performed using a Shimadzu HPLC system (Kyoto, Japan) and Phenomenex C18 HPLC column (50 mm × 4.6 mm). API 3200 triple quadrupole mass spectrometer equipped with an electrospray ionization interface was used (Applied Biosystems/MDS Sciex) as the detector. The mobile phase A and B consisted of 0.1% formic acid/water (% v/v) and 0.1% formic acid/methanol (% v/v), respectively. Total run time was 5 min. The Q1–Q3 ion transitions were 259.3/214, 259.3/228, 245.3/70.2, 231.3/70.0, 245.2/84.2, 189/70.0, 232.3/113, and 266.1/221 for ADMA, SDMA, L-NMMA, Arg, hArg, ornithine, citrulline, and d7-ADMA, respectively. Ionspray voltage, source temperature, curtain, ion source (GS1), and ion source (GS2) gas values were adjusted to 5000 V, 350 °C, 20, 40, and 60 psi, respectively. Intra- and inter-assay CV% values were less than 9.8% and extraction recoveries were higher than 94% for all metabolites.

Sample preparation

Serum Arg, ADMA, L-NMMA, SDMA, hArg, citrulline, and ornithine levels were measured with the developed method by Eryavuz Onmaz et al. (Eryavuz Onmaz et al. 2021). Briefly, 200 µL of serum sample, 100 µL of d7-ADMA, and 1000 µL of methanol were added to the eppendorf tubes and vortexed for 30 s. The mixture was centrifuged at 13,000 rpm for 10 min and 800 µL of the supernatants were evaporated at 60 °C under nitrogen gas. 200 µL of a freshly prepared butanol solution including 5% (v/v %) acetyl chloride was added for derivatization. The capped reaction tubes were incubated at 60 °C for 30 min, and then, mixtures were evaporated with nitrogen gas. The residues were dissolved in 200 µL of water–methanol (90:10, v/v%) mixture including 0.1% (% v/v) formic acid. 30 µL was injected into LC–MS/MS system. GABR was calculated by the formula as [Arg/(Citrulline + Ornithine)] (Sourij et al. 2011). Total methylated arginine load was calculated as the sum of ADMA, SDMA, and L-NMMA levels (Hosaf et al. 2020).

Clinical and routine laboratory parameters

Serum glucose, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride levels were analyzed with BeckmanCoulter AU 5800 (Beckman Coulter, Brea, USA) according to the manufacturer's instructions. Serum high-sensitivity-CRP (hs-CRP) levels and erythrocyte sedimentation rate (ESR) were measured with IMMAGE 800 (Beckman Coulter, Brea, USA) and Alifax (Padova, Italy) systems, respectively. Patients were also evaluated by clinicians with the Ankylosing Spondylitis Metrology Index (BASMI), Bath Ankylosing Spondylitis Functional Index (BASFI), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI).

Statistical analysis

Statistical analysis was performed with SPSS statistical software package version 21.0. One-sample Kolmogorov–Smirnov test was performed to find out the distribution. Student's *t* and Mann–Whitney *U* tests were used to compare the mean and median values between two groups,

Table 1 The basic demographic and clinical characteristics of the participants

Characteristics	AS ($n=60$)	Control ($n=60$)
Age (years)	42.1 ± 8.1	42.9 ± 8.5
Sex (male/female)	38/22	35/25
Disease duration (years)	9.7 ± 7.8	
BMI (kg/m^2)	27.9 ± 5.2	27.1 ± 4.0
BASDAI	4.9 ± 1.7	
BASMI	1.5 ± 1.1	
BASFI	3.7 ± 1.9	
ASQoL	8.7 ± 4.9	
Effusion positive (%)	55	
Enthesitis positive (%)	39	
Erosion positive (%)	20	
Sclerosis positive (%)	18	
Ankylosis positive (%)	0	
Synovitis positive (%)	5	
Drugs (%)		
DMARD plus NSAID	10	
NSAID alone	8	
DMARD alone	17	
Anti-TNF-α alone	25	

