THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

VASCULAR DISEASE IN ANKYLOSING SPONDYLITIS

by

Nóra Bodnár, MD

Supervisor: Sándor Szántó, MD, PhD

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF CLINICAL MEDICINE

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TABLE OF CONTENT

1. INTRODUCTION

1.1. Epidemiology of cardiovascular disease in ankylosing spondylitis

Accelerated atherosclerosis and increased cardiovascular (CV) morbidity and mortality have been associated with various inflammatory rheumatic diseases. Most data have been published in rheumatoid arthritis (RA) [reviewed in (1-7)]. Much less information have become available regarding spondyloarthropathies (SpA), such as ankylosing spondylitis (AS) and psoriatic arthritis (PsA). There is a clinically relevant increase in the prevalence of CV diseases (CVD) and higher CV mortality in AS (8-11). In a comparative study, the prevalence ratios of ischemic heart disease, atherosclerosis and cerebrovascular disease were 1.2, 1.5 and 1.7, respectively, in AS in comparison to healthy controls (12). There may be a 4.4-fold increased risk for myocardial infarction in AS (13).

1.2. Mechanisms of accelerated atherosclerosis in spondyloarthropathies

The basic mechanisms of accelerated atherosclerosis and CV disease in AS are similar to those associated with RA (1, 4, 5). It seems to be clear that systemic inflammation associated with the underlying disease is the major driver of accelerated atherosclerosis. Autoimmune-inflammatory mechanisms that link arthritis to atherosclerotic plaque formation include T, B cells and macrophages, pro-inflammatory cytokines (TNF-α, IL-1, IL-6, interferon-γ), chemokines, cellular adhesion molecules (ICAM-1, VCAM-1, E-selectin) (14-19). Adipokines, primarily leptin and resistin have also been implicated int he pathogenesis of AS-associated atherogenesis (20). There are common genes that confer susceptibility to both

arthritis and atherosclerosis (21). Accelerated atherosclerosis and CVD are usually associated with more progressive disease with systemic, extra-articular manifestations (22-24).

Among traditional Framingham risk factors, AS has been associated with high prevalence of metabolic syndrome including dyslipidemia, high LDL-C/HDL-C ratio and hyperhomocysteinemia (25, 26). However, inflammatory mechanisms underlying AS may be the key factors that lead to atherosclerosis and vascular disease (27, 28). Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) may also be involved in this process and TNF blockade leads to the improvement of lipid profile in AS (29).

1.3. Assessment of vascular pathophysiology

Non-invasive angiological methods have been developed in order to evaluate endothelial and vascular function in rheumatic, as well as autoimmune diseases (30). Flow-mediated vasodilation (FMD), common carotid intima-media thickness (ccIMT) and pulse-wave velocity (PWV) determined by ultrasound-based techniques are reliable indicators of endothelium-dependent vascular function, overt atherosclerosis and arterial stiffness, respectively. We and others have utilized these techniques to assess abnormalities of vascular function in RA (3, 31-33). Similar methods can be applied to detect endothelial dysfunction, atherosclerosis and vascular stiffness in AS (11, 24, 27, 28, 34-37).

There have been recent studies that separately assessed FMD, ccIMT or PWV in AS.

Endothelial dysfunction indicated by impaired FMD has been reported by Sari et al (27) in a cohort of 54 AS patients. Impaired FMD did not correlate with age, sex, CRP, ESR, smoking habits or disease activity. Pieringer et al (36) also published preliminary results on impaired FMD in AS.

Sari et al (27) also assessed ccIMT in the same cohort and found no differences between AS and the control group. Other groups, such as Malesci et al (25), Choe et al (35) also reported normal ccIMT in cohorts of 24 to 28 AS patients in comparison to healthy subjects. Very recently, Gonzalez-Juanatey et al (11) assessed ccIMT and carotid plaques. Carotid plaques were more commonly observed in AS compared to controls but no differences in ccIMT were observed. In contrast to these reports, Mathieu et al (28) and Peters et al (13) found higher ccIMT in AS patients in comparison to controls. In the study of Sijl et al (37), the progression of carotid atherosclerosis stopped in patients continuously receiving anti-TNF biological therapies, while ccIMT progressed in those, who discontinued therapy.

Recently, Capkin et al (38, 39) have reported increased PWV in AS patients in comparison to controls. Moreover, anti-TNF therapy did not improve arterial stiffness (39, 40) and there was no difference in PWV between patients treated with anti-TNF therapy or NSAIDs (38).

All studies described above showed data of separate assessments of FMD, ccIMT and PWV. Only Sari et al (27) investigated FMD and ccIMT simultaneously, but they did not assess vascular stiffness. There have been no reports on PWV is AS. In addition, none of the investigators assessed the three vascular parameters (FMD, ccIMT, PWV) together and also in association with numerous other clinical and laboratory parameters.

Therefore, in ***Study 1***, we wished to conduct a complex study and assess endothelial function (FMD), carotid atherosclerosis (ccIMT) and arterial stiffness (PWV) in the same cohort of AS patients with no known history of CVD. In addition, we correlated vascular function with several other clinical and laboratory parameters including age, disease duration, smoking, body mass index (BMI), disease activity and pain intensity determined by the patient (VAS), BASDAI, lumbar spine mobility, chest expansion, wall-occiput distance, BASFI, CRP, ESR and HLA-B27 status. This was possibly the very first study that

investigated endothelial function, atherosclerosis and arterial stiffness in the very same patient cohort and correlated these markers with numerous other indicators.

1.4. The role of arginine derivatives in vascular pathology

Asymmetric dimethylarginine (ADMA) has emerged as a link between insulin resistance, atherosclerosis and vascular disease (41-44). ADMA is the major endogenous inhibitor of soluble nitric oxide (NO) synthase that has been associated with carotid atherosclerosis, acute coronary syndrome and cerebrovascular disease (42-44). As NO promotes vasodilatation, ADMA might be deleterious by inhibiting this effect (45, 46). Homocysteine and LDL-C, two major risk factors for atherosclerosis and CV disease, also leads to increased ADMA production and retention (47-49). Furthermore, statins also seem to be less efficient in patients with high ADMA levels (50, 51). Recently, increased ADMA production was reported in RA patients (52, 53). ADMA levels were inversely correlated with coronary flow reserve in RA (53). Recently, Sari et al (54) reported increased production of ADMA in 48 AS patients without classical CV risk factors in comparison to healthy controls. Erre et al (55) also reported increased ADMA production in very few (n=17) AS patients compared to healthy subjects. However, in these studies AS patients were not compared to osteoarthritic (OA) patients and SDMA and L-arginine were not measured.

Therefore in ***Study 2*** we assessed serum ADMA, as well as arginine and SDMA levels, in association with several clinical and laboratory parameters in AS patients compared to OA controls. We also correlated ADMA levels in AS patients with numerous clinical, imaging and laboratory parameters described in Study 1.

1.5. Anti-MCV and anti-hsp65 antibodies in AS

Anti-citrullinated protein antibodies (ACPAs) including anti-cyclic citrullinated peptide (CCP), anti-mutated citrullinated vimentin (MCV), anti-citrullinated fibrinogen (CF), anti-citrullinated α enolase peptide (CEP) and some others have been implicated in the pathogenesis and outcome of RA (56-68). ACPA production has been associated with interactions of HLA-DRB1 alleles and lifestyle-related factors, such as smoking in RA, as well as more destructive joint damage (56, 57, 67, 69, 70). In addition, citrullination of proteins in the vessel wall has also been implicated in the pathogenesis of arthritis-associated atherosclerosis and CVD (71, 72).