Table 2 The laboratory parameters of patients with AS and control groups

Parameters	AS ($n=60$)	Control ($n=60$)	p
ADMA (μM)	0.46 ± 0.16	0.31 ± 0.13	**< 0.001**
SDMA (μM)	0.18 ± 0.04	0.13 ± 0.04	**< 0.001**
L-NMMA (μM)	0.023 ± 0.005	0.018 ± 0.004	**< 0.001**
Arg (μM)	60.18 ± 23.9	72.31 ± 18.87	**0.004**
hArg (μM)	2.47 ± 0.52	3.48 ± 1.07	**< 0.001**
Citrulline (μM)	22.5 (8.82–47.50)	19.0 (7.0–30.20)	0.169
Ornithine (μM)	42.4 (14.94.20)	35.4 (10.10–65.14)	0.229
Arg/ADMA	117.9 (61.9–283.7)	227.7 (101.2–382.7)	**< 0.001**
hArg/ADMA	5.39 (2.85–10.88)	9.48 (5.13–24.58)	**< 0.001**
SDMA/ADMA	0.37 (0.25–0.75)	0.40 (0.28–0.68)	0.067
GABR	0.95 (0.24–4.60)	1.26 (0.55–14.0)	**0.002**
Total methylated arginine load (μM)	0.64 (0.42–1.04)	0.45 (0.33–0.78)	**< 0.001**
HDL (mg/dL)	46.38 ± 8.48	57.83 ± 9.36	**< 0.001**
LDL mg/dL)	137.29 ± 36.96	96.66 ± 20.38	**< 0.001**
Total cholesterol (mg/dL)	200.16 ± 35.52	171.38 ± 25.87	**0.002**
Triglyceride (mg/dL)	137.52 ± 35.75	96.63 ± 25.65	**< 0.001**
Glucose (mg/dL)	97.41 ± 17.07	85.58 ± 5.97	**0.001**
CRP (mg/L)	5.73 (1.40–50.70)	0.10 (0.02–1.75)	**< 0.001**
ESR (mm/hr)	14.5 (2.0–60.0)	5.50 (2.0–42.0)	**0.004**

Student's t and Mann–Whitney U tests were performed to compare the mean and median values, respectively. $p < 0.05$ statistically significant

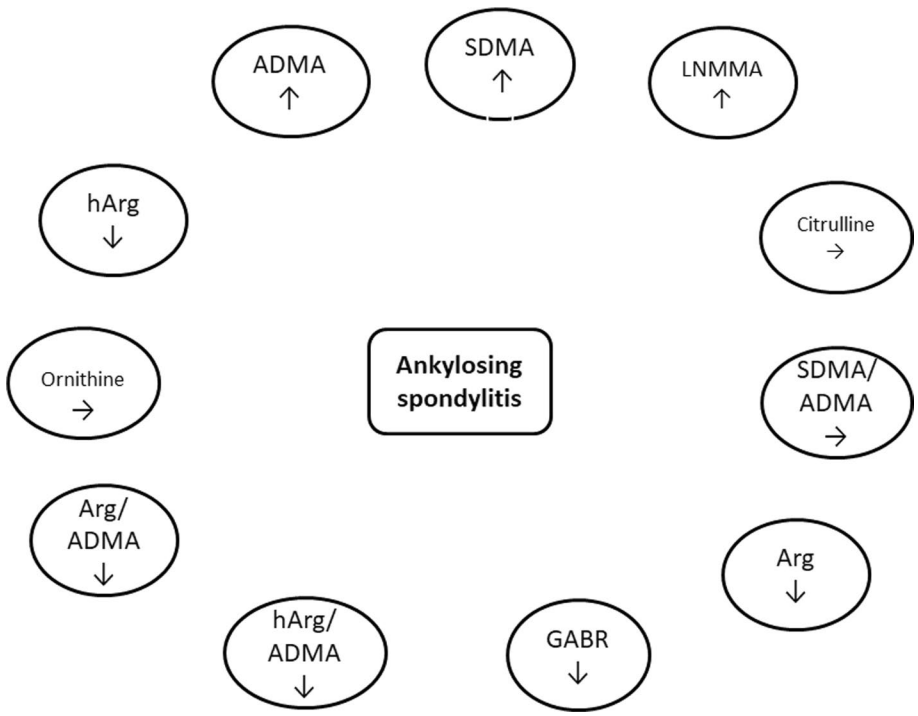

Fig. 1 Changes of methylarginines and related metabolites in AS. ↑: increased; ↓: decreased; →: unchanged

respectively. One-way ANOVA analysis (post hoc analysis with LSD or Tamhane's T^2 tests) and Kruskal–Wallis test (post hoc analysis Mann–Whitney U) were also performed. Spearman correlation was used. $p < 0.05$ was considered as statistically significant.