The anti-Savoie (Sa) antibody was long ago described as specific diagnostic and prognostic marker in RA. It has later been demonstrated that anti-Sa specifically recognizes CV (73). In order to detect antibodies to CV, an ELISA system was developed that contains genetically modified MCV as autoantigen to improve the performance of the test. We and others have shown that anti-MCV ELISA is a very sensitive and specific diagnostic tool in RA. It has also been associated with HLA-DRB1 and radiological progression (56, 63, 66, 68, 74-77).

There have been few data on the possible associations of ACPA with SpA, such as AS. In AS, some HLA-B27 allele variants, specifically HLA-B*2705 and B*2709 may undergo citrullination, which alters their capacity of antigen presentation (78). In another study, 15% of PsA and 14% of AS patients were positive for anti-MCV (79).

Autoantibodies to heat shock proteins (hsp) have been implicated in inflammation, autoimmunity and atherosclerosis. Among inflammatory rheumatic diseases, anti-hsp65 antibodies were detected in the sera of RA patients (80-82). Regarding AS, Mycobacteria have been implicated in the pathogenesis of the disease (83, 84), however, there have been no

reports on anti-hsp65 in relation with other clinical and laboratory markers. In one early study, serum anti-hsp65 was measured in AS, RA patients and controls. Although anti-hsp65 was elevated in 19/59 patients (32%), the level of elevation was not significant. In contrast, significantly elevated IgA anti-hsp65 was observed in RA (85). No other reports on anti-hsp65 in relation to AS have yet become available. Anti-hsp65 antibodies have also been implicated in the pathogenesis of atherosclerosis and CVD (86-88). Anti-hsp65 antibodies may be able to differentiate between atherosclerosis in RA and otherwise healthy subjects (87).

Thus, there have been no studies assessing anti-MCV and anti-Mycobacterial hsp65 production in AS in association with other clinical and laboratory parameters. Based on data on the possible role of citrullination and Mycobacterial infection in AS, as well as RA, our hypothesis was that antibodies to citrullinated proteins and hsp may be associated with AS. Therefore, in ***Study 3*** we assessed anti-MCV and anti-hsp65 levels in the sera of AS patients and healthy controls. In AS, we correlated anti-MCV and anti-hsp65 with each other, as well as various clinical and laboratory biomarkers described in Study 1. As ACPA production has been associated with smoking in RA (70), we also correlated antibody production with the smoking habits of AS patients. In this study, we did not have the opportunity to perform CV assessment in the AS patients. This will be completed in the close future.

2. RESEARCH AIMS

In these studies, we conducted a complex assessment of endothelial function (FMD), carotid atherosclerosis (ccIMT) and arterial stiffness (PWV) in the very same cohort of AS patients (***Study 1***). We also assessed the production of ADMA, a known pro-atherogenic biomarker in AS (***Study 2***). Finally, as autoimmunity may also be involved in AS-associated CVD, we determined the production of anti-MCV and anti-hsp65 antibodies in this disease (***Study 3***).

Our specific aims were as follows:

Study 1. Non-invasive assessment of vascular pathophysiology in AS

- assessment of FMD, ccIMT and PWV in AS patients and controls
- correlation of vascular function with clinical parameters including age, disease duration, smoking, body mass index (BMI), disease activity and pain intensity determined by the patient (VAS), BASDAI, lumbar spine mobility, chest expansion, wall-occiput distance and BASFI
- correlation of vascular function with laboratory biomarkers, such as CRP, ESR and HLA-B27 status

Study 2. Determination of the possible role of ADMA in AS

- assessment of ADMA, as well as arginine and SDMA levels in AS patients and OA controls

- correlation of ADMA production with clinical, imaging and laboratory parameters described in Study 1.

Study 3. Assessment of anti-MCV and anti-hsp65 antibodies in AS compared to controls

- assessment of anti-MCV and anti-hsp65 antibodies in AS patients and controls
- correlation of anti-MCV and anti-hsp65 antibody levels with each other, as well as with other clinical and laboratory parameters described in Study 1.

3. PATIENTS, MATERIALS AND METHODS

3.1. Study 1: Non-invasive assessment of vascular pathophysiology in AS

3.1.1. Patients

Altogether 43 AS patients (31 males – 72% and 12 females - 28%; mean age: 45.4 ± 11.8 years, range: 26-75 years; all Caucasians) were included in the study. The diagnosis of AS was based on the modified New York criteria (89). Among the 43 patients, 33 (76.7%) had only axial involvement, while 10 (23.3%) also had peripheral arthritis. Other information on the AS group is included in *Table 1*. Altogether 36 patients (83.7%) were HLA-B27 positive. Fourteen out of the 43 AS patients (32.6%) were in active state of disease (BASDAI >40) (90). Most patients (37/43, 86%) received non-steroidal anti-iflammatory drugs (NSAID). The possible CV effects of NSAIDs could not be fully ruled out, however, as none of the AS patients had clinical CV disease (see below), this possible effect was not observed on the clinical level. Among the 10 AS patients with peripheral involvement, 6 (60%) received conventional DMARDs including methotrexate or sulfasalazine. Altogether 28 patients (65.1%) currently received anti-TNF-α biologics. We recruited 40 age- and sex-matched healthy control subjects from volunteering hospital workers, visitors and relatives (27 males - 67.5% and 13 females - 32.5%; mean age: 48.2 ±13.2 years, range: 24-80 years; all Caucasians). None of the AS patients received systemic or local corticosteroids at the time of and at least 3 months prior to the study. None of the patients and controls received any vasoactive drugs or anticoagulants, such as aspirin, clopidogrel, heparin, warfarin/acenocoumarol, statins, ACE inhibitors or calcium channel blockers.

Table 1. Description of the AS population in Study 1 and Study 3

Variable (unit)	Mean±SD	Range
Age (years)	45.4±11.8	26-75
Male:female ratio	31:12	.
Age at diagnosis (years)	32.8±10.5	16-57
BMI (kg/m^2)	25.0±3.8	19-33
Disease duration (years)	13.2±10.6	2-40
Axial:peripheral ratio	33:10	-
ESR (mm/h)	15.5±15.6	2-68
CRP (mg/l)	9.00±11.5	0.5-56.7
HLA-B27 positivity (%)	83.7	-
Current smokers (%)	30.2	-
Pain on VAS (mm)	51.1±31.9	12-90
Disease activity on VAS (mm)	49.7±28.5	8-93
Active disease (BASDAI>40; %)	32.6	-
BASDAI (mm)	50.4±19.1	19-80
BASFI	45.4±11.8	
BASMI	45.4±11.8	
Lumbar spine mobility (Schober; cm)	2.2±1.7	0.5-5.5
Chest expansion (cm)	2.3±1.2	1.1-4.5
Wall-occiput distance (cm)	8.2±8.5	0-22
Current NSAID therapy (%)	86%	-
Current DMARD therapy (%)	14%	-
Current anti-TNF therapy (%)	65%	-

BASDAI: Bath disease activity index; BASFI: Bath functional index; BASMI: Bath metric index; BMI: body mass index; CRP: C-reactive protein;DMARD: disease-modifying antirheumatic drug; ESR: erythrocyte sedimentation rate; NSAID: non-steroidla anti-inflammatory drug; TNF: tumor necrosis factor; VAS: visual analogue scale

Informed consent was obtained from each AS patient and healthy control subject according to the Declaration of Helsinki. For this study we also obtained local ethical committee approval at the University of Debrecen. Serum samples were then obtained from all subjects and kept frozen at -70^{o}C until further use.

3.1.2. Clinical and laboratory parameters

The age, disease duration and BMI of all AS patients were recorded. Pain intensity and disease activity was determined by the patient on 10 cm VAS. Disease activity and functional capacity mobility were also tested by obtaining BASDAI and BASFI respectively. Metric measurements including lumbar spine mobility assessed by Schober's test, chest expansion and wall-occiput distance were also recorded.

Among laboratory indicators, erythrocyte sedimentation rate (ESR; mm/h) was assessed by the Westergren method. Serum C reactive protein (CRP; mg/l) was measured by quantitative nephelometry (Cobas Mira Plus-Roche), using CRP reagents (Dialab, Austria). After overnight fasting, blood samples were taken from the patients and controls for serum glucose, total cholesterol, LDL-C, HDL-C, triglyceride, renal and liver function tests and full blood count. Urinary samples were tested by Uricont-S. HLA-B27 genotyping was performed by using polymerase chain reaction-sequence specific primer (PCR-SSP) technique (HISTO TYPE B27 High resolution kit, BAG, Lich, Germany).

3.1.3. Clinical assessment of cardiovascular manifestations

All AS patients and healthy controls had a negative history for previous CV, cerebrovascular or peripheral arterial disease. The AS and control groups were comparable in lipid levels, BMI, blood pressure and other traditional CV risk factors (*Table 2*). Thirteen out of the 43 AS patients (30.2%) and 14 out of 40 controls (35.0%) were current smokers. Regarding current tobacco smoking, we applied the cut-off points published by Pedersen et al (91) in RA (≥ 20 pack-years). Cumulative tobacco intake was also calculated by multiplying the mean daily intake by the duration of consumption in years. Patients with traditional CV

risk factors other than smoking, such as hypertension (blood pressure >140/90 mmHg), diabetes mellitus and those with any vasculitis, current infectious disease or renal failure (serum creatinine ≥ 117 mmol/l) were excluded from the study. CV involvement was also assessed by ECG and echocardiography performed in all AS patients and controls. Based on all these data, all AS patients or controls were considered free of clinically relevant vascular disease.

Table 2. Traditional CV risk factors in AS patients and controls*

Risk factors	**AS (n=43)**	**Controls (n=40)**	**p value**
Age (years)	45.4 ± 11.8	48.2 ± 13.2	0.78
Systolic blood pressure (mmHg)	130.7 ± 11.8	132.0 ± 12.9	0.82
Diastolic blood pressure (mmHg)	86.1 ± 9.2	84.3 ± 6.9	0.75
Total cholesterol (mmol/l)	5.52 ± 1.22	5.45 ± 0.91	0.69
LDL cholesterol (mmol/l)	3.25 ± 1.03	3.30 ± 0.82	0.91
HDL cholesterol (mmol/l)	1.58 ± 0.55	1.65 ± 0.39	0.77
Triglyceride (mmol/l)	1.49 ± 0.69	1.39 ± 0.78	0.68
BMI (kg/m^2)	25.0 ± 3.8	24.7 ± 4.8	0.88
Current smokers (%)	30.2	35.0	-

*Values are mean ± SD. AS: ankylosing spondylitis; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein.

3.1.4. Assessment of brachial artery flow-mediated vasodilation

Brachial artery FMD was assessed as described by us and others previously (31, 92). Briefly, ultrasound examination was performed on the right arm using 10 MHz linear array transducer (ultrasound system: HP Sonos 5500) by a single trained sonographer after 30 minutes resting in a temperature-controlled room (basal value for FMD). A B-mode longitudinal section was obtained of the brachial artery above the antecubital fossa. In order to assess FMD, reactive hyperemia was induced by release of a pneumatic cuff around the forearm inflated to suprasystolic pressure for 4.5 minutes. After deflation the maximal flow velocity and the arterial diameter was 90 minutes long continuously recorded. Flow velocities, the baseline diameter and FMD were ECG gated and detected offline. Three repeated FMD measurements were performed in each patient or control subject. Mean FMD values were expressed as % change from baseline (resting) value.

3.1.5. Determination of common carotid atherosclerosis

The ccIMT measurements were carried out as described before by us and others (31, 93). Briefly, a duplex utrasound system (HP Sonos 5500, 10 MHz linear array transducer) was used to assess the common carotid arteries by a single observer. Longitudinal high-resolution B-mode ultrasound scan were employed over both right and left common carotid arteries and were R-synchronized and recorded. The offline measurements were performed 1 cm proximal to the carotid bulb in the far wall. Sites of carotid plaques were avoided. The ccIMT was

defined as the distance between the first and second echogenic lines from the lumen taking the average of 10-10 measurements on both sides. ccIMT values were expressed in mm.

3.1.6. Assessment of pulse-wave velocity as a stiffness parameter

Determination of arterial stiffness were carried out after a modification of our previously used and validated technique (32). The assessment of PWV is based on the fact that the contraction of the myocardium initiates pulse waves in the aorta. The first wave becomes reflected from the aortic wall at the bifurcation, therefore a second, reflected wave appears as a late systolic peak. The morphology of this second, reflected wave depends on the stiffness of the large artery. Thus, we acquired suprasternal and femoral images and pulse wave Doppler signals (HP Sonos 5500, 2-4 MHz phased array and 5-10 MHz linear array transducers.) Simultaneous ECG recording was performed. ECG gated Doppler analyses were performed over the beginning of the descending aorta and over the common femoral artery at the level of the inguinal ligament. Pulse-wave Doppler signals were recorded over ten cardiac cycles at 150 mm/sec sweep speed. Distances between the suprasternal notch and the two sampling sites were also measured and pulse transit times were recorded. The time delay was derived from the difference between the two transit times and PWV in m/s was calculated as distance divided by time delay.

In order to have reproducible results, all subjects needed a rest for at least 5 minutes prior to FMD and PWV assessments.

3.1.7. Statistical analysis and reproducibility

For the analysis of FMD and PWV, Kolmogorov-Smirnov and Lilliefors-tests were used. Subsequently we performed correlation analyses. In cases of normal distribution (parametric) Pearson's test, while in cases of non-normal distribution (non-parametric) Spearman's test were performed. R values of theses correlations were determined and corresponding P values < 0.05 were considered significant.

Regarding reproducibility, all assessments were performed by a single observer (György Kerekes). Intraobserver variability of FMD, ccIMT and PWV measurements were calculated as 5%, 4.2% and 3.3%, respectively. The "stability" of measurements is indicated by the reproducibility for month-to-month repeated assessments of FMD, ccIMT or PWV. According to the Brand-Altman analysis, the 95% limits of agreement ranged between -1.6% and 1.9% for all assessments.

3.2. Study 2. Determination of the possible role of ADMA in AS

3.2.1. Patient groups

In this study, consecutive 61 AS patients (46 males and 15 females; mean age: 44.5±10.6 years, range: 19-76 years) undergoing regular follow-ups at the Department of Rheumatology and 26 patients with hip or knee osteoarthritis (OA; 22 females and 4 males; mean age: 54.2±11.5 years, range: 30-76 years) were included in this study. AS was diagnosed according to the New York criteria (94), while hip and knee OA was diagnosed according to the corresponding classification criteria of the American College of Rheumatology (95, 96). The mean disease duration of AS patients was 12.6±6.4 years.