Results

The basic demographic and clinical characteristics of the participants were shown in Table 1.

Serum ADMA ($p<0.001$), SDMA ($p<0.001$), L-NMMA ($p<0.001$), total methylated arginine load ($p<0.001$), ESR ($p=0.004$), CRP ($p<0.001$), total cholesterol ($p=0.002$), triglyceride ($p<0.001$), LDL ($p<0.001$), and glucose ($p=0.001$) levels were found to be statistically significant higher in patients with AS compared to the control group, while Arg ($p=0.004$), hArg ($p<0.001$), HDL ($p<0.001$), GABR ($p=0.002$) levels, Arg/ADMA ($p<0.001$), and hArg/ADMA ($p<0.001$) ratios were lower (Table 2). The laboratory parameters of patients with AS and control group are described in Table 2, and the changes of methylarginines and related metabolites in AS are summarized in Fig. 1.

Participants were divided into three groups as follows according to the treatment regimen: anti-TNF-α ($n=25$), conventional therapy (non-steroidal anti-inflammatory drugs and/or disease-modifying drugs, $n=35$), and control group ($n=60$). Patients under TNF-α therapy had not previously received conventional therapy. These groups were compared in terms of serum levels of methylarginine pathway metabolites and various biochemical parameters. Serum ADMA ($p=0.026$), SDMA ($p=0.032$), total methylated arginine load ($p=0.042$), and CRP ($p=0.038$) levels were lower in the TNF-α group compared to the conventional therapy group. Serum ADMA, SDMA, L-NMMA, LDL, total cholesterol, triglyceride, glucose, ESR, CRP levels, and total methylated arginine load were significantly lower in the control group than in both treatment groups ($p<0.05$), while serum hArg, HDL levels, Arg/ADMA, hArg/ADMA ratios, and GABR were higher ($p<0.05$) (Table 3).

Correlation analysis showed that there was a positive correlation between total methylated arginine load and serum triglyceride, LDL, total cholesterol, glucose, ESR, CRP levels, and a negative correlation with HDL. Arg/ADMA ratio was positively correlated with HDL levels and negatively correlated with serum glucose, ESR, total cholesterol, triglyceride, and LDL levels (Table 4). On the other hand, there was only a negative correlation between BASDAI and serum Arg levels among the disease activity indices (Table 5). Relevant correlations between serum levels of methylarginines and related metabolites and various laboratory or clinical parameters are described in Tables 4, 5, respectively.

Discussion

AS is associated with increased cardiovascular morbidity and mortality (Batko et al. 2018). Accelerated atherosclerosis and endothelial dysfunction have been detected in patients with AS. However, the potential mechanisms between cardiovascular events and AS have not yet been fully elucidated (Łosińska et al. 2019). Increased plasma ADMA levels have been reported in various inflammatory rheumatic diseases such as rheumatoid arthritis (Di Franco et al. 2018), systemic lupus (Perna et al. 2010), scleroderma (Zhang et al. 2015), and Behçet's disease (Sahin et al. 2006). In addition, there are few studies investigating serum ADMA levels in patients with AS (Sari et al. 2009; Kemény– Beke et al. 2011; Erre et al. 2011; Inci et al. 2017; Berg et al. 2015). Sari et al. reported that serum ADMA and acute phase reactants levels (CRP and ESR) were higher in young AS patients without classical cardiovascular risk factors compared to the control group, and there was a positive correlation between ADMA and CRP levels. There was no correlation between serum ADMA levels, disease activity indices, and ESR (Sari et al. 2009). Beke et al. reported that serum ADMA levels of patients with AS were statistically significantly higher than patients with osteoarthritis, and there was no significant difference between serum SDMA and Arg levels. Serum ADMA levels were positively correlated with ESR and chest expansion, but there was no correlation between ADMA levels and disease activity indices and CRP levels (Kemény-Beke et al. 2011). Erre et al. reported that plasma ADMA levels in AS patients without any cardiovascular disease were higher than in the control group, but there was no correlation between ADMA levels with carotid artery intima-media thickness (CCA-IMT), flow-mediated dilatation (FMD), and arterial stiffness (aS) (Erre et al. 2011). Inci et al. reported that CRP, ESR, and ADMA levels were higher in AS patients without classical cardiovascular risk factors compared to the control group (Inci et al. 2017). Our findings showed that in addition to biochemical parameters such as total cholesterol, triglyceride, LDL, and glucose, total methylated arginine load and acute phase reactants (ESR and CRP) levels were higher in patients with AS without any risk of cardiovascular disease compared to the control group (Table 2). ADMA is a major inhibitor of NO synthesis and causes a decrease in NO levels with other methylarginine derivatives (SDMA, L-NMMA), so they are a risk factor for cardiovascular diseases such as hypertension, atherosclerosis, diabetes mellitus, pulmonary embolism, heart failure, and stroke (Tousoulis et al. 2015; Liu et al. 2018; Zobel et al. 2017). The endothelium releases NO, which is a potent vasodilator and has antiatherogenic properties; it also regulates blood pressure and vascular tone, inhibits platelet aggregation and leukocyte adhesion, and prevents proliferation of smooth muscle cells. Therefore, NO plays a key role in the protection against the onset and progression of cardiovascular disease (Förstermann and Sessa 2012). However, high levels of ADMA, SDMA, and L-NMMA directly or indirectly reduce NO production and bioavailability. Decreased NO synthesis or bioavailability is a risk factor for cardiovascular diseases. Methylarginine derivatives are synthesized by the PRMT enzymes, especially ADMA and L-NMMA, are metabolized by the dimethylarginine dimethylaminohydrolase (DDAH) enzyme. DDAH and PRMT activities

Table 3 Comparison of AS patients receiving TNF-α inhibitor and conventional therapy with the control group in terms of laboratory parameters

Parameters	Anti-TNF-α ($n=25$)	Conventional therapy ($n=35$)	Control ($n=60$)	p
ADMA (μM)	0.43 ± 0.074	0.48 ± 0.11	0.31 ± 0.07	**a:0.026** **b: < 0.001** **c: < 0.001**
SDMA (μM)	0.16 ± 0.033	0.18 ± 0.034	0.13 ± 0.036	**a:0.032** **b: < 0.001** **c: < 0.001**
L-NMMA (μM)	0.022 ± 0.004	0.023 ± 0.005	0.018 ± 0.004	a:0.406 **b: < 0.001** **c: < 0.001**
Arg (μM)	53.65 ± 16.25	65.90 ± 25.02	72.86 ± 20.29	a:0.104 **b:0.002** c:0.557
hArg (μM)	2.31 ± 0.56	2.61 ± 0.44	3.36 ± 1.09	a:0.454 **b: < 0.001** **c: < 0.001**
Citrulline (μM)	19.10 (9.45–42.50)	23.75 (8.82–47.50)	18.90 (7.0–30.20)	a:0.358 b:0.660 **c:0.071**
Ornithine (μM)	37.90 (18.90–85.0)	45.0 (14.46–94.20)	35.40 (10.5–65.80)	a:0.500 b:0.585 c:0.149
Arg/ADMA	127.4 (61.9–247.7)	116.7 (72.9–283.7)	227.7 (101.2–382.7)	a:0.836 **b: < 0.001** **c: < 0.001**
hArg/ADMA	5.05 (3.05–10.88)	5.63 (2.85–9.31)	9.48 (5.13–24.58)	a:0.563 **b: < 0.001** **c: < 0.001**
SDMA/ADMA	0.39 (0.25–0.75)	0.36 (0.25–0.59)	0.40 (0.28–0.68)	a:0.728 b:0.282 c:0.283
GABR	0.90 (0.31–2.38)	0.99 (0.24–4.60)	1.27 (0.55–14.0)	a:0.630 **b:0.005** **c:0.044**
Total methylated arginine load (μM)	0.60 (0.42–0.77)	0.67 (0.49–1.04)	0.45 (0.33–0.78)	a:0.042 **b: < 0.001** **c: < 0.001**
HDL (mg/dL)	47.12 ± 12.16	45.6 ± 11.08	57.83 ± 9.36	a = 0.687 **b: < 0.001** **c: < 0.001**
LDL (mg/dL)	145.81 ± 38.30	128.2 ± 34.42	96.66 ± 20.38	a:0.282 **b:0.002** **c: < 0.003**
Total cholesterol (mg/dL)	201.35 ± 32.20	199.15 ± 39.23	171.38 ± 25.87	a:0.859 **b:0.025** **c:0.029**
Triglyceride (mg/dL)	142.35 ± 43.19	133.21 ± 49.06	96.63 ± 25.65	a:0.532 **b:0.002** **c:0.013**