Regarding drug therapy, 31 out of the 61 AS patients received one of the three "classical" TNF blockers (infliximab, adalimumab or etanercept) within 3 months prior to this study according to routine protocol. None of the AS patients received traditional DMARDs or systemic corticosteroid therapy. Altogether 16 AS and 10 OA patients received NSAIDs at the time of the study. None of the OA patients received any drugs other than NSAIDs. Serum samples were obtained from all subjects and kept frozen at -70^{o}C until further use.

3.2.2. Clinical and laboratory parameters

All clinical and laboratory tests were performed as described in 3.1.2.

3.2.3. Assessment of cardiovascular involvement

All AS patients and OA controls had a negative history for traditional CV risk factors including smoking, previous CV, cerebrovascular or peripheral arterial disease. The AS and OA groups were comparable in lipid levels, BMI, blood pressure and other traditional CV risk factors (data not shown). Thus, patients with known vascular diseases, hypertension (blood pressure >140/90 mmHg), diabetes mellitus, smoking, obesity (BMI $\geq$ 30 kg/m^2), vasculitis, current infectious disease or renal failure (serum creatinine $\geq$ 117 mmol/l) were excluded from the study. Thus, altogether 77 AS and 39 OA patients were initially screened and 16 AS and 13 OA patients were dropped due to exclusion criteria.

CV involvement was also assessed by ECG and echocardiography performed in all AS and OA patients. In order to assess endothelial function and overt atherosclerosis in the AS patients, we also measured FMD of the brachial artery and ccIMT, respectively, using standard ultrasonography techniques, as described in 3.1.4. and 3.1.5., as well as in (31). FMD

and ccIMT values of AS patients were compared to measurement results obtained from our database of 40 healthy volunteer controls (31).

3.2.4. Determination of arginine derivatives

Quantification of arginine, ADMA, and symmetric dimethylarginine (SDMA; the inactive form of ADMA) was performed as previously described by Zsuga et al (42, 97) in detail. The solid phase extractions (SPE) were achieved based on the method of Nonaka et al. (98). Serum samples (250 µL) were mixed with 50 µL L-homoarginine hydrochloride (Sigma, HArg) as internal standard (1000 µmol/L) and 700 µL borate buffer (pH 9.00) then the solutions were passed through the SPE cartridges (OASIS® MCX 3cc) using a 12-column manifold (J. T. Baker). After the washing procedure the arginine derivatives were eluted with solution of cc ammonia-water-methanol (10/40/50, v/v/v) using ammonia solution (Reanal) and methanol (Scharlau). The solvent was evaporated to dryness at 60 °C in vacuum, then it was dissolved in 200 µL deionized water and used for derivatization as described by Vasanits et al (99). The samples of 200 µL were mixed with 63 µL OPA/MPA (*ortho*-phthaldialdehyde [Fluka]/3-mercaptopropionic acid [Aldrich]) reagent solution. Samples were then incubated at 22 °C for 10 min then were cooled down to 5 °C. For chromatography, 20 µL of the samples was injected into the chromatographic system consisting of a Waters 2695 Separations Module equipped with thermostable autosampler (5 °C) and column module (35 °C), a Waters 2745 Fluorescent detector with a Waters Symmetry C-18 (4.6 x 150 mm, 3.5 µm) column, (each from Waters Milford, MA, USA). Gradient elution at a flow rate of 1 mL/min was applied using mobile phase A (20 mM $(NH_4)_2CO_3$ in water, pH adjusted 7.50 ± 0.05) and mobile phase B (acetonitrile). The gradient condition was as follows: first 0-13 min 90% A and 10% B, 13-15 min linear change to 70 % A and 30 % B and hold this setting for

additional 5 min (i.e. 15-20 min). Then 20-22 min linear change to 90 % A, 10% B and hold until 30 min. Analytes were detected at λ_{ex} =337 nm, λ_{em}=520 nm was used for arginine and homoarginine, and λ_{em}=454 nm for ADMA and SDMA. Baseline separation was obtained.

3.2.5. Statistical analysis

The descriptive data of normal variables are expressed as the mean ± SD. Statistical analysis was carried out by paired two-tailed t-test. Correlations between variables were determined using Pearson correlation analysis for normally distributed values and Spearman correlation analysis as non-parametric test. R values of theses correlations were determined and corresponding P values < 0.05 were considered significant.

3.3. Study 3. Assessment of anti-MCV and anti-hsp65 antibodies in AS compared to controls

3.3.1. Patients and controls

The same 43 AS patients described in Study 1 were also included in Study 3. Patient characteristics are seen in 3.1.1. and in *Table 1*.

For comparisons, we also tested 44 healthy volunteers (28 males – 64% and 16 females - 36%; mean age: 42.7 ± 9.2 years) for anti-MCV and 11 patients with low back pain but no AS (7 males – 64% and 4 females – 36%; mean age: 46.3 ± 11.4 years) for anti-hsp65.

All AS patients and controls had a negative history for previous CV, cerebrovascular or peripheral arterial disease. Thirteen out of the 43 AS patients (30.2%) and 15 out of 44 controls (34.0%) were current smokers. Regarding current tobacco smoking, we applied the

cut-off points published by Pedersen et al (91) in RA (≥ 20 pack-years). Cumulative tobacco intake was calculated by multiplying the mean daily intake by the duration of consumption in years.

Again, informed consent was obtained from each AS patient and control subject according to the Declaration of Helsinki. For this study we also obtained local ethical committee approval at the University of Debrecen. Serum samples were then obtained from all subjects and kept frozen at -70^{o}C until further use.

3.3.2. Clinical and laboratory parameters

All clinical and laboratory tests were performed as described in 3.1.2.

3.3.3. Determination of anti-MCV and anti-hsp65 antibody levels

Anti-MCV IgG antibodies were assessed by ELISA (OrgenTec Diagnostika GmbH, Mainz, Germany) as described previously (63). This assay contains recombinant MCV as antigen. The test was performed according to the manufacturer's instructions. The cut-off value for anti-MCV antibodies was 20 U/ml.

Amounts of IgG antibodies reacting with recombinant M. bovis hsp65 (Lionex, Braunschweig, Germany) were assessed by ELISA as described previously (100). Data obtained as optical density values were calculated as arbitrary unit per ml (AU/ml) values related to standard.

3.3.4. Statistical analysis

Antibody levels between different groups were compared by the non-parametric Mann Whitney U test. Spearman's rank correlation was used to assess the relationship between anti-MCV, anti-hsp65 levels and other parameters described above. P values < 0.05 were considered significant. All statistical analyses were performed using the SPSS for Windows 11.0 statistical package.

4. RESULTS

4.1. Study 1: Non-invasive assessment of vascular pathophysiology in AS

4.1.1. Assessment of FMD, ccIMT and PWV in AS and healthy subjects

In order to assess endothelial function, brachial artery FMD was measured by high resolution B-mode ultrasonography. FMD in AS patients expressed in % of the basal value was significantly lower (6.85±2.98 %) in comparison to controls (8.30±3.96 %) (p=0.005) (*Figure 1A*).

ccIMT was asessed using a duplex ultrasound system. ccIMT was significantly higher in AS patients (0.65±0.15 mm) in comparison to controls (0.54±0.15 mm) (p=0.01) (*Figure 1B*).

PWV, an indicator of aortic stiffness was significantly increased in AS patients (8.64± 2.44 m/s) in comparison to healthy subjects (8.00±1.46 m/s; p=0.03) (*Figure 1C*).

4.1.2. Correlations between FMD, ccIMT, PWV and clinical and laboratory markers in AS patients

Within the AS patient population, FMD, ccIMT and PWV values were correlated with each other, as well as with other clinical and laboratory indicators described above (*Table 3*). ccIMT and FMD negatively correlated with each other (r=-0.563; p=0.0001). PWV also correlated with ccIMT (r=0.374; p=0.018) (*Table 3*).

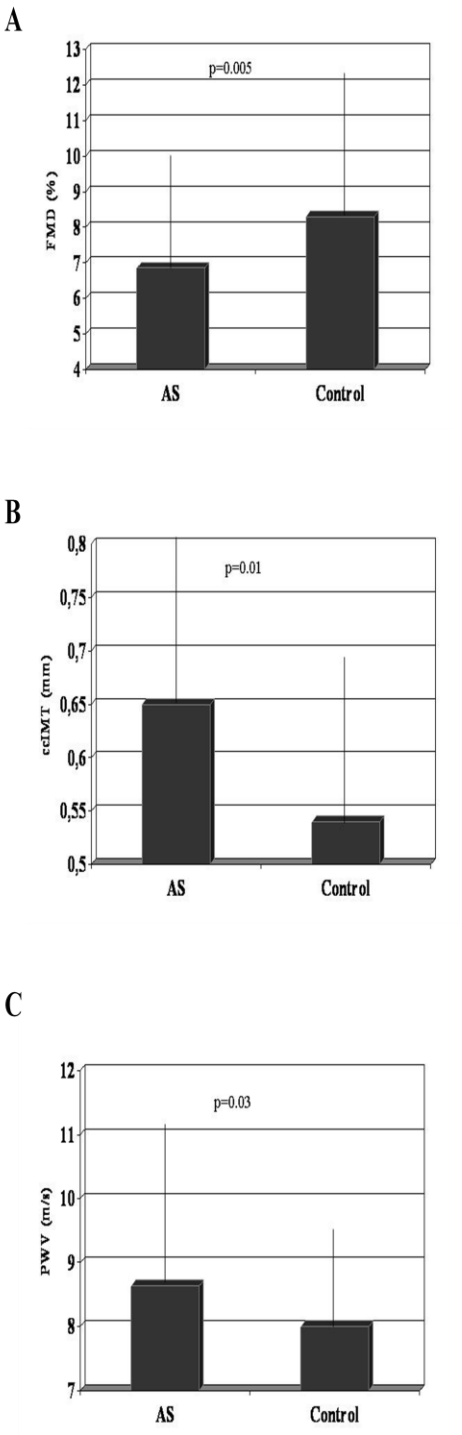

Figure 1. Mean FMD (%; Fig 1A), ccIMT (mm; Fig 1B) and PWV (m/s; Fig 1C) values in AS patients (n=43) and controls (n=40). There is a significant impairment of FMD, as well as significantly increased ccIMT and PWV in AS patients in comparison to healthy subjects. See text for abbreviations.

Both ccIMT and PWV exerted positive correlations with disease duration (r=0.559; p=0.013 and r=0.520; p=0.022, respectively), but FMD did not. PWV also correlated with age (r=0.382; p=0.016). Higher ccIMT or PWV also correlated with increased BASFI (r=0.691; p=0.003 and r=0.654; p=0.006, respectively), negatively correlated with lumbar spine mobility (r=-0.656; p=0.006 and r=-0.604; p=0.013, respectively) and chest expansion (r=-0.502; p=0.047 and r=-0.613; p=0.012, respectively) and positively correlated with wall-occiput distance (r=0.509; p=0.044 and r=0.614; p=0.011, respectively) (*Table 3*). FMD did not.correlate with any functional or metric parameters (data not shown).

Table 3. Relevant correlations between vascular and other parameters in AS patients (n=43)

Vascular parameter	**Clinical/laboratory parameter**	**r value**	**p value**
ccIMT	FMD	-0.563	0.0001
ccIMT	PWV	0.374	0.018
ccIMT	Disease duration	0.559	0.013
ccIMT	BASFI	0.691	0.003
ccIMT	Lumbar spine mobility	-0.656	0.006
ccIMT	Chest expansion	-0.502	0.047
ccIMT	Wall-occiput distance	0.509	0.044
PWV	Age	0.382	0.016
PWV	Disease duration	0.520	0.022
PWV	Lumbar spine mobility	-0.604	0.013
PWV	Chest expansion	-0.613	0.012
PWV	Wall-occiput distance	0.614	0.011

As an internal control, the widely used disease activity scale, BASDAI strongly correlated with the patient's assessment of activity on VAS (r=0.922; p=0.0001) indicating that patient's VAS may be a simple and useful assessment tool in this respect. However, none of the vascular parameters showed any correlation with disease activity markers, such as BASDAI, ESR, CRP or patient's assessment of activity on VAS (data not shown). Furthermore, no significant associations were observed in AS patients between FMD, ccIMT or PWV values in comparison to sex distribution, drug treatment modalities, BMI, HLA-B27 status, current smoking or patient's assessment of pain on VAS (data not shown).

4.2. Study 2. Determination of the possible role of ADMA in AS

4.2.1. Arginine and its derivatives in AS and controls

Serum ADMA levels were significantly increased in AS patients (0.95±0.17 μM) in comparison to OA patients (0.70±0.25 μM) ($p<0.001$). In contrast, there was no difference between AS and OA patients in serum arginine (116.1±32.0 μM versus 111.7±25.6 μM; p=0.53) and SDMA levels (0.52±0.12 μM versus 0.54±0.20 μM; p=0.71) (*Figure 2*).

When AS patients currently receiving anti-TNF agents (n=31) were compared to those not receiving biologics (n=30), no significant differences were observed in ADMA levels between the two groups (0.95±0.22 versus 0.94±0.20 μM).

4.2.2. Correlations between clinical, imaging and laboratory markers and ADMA levels in AS

Serum ADMA levels positively correlated with age (r=0.258; p=0.043), BMI (r=0.368; p=0.003), ESR (r=0.329; p=0.009). Also, ADMA levels negative correlated with chest expansion (r=-0.251; p=0.04) and lumbar spine mobility (r=-0.256; p=0.04) (*Table 4*).

No correlations were found between ADMA levels and disease duration, pain intensity on VAS, use of NSAIDs, CRP, BASDAI, BASFI, BASMI, quality of life (EQ5D), HLA-B27 positivity, FMD or ccIMT (data not shown).