Table 3 (continued)

Parameters	Anti-TNF-α ($n=25$)	Conventional therapy ($n=35$)	Control ($n=60$)	p
Glucose (mg/dL)	94.43 ± 9.75	100.6 ± 12.40	85.58 ± 5.98	**b:0.020** **c:0.001** **a:0.038**
CRP (mg/L)	4.65 (1.40–18.10)	7.40 (1.60–50.70)	0.10 (0.02–1.75)	**b: < 0.001** **c: < 0.001** a:0.137
ESR (mm/hr)	12.50 (2.0–60.0)	19.0 (2.0–50.0)	5.50 (2.0–42.0)	**b:0.047** **c: < 0.001**

a: anti-TNF-α vs. conventional therapy group

b: anti-TNF-α vs. control group

c: conventional therapy group vs. control group comparison

One-way ANOVA analysis (post hoc analysis with LSD or Tamhane's T^2 tests) and Mann–Whitney U analysis were performed. $p<0.05$ Statistically significant

Table 4 Correlations between methylarginine pathway metabolites and various laboratory parameters

Parameters	HDL (mg/dL)		LDL (mg/dL)		TC (mg/dL)		Triglyceride (mg/dL)		Glucose (mg/dL)		CRP (mg/L)		ESR (mm/hr)	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
ADMA (μM)	**−0.323**	**0.008**	**0.373**	**0.002**	**0.317**	**0.006**	**0.297**	**0.011**	**0.308**	**0.011**	**0.295**	**0.034**	**0.301**	**0.037**
SDMA (μM)	**−0.442**	**< 0.001**	**0.308**	**0.011**	0.152	0.199	0.191	0.108	0.139	0.261	**0.364**	**0.009**	0.179	0.213
L-NMMA (μM)	**−0.376**	**0.002**	0.156	0.209	0.132	0.266	0.196	0.099	0.086	0.490	**0.328**	**0.020**	0.170	0.237
Arg (μM)	**0.323**	**0.008**	**−0.340**	**0.005**	−0.187	0.112	**−0.255**	**0.030**	**−0.485**	**< 0.001**	−0.100	0.484	− 0.190	0.179
hArg (μM)	0.149	0.230	**−0.250**	**0.042**	−0.100	0.399	−0.032	0.788	−0.215	0.081	0.019	0.898	− 0.070	0.615
Citrulline (μM)	0.015	0.907	−0.200	0.110	−0.126	0.289	−0.061	0.611	−0.002	0.987	0.008	0.957	− 0.180	0.211
Ornithine (μM)	−0.161	0.193	−0.180	0.145	−0.039	0.745	0.090	0.451	0.082	0.510	0.014	0.921	− 0.160	0.262
Arg/ADMA	**0.399**	**0.001**	**−0.460**	**< 0.001**	**−0.364**	**0.002**	**− 0.389**	**0.001**	**−0.514**	**< 0.001**	−0.220	0.131	**− 0.350**	**0.012**
hArg/ADMA	**0.324**	**0.007**	**−0.360**	**0.003**	**−0.246**	**0.036**	**− 0.215**	**0.070**	**−0.339**	**0.005**	−0.180	0.224	− 0.260	0.072
SDMA/ADMA	−0.016	0.897	−0.130	0.315	−0.209	0.076	**− 0.236**	**0.046**	−0.213	0.083	−0.070	0.648	− 0.190	0.182
GABR	0.161	0.194	−0.040	0.768	−0.016	0.894	−0.030	0.803	**−0.231**	**0.060**	−0.050	0.726	0.022	0.881
TMAL(μM)	**−0.415**	**< 0.001**	**0.377**	**0.002**	**0.284**	**0.015**	**0.312**	**0.008**	**0.290**	**0.017**	**0.340**	**0.016**	**0.319**	**0.024**