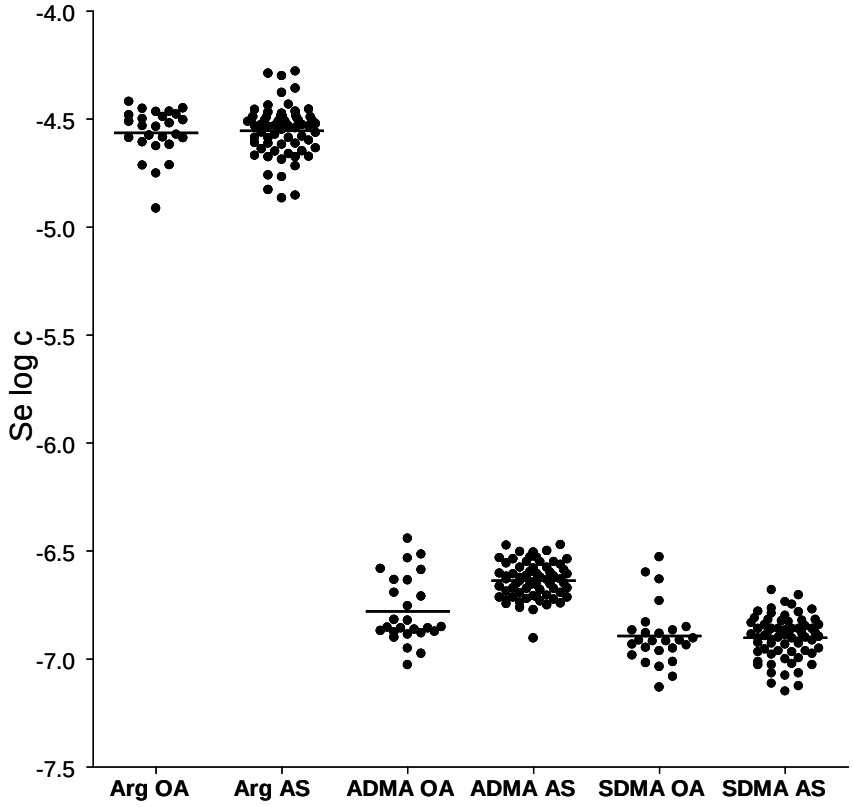

Figure 2. Serum arginine (Arg), ADMA and SDMA levels in ankylosing spondylitis (AS) and osteoarthritic (OA) patients. Values are plotted on a log scale. There is significantly elevated levels of ADMA in AS compared to OA ($p<0.001$), while there is no difference between serum Arg or SDMA levels in AS vs OA patients.

Table 4. Relevant correlations between ADMA levels and other clinical or laboratory parameters in AS patients (n=61)

Corresponding parameter	r value	p value
Clinical parameters		
Age	0.258	0.04
BMI	0.368	0.003
Chest expansion	-0.251	0.04
Lumbar spine mobility	-0.256	0.04
Laboratory parameters		
ESR	0.329	0.009

4.3. Study 3. Assessment of anti-MCV and anti-hsp65 antibodies in AS compared to controls

4.3.1. Anti-MCV positivity and absolute levels in the study population

Patients with AS had significantly median higher serum anti-MCV levels (17.3 U/ml, range: 8.3-31.5 U/ml) in comparison to healthy subjects (8.9 U/ml, range: 5.4-13.3 U/ml) ($p<0.01$) (*Figure 3*).

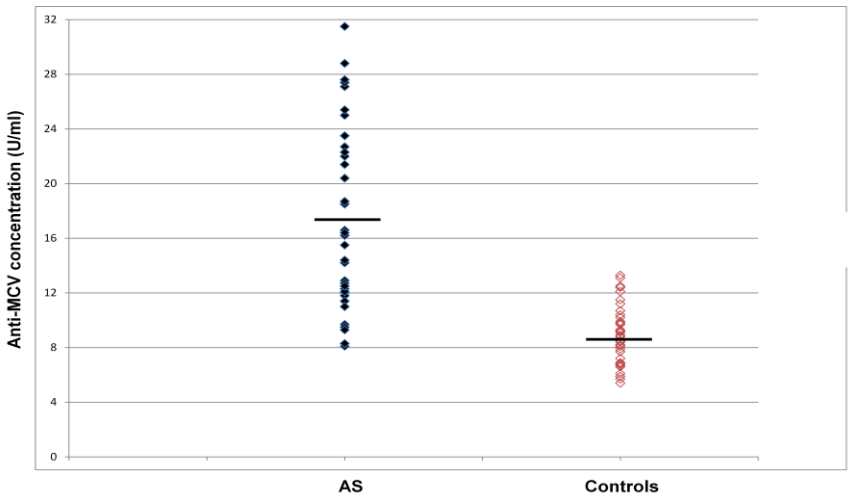

Figure 3. Scatter plot showing anti-MCV levels in the sera of AS patients and controls. There are increased serum levels of anti-MCV in AS versus controls.

Regarding anti-MCV positivity, 16 of the 43 AS patients (37%) and none of the 44 healthy controls (0%) were anti-MCV positive using the cut-off value recommended by the manufacturer (> 20 U/ml).

Patients with axial versus peripheral AS, those with versus without psoriasis, uveitis or inflammatory bowel disease (IBD) did not differ in anti-MCV levels (data not shown).

4.3.2. Anti-hsp65 levels in AS and controls

The median anti-hsp65 concentration in the sera of AS patients was 124.8 AU/ml (range: 27.2-1000 AU/ml), while the non-AS low back pain controls exerted significantly lower anti-hsp65 levels (median: 51.8 AU/ml; range: 22.5-88.5 AU/ml) ($p<0.001$) (*Figure 4*).

Again, patients with axial versus peripheral AS, those with versus without psoriasis, uveitis or IBD did not differ in anti-hsp65 levels (data not shown).

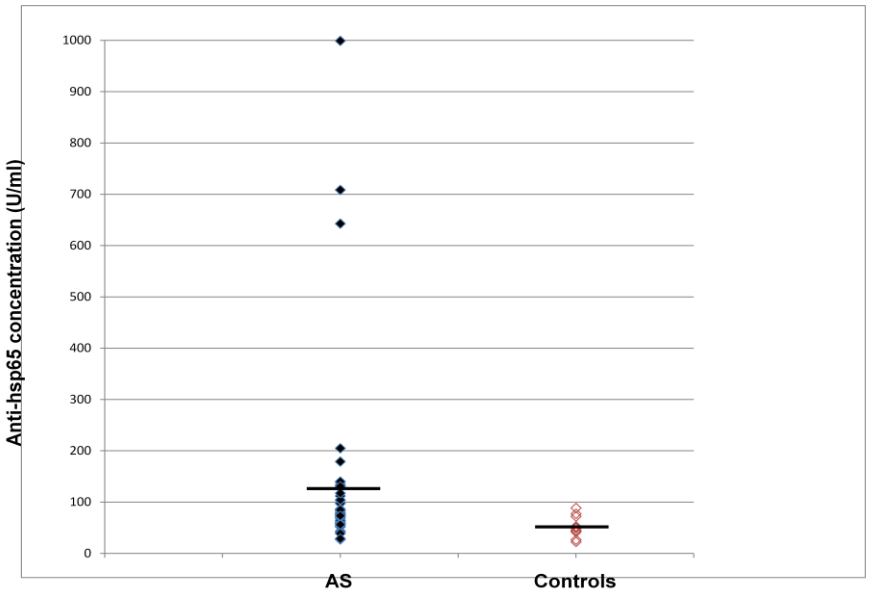

Figure 4. Scatter plot showing anti-hsp65 levels in the sera of AS patients and controls. There are increased serum levels of anti-hsp65 in AS versus controls.

4.3.3. Relationship between anti-MCV, anti-hsp65 antibody levels and other parameters

Interestingly, both anti-MCV positivity ($r=0.613$; $p=0.012$) and absolute serum anti-MCV levels ($r=0,553$; $p=0.021$) exerted significant postive correlations with anti-hsp65 levels. Anti-MCV positivity also correlated with ESR ($r= 0.437$; $p=0.03$).

Neither anti-MCV, nor anti-hsp65 correlated with age, disease duration, CRP, HLA-B27 status, smoking habits, pain intensity (VAS), BASDAI, BASFI or BASMI (data not shown).

5. DISCUSSION

CV and cerebrovascular diseases are major causes of morbidity and mortality in the general population, as well as in autoimmune-inflammatory diseases [reviewed in (1, 2, 4, 5)]. The early identification of patients with higher risk for vascular disorders allows us to introduce primary prevention or effective pharmacological treatment. Assessing risk for future vascular events include non-invasive determination of endothelial function (indicated by FMD), overt carotid atherosclerosis (ccIMT) and arterial stiffness (PWV), as well as several laboratory biomarkers of inflammation and atherosclerosis (31, 32, 92, 93, 101). There have been numerous recent studies showing increased ccIMT and PWV, as well as impaired FMD in RA (2, 3, 31, 32, 101-105).

There have been relatively few and somewhat controversial data regarding vascular function in AS [reviewed in (10)]. Most studies separately assessed FMD, ccIMT or PWV in AS. Sari et al (27), as well as Pieringer et al (36) found impaired FMD in AS. In our ***Study 1***, we also found impaired FMD in 43 AS patients without history of CVD in comparison to controls. In addition, decreased FMD correlated with increased ccIMT in AS. We found no associations between FMD and functional or metric parameters of AS suggesting that FMD is a „snapshot" of endothelial function, while structural and functional damage of the musculoskeletal system may occur after many years

Most groups including Sari et al (27), Malesci et al (25) and Choe et al (35) did not find any differences in ccIMT between AS and control groups. These AS cohorts consisted of 26-28 patients. Gonzalez-Juanatey et al (11) reported more carotid plaques, but unchanged ccIMT in AS. In contrast, similarly to us, Mathieu et al (28) and Peters et al (13) found significantly higher ccIMT in AS compared to controls. In the latter study, more than 50 AS patients were recruited. Here we also report significantly increased ccIMT in AS compared to

controls. Our results in ***Study 1*** are similar to those published by Peters et al (24), but are different from the other studies described above. The studies of Malesci et al (25) and Choe et al (35) included a much lower number of patients than our study. Although Sari et al (27) reported normal ccIMT in AS patients there could be other differences between that cohort and ours. Also, Sari et al (27) published their data in 2006, while Peters el al (24) and Mathieu et al (28) reported their results that are very similar to ours in 2008-2009. Further studies are needed to come to a decision regarding carotid atherosclerosis in AS. Regarding possible correlations, ccIMT correlated with impaired FMD, increased aortic stiffness and also correlated with disese duration, BASFI and metric parameters including lumbar spine mobility, chest expansion and wall-occiput distance. Other investigators assessing ccIMT did not report associations of ccIMT with any of these functional or metric parameters. Our results indicate that the development of atherosclerosis occurs over a longer period of time and is associated with longer duration of AS and the development of more severe structural and functional disability.

Regarding arterial stiffness, there have been very little data on PWV. Only the group of Capkin et al (38, 39) have reported increased PWV in AS patients in comparison to controls. In addition, Choe et al (35) reported normal, while Moyssakis et al (106) found impaired elasticity in AS. Yet, elasticity and stiffness are not the same. In ***Study 1***, we have been the first showing PWV together with ccIMT and FMD data in AS. PWV may be the most widely determined and most reliable indicator of arterial stuffness (107, 108). We found significantly increased aortic stiffness indicated by increased PWV in AS compared to healthy volunteers. Moreover, PWV correlated with ccIMT, as well as with age, disease duration, BASFI and all metric parameters described above. Thus, aortic stiffness, similarly to carotid atherosclerosis, develops in parallel with the progression of AS. Furthermore, as PWV was correlated with ccIMT, increased stiffness may be a consequence of atherosclerosis.

ADMA, a major inhibitor of NO synthesis, has been associated with atherosclerosis, insulin resistance and vascular diseases (41-44, 54, 97). Elevated plasma ADMA levels were detected in RA patients and increased ADMA production was linked to accelerated atherosclerosis observed in RA (52, 53). To date, there has been only very few reports on ADMA production in AS. Sari et al (54) showed high serum ADMA levels in AS patients. Erre et al (55) also reported increased ADMA production in very few (n=17) AS patients compared to healthy subjects. However, these investigators compared AS patients to healthy subjects and not to OA patients. Moreover, those studies did not include measurements of SDMA and L-arginine and also did not correlate ADMA with FMD or ccIMT.

In ***Study 2***, we assessed ADMA production in 61 AS patients in comparison to 26 OA patients. Significantly elevated serum ADMA, but not serum arginine and SDMA levels were detected in AS compared to OA patients. This comparison may be important as it shows that increased production of ADMA may be associated with AS or RA (52), but not with OA. In addition, ADMA levels correlated with age and BMI suggesting its possible association with age-dependent atherosclerosis and obesity. Interestingly, ADMA also correlated with ESR suggesting that serum ADMA may also be a marker of systemic inflammation. Serum ADMA levels inversely correlated with chest expansion and lumbar spine mobility, which suggests an association with worse functional capacity.

Sari et al (54) reported correlation of ADMA levels with CRP, LDL-C, HDL-C and triglycerides. We did not correlate ADMA with lipids, however, we found correlation between ADMA and ESR. Although we did not find correlation between ADMA and CRP, ESR also reflects systemic inflammation. In addition, while Sari et al (54) reported no correlation of ADMA with lumbar spine mobility, we did find inverse correlation between ADMA and lumbar spine involvement. Neither Sari et al (54) nor we found correlations

between ADMA and BASDAI or BASFI. Also, while Sari et al (54) associated high ADMA levels with conventional drug therapy but not with anti-TNF biologics, we did not find any correlations between ADMA production and treatment modalities whatsoever.

In ***Study 2***, ADMA did not correlate with endothelial dysfunction indicated by FMD or carotid atherosclerosis in AS. There have been no reports on possible associations between ADMA and FMD or ccIMT in AS. In one early RA study, ADMA was associated with impaired coronary flow reserve but not with ccIMT (53). Further investigations regarding ADMA production in AS with respect to AS clinical activity, functional impairment, quality of life, as well as AS-associated vascular disease are needed.

ACPAs are considered to be specific and sensitive diagnostic markers of RA (63, 64, 109, 110). While numerous autoantibodies of pathogenic, diagnostic and prognostic significance are available in other autoimmune-inflammatory diseases, AS has not yet been associated with such antibodies. Anti-MCV antibody production has been investigated in very few SpA studies. In one recent study, Damjanovska et al (79) compared anti-MCV production in 917 patients with recent onset arthritis. This population included early RA, AS and PsA patients. The anti-MCV test had a higher sensitivity than two anti-CCP tests. In addition, while >80% of early RA patients were anti-MCV positive, only 15% of PsA and 14% of AS patients exerted anti-MCV seropositivity. In ***Study 3***, 37% of AS patients but only 4.5% of healthy controls were anti-MCV positive. Moreover, AS patients had significantly higher serum anti-MCV levels than controls. Anti-MCV positivity in AS also correlated with acute phase protein production indicated by ESR. In contrast, anti-MCV did not correlate with HLA-B27 status, disease activity, functional and metric indices or smoking habits among AS patients.

Heat shock proteins, as well as antibodies against the Mycobacterial hsp65 have been implicated in the pathogenesis of vascular and autoimmune diseases (81, 84, 85). There has

been only one study reporting non-significant elevation of anti-hsp65 in 19 out of 59 AS patients (85), however anti-hsp65 was not assessed in association with other clinical or laboratory parameters. In ***Study 3***, we also found significantly elevated serum anti-hsp65 levels in AS. Our results are somewhat different from those published by McLean et al (85) as in their study, the elevation of anti-hsp65 in AS compared to controls was not statistically significant.