TMAL total methylated arginine load, *TC* total cholesterol, *CRP* C– reactive protein, *ESR* erythrocyte sedimentation rate. Spearman correlation was used, $p<0.05$ statistically significant

are modulated by oxidative stress and inflammation (Tain and Hsu 2017). Oxidative stress decreases DDAH activity, while it increases PRMT activity (Unlu et al. 2020). AS is a chronic inflammatory disease and elevated ESR and CRP levels indicate inflammatory states in patients with AS. The formation of reactive oxygen species is an important initial event of inflammation (Tripepi et al. 2011). Therefore, we think that increased methylarginine load in patients with AS may be associated with altered PRMT and DDAH enzyme activity due to oxidative stress and inflammation. Elevated methylated arginine load may play a role in the pathogenesis of cardiovascular events in AS by causing a decrease in NO production and bioavailability. In addition, our findings suggested that Arg, hArg levels, Arg/ADMA, and hArg/ADMA ratios and GABR were lower in patients with AS compared to the control group (Table 2). GABR is a newly developed approach used to demonstrate the NO production capacity of a system. This ratio provides a more precise estimate of arginine bioavailability, NO-generating capacity and is used as a marker for endothelial dysfunction and cardiovascular diseases (Ali-Sisto et al. 2017). Therefore, decreased Arg, Arg/ADMA, and GABR levels in patients with AS (Table 2) support the finding that NO production is decreased in these patients, and decreased NO levels may be associated with the pathogenesis of cardiovascular diseases in patients with AS. hArg is a cationic amino acid derived from lysine and can increase NO levels in two ways. First, hArg serves as an alternative substrate of NOS and a precursor of NO. Second,

Table 5 Correlations between methylarginine pathway metabolites and AS disease activity indices

Parameters	BASDAI		BASMI		BASFI		ASQol	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
ADMA (μM)	−0.075	0.570	−0.229	0.075	−0.076	0.563	−0.147	0.262
SDMA (μM)	−0.015	0.907	−0.097	0.463	−0.113	0.389	−0.098	0.456
L−NMMA (μM)	−0.139	0.288	−0.197	0.132	−0.167	0.202	−0.188	0.150
Arg (μM)	**−0.269**	**0.038**	−0.126	0.336	−0.223	0.087	−0.082	0.534
hArg (μM)	0.206	0.114	0.228	0.079	0.080	0.541	0.046	0.618
Citrulline (μM)	−0.090	0.494	−0.065	0.654	−0.175	0.224	−0.193	0.140
Ornithine (μM)	0.009	0.947	0.084	0.525	−0.043	0.745	−0.017	0.895
Arg/ADMA	−0.198	0.129	−0.013	0.920	−0.185	0.157	−0.018	0.890
hArg/ADMA	0.179	0.170	0.198	0.135	0.105	0.426	0.030	0.822
SDMA/ADMA	0.094	0.475	0.138	0.292	0.071	0.589	0.104	0.429
GABR	−0.123	0.348	−0.143	0.274	−0.093	0.480	0.067	0.613
TMAL (μM)	−0.048	0.717	−0.250	0.054	−0.094	0.477	−0.177	0.177
CRP (mg/L)	0.247	0.084	−0.065	0.654	−0.175	0.224	−0.151	0.296
ESR (mm/hr)	0.220	0.130	0.030	0.838	0.041	0.777	0.063	0.665

TMAL total methylated arginine load, *TC* total cholesterol, *CRP* C– reactive protein, *ESR* erythrocyte sedimentation rate

Spearman correlation was used, $p < 0.05$ statistically significant

it increases intracellular Arg concentrations by inhibiting arginase, the enzyme that competes with NOS for Arg, the major substrate of NO (Karetnikova et al. 2019). Therefore, decreased serum hArg levels have also been identified as a risk factor for cardiovascular diseases. Reduced serum hArg levels and hArg/ADMA ratio in patients with AS (Table 2) also strengthen the finding that the increased risk of cardiovascular disease in patients with AS may be associated with decreased NO availability.