In ***Study 3*** we also correlated anti-hsp65 and anti-MCV levels for the first time in the literature. There have been no reports on direct links between ACPA and anti-hsp autoantibody production in AS. As described above, Mycobacteria, and thus Mycobacterial hsp65 have been implicated in the pathogenesis of AS (83, 84). Among citrullinated proteins, citrullinated vimentin has also been detected in the synovial tissues of SpA, as well as RA patients (111). Thus, according to the molecular mimicry theory, AS induced by infectious agents including Mycobacteria may trigger synovial inflammation and synovitis in AS may also be associated with increased citrullination of synovial proteins (83, 84, 111). It is not clear whether there would be a direct cross-reactivity between anti-MCV and anti-hsp65 antibodies in AS. Anti-MCV and anti-hsp65 antibodies were not studied here in context with AS, atherosclerosis and CVD. We wish to further investigate on that in future studies.

In conclusion, AS patients may be screened for atherosclerosis and subclinical vasculopathy using these non-invasive imaging techniques, as well as laboratory biomarkers including ADMA, anti-MCV and anti-hsp65 antibodies.

Although we did not study the effects of therapy in these three studies, according to the recent CV recommendations of the European League Against Rheumatism (EULAR) study group, patients at high risk should undergo early primary or secondary prevention (112). Statins might be beneficial for patients with AS (113). There have been many recent reports on the possible vasculoprotective effects of anti-TNF biologics in RA [reviewed in (114,

115)]. Recent reports have suggested that TNF blockade might improve dyslipidemia and microvascular function in AS (29, 114-118). The EULAR recommendations mentioned above suggest that every effort should be made in order to identify high-risk patients early. As systemic inflammation is a major driver of accelerated atherosclerosis and CVD in arthritides including AS, the aggressive suppression of inflammation by DMARDs or biologics may also lower the risk for vascular diseases in AS (112, 114, 116, 118). Certainly, the management of traditional CV risk factors (obesity, smoking, hypertension, etc), as well as the use of vasculoprotective agents including aspirin, calcium channel blockers, ACE inhibitors, ARBs and statins are also recommended (112).

6. SUMMARY

Our research group wished to assess accelerated atherosclerosis and vascular involvement in AS by studying vascular pathophysiology, as well as mediators and antibodies involved in the pathogenesis of both arthritis and atherosclerosis.

Our results are summarized below:

1. Impaired endothelial function and increased carotid atherosclerosis and arterial stiffness were found in AS as determined by FMD, ccIMT and PWV measurements, respectively.
2. ccIMT negatively correlated with FMD and positively correlated with PWV. In addition, both carotid atherosclerosis and arterial stiffness correlated with disease duration, BASFI and various functional parameters that are impaired in AS.
3. Increased production of ADMA, a biomarker of atherosclerosis and CVD, was found in AS compared to OA patients.
4. Serum ADMA levels positively correlated with age, BMI and ESR. ADMA also negatively correlated with chest expansion.
5. Increased release of anti-MCV and anti-hsp65 antibodies were detected in AS compared to healthy controls. These results suggest the involvement of citrullinated autoantiogens and Mycobacterial hsps in the development of AS.
6. Both anti-MCV positivity and absolute serum anti-MCV levels correlated with anti-hsp65 levels. Anti-MCV positivity also correlated with ESR.

6. ÖSSZEFOGLALALÁS

Kutatócsoportunk meg kívánta vizsgálni SPA-ban a felgyorsult atherosclerosist és a vascularis érintettséget elemezve mind a vascularis patofiziológiát, mind az arthritis és az atherosclerosis patogenezisében szerepet játszó mediátorokat és antitesteket.

Eredményeink a következőkben foglalhatók össze:

1. Az FMD, ccIMT és PWV meghatározása révén károsodott endothelialis funkciót, megnövekedett carotis atherosclerosist és artériás merevséget találtunk SPA-ban.

2. A ccIMT negatívan korrelált az FMD-el és pozitívan a PWV-vel. Ráadásul mind a carotis atherosclerosis, mind az artériás merevség öszefüggést mutatott a betegségfennállási idővel, a BASFI-val és különböző funkcionális paraméterekkel, melyek károsodnak SPA-ban.

3. Az ADMA, mint az atherosclerosis és a CVB-ek biomarkere, fokozott termelődését tapasztaltuk SPA-ban OA-es betegekkel összehasonlítva.

4. A szérum ADMA szintek pozitívan korreláltak az életkorral, a BMI-vel és a We-nel. Az ADMA emellett negatívan korrelált a mellkas kitéréssel.

5. Az anti-MCV és anti-hsp65 megnövekedett termelődése volt igazolható SPA-sokban egészséges kontrollokkal összehasonlítva. Ezek az eredmények a citrullinált autoantigének és a Mycobacterialis hősokk proteinek szerepére utalnak az SPA kialakulásában.

6. Mind az anti-MCV pozitivitás, mind az abszolút szérumszintek korreláltak az anti-hsp65 szintekkel. Az anti-MCV pozititás szintén összefüggést mutatott a We-nel.

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8. KEYWORDS AND LIST OF ABBREVIATIONS

Keywords: spondyloarthropathies, ankylosing spondylitis, atherosclerosis, cardiovascular disease, endothelial dysfunction, arterial stiffness, ultrasound, arginine, ADMA, anti-MCV, anti-hsp65

Tárgyszavak: spondylarthropathiák, spondylitis ankylopoetica, atherosclerosis, cardiovascularis betegség, endothel dysfunctio, arteria merevség, ultrahang, arginin, ADMA, anti-MCV, anti-hsp65

Abbreviations:

ACE	angiotensin converting enzyme
ACPA	anti-citrullinated protein antibody
ADMA	asymmetric dimethylarginine
ARB	angiotensin receptor blocker
AS	ankylosing spondylitis
BASDAI	Bath ankylosing spondylitis disease activity index
BASFI	Bath ankylosing spondylitis functional index
BASMI	Bath ankylosing spondylitis metric index
BMI	body mass index
ccIMT	common carotid intima-media thickness
CCP	cyclic citrullinated peptide
CEP	citrullinated enolase peptide
CF	citrullinated fibrinogen
CRP	C reactive protein
CV	cardiovascular
CVD	cardiovascular disease
DMARD	disease-modifying antirheumatic drug
ECG	electrocardiography
ELISA	enzyme-linked immunosorbent assay
EQ5D	quality of life questionnaire – 5 domains
ESR	erythrocyte sedimentation rate

EULAR	European League Against Rheumatism
FMD	flow-mediated vasodilation
HDL-C	high density lipoprotein cholesterol
HLA-B27	human leukocyte antigen B27
hsp	heat shock protein
IBD	inflammatory bowel disease
LDL-C	low-density lipoprotein cholesterol
MCV	mutated citrullinated vimentin
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
PsA	psoriatic arthritis
PWV	pulse-wave velocity
RA	rheumatoid arthritis
SD	standard deviation
SDMA	symmetric dimethylarginine
SpA	spondylarthritis (spondylarthropathy)
SPE	solid phase extraction
TNF	tumor necrosis factor
VAS	visual analogue scale

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List of publications related to the dissertation

1. **Bodnár, N.**, Szekanecz, Z., Prohászka, Z., Kemény-Beke, Á., Némethné-Gyurcsik, Z., Gulyás, K., Lakos, G., Sipka, S., Szántó, S.: Anti-mutated citrullinated vimentin (anti-MCV) and anti-65kDa heat shock protein (anti-hsp65): New biomarkers in ankylosing spondylitis.
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