Sari et al. reported that there was no significant difference between the ADMA levels of the anti-TNF-α and control groups, and that the serum ADMA levels of the patients receiving conventional therapy were higher than the control group. Serum ADMA levels were lower in the anti-TNF-α group compared to the conventional therapy group, but this difference was not statistically significant (Sari et al. 2009). Inci et al. reported that serum ADMA levels were statistically significantly lower in the anti-TNF-α group compared to the conventional therapy group. Serum ADMA levels of the control group were lower than both therapy groups (Inci et al. 2017). Similarly, our findings reveal that serum ADMA, SDMA levels, and also total methylated arginine load were lower in the anti-TNF-α group than in the conventional therapy group, and that the total methylated arginine load was lower in the control group compared to both therapy groups (Table 3). TNF-α is a pleiotropic inflammatory cytokine that causes the production of reactive oxygen derivatives (ROS) in endothelial cells. It has been shown that TNF-α is both secreted by endothelial cells and induces ROS production in these cells (Chen et al. 2008). It is known that excessive production of ROS increases PRMT activity and causes a decrease in DDAH activity (Unlu et al. 2020). Therefore, reduced serum ADMA and SDMA levels and total methylated arginine load in the anti-TNF-α group compared to the conventional therapy group may be associated with the alleviation of TNF-α-induced oxidative stress. However, total methylated arginine loads of the two groups were slightly different. Therefore, the use of TNF-α inhibitors in the treatment of AS may contribute to the reduction of vascular damage, but further studies are needed in a larger population.

Moreover, there was a positive correlation between total methylated arginine load with CRP and ESR levels, while a negative correlation was found between Arg/ADMA ratio and ESR levels (Table 4). These correlations show that the production of ADMA, SDMA, and L-NMMA is induced by the chronic inflammatory process in AS, and also suggest that methylarginine derivatives may contribute to the inflammatory process. Inflammatory process and oxidative stress-mediated mechanisms may affect PRMT and DDAH activities, leading to an increase in methylarginine levels (Mookerjee et al. 2007). On the other hand, ADMA has been shown to increase the production of proinflammatory cytokines such as TNF-α and interleukin 8 (IL-8) via the ROS/NF-κB-dependent pathway in vitro studies (Tripepi et al. 2011). Similarly, in a study conducted with chronic kidney disease patients, SDMA was shown to increase IL-6 and TNF-α levels by activating NF-κB, thus contributing to the inflammatory process (Schepers et al. 2011). The evaluation of all these findings, together with the correlations between methylarginine derivatives and systemic inflammation markers, indicates that methylarginine derivatives may

also be potential markers for chronic systemic inflammation. There was no correlation between disease activity indices and methylarginine derivatives or total methylated arginine load, while a negative weak correlation was found between Arg and BASDAI score which reflects the intensity of disease activity. However, no correlation was found between serum Arg levels with BASMI, BASFI, ASQol, CRP, and ESR levels (Table 5). These findings show that the decrease in serum arginine levels may be associated with an increase in AS disease activity, but further studies are needed. Various studies have shown that serum LDL and triglyceride levels are higher in patients with AS compared to the control group, while HDL levels are lower (Kucuk et al. 2017; Malesci et al. 2007). Kucuk et al. reported that the LDL/HDL ratio in patients with AS was higher than the control and correlated with the CIMT, and therefore, the LDL/HDL ratio could be a reliable marker in predicting atherosclerotic heart diseases in patients with AS (Kucuk et al. 2017). In our study, serum LDL, triglyceride, total cholesterol, and glucose levels were higher in patients with AS compared to the control group, while HDL levels were lower. Moreover, there was a positive correlation between total methylated arginine load and LDL, total cholesterol, triglyceride, and glucose levels, while a negative correlation was found with HDL. It is known that increased triglyceride, LDL levels, and decreased HDL levels are risk factors for endothelial dysfunction and atherosclerotic cardiovascular disease. The correlations between well-defined atherosclerosis risk factors and total methylarginine levels support the finding that impaired methylarginine and NO metabolism in patients with AS are associated with cardiovascular events in AS.

Conclusions

To the best of our knowledge, this is the first study to comprehensively investigate serum ADMA, SDMA, L-NMMA, arginine, citrulline, ornithine, and homoarginine levels, which are metabolites associated with NO metabolism, in patients with AS. Elevated total methylated arginine load (the sum of ADMA, SDMA, and L-NMMA levels) and decreased Arg, hArg, GABR, Arg/ADMA, and hArg/ADMA levels indicate that NO metabolism is impaired in patients with AS without cardiovascular risk factors. Therefore, imbalances in the levels of methylarginine pathway metabolites and altered NO levels and bioavailability in patients with AS may be associated with cardiovascular events. However, our study has disadvantages in terms of the limited number of patients and lack of measurement of NO levels, and further studies are needed in a larger population.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest relevant to the content of this manuscript.

Informed consent Informed consent was obtained from all individual participants included in the study.

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